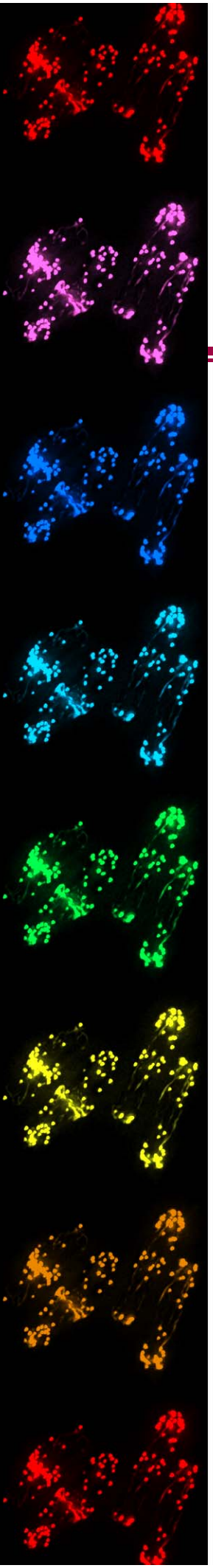

Universidad de Salamanca

Departamento de Microbiología y Genética
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Papel de los reguladores de Rho-GTPasas, Rgf1p y Rgf2p, en el control de la integridad y la polaridad en *Schizosaccharomyces pombe*

Patricia García Rodríguez

2009

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Memoria presentada por **Patricia García Rodríguez**
para optar al grado de Doctor en Bioquímica

Salamanca, Febrero 2009

DR. ÁNGEL DOMÍNGUEZ OLAVARRI, CATEDRÁTICO DE MICROBIOLOGÍA Y DIRECTOR DEL DEPARTAMENTO DE MICROBIOLOGÍA Y GENÉTICA DE LA UNIVERSIDAD DE SALAMANCA,

CERTIFICA:

Que la memoria titulada "**Papel de los reguladores de Rho-GTPasas, Rgf1p y Rgf2p, en el control de la integridad y la polaridad en *Schizosaccharomyces pombe***", presentada por la licenciada **Patricia García Rodríguez**, para optar al Grado de **Doctor en Bioquímica**, ha sido realizada bajo la dirección de la Dra. Yolanda Sánchez Martín en el Instituto de Microbiología Bioquímica, centro mixto de la Universidad de Salamanca (Departamento de Microbiología y Genética) y el Consejo Superior de Investigaciones Científicas.

Y para autorizar su presentación y evaluación por el tribunal correspondiente, firmo el presente certificado en Salamanca a ___ de Febrero de 2009.

Fdo. Dr. Ángel Domínguez Olavarri

DRA. YOLANDA SÁNCHEZ MARTÍN, PROFESORA TITULAR DEL DEPARTAMENTO DE MICROBIOLOGÍA Y GENÉTICA DE LA UNIVERSIDAD DE SALAMANCA,

CERTIFICA:

Que la licenciada **Patricia García Rodríguez** ha realizado el trabajo titulado "**Papel de los reguladores de Rho-GTPasas, Rgf1p y Rgf2p, en el control de la integridad y la polaridad en *Schizosaccharomyces pombe***", bajo su dirección, en el Instituto de Microbiología Bioquímica, centro mixto de la Universidad de Salamanca (Departamento de Microbiología y Genética) y el Consejo Superior de Investigaciones Científicas.

Y para autorizar su presentación y evaluación por el tribunal correspondiente, firmo el presente certificado en Salamanca a ___ de Febrero de 2009.

Fdo. Dra. Yolanda Sánchez Martín

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ABREVIATURAS

ATP	Adenosina 5'-trifosfato
CFP	Proteína azul fluorescente (*Cyan Fluorescent Protein*)
Csp	Caspofungina (Cancidas)
DAPI	4', 6-diamidino-2-fenilindol
DH	Dominio de proteínas con homología a Dbl (*Dbl Homology*)
DNA	Ácido desoxirribonucleico
GAP	Proteína activadora de la actividad GTPásica (*GTPase Acivating Protein*)
GDI	Inhibidor de la disociación de GDP (*GDP Dissociation Inhibitor*)
GDP	Guanina 5'-difosfato
GEF	Factor intercambiador de nucleótidos de guanina (*Guanine Exchange Factor)*
GFP	Proteína verde fluorescente (*Green Fluorescent Protein*)
GS	Glucán sintasa
GTP	Guanina 5'-trifosfato
HA	Epítopo procedente de la hemaglutinina del virus humano de la gripe
MAP	Proteína activada por mitógeno
MAPK	Quinasa activada por mitógeno
MM	Medio mínimo para *S. pombe*
MTs	Microtúbulos
NETO	Comienzo del crecimiento por el polo celular nuevo (*New End Take Off*)
OETO	Comienzo del crecimiento por el polo celular antiguo (*Old End Take Off*)
ORF	Fase de lectura abierta
PCR	Reacción en cadena de la polimerasa
PKC	Proteína Quinasa C
T	Tiamina
wt	Estirpe silvestre
YES	Medio rico para *S. pombe*
YFP	Proteína amarilla fluorescente (*Yellow Fluorescent Protein*)

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INTRODUCCIÓN

El organismo elegido para realizar este trabajo ha sido la levadura de fisión *Schizosaccharomyces pombe*. Esta levadura fue descrita por primera vez por P. Lindner en 1893 y su nombre proviene de la palabra Swahili "pombe" que significa cerveza, de donde fue aislada.

En las últimas décadas *S. pombe* se ha convertido en un organismo de referencia para el análisis genético por su facilidad de crecimiento, su estilo de vida haploide y su accesibilidad para el análisis molecular. Además, posee una característica que hace a esta levadura ideal para el estudio del control de la polaridad, y es que su forma, tamaño y modo de división son extremadamente reproducibles (Chang, 2001).

Desde el año 2002 el genoma de esta levadura está totalmente secuenciado (Wood et al., 2002) y el acceso público a esta información ha facilitado enormemente el trabajo con este organismo (http://www.genedb.org/genedb/pombe/index.jsp).

S. pombe pertenece a los ascomicetos al igual que *Saccharomyces cerevisiae*, sin embargo las diferencias entre estas dos levaduras son importantes. *S. pombe* tiene una forma cilíndrica (**Figura 1**) y se divide por fisión transversal, frente a *S. cerevisiae* que presenta forma elíptica y se reproduce por gemación. La frecuencia de genes por unidad de longitud de DNA es mayor en *S. pombe* y también lo son las regiones que preceden a los genes, lo que podría indicar una mayor región de control (Yanagida, 2002).

Si comparamos su secuencia genómica, las dos levaduras parecen haberse separado hace unos 330-420 millones de años, y de los metazoos y plantas hace unos 1000-2000 millones de años. Como la divergencia parece ser más rápida en los hongos, estas levaduras son probablemente tan divergentes entre sí, como lo pueden ser cada una de ellas con respecto a los eucariotas superiores (Yanagida, 2002).

1. Ciclo de vida de *S. pombe*

1.1. Ciclo vegetativo

En el ciclo biológico de *S. pombe* la fase haploide es la que predomina. Las células de *S. pombe* presentan una forma cilíndrica con extremos redondeados, que al nacer tienen un tamaño aproximado de 3-4 µm de diámetro y 7-8 µm de longitud. Durante el crecimiento, las células mantienen un diámetro constante y aumentan en longitud hasta alcanzar 12-15 µm, que es el tamaño necesario para formar el tabique o septo, que divide a la célula madre en dos células hijas del mismo tamaño.

Las cepas de *S. pombe* utilizadas en el laboratorio son heterotálicas, a diferencia de las aisladas de la naturaleza, que son homotálicas y se denominan h^{90} porque forman aproximadamente un 90% de esporas en cultivo puro (Leupold, 1950). Las cepas del laboratorio, por tanto, se pueden mantener de forma continua en estado haploide.

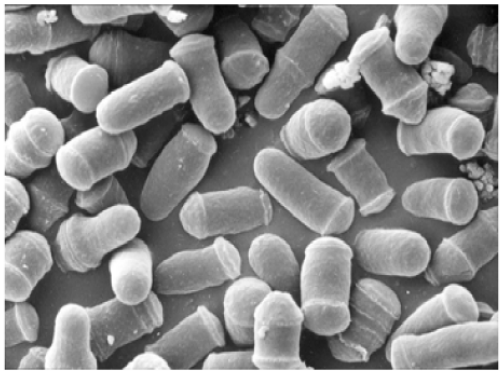

Figura 1: Morfología de *S. pombe* al microscopio electrónico de barrido.

En la naturaleza, es bastante frecuente que *S. pombe* se desarrolle en medios donde los nutrientes escasean. En este caso, las células pierden su aspecto levaduriforme y pasan a formar pseudohifas en las que las diferentes células hijas permanecen unidas unas a otras. La formación de pseudohifas permite a las células buscar nutrientes de una forma más eficaz.

1.2. Ciclo sexual

Las células haploides de *S. pombe* presentan dos tipos sexuales diferentes: h^+ y h^- (Leupold, 1950).

Cuando células de tipos sexuales diferentes se encuentran en un medio desfavorable, sobre todo pobre en nitrógeno, comienzan a secretar feromonas características de su tipo sexual y a producir receptores para la hormona del tipo sexual complementario. La unión de las feromonas a sus receptores desencadena el proceso de conjugación. Éste empieza con la formación de una proyección denominada "*schmoo*" en un extremo de la célula, hacia la fuente de feromonas del tipo sexual complementario (**Figura 2**). A continuación, las células se unen por parejas y finalmente se fusionan formando un zigoto diploide (Egel, 1994). Los zigotos así formados pueden ser mantenidos como células diploides si se reinoculan inmediatamente en un medio rico. Estas células diploides presentan un tamaño mayor que las haploides, siendo de 11-14 µm de longitud al formarse y de 20-25 µm antes de la división.

Las células diploides de *S. pombe* son muy inestables y en medios pobres suelen sufrir inmediatamente meiosis, que conduce a la formación de cuatro núcleos haploides. Alrededor de los núcleos

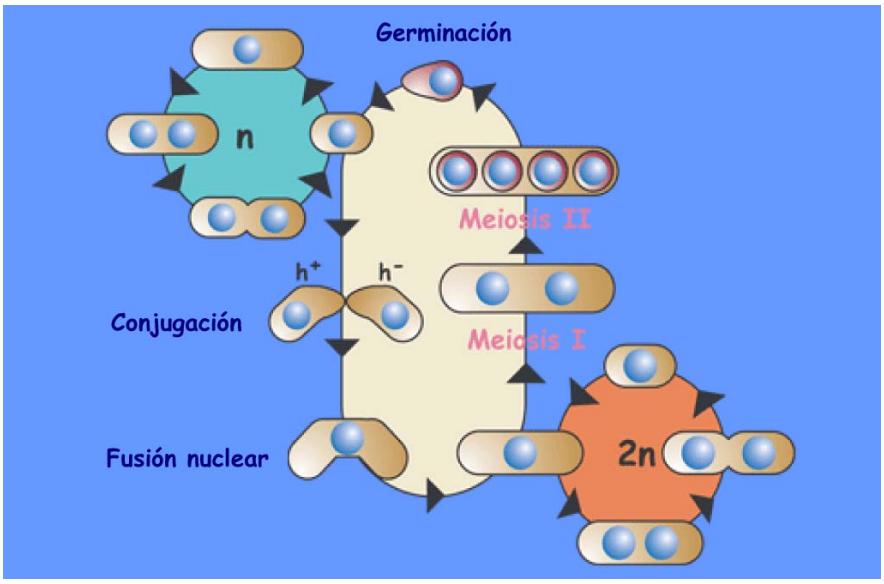

Figura 2:
Ciclo de vida de *S. pombe*. Ciclo vegetativo haploide (señalado en verde) y diploide (señalado en naranja) de *S. pombe*. Las células vegetativas se dividen por fisión transversal dando lugar a dos células hijas de igual tamaño. Cuando las condiciones del medio no son óptimas *S. pombe* cambia a un ciclo sexual en el que se produce la formación de un zigoto diploide que posteriormente sufrirá meiosis y esporulación, dando lugar a 4 ascosporas haploides que quedan incluidas en el interior de un asca.

se sintetizan unas capas protectoras de naturaleza similar a las de la pared celular, formando cuatro estructuras individuales denominadas ascosporas, que a su vez quedan englobadas dentro de un asca.

Finalmente, la pared del asca se rompe y libera las esporas haploides que permanecen durmientes hasta que encuentran condiciones favorables, entonces germinan formando células vegetativas y reiniciando el ciclo (**Figura 2**).

2. Morfogénesis y Control de la Polaridad

2.1. Ciclo celular y ciclo morfogenético en *S. pombe*

La levadura de fisión *S. pombe* se ha convertido en un sistema genético excelente para el estudio de la morfogénesis celular. Este organismo presenta células cilíndricas simples, que crecen por los polos y se dividen mediante fisión transversal.

El ciclo celular de *S. pombe* se caracteriza por presentar una fase G2 muy amplia que abarca aproximadamente el 70% del ciclo (**Figura 3**). Cuando la célula ha duplicado su material genético y ha alcanzado un tamaño mínimo determinado, sufre mitosis y sus núcleos se separan. En este momento comienza a formarse el septo de división, mientras las células, aún unidas, entran en una fase G1 muy corta; posteriormente vuelven a duplicar su material genético (fase S) coincidiendo con la septación, de forma que las dos células hijas recién separadas emergen en la fase G2 de su ciclo celular (Chang and Verde, 2004).

Las células de *S. pombe* sufren tres transiciones morfológicas importantes durante su ciclo de vida vegetativo (**Figura 3**):

Crecimiento monopolar: tras la citoquinesis, en el momento denominado OETO (*Old End Take Off*), las dos células hijas idénticas en tamaño inician un crecimiento polarizado, alargándose sólo por el polo que ya existía en la célula madre (“polo antiguo”). Este patrón de crecimiento se mantiene durante los primeros momentos de la fase G2.

Crecimiento bipolar: también llamado NETO (*New End Take Off*), en este momento se produce una transición desde el crecimiento monopolar a un crecimiento bipolar, y el polo creado en la división anterior (“polo nuevo”) también comienza a alargarse (Mitchison and Nurse, 1985).

Separación celular: finalmente, cuando la célula alcanza su tamaño máximo, cesa el alargamiento por los polos y se produce la formación de un septo de división central y la posterior separación de las células hijas.

2.2. Regulación de la polaridad celular por microtúbulos

Los microtúbulos son cilindros de aproximadamente 25 nm compuestos por dímeros de α /β tubulina (Streiblova, 1980). Normalmente se polimerizan desde un Centro Organizador de Microtúbulos (MTOC) y tienen un extremo positivo y uno negativo con diferentes características.

Los microtúbulos interfásicos están formando de 3 a 5 haces que se extienden a lo largo del eje longitudinal de la célula, formando una cesta alrededor del núcleo (Hagan, 1998; Hagan and Hyams, 1988; Sawin and Tran, 2006). El extremo positivo del microtúbulo se encuentra situado próximo al polo y el

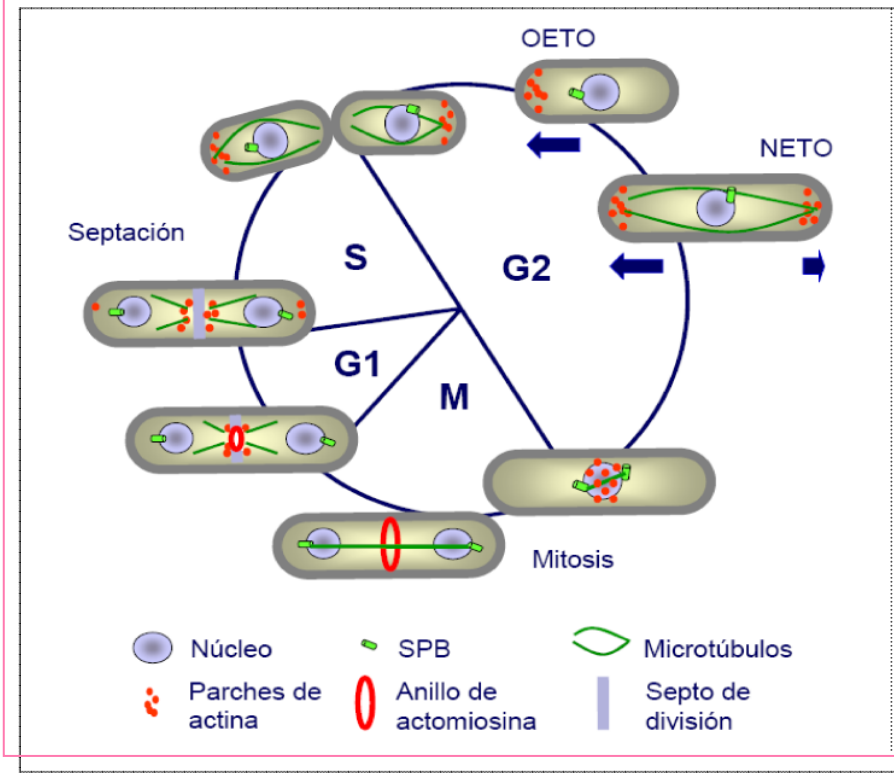

Figura 3:
Ciclo morfogenético y ciclo celular de *S. pombe*. La fase G1 del ciclo celular de *S. pombe* es muy corta y tiene lugar cuando las células hijas aún no se han separado, al igual que la fase S, que coincide aproximadamente con el momento de la separación. A lo largo del ciclo celular se produce una reorganización del citoesqueleto de actina y microtúbulos. Los parches de actina se localizan en los polos en crecimiento durante la interfase y en la región del septo durante la división celular, junto con el anillo de actomiosina. Los MTs forman haces que se distribuyen a lo largo de la célula en interfase. Durante la mitosis se forma el huso mitótico para la segregación cromosómica y posteriormente el huso se desensambla para formar el "*post anafase array*" durante la formación del septo y la contracción del anillo.

negativo hacia la zona media de la célula. Normalmente cada haz de microtúbulos individual crece hasta el polo celular y una vez que lo alcanza, se produce un desensamblaje que lo hace retroceder hacia la zona media, volviendo a crecer después hacia el polo de nuevo (Drummond and Cross, 2000; Tran et al., 2001).

Otra característica de los microtúbulos es que normalmente sólo se retraen después de alcanzar el polo celular y no lo hacen cuando alcanzan los lados de la célula (Brunner and Nurse, 2000). Esta dinámica podría determinar la posición del núcleo, produciendo fuerzas de empuje frecuentes y transitorias desde los polos celulares sobre el núcleo. Este sería un mecanismo simple para explicar cómo las células sienten la posición de sus polos y centran el núcleo entre ellos (Tran et al., 2001).

En *S. pombe* se han encontrado numerosos mutantes con un eje longitudinal alterado, éstos se agrupan en dos clases principalmente: mutantes que forman esferas porque no son capaces de organizar zonas de crecimiento polarizado y mutantes con forma de T, porque no pueden mantener las zonas de crecimiento en los dos polos opuestos de la célula.

El estudio de algunos de estos mutantes ha sido muy útil para la identificación de proteínas de polaridad como Tip1p y Tea1p, que sirven para reconocer los polos y para marcar las zonas de crecimiento activo. En ausencia de Tip1p el retroceso de los microtúbulos no se restringe a los polos de la célula, sino que ocurre en cualquier región del córtex, por lo tanto Tip1p es necesaria para regular la dinámica de microtúbulos y discriminar entre diferentes regiones del córtex celular (Brunner and Nurse, 2000).

Tea1p es considerado un marcador de la geometría celular. Esta proteína viaja hacia los polos celulares en los extremos positivos de los microtúbulos y se localiza en ambos polos, incluso cuando las células crecen sólo por uno de ellos (**Figura 4**) (Mata and Nurse, 1997). En los mutantes *tea1*Δ, los microtúbulos no retroceden al alcanzar el polo y a veces se curvan en este punto, por lo que Tea1p también influye en la habilidad de los microtúbulos para reconocer el polo y dejar de crecer (Mata and Nurse, 1997).

Los microtúbulos son importantes como señalizadores o marcadores de los puntos de crecimiento. Durante la activación del crecimiento bipolar (NETO), los extremos de los microtúbulos contactan con el polo y depositan a Tea1p junto con Tea4p, que se unen a la formina For3p y a otras proteínas, y promueven la nucleación de los cables y parches de actina en el polo nuevo (ver Apartado 2.4). Sin embargo, una vez elegidos estos lugares, los MTs no son esenciales para el mantenimiento de la polaridad o para la correcta localización de los parches de actina (Pelham and Chang, 2001; Sawin and Nurse, 1998). Se ha observado, que mutantes que presentan defectos en la organización de los microtúbulos

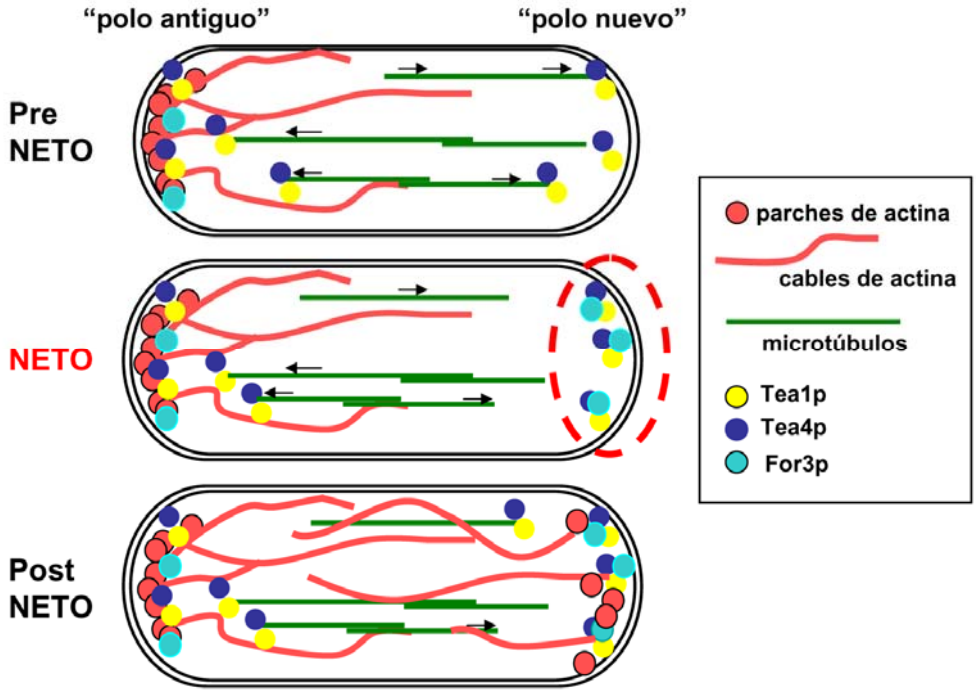

Figura 4: Modelo molecular de la transición de crecimiento monopolar a bipolar. Figura adaptada de (Martin and Chang, 2005).

todavía muestran un crecimiento polarizado, aunque presentan células curvadas o ramificadas, o con crecimiento monopolar (Sawin and Nurse, 1998).

2.3. Regulación de la polaridad celular por actina

La localización del citoesqueleto de actina se correlaciona con los lugares de crecimiento celular polarizado: septo y polos.

Los filamentos de actina se organizan en tres estructuras diferentes dentro de la célula: parches de actina, cables de actina y anillos contráctiles de actomiosina.

Durante la interfase, los parches de actina se concentran en los sitios de crecimiento celular. Los cables corren a lo largo del eje longitudinal de la célula y normalmente acaban en los polos. En mitosis, la actina se reorganiza en un anillo central de actomiosina que se contrae durante la citoquinesis. En este momento, los parches y los cables se concentran en la zona media y permanecen allí durante la formación del septo de división; revisado en (Chang and Verde, 2004; Pollard, 2008).

Los **parches** de actina se han usado habitualmente como marcadores de los sitios de crecimiento activo de la célula, y se asume que funcionan en el crecimiento polarizado y en endocitosis, pero su función real es todavía desconocida. Los parches de actina contienen proteínas relacionadas con la organización de la actina, como los componentes del complejo Arp2/3 y parecen ser los sitios primarios de nucleación de actina en la célula en interfase (McCollum et al., 1996; Nolen and Pollard, 2008).

Se cree que los **cables** de actina que se proyectan de los polos celulares participan directamente en el crecimiento polarizado. Una de sus funciones es dirigir junto con las miosinas de tipo V, los movimientos de las vesículas secretoras que proporcionan membranas y componentes necesarios para la síntesis de la pared celular en los polos de crecimiento (Motegi et al., 2001; Mulvihill et al., 2006).

Pero, ¿Cómo se forman los cables de actina? Se sabe que la formina For3p y la proteína de unión a actina, tropomiosina (Cdc8p), son necesarias para el ensamblaje de estos cables (Feierbach and Chang, 2001; Martin and Chang, 2006). La tropomiosina es una proteína que estabiliza los filamentos de actina e influye en la interacción de la actina con las miosinas (Balasubramanian et al., 1992; Skoumpla et al., 2007). En un mutante *for3*Δ, no existen cables de actina, pero las células aún mantienen cierto grado de crecimiento polarizado y son viables, aunque crecen lentamente y muchas de forma monopolar. Por lo tanto, aunque los cables de actina no sean esenciales para el crecimiento polarizado, deben funcionar localizando o reciclando

otros factores de crecimiento en el extremo celular; revisado en (Chang and Verde, 2004).

Durante la mitosis temprana la actina se ensambla en el centro de la célula formando un **anillo** de actina y miosina. En mitosis tardía, este anillo marca el sitio de formación del septo de pared celular, y se contrae cercano a la membrana y guiando el proceso de septación (Chang et al., 1996; Marks and Hyams, 1985; Wu et al., 2003).

Existe una estrecha relación funcional entre el citoesqueleto de actina y el crecimiento polarizado, una de cuyas últimas consecuencias es la síntesis de la pared celular. Por ejemplo, en los experimentos de regeneración de protoplastos (células esféricas a las que se ha eliminado la pared) se ha visto que la formación de nuevo material de pared depende del citoesqueleto de actina (Osumi et al., 1989).

Otros factores que actúan en polaridad, son las GTPasas de la familia Rho. Éstas regulan tanto la polimerización de la actina como su organización en todos los organismos estudiados (Jaffe and Hall, 2005). En *S. pombe*, al menos tres GTPasas de esta familia se han relacionado con el control de la polimerización y la localización del citoesqueleto de actina, son Cdc42p, Rho1p y Rho3p (este tema se abordará con mayor profundidad en el Apartado 4.4).

2.4. Establecimiento de NETO

Las células de la levadura de fisión después de la citoquinesis, adquieren un patrón de crecimiento monopolar únicamente por el polo preexistente. A continuación, estas células muestran un cambio en el crecimiento polarizado conocido como *New End Take Off* (NETO), en el que activan el crecimiento del polo nuevo e inician un crecimiento bipolar (Mitchison and Nurse, 1985).

A partir del estudio de mutantes que presentan crecimiento monopolar se han identificado numerosos genes con funciones en NETO, estos factores se pueden englobar en tres grandes categorías:

-Proteínas quinasas: constituye el grupo más grande y en él se encuentran Kin1p, Pom1p, Orb2p, Orb6p y Ssp1p (Bähler and Pringle, 1998; Drewes and Nurse, 2003; Rupes et al., 1999; Sawin, 1999; Verde et al, 1998).

-Proteínas de unión a actina: Bud6p, Sla2p y For3p (Castagnetti et al., 2005; Feierbach and Chang, 2001; Feierbach et al., 2004; Glynn et al., 2001).

-Proteínas que contienen múltiples dominios de interacción proteína-proteína: Tea1p, Tea3p y Tea4p, que pueden actuar como proteínas de anclaje para la formación de complejos multiproteicos (Martin and Chang, 2005) y el factor de ADP-ribosilación Arf6 (Fujita, 2008).

Gran parte del trabajo realizado en NETO se ha centrado en el estudio de la proteína Tea1p (**Figura 4**). Esta proteína se localiza en los extremos positivos de los MTs y va acompañada de otras proteínas +TIP como Tip1p y Tea2p, que se encargan de que su localización sea correcta (Browning et al., 2000; Brunner and Nurse, 2000).

Una vez en el polo, Tea1p es depositada allí cuando el MT comienza a retraerse y se ancla al extremo celular gracias a su asociación con Mod5p (Snaith and Sawin, 2003).

Tea4p es otra proteína que se une directamente a Tea1p y que funciona como puente de unión entre esta proteína y la formina For3p, y que además podría funcionar de alguna manera activando a la formina. Estas tres proteínas se asocian entre sí y probablemente con otras, formando un gran complejo multiproteico denominado "polarisoma", que podría coordinar muchos aspectos necesarios para el crecimiento polarizado, como el ensamblaje de la actina y la localización de la secreción (Martin and Chang, 2005; Martin et al., 2005).

Parece probable que uno de los pasos clave para que se desencadene NETO sea el reclutamiento de For3p en el polo nuevo (Martin et al., 2005). La función de las forminas es nuclear y alargar filamentos lineales de actina, y su actividad necesita una regulación cuidadosa *in vivo* para generar la estructura de actina correcta, en el sitio y tiempo adecuados. Se ha propuesto la autoinhibición como un mecanismo para controlar la actividad de la formina en muchos organismos. Este mecanismo consiste en la interacción de un motivo de autorregulación C-terminal, llamado DAD, con un dominio de inhibición en la región N-terminal, denominado DID (Martin et al., 2007). La autoinhibición de muchas forminas es regulada por pequeñas GTPasas, por ejemplo, en *S. cerevisiae* Rho3p y Rho4p regulan a la formina Bni1p (Dong, 2003).

En *S. pombe*, recientemente se ha encontrado un mecanismo de autoinhibición de For3p por unión intramolecular. Se ha visto que tanto la GTPasa Cdc42p como la proteína Bud6p, son necesarias para eliminar esta autoinhibición y conseguir que la formina se localice correctamente en los polos celulares. La prevención de la autoinhibición de For3p, es capaz de suprimir el defecto en el establecimiento del crecimiento bipolar de la cepa que carece de Bud6p (Martin et al., 2007).

Otra proteína relacionada con NETO es Sla2p, homóloga a las *huntingtin interacting proteins* en humanos, Hip1 y Hip1R, y necesaria para reclutar la actina y la maquinaria de crecimiento en el polo nuevo. Esta proteína podría representar una unión entre NETO y los parches de actina, más relacionados con el

proceso de secreción (Castagnetti et al., 2005; Iwaki et al., 2003).

Durante las transiciones morfológicas comentadas hasta ahora, es esencial el mantenimiento de la integridad celular. Esto requiere que la secreción y el crecimiento polarizados estén perfectamente coordinados con la biosíntesis de la pared celular y que exista una estricta regulación de las rutas de biosíntesis y degradación de los polímeros que la forman; revisado en (Durán and Pérez, 2004; Fischer et al., 2008; Latgé, 2007; Lessage and Bussey, 2006; Levin, 2005; Moseley and Goode, 2006; Pruyne and Bretscher, 2000).

3. Pared Celular

La pared celular es una estructura rígida que proporciona a la célula una protección mecánica y resistencia ante la presión osmótica interna. Sin embargo, también es una estructura dinámica que se adapta a los cambios morfológicos que ocurren durante el ciclo de vida de la levadura. Todos estos cambios exigen una remodelación de la pared celular y se correlacionan con cambios en el citoesqueleto de actina; revisado en (Durán and Pérez, 2004).

3.1. Composición y síntesis de la pared de la espora

La formación de las ascosporas es un proceso morfogenético que supone cambios muy drásticos para la célula. Los núcleos formados tras la meiosis deben empaquetarse, junto con algunos orgánulos y parte del citosol. La etapa final del empaquetamiento consiste en la síntesis de una pared celular alrededor de cada espora. Esta pared celular les proporciona resistencia a ciertas condiciones ambientales de estrés, a las que las células vegetativas son sensibles.

El primer paso en el desarrollo de las ascosporas es la formación de las proesporas, que son los precursores de las ascosporas. Al principio, la membrana de la proespora se localiza en la región donde se encuentran los cuerpos polares del huso, formando una estructura en forma de cazo. Después, esta doble membrana sigue creciendo englobando poco a poco al núcleo hasta que la proespora esté completa. En el lumen de esta membrana, es dónde se produce la acumulación de la pared celular que dará lugar a las ascosporas maduras (**Figura 5**) (Shimoda, 2004; Tanaka and Hirata, 1982).

La membrana que queda en el interior será la membrana de la ascospora, pero aún no está claro el destino de la membrana externa, que parece perderse.

La composición de la pared de la ascospora de *S. pombe* apenas se conoce, pero debe ser diferente a la de la pared de las células vegetativas, puesto que está

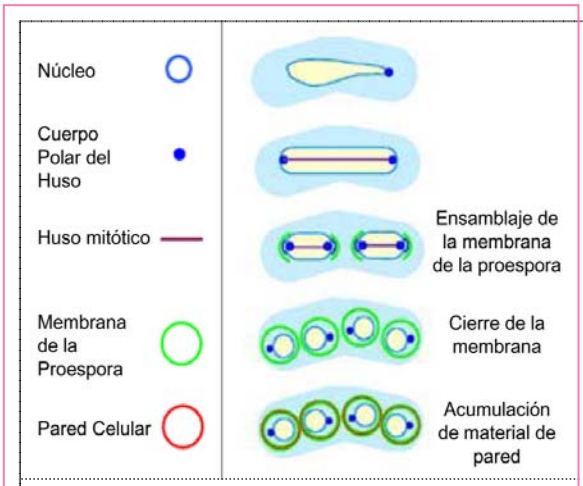

Figura 5: Proceso de esporulación en *S. pombe*. Durante la meiosis II comienzan a ensamblarse las membranas de la proespora, hasta que poco a poco engloban cada uno de los núcleos. En el espacio luminar de estas membranas se acumula el material de pared celular, que constituirá la pared de la espora.

diseñada para resistir condiciones ambientales a las que las células vegetativas son sensibles. Vista al microscopio electrónico (**Figura 6**), la pared de las esporas de *S. pombe* consta de una capa interna transparente a los electrones y una capa externa densa a los electrones (Johnson et al., 1982; Yoo et al., 1973). Durante la germinación, la capa externa se rompe y la interna se expande y se convierte en la pared celular de la espora germinada. Esto hace pensar que la composición de la capa interna podría ser muy similar a la de la pared de las células vegetativas (α y β-glucanos), mientras la externa podría ser específica de la espora.

Gracias a estudios realizados en nuestro laboratorio, sabemos que la pared de las ascosporas de *S. pombe* presenta al menos dos tipos de α-glucano, uno mayoritario formado principalmente por glucosas unidas por el enlaces α(1,3) y un segundo tipo de

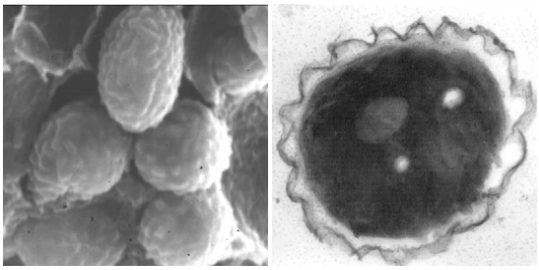

Figura 6: Apariencia de la pared de la espora en *S. pombe*. Fotografía al microscopio electrónico de barrido (izquierda) y de transmisión (derecha), de esporas de *S. pombe* (Nakamura et al., 2004).

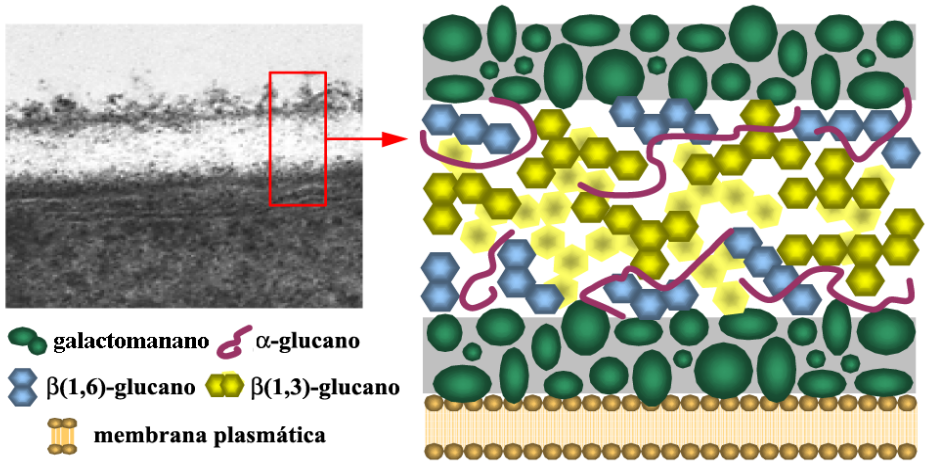

Figura 7: **Estructura de la pared celular de *S. pombe*.** La pared consta de tres capas. La capa intermedia, menos densa a los electrones, está formada por α y β glucanos. Las capas más externas están formadas por galactomanano.

naturaleza amiloidea, formado por enlaces α(1,4) (García et al., 2006). Además, también presenta β-glucano, pero no se sabe si se trata de β-glucano ramificado o lineal (Martín et al., 2000) y muy probablemente quitosán (Arellano et al., 2000).

Aunque los datos sobre la composición química de la pared de las ascosporas de *S. pombe* son escasos, sí se han encontrado posibles enzimas biosintéticas que actúan a lo largo de este proceso:

-***chs1***$^+$ (*chitin synthase*) fue clonado por su similitud con los genes responsables de la síntesis de quitina en *S. cerevisiae*. Chs1p se ha identificado como la quitín sintasa responsable de la síntesis de un compuesto similar a la quitina durante la esporulación (Arellano et al., 2000).

-***bgs2***$^+$ (*β-glucan synthase*) es la única β-glucán sintasa esencial durante el proceso de esporulación y es responsable de la mayor parte de la actividad glucán sintasa durante este proceso (Martín et al., 2000) (ver Apartado 3.3.2.).

-***gas4***$^+$ (*glycolipid anchored surface protein*) codifica una β(1,3)-glucanosiltrasferasa necesaria para la maduración de la pared de la espora. Esta proteína transfiere oligosacáridos de 6-7 glucosas desde el extremo no reductor de una molécula de β-glucano lineal donadora a otra receptora, promoviendo así el alargamiento de las ramificaciones de este polímero (Medina-Redondo et al., 2008).

-***mok12***$^+$ (*morphological and kinase inhibitor supersensitive)* es uno de los genes, junto con *mok13*$^+$, responsable de la biosíntesis del α(1,3)-glucano de la pared de las ascosporas. Además, es esencial para la viabilidad de éstas (García et al., 2006).

-***mok13***$^+$ es necesario para el correcto ensamblaje de la pared de la espora. El mutante *mok13*Δ presenta unas esporas viables, aunque mucho más sensibles a diferentes estreses que las silvestres. Las esporas de este mutante, al igual que las de *mok12* Δ presentan unos niveles de α-glucano muy bajos (García et al., 2006).

-***mok14***$^+$ es responsable de la síntesis de un polímero de naturaleza amiloidea específico de esporulación y compuesto mayoritariamente por enlaces α(1,4). Este polímero y la proteína Mok14p se distribuyen entre la envuelta de la ascospora y la del asca, y es responsable del color pardusco de las esporas de *S. pombe* en presencia de vapores de iodo (García et al., 2006).

Curiosamente, en las células vegetativas el β-glucano es más abundante que el α-glucano (55% frente a 28%), mientras que en las esporas esta relación se invierte (38% frente a un 46%) (García et al., 2006). Este dato se corresponde con el número de GS descritas en ambos procesos, hay 3 βGS que actúan durante el crecimiento vegetativo y sólo una durante la esporulación, mientras que hay 4 α-glucán sintasas en esporulación (Mok11p, Mok12p, Mok13p y Mok14p) y sólo una en crecimiento vegetativo (Mok1p) (Katayama et al., 1999).

3.2. Composición de la pared celular en células vegetativas

Cuando se observa la pared celular de *S. pombe* con microscopía electrónica de transmisión, se puede apreciar una estructura trilaminar, formada por dos capas densas a los electrones separadas por una capa menos densa (**Figura 7**). Las capas más externas están compuestas por glicoproteínas (9-14% de galactomanano) y la capa interna por carbohidratos (Osumi, 1998).

Esta capa de carbohidratos está formada por β (1,3)-glucanos (48-54%) interconectados en una maraña fibrilar con α(1,3)-glucanos (18-28%), y con una pequeña proporción de β(1,6)-glucanos (2-4%). (Bush et al., 1974; Kopecka et al., 1995; Manners and Meyer, 1977).

3.3. Biosíntesis de β-glucano

El β(1,3)-glucano es un polímero formado por glucosas unidas por enlaces β(1,3) y es el compuesto más abundante de la pared de *S. pombe* (Manners and Meyer, 1977). El β-glucano es el primer polisacárido que se deposita en la nueva pared en formación durante la regeneración de protoplastos y forma una red de microfibrillas. En este sistema, los protoplastos se regeneran de forma polarizada y las microfibrillas de β-glucano aparecen en un polo, extendiéndose hasta cubrir la superficie del protoplasto y restablecer la típica forma cilíndrica de *S. pombe* (Osumi et al., 1998). En torno a esta red de β-glucano se ensamblan el α-glucano y el galactomanano.

Mediante experimentos de inmuno-microscopía electrónica, se ha podido averiguar la localización de tres tipos de β-glucano distintos (**Figura 8**): β(1,3)-glucano con ramificaciones en β(1,6), β(1,6)-glucano y β(1,3)-glucano lineal (Humbel et al., 2001).

El β(1,3)-glucano ramificado se localiza por toda la pared celular, en el septo primario (capa interna del septo menos densa a los electrones) y en el septo secundario (capa más densa a los electrones situada a ambos lados del septo primario).

El β(1,6)-glucano se distribuye por toda la pared celular, situándose más próximo a la capa de galactomanano externa; en la región del septo se sitúa exclusivamente en el septo secundario.

El β(1,3)-glucano lineal se localiza exclusivamente en el septo primario. Por lo que, aparentemente no existe colocalización de estos dos últimos tipos de glucano en la célula (Humbel et al., 2001).

Aunque acabamos de describir distintos tipos de β-glucano, sólo se conoce una actividad β(1,3)-glucán sintasa (E.C.2.4.1.34, UDP-glucosa: (1,3)-β-D-glucano-3-β-gucosiltransferasa). Esta enzima se encuentra asociada a la cara interna de la membrana plasmática y emplea como sustrato UDP-glucosa, formando cadenas lineales de unos 60-700 residuos de glucosa. Se ha desarrollado un sistema *in vitro* para la síntesis de β-glucano; la reacción necesita ATP y es estimulada por GTP. Gracias en parte a este sistema hoy sabemos que la actividad βGS está constituida por, al menos, dos componentes: una fracción catalítica y una reguladora; revisado en (Cabib et al., 1998; Douglas, 2001).

En *S. pombe* se han identificado cuatro genes que codifican posibles subunidades catalíticas del complejo β-glucán sintasa: (*bgs1*$^+$, *bgs2*$^+$, *bgs3*$^+$ *y bgs4*$^+$ (de (1,3)-*beta-glucan synthesis*). Todos ellos son esenciales en distintos momentos del ciclo de vida de *S. pombe* (Cortés et al., 2005; Cortés et al., 2002; Liu et al., 2000; Liu et al., 1999; Martín et al., 2003; Martín et al., 2000).

La subunidad reguladora está codificada por el gen *rho1*$^+$, que también es esencial. Rho1p es una GTPasa de bajo peso molecular que en su forma activa (unida a GTP) y prenilada (asociada a la membrana) activa a la subunidad catalítica de la glucán sintasa (Arellano et al., 1996).

3.3.1. *bgs1*$^+$

La primera posible subunidad catalítica identificada en *S. pombe* fue Bgs1p/Cps1p, muy similar a Fks1p y Fks2p, las subunidades catalíticas responsables de la actividad βGS en *S. cerevisiae* (Ishiguro et al., 1997). Fue descrita como una posible

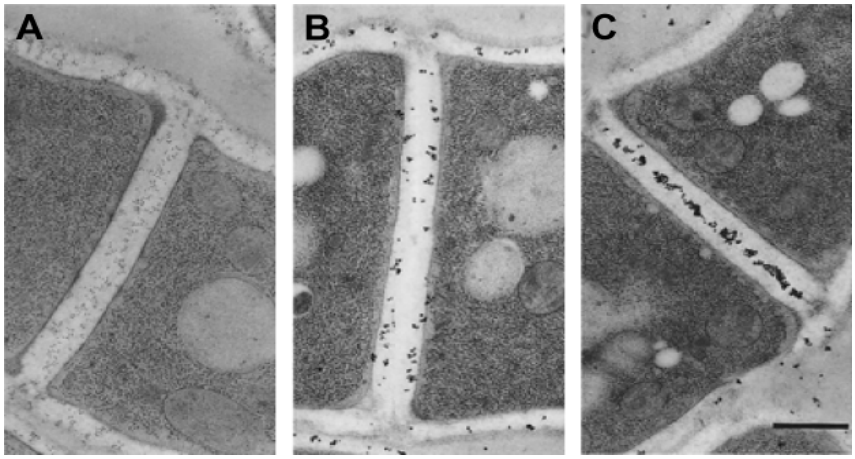

Figura 8:
Inmunolocalización de los distintos tipos de β-glucano de la pared celular de *S. pombe*. Se obtuvieron anticuerpos específicos para cada tipo de β-glucano, marcados con oro coloidal, y se usaron en un experimento de inmunolocalización.
A β(1,3)-glucano ramificado en β(1,6), **B** β(1,6)-glucano, **C** β (1,3)-glucano lineal (Humbel et al., 2001).

subunidad catalítica de la β(1,3)-glucán sintasa implicada en el proceso de división celular.

Algunos mutantes de *bgs1*$^+$ no forman el septo de división y se paran en fase G2 del ciclo celular. Otros, presentan defectos de polaridad, por lo que además de esencial en el proceso de división, Bgs1p podría ser necesaria para el mantenimiento de la polaridad (Cortés et al., 2007; Ishiguro et al., 1997; Le Goff et al., 1999; Liu et al., 2000b; Liu et al., 1999).

Bgs1p se localiza en el septo durante la citoquinesis formando un anillo que aparece en la región interna de la marca de Calcoflúor que delimita el septo, pero también se encuentra en los polos, en las proyecciones de conjugación y en las ascosporas (Cortés et al., 2002). Además, se ha visto que las esporas carentes de *bgs1*$^+$ al germinar forman septos aberrantes que no se tiñen con Calcoflúor y no poseen β(1,3)-glucano lineal, lo que podría ser la prueba definitiva de que Bgs1p es la subunidad responsable de la síntesis del septo primario (Cortés et al., 2007).

3.3.2. *bgs2*$^+$

En el genoma de *S. pombe* se encontraron otros tres genes similares a *bgs1*$^+$, denominados *bgs2*$^+$, *bgs3*$^+$ *y bgs4*$^+$.

Bgs2p es una subunidad catalítica de la β-glucán sintasa, esencial en el ensamblaje y la maduración de la pared de las ascosporas. Varios datos apoyan esta afirmación: en primer lugar, la expresión de *bgs2*$^+$ es inducida durante el proceso de esporulación. En segundo lugar, las ascas que carecen de este gen no liberan espontáneamente las esporas, y además, éstas son inmaduras e incapaces de germinar. Por último, en células diploides *bgs2Δ/bgs2Δ*, la actividad βGS medida durante el proceso de esporulación es mínima comparada con la de las células diploides silvestres (Liu et al., 2000; Martín et al., 2000).

Además, la proteína Bgs2p se localiza en la periferia de las ascosporas que ya han completado la meiosis I y II, probablemente asociada a la membrana de la proespora (Martín et al., 2000).

3.3.3. *bgs3*$^+$

El gen *bgs3*$^+$ fue clonado como supresor del mutante *ehs2-1*, hipersensible a Equinocandina, una droga antifúngica que afecta a las síntesis del β-glucano. *bgs3*$^+$ es un gen esencial y las células con niveles muy bajos de Bgs3p son redondeadas y mucho más cortas que las de la estirpe silvestre. Existen evidencias que hacen pensar, que *bgs3*$^+$ podría estar relacionado con la síntesis de la pared celular durante la elongación de la célula (Martín et al., 2003).

Su localización se corresponde con los sitios de crecimiento celular en vegetativo: polos y septo, y también se observa durante la conjugación y la germinación, situándose en las proyecciones tanto de los "*schmoos*", como de las esporas en germinación.

3.3.4. *bgs4*$^+$

El último gen identificado como posible subunidad de la βGS fue el gen *bgs4*$^+$. También es un gen esencial y su eliminación produce lisis celular en los polos de crecimiento y en el septo antes de la citoquinesis. Bgs4p es responsable de la mayor parte de la actividad βGS medida *in vitro* (Cortés et al., 2005).

Esta proteína se localiza en los mismos lugares que Bgs1p y Bgs3p, pero a la zona del septo llega más tarde que Bgs1p. Es una βGS mayoritaria en cuanto a su expresión y podría estar involucrada en la síntesis del septo secundario; (Cortés et al., 2005) y J. C. Ribas, comunicación personal.

3.3.5. *rho1*$^+$

El producto del gen *rho1*$^+$ se identificó como una subunidad reguladora de la enzima β-glucán sintasa, clave para su actividad y miembro del complejo enzimático (Arellano et al., 1996); de la que hablaremos en detalle en el Apartado 4.4.5.

La síntesis de los polisacáridos de la pared celular está regulada finamente. Para controlar la síntesis de β(1,3)-glucano las células pueden modular la actividad catalítica directamente, controlando la cantidad o localización en la membrana de Rho1p activo, o indirectamente activando o inhibiendo la transcripción de sus subunidades catalíticas; revisado en (Douglas, 2001).

3.4. Biosíntesis de α-glucano

El α-glucano está presente en la pared celular de *S. pombe* y otros hongos dimórficos y filamentosos, como *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* y *Blastomyces dermatitidis*, aunque curiosamente se encuentra ausente en la pared celular de *S. cerevisiae* (Bobbit et al., 1977; Bush et al., 1974; Hogan and Klein, 1994; Klimpel and Goldman, 1988; San-Blas et al., 1977).

Este polímero tiene un papel esencial en el mantenimiento de la forma celular y en el control de la polaridad. Tratamientos combinados de β-glucanasa y α-glucanasa producen protoplastos redondeados, que no se consiguen tratando sólo con β-glucanasa (Alfa et al., 1993). Además, algunos organismos patógenos como *Cryptococcus neoformans* necesitan el α-glucano para que se ancle la cápsula, que es un determinante de la virulencia (Reese and Doering, 2003).

En *S. pombe* las cadenas de α-glucano están formadas por D-glucosas unidas por enlaces α(1,3) con

un 7% de enlaces α(1,4) (Manners and Meyer, 1977) y se distribuyen por toda la pared celular y en la región del septo, sobre todo en las regiones adyacentes a la membrana plasmática (Sugawara et al., 2003).

Se han identificado varios genes que codifican posibles subunidades catalíticas de la αGS, denominados genes *mok* (*morphological and kinase inhibitor supersensitive*). De ellos, sólo *mok1*$^+$ parece estar relacionado con la síntesis de α-glucano durante el crecimiento vegetativo, mientras que los otros, *mok11*$^+$, *mok12*$^+$, *mok13*$^+$ y *mok14*$^+$, participan en el proceso de esporulación (ver Apartado 3.1).

mok1$^+$ es esencial para la viabilidad y para el mantenimiento de la polaridad celular durante el crecimiento vegetativo (Hochstenbach et al., 1998; Katayama et al., 1999). Un mutante termosensible en este gen, presenta una reducción de α-glucano en la pared celular y posee una morfología redondeada. La sobreexpresión de *mok1*$^+$ también produce un incremento en la cantidad de α-glucano en la pared celular (Katayama et al., 1999).

3.5. Biosíntesis de galactomanoproteínas

En *S. pombe* se sabe muy poco del proceso de síntesis del galactomanano en general.

Las galactomanoproteínas representan del 9 al 14% de los azúcares de la pared celular. Son polímeros de naturaleza glicoproteica que se encuentran formados por un núcleo central de residuos de manosa unidas por enlaces α(1,6) y con cadenas laterales de manosa con enlaces α(1,2), terminadas por un resto de galactosa en los extremos no reductores (Bush et al., 1974; Horisberger et al., 1978; Moreno et al., 1985).

3.6. Regulación de la biosíntesis de la pared celular

3.6.1. Regulación de la síntesis de β-glucano

Rho1p es la molécula principal en la regulación coordinada de la biosíntesis del β(1,3)-glucano y otros procesos que participan en la secreción polarizada (Arellano et al., 1999a).

Esta GTPasa regula la biosíntesis de β-glucano por dos vías, directamente como activador de la βGS (Arellano et al., 1999a) y a través de su interacción con dos proteínas Pck1p y Pck2p (homólogas a PKC de mamíferos). Ambas quinasas se unen a Rho1-GTP por su extremo N-terminal y esta unión las estabiliza y permite que aumente su concentración en las zonas de crecimiento (Sayers et al., 2000). Además, Pck2p es un activador de la enzima β(1,3)-glucán sintasa, por lo que Rho1p podría activar a esta enzima a través de Pck2p y en menor medida de Pck1p (Arellano et al., 1999b).

En *S. cerevisiae* Rho1p activa a Pkc1p (homóloga a Pck1p y Pck2p de *S. pombe*) y ésta a su vez activa la ruta de MAP quinasas implicada en el mantenimiento de la integridad celular (Kamada et al., 1995; Levin and Errede, 1995; Nonaka et al., 1995). Recientemente, también se ha demostrado la participación de Pck2p como activador de una ruta de MAPKs necesaria para el mantenimiento de la integridad en *S. pombe* (Barba et al., 2008; Ma et al., 2006).

3.6.2. Regulación de la síntesis de α-glucano

Otra GTPasa perteneciente a la familia Rho, Rho2p, parece ser la responsable de la regulación de la

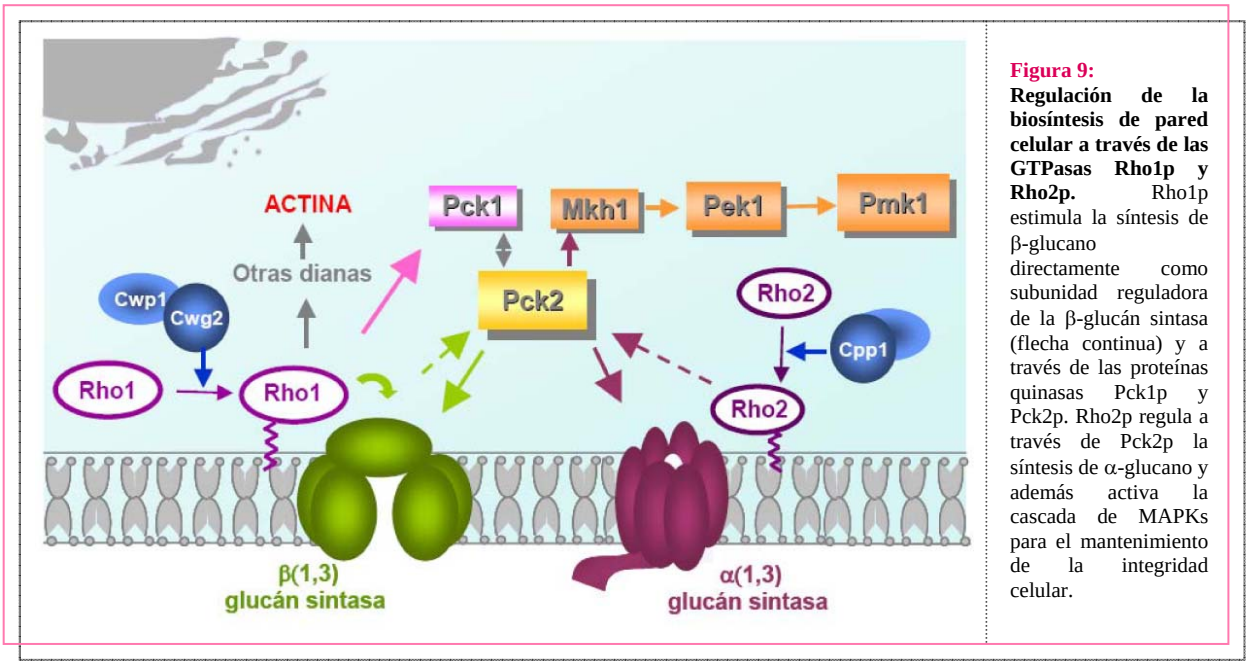

Figura 9: **Regulación de la biosíntesis de pared celular a través de las GTPasas Rho1p y Rho2p.** Rho1p estimula la síntesis de β-glucano directamente como subunidad reguladora de la β-glucán sintasa (flecha continua) y a través de las proteínas quinasas Pck1p y Pck2p. Rho2p regula a través de Pck2p la síntesis de α-glucano y además activa la cascada de MAPKs para el mantenimiento de la integridad celular.

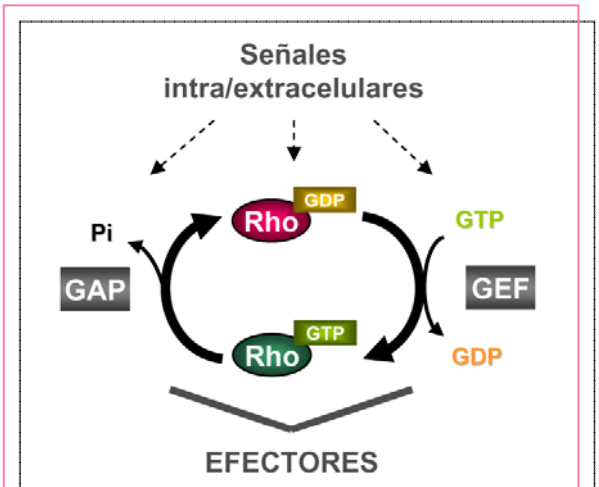

Figura 10: Regulación de las GTPasas. Las proteínas con actividad GEF favorecen el intercambio de GDP por GTP y activan las GTPasas, permitiendo así la unión de éstas a sus efectores. Las proteínas con actividad GAP inactivan a las GTPasas, estimulando la actividad GTPásica intrínseca de éstas.

biosíntesis del α(1,3)-glucano en *S. pombe*. Existen una serie de evidencias que relacionan a Rho2p con la biosíntesis de este compuesto. La sobreexpresión de *rho2*$^{+}$, a diferencia de la de *rho1*$^{+}$, produce un aumento en la cantidad de α(1,3)-glucano presente en la pared (Calonge et al., 2000). Por otro lado, Pck2p es necesaria para que la α(1,3)-glucán sintasa, Mok1p, se localice correctamente y desarrolle su actividad catalítica (Katayama et al., 1999).

Además, se sabe que Rho2p y Pck2p están íntimamente relacionadas, ya que interaccionan físicamente y los mutantes en estos dos genes presentan fenotipos similares (Arellano et al., 1999b; Calonge et al., 2000).

El modo en que Rho2p interacciona con Mok1p no está del todo claro, sin embargo todos los datos apuntan a que esta interacción se produce a través de Pck2p (Arellano et al., 1999b; Calonge et al., 2000; Katayama et al., 1999) (**Figura 9**).

4. GTPasas de la familia Rho

Las GTPasas Rho (*Ras Homology*) constituyen una familia dentro de la superfamilia de pequeñas GTPasas relacionadas con Ras y se encuentran en todas las células eucariotas. La familia de GTPasas Rho incluye tres subfamilias: Rho, Rac y Cdc42; revisado en (Arellano et al., 1999a; Heasman and Ridly, 2008; Iden and Collard, 2008; Jaffe and Hall, 2005; Park and Bi, 2007).

Las proteínas Rho comparten con los demás miembros de la superfamilia Ras un diseño estructural y un mecanismo molecular por el cual, se activan cuando se unen a GTP y se inactivan cuando este compuesto es hidrolizado a GDP (**Figura 10**).

En su estado unido a GTP estas GTPasas se unen a una gran cantidad de moléculas efectoras, que permiten la activación de las cascadas de señalización, que a su vez promueven respuestas celulares generales, como cambios del citoesqueleto, dinámica de microtúbulos, tráfico de vesículas, polaridad celular y progresión del ciclo celular. La plasticidad de las proteínas Rho en términos de localización subcelular, regulación, unión a los efectores y relación con proteínas de otras rutas celulares, sitúan a estas GTPasas en un punto central de la regulación de una gran cantidad de procesos celulares; revisado en (Bustelo et al., 2007; García et al., 2006b; Heasman and Ridly, 2008; Iden and Collard, 2008; Park and Bi, 2007).

4.1. Moléculas efectoras de Rho-GTPasas

Una vez activadas y translocadas a sus sitios específicos de localización, las proteínas Rho interaccionan con sus efectores moleculares para aumentar la señal específica de las cascadas de transducción de señales. Estructuralmente, estos efectores utilizan diferentes residuos para su interacción, siendo las regiones switch I y II de la GTPasa los sitios principales de reconocimiento.

El anclaje a la membrana de los efectores es parte del mecanismo por el cual empiezan a ser activados. La mayoría de las interacciones entre las GTPasas Rho y sus efectores se pueden englobar en los tres modelos siguientes:

-La interacción de la GTPasa con su efector provoca un cambio de conformación en éste que le permite salir de una conformación autoinhibida, dando lugar a una estructura totalmente activa.

-Otros efectores son activados mediante la liberación de factores inhibitorios a los que estaban asociados, gracias a la unión de la GTPasa.

-En otras ocasiones la GTPasa produce la inhibición y no la activación, de la proteína efectora.

El resultado final de la regulación de estas moléculas efectoras es la generación de señales multirramificadas que producen, entre otras respuestas, cambios en el citoesqueleto, tráfico de vesículas y progresión del ciclo celular.

Cabe destacar que entre las funciones de los efectores en muchos casos también se encuentra la de regular a las propias GTPasas, lo que contribuye a una señalización por GTPasas coordinada y equilibrada. Esto pone de manifiesto el alto nivel de plasticidad que tienen lugar en las rutas de transducción de señales de GTPasas; revisado en (Bustelo et al., 2007; Park and Bi, 2007).

4.2. Regulación de Rho-GTPasas

Las células controlan la actividad de estas proteínas a través de varios tipos de regulación.

4.2.1. Regulación por cambio de nucleótido

Las GTPasas de la familia Rho alternan entre un estado unido a GTP (activo) y un estado unido a GDP (inactivo) y su actividad está regulada por dos tipos de proteínas: GEFs y GAPs (**Figura 10**). Los GEFs (*Guanine Exchange Factor)* facilitan el cambio de GDP por GTP, activando a las GTPasas (ver Apartado 4.3). Las proteínas GAPs (*GTPase Acivating Protein*) producen la hidrólisis del GTP, permitiendo a la GTPasa volver a su estado inactivo.

La simplicidad de este modelo contrasta con la complejidad de las rutas reguladas por estas proteínas; revisado en (Arellano et al., 1999a; Bustelo et al., 2007). De hecho, el estudio de las proteínas que controlan el intercambio de nucleótido de las GTPasas ha sido crucial para entender esta paradoja. Tanto GEFs como GAPs son proteínas mayores y más complejas que las GTPasas en sí mismas y contienen múltiples dominios capaces de interaccionar con otras proteínas y con lípidos, indicando que pueden actuar como señales de localización o como adaptadores en complejos multiproteicos; revisado en (Bos et al., 2007; Gulli and Peter, 2001; Moon and Zheng, 2003; Rossman et al., 2005; Schmidt and Hall, 2002).

4.2.2. Regulación por cambios en su localización subcelular

Muchas proteínas Rho deben anclarse a la membrana para poder realizar sus funciones biológicas y la condición esencial para ello es la incorporación de un grupo de naturaleza isoprenoide.

En primer lugar, se incorpora un grupo geranil-geranilo, o menos frecuentemente farnesilo a la secuencia denominada "Caja CAAX". La incorporación de este grupo isoprenoide promueve la translocación de la GTPasa al Retículo Endoplásmico, donde se elimina el tripéptido "AAX". Por último, la cisteína que queda es metilada; revisado en (Bustelo et al., 2007).

Las proteínas GDIs (*Guanine Dissociation Inhibitors*) también juegan un papel importante en la regulación del anclaje a la membrana de estas proteínas. Se unen a los grupos isoprenoides de la GTPasa lo que impide el intercambio de GDP por GTP y favorece el secuestro de la GTPasa inactiva en el citosol.

La función de los GDIs también es importante para la liberación de estas proteínas de la membrana cuando termina el proceso de señalización. La disociación de los GDIs y las GTPasas, es un requisito esencial para que se produzca la activación de éstas por parte de los GEFs y su subsiguiente asociación con las membranas.

4.2.3. Otros tipos de regulación

En mamíferos, muchas GTPasas de la familia Rho presentan una expresión específica dependiente del tipo celular o de diferentes estímulos. Otras son reguladas mediante degradación en sitios específicos de la célula, por ubiquitinación, degradación por caspasas, etc.

Otra forma de regulación de ciertas proteínas Rho se produce a través de un posible dominio de autoinhibición en su extremo N-terminal. Dicha autoinhibición puede ser desactivada mediante la unión de una proteína adaptadora cuya interacción no altera el intercambio GDP/GTP de la GTPasa.

Las GTPasas también pueden estar fosforiladas en residuos específicos, esta modificación post-transcripcional puede influir en su interacción con los GDIs, su estabilidad en la membrana y sus funciones efectoras; revisado en (Bustelo et al., 2007).

4.3. GEFs de GTPasas de la familia Rho

Las proteínas Rho ciclan entre una forma inactiva (unida a GDP) y una forma activa (unida a GTP). Las células regulan a estas proteínas modulando la interconversión y accesibilidad de estas dos isoformas. Los factores GEFs se unen a la GTPasa unida a GDP y desestabilizan este complejo, mientras que estabilizan el intermediario de reacción libre de nucleótido (Bos et al., 2007). Gracias a la mayor concentración de GTP en la célula, el GDP es

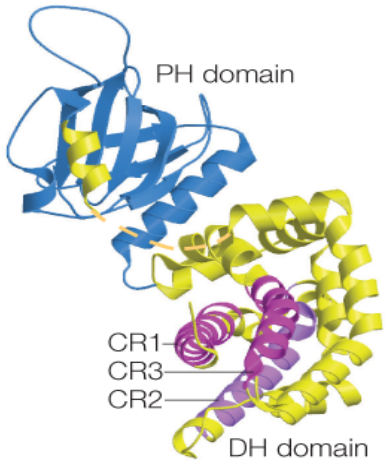

Figura 11: Estructura secundaria y terciaria de los dominios DH y PH de Sos1, un GEF de humanos. Dentro del dominio DH se pueden distinguir las regiones conservadas CR1, CR2 y CR3. Las regiones CR1 Y CR3 (rosa oscuro) forman el núcleo catalítico del dominio DH donde se une la GTPasa. Imagen tomada de (Rossman et al., 2005).

reemplazado por GTP, dando lugar a la activación de la GTPasa, que es así capaz de reconocer sus dianas moleculares o efectores. Los GAPs aceleran la actividad GTPasa intrínseca de las Rho-GTPasas para inactivarlas.

En teoría, la activación de una GTPasa podría ocurrir por estimulación de un GEF o por inhibición de un GAP, pero en la práctica, todo apunta a que las proteínas GEFs son los mediadores críticos en la activación de las GTPasas de la familia Rho; revisado en (Gulli and Peter, 2001; Rossman et al., 2005; Schmidt and Hall, 2002).

El número de reguladores GEFs supera al de GTPasas en todos los organismos estudiados. Se ha propuesto que los Rho-GEFs podrían estar regulando la especificidad de activación de unos efectores u otros, debido a su capacidad de unión a diferentes proteínas y al reclutamiento o activación de las GTPasas en diferentes lugares de la célula. De esta forma los factores GEFs se comportan como integradores de señales para la activación de respuestas celulares concretas, y lo hacen mediante la unión a determinadas proteínas en complejos macromoleculares (Papadaki et al., 2002; Schmidt and Hall, 2002).

4.3.1. Características estructurales de las proteínas Rho-GEFs

Los factores Rho-GEFs se caracterizan por la presencia en su secuencia proteica de un dominio denominado DH (*Dbl homology*) descrito por primera vez en la proteína Dbl (el primer GEF de mamíferos aislado como un oncogen a partir de DNA de *diffuse B cell lynphome*; Eva and Aaronson, 1985). Este dominio es imprescindible para la actividad GEF.

Los dominios DH de distintos GEFs presentan un bajo grado de identidad a nivel de secuencia, incluso entre GEFs de la misma GTPasa. Sin embargo, la estructura tridimensional de estos dominios está muy conservada (Liu et al., 1998; Soisson et al., 1998; Worthylake et al., 2000) (**Figura 11**). El domino DH presenta tres regiones muy conservadas denominadas CR1, CR2 y CR3. De ellas, CR1 y CR3 están expuestas hacia la superficie y forman el núcleo en el que se une la GTPasa. Mutaciones en esta región afectan a la actividad catalítica de las proteínas GEFs (García et al., 2006b; Liu et al., 1998; Schmidt and Hall, 2002; Soisson et al., 1998).

Casi todos los factores Rho-GEFs presentan un dominio PH (*Plekstrin homology*), adyacente al dominio DH hacia su extremo carboxilo-terminal. En muchos casos el módulo DH-PH es suficiente para promover el intercambio de GDP por GTP *in vivo*. El dominio PH es capaz de unirse a fosfolípidos de membrana y también a otras proteínas y regula de esta manera la localización de los Rho-GEFs, su actividad catalítica y la unión a la GTPasa (Rossman et al., 2005).

Además del módulo DH-PH la mayoría de los GEFs poseen dominios proteicos adicionales que incluyen dominios SH3, SH2, PDZ, DEP, Ser/Thr Kinase, PH adicionales, etc. Éstos probablemente están implicados en interacciones con otras proteínas de cascadas de señalización, proteínas quinasas, entre otras; revisado en (Rossman et al., 2005; Schmidt and Hall, 2002).

4.3.2. Regulación de los factores Rho-GEFs

Las proteínas GEFs por sí mimas también son finamente reguladas y cada miembro de esta familia parece poseer un mecanismo de activación y desactivación único. Sin embargo, se han podido describir algunos principios generales que se detallan a continuación (**Figura 12**) (Schmidt and Hall, 2002).

Inhibición intramolecular:

Algunos GEFs poseen un dominio regulador que bloquea la actividad de la proteína mediante una interacción intramolecular, de forma que se bloquea el sitio activo en el dominio DH. La liberación de la autoinhibición se puede producir mediante la unión a otras proteínas, por fosforilación o por otros mecanismos (**Figura 12A**) (Bustelo, 2000). Incluso se ha realizado un estudio en el que el dominio de autoinhibición de un determinado GEF se ha sustituido por otro dominio regulable de forma exógena, de manera que se consigue controlar fácilmente la activación de este nuevo GEF y por tanto cambiar la regulación de su GTPasa diana (Yeh et al., 2007).

Interacción proteína-proteína:

Muchos GEFs son estimulados por la interacción con otras proteínas o por fosforilación, sin necesidad de eliminar ningún mecanismo autoinhibitorio. Otra variación es la oligomerización mediada por el dominio DH, en la que es necesaria la secuencia conservada CR2 (Schmidt and Hall, 2002).

Regulación por localización:

Muchas de las funciones asociadas a las GTPasas dependen del control espacial de su activación. La localización subcelular de los GEFs es un aspecto importante de su actividad. Existen Rho-GEFs que ante determinados estímulos cambian su localización subcelular desde el núcleo, el citosol o el citoesqueleto, a las membranas para activar a las GTPasas (Rossman et al., 2005; Schmidt and Hall, 2002).

Desactivación de los factores GEFs:

Se sabe muy poco acerca de la desactivación de

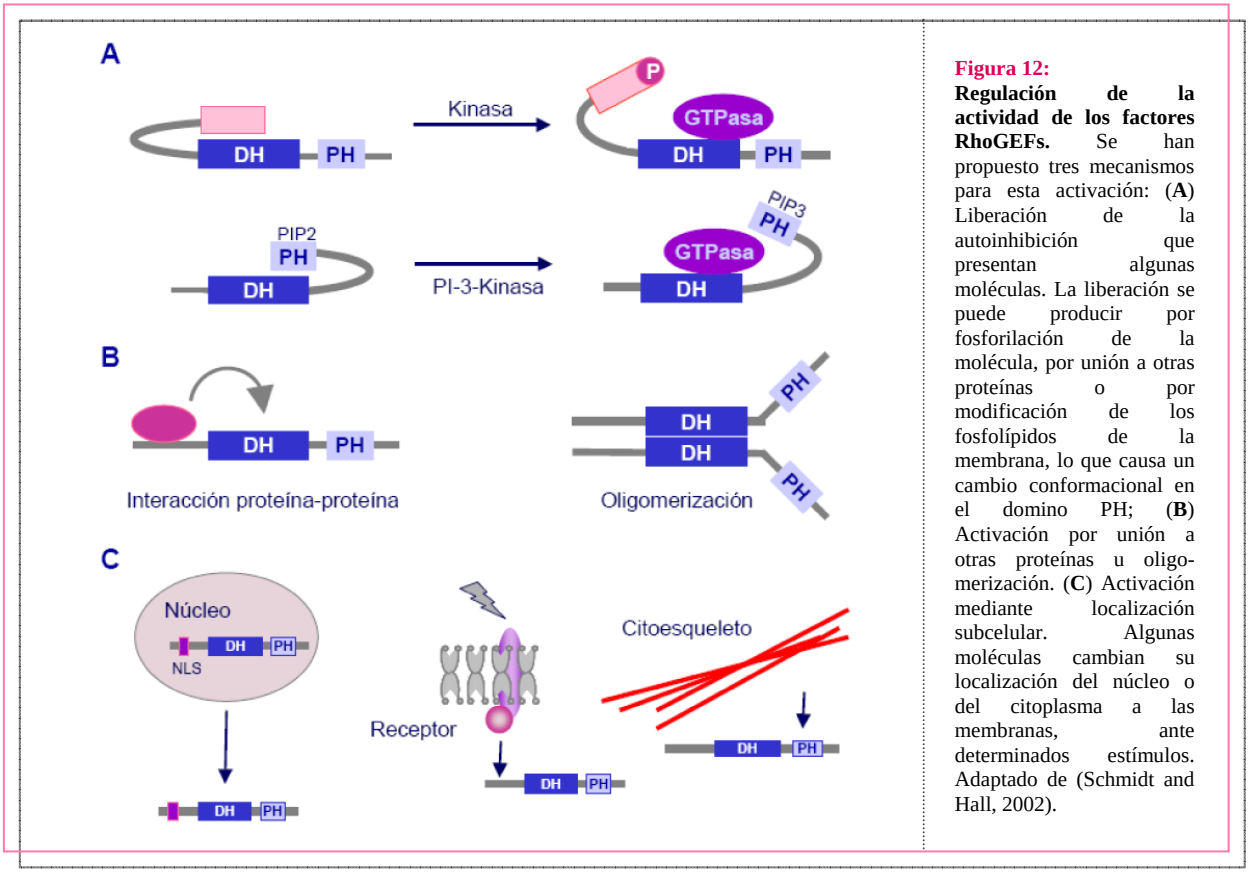

Figura 12:
Regulación de la actividad de los factores RhoGEFs. Se han propuesto tres mecanismos para esta activación: (**A**) Liberación de la autoinhibición que presentan algunas moléculas. La liberación se puede producir por fosforilación de la molécula, por unión a otras proteínas o por modificación de los fosfolípidos de la membrana, lo que causa un cambio conformacional en el domino PH; (**B**) Activación por unión a otras proteínas u oligomerización. (**C**) Activación mediante localización subcelular. Algunas moléculas cambian su localización del núcleo o del citoplasma a las membranas, ante determinados estímulos. Adaptado de (Schmidt and Hall, 2002).

estas proteínas. Se pueden revertir los mecanismos de activación, a través de la eliminación de la fosforilación o de la unión a la proteína activadora. Se ha propuesto también que la unión a determinados reguladores podría favorecer su poliubiquitinación y degradación (Schmidt and Hall, 2002).

4.4. GTPasas de la familia Rho en *S. pombe*

Un gran número de estudios en diversos organismos han desvelado que las GTPasas de bajo peso molecular funcionan como moléculas de señalización claves en el desarrollo polarizado. Además, están muy conservadas desde levaduras hasta humanos, tanto a nivel estructural como funcional (Bourne et al., 1991; Bourne et al., 1990; Etienne-Manneville and Hall, 2002; Hall, 1998).

La familia Rho en *S. pombe* está compuesta por seis miembros: Cdc42p, Rho1p, Rho2p, Rho3p, Rho4p y Rho5p (Arellano et al., 1999a). De los cuales sólo dos, Cdc42p y Rho1p, son esenciales para la supervivencia de la célula (Miller and Johnson, 1994; Nakano et al., 1997).

4.4.1. Cdc42

En *S. cerevisiae*, una proteína clave en el establecimiento de la polaridad es Cdc42p, que está involucrada en la organización de la actina, de las septinas y en exocitosis (Bi et al., 2000; Park and Bi, 2007).

En *S. pombe*, Cdc42p participa en el control del crecimiento celular polarizado y es necesaria para mantener la morfología cilíndrica típica de esta levadura (Miller and Johnson, 1994). Los mutantes de algunos activadores y efectores de Cdc42p descritos hasta el momento presentan una morfología redondeada y defectos en la polarización de los parches de actina (Hirota et al., 2003; Murray and Johnson, 2001; Ottilie et al., 1995; Sells et al., 1998).

En *S. pombe* el ensamblaje de los parches de actina tiene lugar por dos vías diferentes; una depende de Wsp1p y la verprolina Vrp1p, y la otra depende de la miosina Myo1p, ambas rutas convergen para activar al complejo Arp2/3 (García et al., 2006b; Sirotkin et al., 2005). Cdc42p puede promover el crecimiento polarizado controlando la fosforilación de la miosina, pero no se ha encontrado interacción entre Cdc42p y Wsp1p.

Otra función importante de Cdc42p está relacionada con el ensamblaje de los cables de actina. Como ya se ha comentado (ver Apartado 2.4) Cdc42p es necesaria para activar y localizar a la formina For3p encargada de la formación de estos cables. Un mutante puntual en esta GTPasa, *cdc42-1625*, presenta unos

cables de actina muy cortos y finos. Además, en este mutante la proteína For3p no se acumula correctamente en los polos celulares (Martin et al., 2007).

Los efectores de Cdc42p mejor conocidos son las proteínas PAK, Shk1p/Pak1p/Orb2p y Shk2p/Pak2p. *shk1*$^+$ es un gen esencial necesario para el crecimiento polarizado, control de la progresión del ciclo celular, citoquinesis y conjugación (Marcus et al., 1995; Ottilie et al., 1995; Verde et al., 1995; Kim et al., 2003). *shk2*$^+$ no es esencial y sus funciones parecen ser redundantes con respecto a las de *shk1*$^+$ (Merla and Johnson, 2001; Sells et al., 1998; Yang et al., 1998).

Cdc42p es activado al menos por dos GEFs, Scd1p/Ral1p y Gef1p (Coll et al., 2003; Murray and Johnson, 2001).

La disrupción de *scd1*$^+$ produce células redondeadas que son incapaces de conjugar. Cdc42p es un componente del complejo multiproteico que funciona por debajo de Ras1p, regulando la morfología celular y la conjugación. Estas dos proteínas interaccionan a través de Scd1p (Chang et al., 1994; Fukui and Yamamoto, 1988).

La deleción del otro GEF de Cdc42p, Gef1p, es viable pero causa defectos en el crecimiento bipolar y la formación del septo, donde se localiza mayoritariamente (Coll et al., 2003). La deleción de los dos GEFs de Cdc42p a la vez es letal, generando células redondas que mimetizan el fenotipo de la disrupción de *cdc42*$^+$.

4.4.2. Rho2

Rho2p, al igual que Rho1p, es necesaria para el mantenimiento de la integridad celular. Esta proteína interacciona con los homólogos de la Proteína quinasa C, Pck1p y Pck2p (**Figura 9**). Dicha GTPasa interviene en la regulación de la síntesis del α-glucano, mediante la activación y localización de Mok1p (αGS) a través de Pck2p (Calonge et al., 2000) (ver Apartado 3.6.2). De esta forma Rho1p y Rho2p regulan a través de Pck2p la síntesis de los principales polímeros que componen la pared celular, β- y α-glucano (Arellano et al., 1999b; Calonge et al., 2000).

Recientemente, se ha descrito la relación de Rho2p y Pck2p con la activación de la ruta de integridad (Ma et al., 2006). La sobreexpresión de *rho2*$^+$ y *pck2*$^+$ se traduce en un aumento de la fosforilación de Pmk1p, la última de las quinasas de esta ruta. Mientras que la disrupción de estos genes produce una disminución de la cantidad de Pmk1p fosforilada. Esto indica que Rho2p activa la ruta de integridad, a través de Pck2p (Barba et al., 2008; Ma et al., 2006).

4.4.3. Rho3 y Rho4

Rho3p y Rho4p participan en la regulación de la secreción durante la separación celular.

En *S. cerevisiae*, Rho3p está involucrada en el transporte de las vesículas endocíticas a través de los cables de actina y en el anclaje de éstas a la membrana plasmática (Adamo et al., 1999). En *S. pombe*, los mutantes nulos *rho3*Δ presentan células multitabicadas y acumulan vesículas a 37ºC. Rho3p interacciona con la formina For3p y modula las funciones del exocisto (Nakano et al., 2002; Wang et al., 2003).

Rho4p es la única GTPasa de la familia Rho que se localiza exclusivamente en la región de división. Esta proteína es necesaria para la degradación del septo durante la citoquinesis y regula la secreción y localización de las glucanasas Agn1p y Eng1p (Nakano et al., 2003; Santos et al., 2003; Santos et al., 2005).

4.4.4. Rho5

Rho5p es una proteína muy parecida a Rho1p (86% de identidad). Se expresa bajo condiciones de estrés y en ausencia de Rho1p, la sobreexpresión de Rho5p mantiene la actividad βGS y la organización del citoesqueleto de actina, aunque de forma menos eficiente (Nakano et al., 2005; Rincón et al., 2005).

4.4.5. Rho1

El crecimiento celular en las levaduras está necesariamente relacionado con la síntesis de pared celular y con la regulación del citoesqueleto de actina. La GTPasa Rho1p participa en ambos procesos, tanto en *S. cerevisiae* como en *S. pombe*.

En *S. cerevisiae* Rho1p regula la síntesis de β-glucano al menos por dos vías diferentes, como subunidad reguladora de las β-glucán sintasas Fks1p y Fks2p y como activador de la ruta de MAP quinasas que controlan la integridad celular (**Figura 13**) (Levin, 2005).

La ruta de integridad está formada por el homólogo de la Proteína quinasa C, Pkc1p, un módulo de MAP quinasas y los factores de transcripción Rlm1p y Swi4/6, que regulan la expresión de genes necesarios para la transición G1/S y para la biosíntesis de la pared; revisado por (Levin, 2005; Park and Bi, 2007). En *S. pombe* Rho1p no se ha relacionado con la cascada de quinasas de la ruta de integridad Mkh1p-Pek1p-Pmk1p/Spm1p (Toda et al., 1996), aunque si tenemos en cuenta que Pck2p es un efector de Rho1p y que esta proteína a su vez activa dicha cascada, no se descarta esta posibilidad (Ma et al., 2006).

En *S. cerevisiae*, Rho1p participa en el proceso de exocitosis polarizada, regula la formación de filamentos de actina y del anillo contráctil de actomiosina, a través de las forminas Bni1p y Bnr1p e interacciona con Sec3p, un componente del exocisto

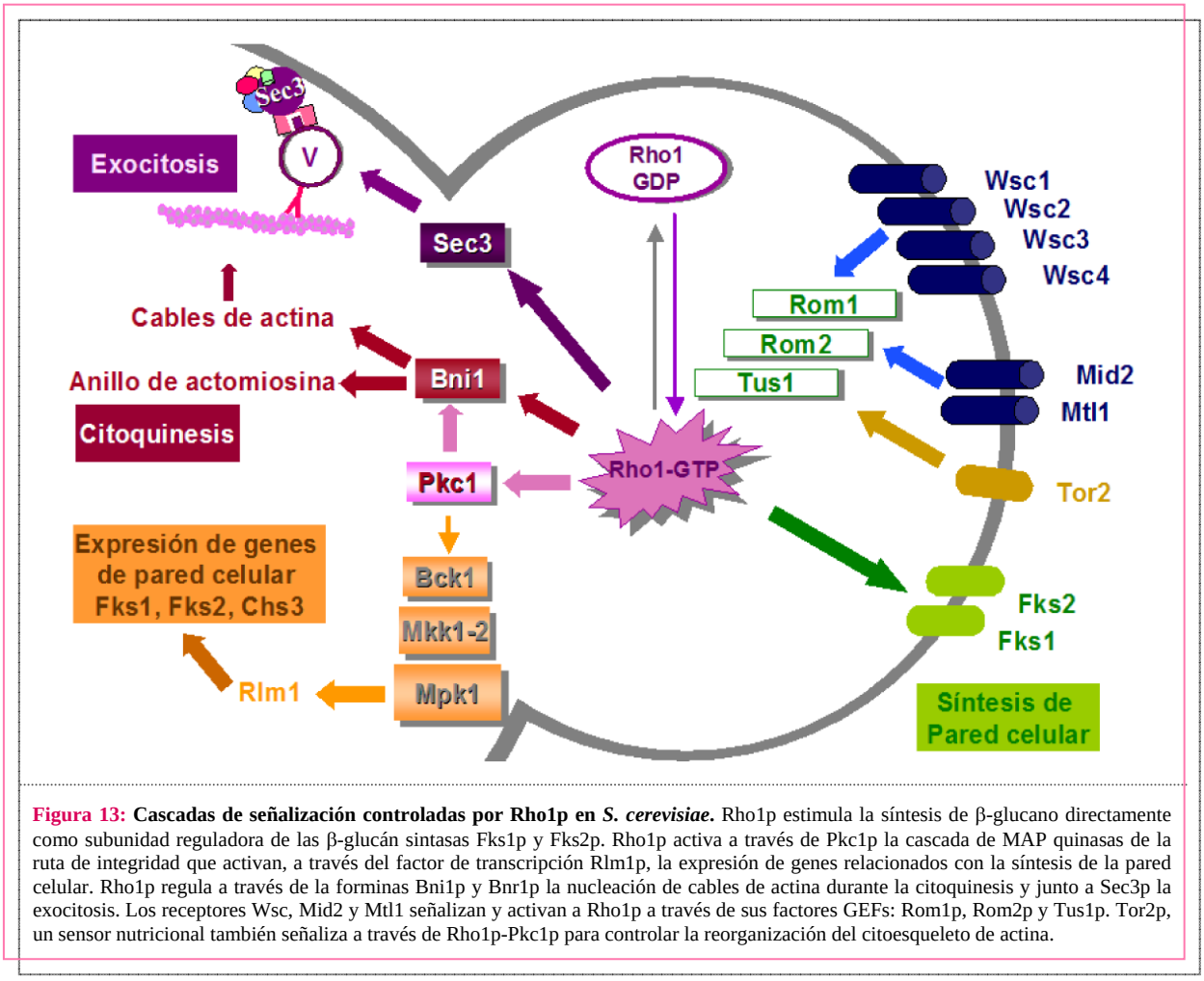

Figura 13: Cascadas de señalización controladas por Rho1p en *S. cerevisiae*. Rho1p estimula la síntesis de β-glucano directamente como subunidad reguladora de las β-glucán sintasas Fks1p y Fks2p. Rho1p activa a través de Pkc1p la cascada de MAP quinasas de la ruta de integridad que activan, a través del factor de transcripción Rlm1p, la expresión de genes relacionados con la síntesis de la pared celular. Rho1p regula a través de la forminas Bni1p y Bnr1p la nucleación de cables de actina durante la citoquinesis y junto a Sec3p la exocitosis. Los receptores Wsc, Mid2 y Mtl1 señalizan y activan a Rho1p a través de sus factores GEFs: Rom1p, Rom2p y Tus1p. Tor2p, un sensor nutricional también señaliza a través de Rho1p-Pkc1p para controlar la reorganización del citoesqueleto de actina.

(Imamura et al., 1997; Kohno et al., 1996; Guo et al., 2001). (**Figura 13**)

En *S. pombe* Rho1p también presenta funciones relacionadas con la polarización de la actina y la biosíntesis de la pared celular.

Cuando se elimina la expresión de *rho1*$^+$ durante el crecimiento vegetativo, las células pierden la integridad y se lisan, la mayoría como parejas en el momento de la separación. Además la actividad βGS también disminuye drásticamente (Arellano et al., 1999a; Arellano et al., 1997). De acuerdo con esto, los efectores de Rho1p mejor conocidos son enzimas relacionadas con la síntesis de la pared celular: la β (1,3)-glucán sintasa y las proteínas quinasas C, Pck1p y Pck2p (Arellano et al., 1999b; Sayers et al., 2000).

Rho1p funciona por debajo del marcador de polaridad Tea1p, y se localiza en los lugares de crecimiento celular, polos y septo. La disminución progresiva de Rho1p en las células produce la desaparición de la actina polimerizada, mientras que un incremento de su expresión da lugar a puntos de actina mayores y distribuidos aleatoriamente por toda la célula.

Parece ser que un equilibrio adecuado en la activación de Rho1p es importante para la regulación del citoesqueleto de actina, sin embargo aún no se ha descrito ninguna proteína que medie en este proceso. No está claro si alguna de las forminas de *S. pombe* interacciona con Rho1p y tampoco hay ningún estudio que relacione a Rho1p con el complejo Arp2/3; revisado en (García et al., 2006b).

Hasta ahora se han identificado tres GEFs de Rho1p, denominados Rgf1p, Rgf2p y Rgf3p, tres reguladores negativos, Rga1p, Rga5p y Rga8p y una proteína GDI, Rdi1p (Calonge et al., 2003; Nakano et al., 2003; Nakano et al., 2001; Yang et al., 2003)

rgf3$^+$ fue clonado en nuestro laboratorio por complementación de un mutante hipersensible a drogas que interfieren con la biosíntesis de la pared celular. Es un gen esencial y la disminución de su expresión causa lisis celular, con un fenotipo muy similar a la disrupción de *rho1*$^+$. La expresión de *rgf3*$^+$ presenta un pico durante la septación de una manera dependiente

de Ace2p y la proteína se localiza exclusivamente en la región del septo, como un anillo que se contrae (Iwaki et al., 2003; Morrell-Falvey et al., 2005; Mutoh et al., 2005; Tajadura et al., 2004).

Se ha visto que Rgf3p activa la βGS y que controla la cantidad de β-glucano de la pared celular. Es probable que Rgf3p estimule la activación, a través de Rho1p, de una GS cuya función sea crucial para la correcta septación. También, podría ser necesaria para el reclutamiento de Rho1p en esa zona (Tajadura et al., 2004).

rgf1^{+} y *rgf2*$^{+}$, como se describirá más adelante en esta memoria, también son activadores de Rho1p (García et al., 2006a; Mutoh et al., 2005). Rgf1p es necesario para la activación del crecimiento en el polo nuevo durante NETO, mientras que Rgf2p es esencial durante el proceso de esporulación.

En cuanto a los GAPs conocidos de Rho1p, se ha visto que la eliminación de *rga1*$^{+}$ ralentiza el crecimiento y produce alteraciones morfológicas similares a las producidas por la sobreexpresión de Rho1p (Nakano et al., 2001). *rga5*$^{+}$ participa en la regulación de la actividad βGS y en el mantenimiento de la integridad celular (Calonge et al., 2003). Por su parte, *rga8*$^{+}$ se aisló a partir de una búsqueda de proteínas que interaccionan con Pak1/Skh1p pero su función apenas se conoce (Yang et al., 2003).

OBJETIVOS

OBJETIVOS

El objetivo general de esta tesis ha sido la caracterización de las proteínas Rgf1p y Rgf2p, posibles reguladores de GTPasas de la familia Rho.

Objetivos específicos:

- Establecer la relación que pueda existir entre las proteínas codificadas por los genes *rgf1*$^{+}$ y *rgf2*$^{+}$ con la biosíntesis de la pared celular en *S. pombe*.

- Determinar si Rgf1p y Rgf2p actúan como reguladores de alguna de las GTPasas de la familia Rho y averiguar en cuáles de los procesos regulados por dichas GTPasas intervienen y de qué forma.

METODOLOGÍA , RESULTADOS Y DISCUSIÓN

En esta sección de la memoria se desarrolla el trabajo experimental llevado a cabo, incluyendo la metodología empleada y la discusión surgida del mismo. Todo ello se ha dividido en cuatro capítulos, cada uno de los cuales supone una línea de investigación independiente, aunque todas ellas se encuentran relacionadas entre sí e incluidas dentro del objetivo global de este trabajo, estudiar el papel de los reguladores de Rho-GTPasas, Rgf1p y Rgf2p, en el control de la integridad y la polaridad en *Schizosaccharomyces pombe*. Una discusión general, con datos adicionales y que engloba al conjunto de la investigación, se abordará con posterioridad en otra sección independiente de la memoria.

Tres de los capítulos están constituidos por artículos publicados en prensa y que son el resultado de cada línea de investigación. Además, cada artículo está precedido de un pequeño resumen en castellano de los resultados obtenidos, que facilitará la revisión rápida de la información contenida en los mismos. Por otro lado, el Capítulo II consta de una serie de resultados no publicados en prensa, pero que nos pareció interesante reflejar en esta memoria para completar el estudio de la proteína Rgf1p.

Los capítulos incluidos en la memoria son:

CAPÍTULO I

La proteína Rgf1p es un GEF específico de Rho1p que coordina la polarización celular con la biosíntesis de la pared celular en la levadura de fisión.
García, P., Tajadura, V., García, I., and Sánchez, Y. (2006). Rgf1p is a specific Rho1-GEF that coordinates cell polarization with cell wall biogenesis in fission yeast. *Mol. Biol. Cell* **17**, 1620-1631.

CAPÍTULO II

La proteína Rgf1p participa en el establecimiento del crecimiento bipolar en *S. pombe*.

CAPÍTULO III

El GEF de Rho1p, Rgf1p, señaliza por encima de la cascada de proteínas quinasas activadas por mitógeno de Pmk1p en la levadura de fisión.
García, P., Tajadura, V. and Sánchez, Y. (2009). The Rho1p exchange factor Rgf1p signals upstream from the Pmk1 Mitogen-activated protein kinase pathway in fission yeast. *Mol. Biol. Cell* **20**, 721-731.

CAPÍTULO IV

En *Schizosaccharomyces pombe*, la proteína Rgf2p es un factor intercambiador de nucleótidos de guanina de Rho1p, necesario para la maduración de la pared de la espora y el mantenimiento de la integridad celular en ausencia de Rgf1p.
García, P., García, I., Marcos, F., de Garibay, G. R. and Sánchez, Y. (2009). Fission yeast Rgf2p is a Rho1p guanine nucleotide exchange factor required for spore wall maturation and for the maintenance of cell integrity in the absence of Rgf1p. *Genetics* **181**.

CAPÍTULO I

La proteína Rgf1p es un GEF específico de Rho1p que coordina la polarización celular con la biosíntesis de la pared celular en la levadura de fisión

Antecedentes

La síntesis de β-glucano debe estar regulada temporal y espacialmente, para mantener la integridad celular durante los cambios morfogenéticos que tienen lugar a lo largo del ciclo de vida de *S. pombe*.

Con objeto de identificar nuevos genes que participen en la biosíntesis y regulación del β-glucano, se obtuvieron una serie de mutantes hipersensibles a los antifúngicos Calcoflúor y Equinocandina (Carnero et al., 2000). Ambos compuestos actúan sobre los polímeros de la pared de la levadura. El Calcoflúor interacciona con polímeros lineales de β(1,3) y β(1,4)-glucano y los desorganiza (Nicholas et al., 1994). Las Equinocandinas son antibióticos lipopeptídicos de origen natural que inhiben específicamente la síntesis de β(1,3)-glucano tanto *in vitro* (Douglas et al., 1994) como *in vivo* (Abruzzo et al., 2000). La base de este método reside en que aquellas células con una pared defectuosa, no podrán crecer en presencia de concentraciones del antifúngico a las que la cepa silvestre es perfectamente viable.

Uno de estos mutantes, *ehs2-1* (*Echinocandin hypersensitive*) es termosensible, cuando crece en medio líquido a 37ºC aproximadamente el 60% de las células se lisan como dobletes. Este fenotipo es suprimido por estabilización osmótica.

Las características de este mutante sugieren un defecto en la síntesis de la pared celular, hipótesis que se vio confirmada al comprobar que esta cepa presentaba una disminución del 50% en la actividad glucán sintasa, con respecto a un silvestre.

Trabajo Experimental

1. Identificación de *rgf1*$^+$

En el proceso de identificación del gen afectado en el mutante *ehs2-1* se encontraron dos ORFs consecutivas: *rgf1*$^+$ y *rgf3*$^+$. Ambos genes codifican dos proteínas con un dominio RhoGEF. Se descubrió que *rgf3*$^+$ era el gen estructural defectuoso en el mutante *ehs2-1*, mientras que *rgf1*$^+$ suprime parcialmente el defecto de crecimiento en presencia de Calcoflúor y Equinocandina, pero no la lisis a 37ºC (Tajadura et al., 2004).

2. Las células *rgf1*Δ muestran defectos en integridad celular y crecimiento bipolar

La aproximación al estudio de la función del gen *rgf1*$^+$ comenzó con la eliminación del gen y la caracterización del mutante *rgf1*Δ. En un cultivo líquido, aproximadamente el 30% de las células se encuentran lisadas y cuando se analiza la viabilidad en medio sólido, sólo forman colonia el 55%. Este fenotipo de lisis es similar al observado en un mutante condicional de *rho1*$^+$ (Arellano et al., 1997), y es suprimido en presencia de un estabilizador osmótico en el medio. Esto sugería que este mutante podría estar afectado en la organización de la pared celular, por lo que analizamos su crecimiento en presencia de Caspofungina (Csp). La cepa *rgf1*Δ resultó ser extremadamente sensible a este compuesto.

En *S. pombe* la actina se organiza como cables longitudinales y como parches situados en los sitios de crecimiento (Marks and Hyams, 1985). La activación de las GTPasas de la familia Rho es necesaria para el ensamblaje de filamentos contráctiles de actomiosina en muchos organismos (Jaffe and Hall, 2005). Para determinar si Rgf1p participaba en alguno de estos procesos, analizamos el patrón de crecimiento y la organización del citoesqueleto de actina en el mutante *rgf1*Δ. El 80% de estas células muestran un crecimiento monopolar, comparado con el 20% que presenta la cepa silvestre. Además, esto se correlaciona con un defecto en la organización de los parches de actina en el polo que no está creciendo.

Mediante el uso de cultivos sincronizados en fase G2, pudimos comprobar que las células no presentan defectos en la dinámica de septación, pero sí un mayor porcentaje de lisis en los momentos previos a ésta, es decir cuando las células empiezan a crecer de forma bipolar. Esto indica que Rgf1p participa en la organización del citoesqueleto de actina, durante la activación del crecimiento bipolar en *S. pombe*.

3. Rgf1p actúa como regulador positivo de Rho1p

Varios datos indican que Rgf1p actúa como GEF de Rho1p:

-Entre todas las GTPasas de la familia Rho, sólo la sobreexpresión de Rho1p fue capaz de suprimir la hipersensibilidad a Csp y la lisis de la cepa *rgf1*Δ. Por lo que Rgf1p podría actuar en la misma vía que Rho1p.

-Además, la ausencia de *rgf1*$^+$ suprimió parcialmente el defecto de crecimiento del mutante *rga1*Δ, un GAP de Rho1p (Nakano et al., 2001), este resultado podría indicar que Rgf1p y Rga1p realizan funciones opuestas, posiblemente actuando ambas sobre Rho1p. Para comprobarlo, realizamos un ensayo con rhotequina en el que se precipita la GTPasa Rho1p en su estado activo y en presencia de diferentes niveles de Rgf1p. De esta forma, observamos que la cantidad de Rho1p activo (Rho1p-GTP) es directamente proporcional a los niveles de Rgf1p presentes en las células.

-Por otro lado, mediante un ensayo de coinmunoprecipitación, observamos que Rgf1p interacciona físicamente con Rho1p. Además ambas presentan el mismo patrón de localización, en los polos de crecimiento activo y en el septo, formando primero un anillo que se va cerrando hasta convertirse en una

placa (Arellano et al., 1997). Todos estos resultados demuestran que Rgf1p funciona como GEF de Rho1p en *S. pombe*.

4. La sobreexpresión de *rgf1*$^+$ causa un aumento de la actividad β(1,3)-glucán sintasa

La sobreexpresión de *rgf1*$^+$ es letal, las células presentan una morfología aberrante, apareciendo células más grandes, multiseptadas, y con engrosamientos de material de pared que se tiñen con Calcoflúor. Esto está probablemente provocado por un aumento de la actividad β(1,3)-glucán sintasa del orden de 4 veces con respecto a la de la cepa silvestre. Esta actividad es aún mayor cuando se sobreexpresan a la vez *rgf1*$^+$ y *rho1*$^+$.

5. Rgf1p interacciona funcionalmente con Bgs4p y Pck1p

Tres de los efectores mejor conocidos de Rho1p son la βGS y las proteínas quinasas C, Pck1p y Pck2p (Arellano et al., 1999b; Sayers et al., 2000). Para determinar la posible relación de alguno de los efectores de Rho1p con Rgf1p, comprobamos si la sobreexpresión de éstos era capaz de suprimir la hipersensibilidad a Caspofungina de la cepa *rgf1*Δ. Observamos que la expresión moderada de *pck1*$^+$ era capaz de suprimir este defecto. Por otro lado, de todas las posibles subunidades catalíticas de la βGS (Bgs1-4) sólo *bgs4*$^+$ fue capaz de restablecer el crecimiento de las células *rgf1*Δ. Además, el mutante doble *rgf1*Δ *cwg1-1* (afectado en el gen *bgs4*$^+$) presentó un fenotipo similar al mutante sencillo *cwg1-1*. Lo cual podría indicar que ambos genes participan en la misma ruta y que Rgf1p activa específicamente el complejo Rho1p-Bgs4p.

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Rgf1p Is a Specific Rho1-GEF That Coordinates Cell Polarization with Cell Wall Biogenesis in Fission Yeast

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Rho1p regulates cell integrity by controlling the actin cytoskeleton and cell wall synthesis. We have identified a new GEF, designated Rgf1p, which specifically regulates Rho1p during polarized growth. The phenotype of *rgf1* null cells was very similar to that seen after depletion of Rho1p, 30% of cells being lysed. In addition, *rgf1*$^+$ deletion caused hypersensitivity to the antifungal drug Caspofungin and defects in the establishment of bipolar growth. *rho1*$^+$, *but* none of the other GTPases of the Rho-family, suppressed the *rgf1*Δ phenotypes. Moreover, deletion of *rgf1*$^+$ suppressed the severe growth defect in *rga1*$^+$ null mutants (a Rho1-GAP, negative regulator). Rgf1p and Rho1p coimmunoprecipitated and overexpression of *rgf1*$^+$ specifically increased the GTP-bound Rho1p; it caused changes in cell morphology, and a large increase in β(1,3)-glucan synthase activity. These effects were similar to those elicited when the hyperactive *rho1-G15V* allele was expressed. A genetic relationship was observed between Rgf1p, Bgs4p (β[1,3]-glucan synthase), and Pck1p (protein kinase C [PKC] homologue); Bgs4p and Pck1p suppressed the hypersensitivity to Caspofungin in *rgf1*Δ mutants. Rgf1p localized to the growing ends and the septum, where Rho1, Pck1p, and Bgs4p are known to function. Our results suggest that Rgf1p probably activates the Rho functions necessary for coordinating actin deposition with cell wall biosynthesis during bipolar growth, allowing the cells to remodel their wall without risk of rupture.

INTRODUCTION

Fission yeast cells are rod-shaped and grow in a polarized manner at the cell ends. Immediately after cell division, the daughter cells initiate growth in a monopolar manner from the cell end that preexisted before cell division (the old end). After a point in G2, cells initiate growth from the new end (the end created by cell division) in a process known as new end take off (NETO), so that they grow in a bipolar mode up to mitosis (Mitchison and Nurse, 1985; Hayles and Nurse, 2001). Fission yeast is a useful model system for studying cell wall biosynthesis and how this fits in the complex morphogenetic processes required for the cell shape to be attained.

The *Schizosaccharomyces pombe* cell wall consists mainly of three polysaccharides, β(1,3)-glucan, α(1,3)-glucan, and galactomannoproteins, all of which form a large complex. Their coordinated synthesis represents an essential step in the assembly of a functional cell wall to ensure cell integrity (for a review, see Duran and Perez, 2004). Among the polysaccharides, β(1,3)-glucans are the most prevalent (50–54% of the wall) and it is generally accepted that they are the main structural components responsible for cell wall rigidity (Manners and Meyer, 1977). β(1,3)-glucan is the first polymer to be synthesized in *S. pombe* regenerating protoplasts (Osumi *et al.*, 1989) and in the spore wall (Martin *et al.*, 2000) and hence the regulation of this polysaccharide may be a key step in the sequential assembly of the other cell wall components. The enzymatic system that catalyzes the synthesis of this polysaccharide is β(1,3)-glucan synthase (GS). GS is composed of at least two fractions: the catalytic moiety of the enzyme and the regulatory component. The catalytic subunit of GS is encoded by at least four genes: *cps1*$^+$/*bgs1*$^+$ (Le Goff *et al.*, 1999; Liu *et al.*, 2000b, 2002; Cortes *et al.*, 2002), *bgs2*$^+$ (Martin *et al.*, 2000; Liu *et al.*, 2000a), *bgs3*$^+$ (Martin *et al.*, 2003), and *bgs4*$^+$ (Cortes *et al.*, 2005). All of them code for essential proteins at different stages in the cellular life cycle. In addition to the catalytic subunit, the small GTP-binding protein Rho1p is an essential regulatory subunit (Arellano *et al.*, 1996; Nakano *et al.*, 1997). Rho1 acts as a binary switch by cycling between an inactive GDP-bound and an active GTP-bound conformational state. Rho1p stimulates GS in its GTP-bound prenylated form, providing a rationale for an understanding of the mechanism through which the cell can switch β(1,3)-glucan synthesis on and off by interconverting the GDP and GTP forms of Rho1p.

To maintain intracellular osmolarity and to produce cell shapes other than spheres, cell wall expansion must be focused on particular regions. *S. pombe* uses both microtubules and the actin cytoskeleton for this purpose (for reviews, see Yarm *et al.*, 2001; Chang and Verde, 2004; Gachet *et al.*, 2004). It has been proposed that microtubules (MTs) would act to localize key proteins involved in setting up polarized growth or to localize secretion, or even to localize actin itself to the cortex. Actin is strictly required for cell growth and is assembled in three types of structures: actomyosin rings, actin cables, and actin patches. Both cables and patches are reorganized during the cell cycle and are focused around the areas of cell growth (Marks and Hyams, 1985). The cables serve as trackways along which both actin patches (Pelham and Chang, 2001) and presumably also myosin motors with their associated cargos move to the poles or the equator for cell growth (Win *et al.*, 2001). Actin patches are dense membrane-associated structures possibly

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involved in localized cell wall synthesis. In fission yeast regenerating protoplasts, their localization coincides precisely with active sites of cell wall deposition (Kobori *et al.*, 1989).

Rho1p provides a link between polarized growth and cell wall biosynthesis (Arellano *et al.*, 1997; Nakano *et al.*, 1997), and it belongs to a family of small GTPases that are key regulators in morphogenesis, polarity, movement, and division processes (reviewed in Jaffe and Hall, 2005). The fission yeast Rho family includes Cdc42p and Rho1p through Rho5p. Rho1p localizes to sites of polarized growth, the cell poles, and the septum (Arellano *et al.*, 1997; Nakano *et al.*, 1997) and activates the abovementioned cell wall-synthesizing enzyme GS (Arellano *et al.*, 1996); Rho1 also regulates the organization of F-actin patches (Arellano *et al.*, 1997), and it binds directly to the PKC family of protein kinases, Pck1p and Pck2p, functioning as a positive regulator of these (Arellano *et al.*, 1999b; Sayers *et al.*, 2000). However, little is known about the proteins that turn Rho1p on and off in the cell. Rho GTPase regulators such as GEFs (GDP-GTP exchange factors) modify the nucleotide-bound state of the GTPase and contain protein–protein interaction domains that could be important for GTPase localization, activation, and stabilization, and thus for interaction with its effectors (Gulli and Peter, 2001; Rossman *et al.*, 2005). *S. pombe* contains seven genes bearing a Rho-GEF domain: *scd1*$^+$, *gef1*$^+$, *gef2*$^+$, *gef3*$^+$, *rgf1*$^+$, *rgf2*$^+$, and *rgf3*$^+$ (Iwaki *et al.*, 2003). Of these, *scd1*$^+$ and *gef1*$^+$ are Cdc42p-specific GEF(s) and Rgf3p has been described to function as a GEF for Rho1p. *rgf3*$^+$ is an essential gene and regulates cell wall β-glucan biosynthesis through the GTPase Rho1p, in particular during cytokinesis (Tajadura *et al.*, 2004). Previous studies have shown that Rho1p depletion causes cell death that cannot be prevented by an osmotic stabilizer (Arellano *et al.*, 1997). However, Rgf3p depletion was prevented by 1.2 M sorbitol (Tajadura *et al.*, 2004). This intriguing phenomenon suggests that in the absence of Rgf3p, but in the presence of an osmotic support, Rho1p could be activated in some other way. Accordingly, we hypothesized that this function of Rho1p would be regulated by other GEF(s) (Iwaki *et al.*, 2003). Here we demonstrate that Rgf1p specifically activates Rho1p. Our data support a model in which Rgf1p would coordinate actin deposition at polarized sites with cell wall biosynthesis, allowing the cells to remodel their wall without risk of rupture.

MATERIALS AND METHODS

Media, Reagents, and Genetics

The genotypes of the *S. pombe* strains used in this study are listed in Table 1. The complete yeast growth medium (YES), selective medium (MM) supplemented with the appropriate requirements and sporulation medium (MEA) have been described elsewhere (Moreno *et al.*, 1991). Caspofungin (Csp; Deresinski and Stevens, 2003) was stored at −20°C in a stock solution (2.5 μg/ml) in H_2O and was added to the media at the corresponding final concentration after autoclaving. Crosses were performed by mixing appropriate strains directly on MEA plates. Recombinant strains were obtained by tetrad analysis. For overexpression experiments using the *nmt1* promoter, cells were grown in EMM containing 15 μM thiamine up to the logarithmic phase. Then, the cells were harvested, washed three times with water, and inoculated in fresh medium (without thiamine) at an OD_{600} = 0.01.

Table 1. *S. pombe* strains used in this work

Strains	Genotypes
YSM180	h$^-$ 972
PN22	h$^-$ *leu1-32*
GI 1	h$^+$ *leu1-32, ehs2-1*
YS64	h$^-$ *leu1-32 ade6M210 ura4D-18 his3D1*
HVP54	h$^-$ *leu1-32 ade6M210 ura4D-18*
YS165	h$^+$/h$^-$ *leu1-32/leu1-32 ade6M210/ade6M216 ura4D-18/ura4D-18 his3D1/his3D1*
VT14	h$^-$ *leu1-32 ade6M210 ura4D-18 his3D1rgf1::his3*$^+$
VT18	h$^+$ *leu1-32 ade6M210 ura4D-18 his3D1rgf1::his3*$^+$
PG65	h$^-$ *leu1-32 ade6M210 ura4D-18 his3D1rgf1::kanMX6*
PG40	h$^-$ *rgf1::his3 his3D1 leu1-32 ade6M210 ura4D-18 leu1*$^+$*: rgf1*$^+$*-GFP*
PG92	h$^-$ *leu1-32 ade6M210 ura4D-18 his3D1rgf1::his3*$^+$*crn1*$^+$*-GFP:KanMX6*
JCR962	h$^+$ *leu1-32 ura4D-18 ade6 crn1*$^+$*-GFP:KanMX6*
KNG101	h$^+$/h$^-$ *rga1::ura4*$^+$ *leu1-32 ura4-D18 ade6M216/rga1*$^+$ *leu1-32 ura4-D18 ade6M210*
PG72	h$^+$/h$^-$ *rga1::ura4*$^+$ *rgf1::kanMX6 leu1-32 ura4-D18 ade6M216/rga1*$^+$ *rgf1*$^+$ *leu1-32 ura4-D18 ade6M210*
PG73-1c	*rga1::ura4*$^+$ *rgf1::kanMX6 leu1-32 ura4-D18 ade6M216*
PG74-2b	*rga1::ura4*$^+$ *leu1-32 ura4-D18 ade6M216*
PG75-4c	*rga1*$^+$ *leu1-32 ura4-D18 ade6M210*
PG76-5a	*rgf1::kanMX6 leu1-32 ura4-D18 ade6M216*
MS168	h$^-$ *leu1-32 ura4D-18 cdc10-129*
PG88	h$^+$ *leu1-32 ura4D-18 cdc10-129rgf1::his3*$^+$
NG669	h$^-$ *leu1-32 ura4D-18 cdc25-22*
PG43	h$^+$ *leu1-32 ura4D-18 cdc25-22rgf1::his3*$^+$
YSM373	h$^+$/h$^-$ *leu1-32/leu1-32 ade6M210/ade6M216, rgf3::ura4*$^+$*/rgf3*$^+$ *his3D1/his3D1 ura4D-18/ura4D-18*
VT128	h$^-$ *leu1-32 ade6M210 ura4D-18, his3DI leu1*$^+$*: EGFP- rgf3*$^+$
PPG217	h$^-$ *leu1-32 ade6M210 ura4D-18 his3D1 rho1::ura4*$^+$ +pREP41X-*rho1*
JCR132	h$^-$ *leu1-32 cwg1-1*
PG76	h$^+$ *rgf1::kanMX6*
PG82	h$^-$ *cwg1-1 rgf1::kanMX6*

Plasmid and DNA Manipulations

pYS8, containing the *rgf1* ORF, was obtained by inserting a 7-kb EcoRI fragment from cosmid C645 into pAU-KS (Tajadura *et al.*, 2004). An XhoI-NotI fragment from pYS8 (containing the 7-kb EcoRI fragment) was subcloned into the XhoI-NotI sites of pAL-KS, thus affording pAL-*rgf1*$^+$. To tag Rgf1p at the C-terminus with enhanced green fluorescent protein (EGFP) and with the triple repeat of the influenza virus hemagglutinin (HA) epitope (Craven *et al.*, 1998), pAL-*rgf1*$^+$ was modified by site-directed mutagenesis. We destroyed the NotI site at the multiple cloning site and created a NotI site by site-directed mutagenesis just before the TAA stop codon of *rgf1*$^+$ (pGR41). The GFP and HA epitopes were inserted in-frame at the NotI site of pGR41. pGR45 (pAL-*rgf1*$^+$*-GFP*) and pGR46 (pAL-*rgf1*$^+$*-HA*) fully complemented the *rgf1*Δ phenotypes. Strain PG40, with the *rgf1*$^+$*-GFP* integrated under its own promoter, was constructed by subcloning the *rgf1*$^+$ tagged with GFP (from plasmid pAL-*rgf1-GFP*) into the integrative vector pIJ148, resulting in pIJ148-*rgf1*$^+$*-GFP* (pGR49). This plasmid was cut with Eco47III and integrated into the *leu1* locus of strain VT14. The *nmt*1 promoter-containing vectors pREP3X and pREP41X (Forsburg and Sherman, 1997) were used to overexpress *rho1*$^+$ to *rho5*$^+$, *cdc42*$^+$, and *rgf3*$^+$. All GTPases of the Rho family were tagged with two HA epitopes at the 5′ end (Calonge *et al.*, 2003). The *rho* overexpression plasmids were kindly provided by P. Perez and P. M. Coll (Instituto de Microbiologia Bioquimica, Salamanca, Spain). pAL-*bgs1*$^+$, pAL-*bgs2*$^+$, pAL-*bgs3*$^+$, and pAL-*bgs4*$^+$ multicopy plasmids were used to overexpress the β-GS catalytic subunits, each with their own promoter. pAL-*bgs1*$^+$ was kindly provided by J. C. Cortes and J. C. Ribas (Cortes *et al.*, 2002). pAL-*bgs2*$^+$, pAL-*bgs3*$^+$ and pAL-*bgs4*$^+$ have been described previously (Martin *et al.*, 2000, 2003; Cortes *et al.*, 2005). To overexpress *rgf1*$^+$, an XhoI-SmaI fragment containing the *rgf1*$^+$ gene tagged with the HA epitope from plasmid pGR46 was ligated into the XhoI-SmaI sites of plasmid pREP41X (pGR57) and pREP3X (pGR58). pGR33 is pREP3X with an XhoI-SmaI fragment containing the *rgf1*$^+$ ORF without the HA tag.

Rgf1 Deletion

The *rgf1::his3* disruption construct was obtained in a two-step process. The 3′-flanking region (nt 4004–5350) was obtained by PCR, inserting SalI and ApaI sites into the same sites of the SK-*his3*$^+$ vector to yield pVT16. Then, a PCR fragment of the 5′ end of *rgf1*$^+$ (nt −1490 to −62) carrying BamHI and NotI sites at the ends was digested with BamHI, treated with Klenow, and then digested with NotI and ligated into the SmaI and NotI sites of pVT16 to yield pVT2. Plasmid pVT2 was digested with ApaI and NotI, and the linear

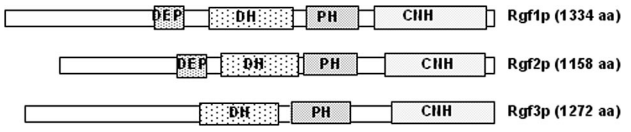

Figure 1. Comparison of structural features of Rgf1, Rgf2, and Rgf3 analyzed by the SMART program (Letunic *et al.*, 2002; http://smart.embl-heidelberg.de/). Domains are indicated: CNH, citron homology domain; DH, Dbl homology domain conserved among GEFs for Rho/Rac/Cdc42-like GTPases; PH, pleckstrin homology domain; DEP, domain of unknown function present in signaling proteins that contain PH, RasGEF, RhoGEF, RhoGAP, RGS, and PDZ domains.

DNA inserted was used to transform a diploid and a haploid strain (YS165 and YS64, respectively). Correct deletion of the *rgf1*$^+$ ORF was confirmed by PCR analysis using the following oligonucleotides: M22 (5′-GTGTTCGCTAATTGCGC-3′) into the *his3*$^+$ gene and R23-e (5′-CAAGGGTATGTGGTCTGG-3′) downstream from the nt 5350 and therefore external to the deletion cassette. A diploid strain heterozygous for the *rgf1*::*his3*$^+$ allele was subjected to tetrad analysis. *his*$^+$/*his*$^-$ segregation in tetrads was regular, indicating that *rgf1*$^+$ is not essential for vegetative growth. Gene replacement was also confirmed by genomic Southern blotting of a tetrad (unpublished data). To make the *rgf1*::*kan* disruption construct (pGR59), pVT2 was cut with SalI and SpeI (to eliminate the *his3* marker) and replaced it by the *kanMX6* gene from pFA6a-kanMX6, (Bähler *et al.*, 1998). Plasmid pGR59 was digested with ApaI and NotI and the linear DNA containing the cassette was used transform a haploid strain (YS64). *rgf1*::*kan*^{R} disruptants were selected as Kanamicin-resistant and Caspofungin-hypersensitive. *cdc25-2 rgf1*Δ and *cdc10-129 rgf1*Δ mutants were obtained by genetic crosses; the offspring were analyzed for *cdc* (ts phenotype) and for *rgf1*Δ kanamicin resistance and Caspofungin hypersensitivity. *cwg1-1 rgf1*Δ mutants were obtained by genetic cross of *cwg1-1* (JCR132) and *rgf1*Δ (PG76) strains and selected from tetrads where NPD (nonparental ditypes) were produced.

Immunoprecipitation

Rho1-GST (in pREP-KZ; a gift from P. Perez and P. M. Coll; Calonge *et al.*, 2003) and pREP41X-*rgf1HA* (pGR57) were used to cotransform *leu1-32 ura4D18 S. pombe* cells and protein expression was induced by growing the cells in the absence of thiamine for 18 h. Extracts from 2×10^8 cells expressing GST-Rho1p/Rgf1p-HA, GST/Rgf1p-HA, and GST-Rho1p/Rgf1p were obtained using 200 μl of lysis buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 137 mM NaCl, 0.5% NP-40, 10% glycerol containing 100 μM *p*-amino-phenyl methanesulfonyl fluoride, leupeptin, and aprotinin). The beads were washed four times with lysis buffer and then resuspended in sample buffer and subjected to 7.5% SDS-PAGE. The separated proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, MA) and blotted to detect Rgf1p-HA with 1:5000 diluted 12CA5 monoclonal antibody (mAb) as primary antibody and the enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ). Total Rgf1p-HA levels were monitored in whole cell extracts (50 μg of total protein) and used directly for Western blot.

Pulldown Assay for GTP-bound Rho Proteins

The expression vector pGEX-C21RBD (rhotekin-binding domain; Reid *et al.*, 1996) was used to transform *Escherichia coli* cells. The fusion protein was produced according to the manufacturer's instructions and immobilized on glutathione-Sepharose 4B beads (Amersham). After incubation, the beads were washed several times, and the bound proteins were analyzed by SDS-PAGE and stained with Coomassie. The amount of GTP-bound Rho proteins was analyzed using the Rho-GTP pulldown assay modified from Ren *et al.* (1999). Briefly, wild-type, *rgf1*$^+$-overexpressing, and *rgf1*Δ mutant cells were transformed with either pREP3X-*HArho1*$^+$ or pREP3X-*HArho4*$^+$ and grown for 18 h in minimal medium without thiamine. Extracts from 10^8 cells were obtained as described previously (Arellano *et al.*, 1997), using 500 μl of lysis buffer (50 mM Tris, pH 7.5, 20 mM NaCl, 0.5% NP-40, 10% glycerol, 0.1 μM dithiothreitol, 1 mM NaF, 2 mM $MgCl_2$, containing 100 μM *p*-aminophenyl methanesulfonyl fluoride, leupeptin, and aprotinin). GST-RBD fusion protein, 100 μg, coupled to glutathione-agarose beads was used to immunoprecipitate 1.5 mg of the cell lysates. The extracts were incubated with GST-RBD beads for 2 h. The beads were washed with lysis buffer four times, and bound proteins were blotted against 1:2000-diluted 12CA5 mAb as primary antibody to detect HA-Rho1p or HA-Rho4p. The total amounts of HA-Rho1p or HA-Rho4p levels were monitored in whole-cell extracts (10 μg of total protein), which were used directly for Western blot and were developed with 12CA5 mAb. Immunodetection was accomplished using the ECL detection kit (Amersham Biosciences).

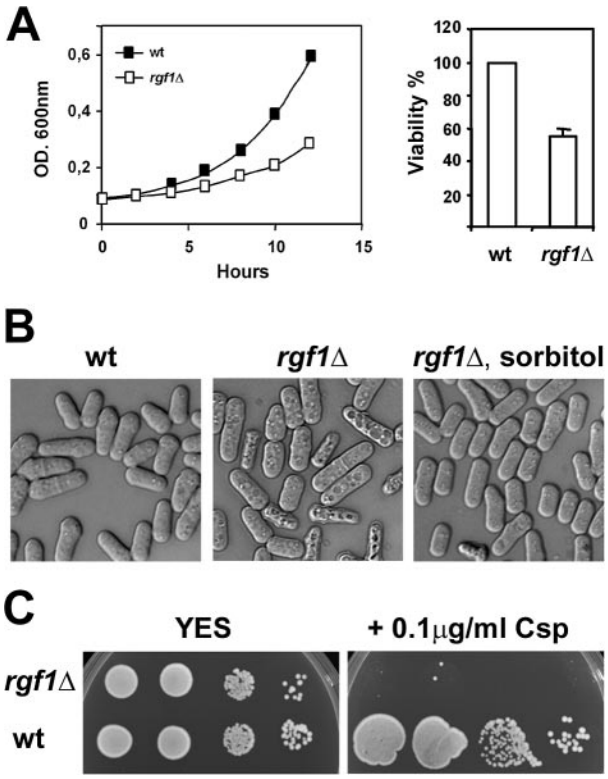

Figure 2. Growth phenotypes of *rgf1*Δ null cells. (A) Left, growth curves of *rgf1*Δ cells (VT14) and the corresponding isogenic wild-type cells (HVP54). Log-phase cells grown at 28°C were diluted to the same optical density and further grown in YES medium. Right, percentage of colony forming units (cfu) of the mutant *rgf1*Δ compared with that of the wild-type isogenic strain. Cells prepared as above were diluted and counted, and the same number of cells were plated on YES medium and incubated for 3 d at 28°C. (B) Morphology of *rgf1*Δ mutant. Differential interference contrast (DIC) micrographs of *S. pombe* wild type (HVP54) and *rgf1*Δ (VT14) grown in YES liquid medium at 28°C; in right panel, *rgf1*Δ cells were grown in the presence of 1.2 M sorbitol for 6 h. (C) *rgf1*Δ mutant cells are hypersensitive to Caspofungin (Csp). Equal number of wild-type and *rgf1*Δ cells were diluted and (4×10^4, 2×10^4, 2×10^2, and 2×10^1 cells, respectively) were spotted onto YES plates with or without 0.1 μg/ml Csp (CANCIDAS). Colony formation was analyzed following 2–3 d of incubation at 28°C.

Cell Wall Analyses

Enzyme preparations and GS assays were performed basically as described previously (Martin *et al.*, 2000). Cell extracts were obtained from early log-phase cells grown in MM as indicated for each case. Standard GS assays contained 15–25 μg protein of enzyme extract (3–5 mg protein/ml) in a total volume of 40 μl and the reaction was incubated at 30°C for 60–90 min. All reactions were carried out in duplicate and the values were calculated from three independent cell cultures. One unit of activity was measured as the amount that catalyzes the incorporation of 1 μmol of substrate (UDP-D-glucose) min^{-1} at 30°C.

Microscopy Techniques

The localization of Rgf1p-GFP, Crn1p-GFP, and Atb2p-GFP was visualized in living cells. For Calcofluor staining, exponentially growing *S. pombe* cells were harvested, washed once, and resuspended in water with Calcofluor (Cfw) at a final concentration 20 μg/ml for 5 min at room temperature. After washing with water, cells were observed under a DMRXA microscope (Leica, Wetzlar, Germany). Actin staining was performed using AlexaFluor 488-phalloidin (Molecular Probes, Eugene, OR).

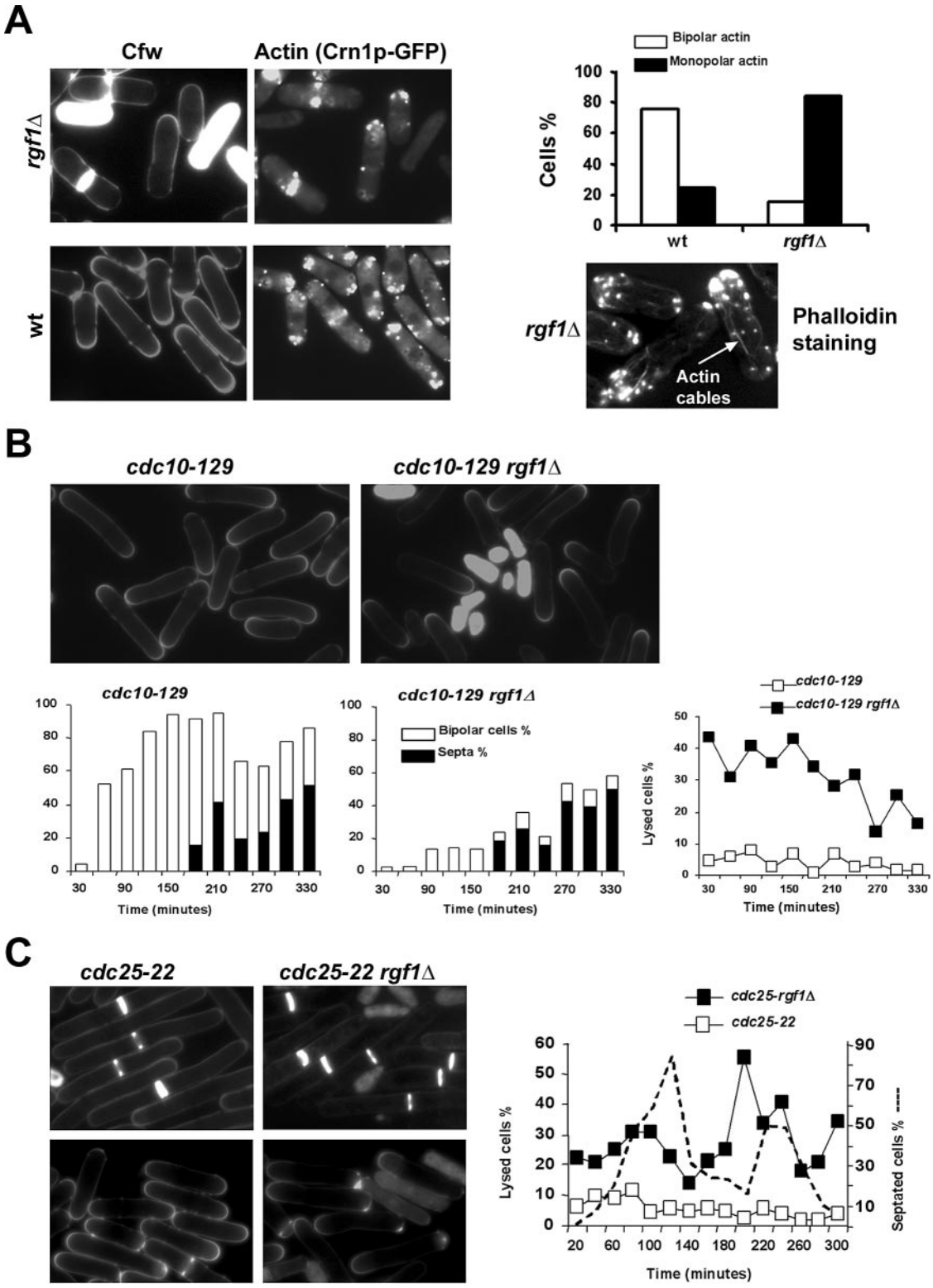

Figure 3. Rgf1p is required for bipolar growth. (A) Actin organization in *rgf1*Δ cells. Left, early-log phase cells *rgf1*$^+$*crn1*$^+$-GFP (JCR962) and *rgf1*Δ *crn1*$^+$-GFP (PG92) grown in YES liquid medium at 28°C were collected and visualized for Calcofluor (Cfw) staining and GFP (actin-associated Crn1p) fluorescence. Cfw was added at 20 μg/ml, followed by immediate examination of the cells. Note that compared with the wild type, in the *rgf1*Δ mutant the actin patches often have a more monopolar distribution. The graphic represents the percentages of monopolar and bipolar actin growth patterns in *rgf1*$^+$ (JCR962, n = 203) and *rgf1*Δ cells (PG92, n = 226) as examined by actin staining. Bottom right, *rgf1*Δ cells were fixed and stained with AlexaFluor-conjugated phalloidin to stain F-actin structures. Actin cables and patches are indicated. (B) *cdc10-129* (MS168) and *cdc10-129 rgf1*Δ (PG88) grown at 25°C to OD_{600} 0.15, shifted to 37°C for 4 h, and then grown at 25°C for 330 min. Aliquots of cells were harvested before and every 30 min after the shift to 25°C. The graphics (on the left) represents the percentage of bipolar cells (□) and septa (■) at each time point. On the right the percentage of lysed cells at each time point is represented.

RESULTS

Identification of rgf1⁺

We originally isolated *rgf1⁺* from the *S. pombe* cosmid SPCC645. In the process of cloning the gene affected in the *ehs2-1* mutation (for Echinocandin hypersensitive) we found two ORFs: *rgf1⁺* (SPCC645.07C) and *rgf3⁺* (SPCC645.06c). Both genes lay consecutive with divergent promoters and coded for proteins containing a Rho-GEF domain (the acronym *rgf* stands for RhoGEF). *rgf3⁺* is the gene affected in the *ehs2-1* mutant, whereas *rgf1⁺* partially suppresses hypersensitivity to Calcofluor (Cfw) and Echinocandin (Ech) but does not rescue lysis at 37°C in the *ehs2-1* mutant (Tajadura *et al.*, 2004).

The *rgf1⁺* gene encodes a protein of 1334 amino acids, with a predicted molecular size of ~150.1 kDa. Structural analysis of Rgf1p revealed that it contains the putative Dbl homology domain (DH; amino acid residues: aa 625-807) and a pleckstrin homology domain (PH; aa 844-973) adjacent to the DH domain characteristic of most Rho-GEFs. The DH-PH tandem is responsible for the activation of Rho-family GTPases in response to diverse extracellular stimuli (reviewed in Gulli and Peter, 2001; Rossman *et al.*, 2005). A DEP (Dishevelled, Egl-10, and Pleckstrin) domain (aa 424-497) and a CNH (Citron and NIK1-like kinase homology domain; aa 997-1293) were also found. Their function is not clear, but in most cases they act as regulatory domains involved in macromolecular interactions (http://www.genedb. org/genedb/pombe/index.jsp; Figure 1). There are seven genes that encode a putative Rho GEF domain in *S. pombe* (Coll *et al.*, 2003; Hirota *et al.*, 2003; Iwaki *et al.*, 2003; Tajadura *et al.*, 2004); among them, the closest homolog to *rgf1⁺* is *rgf2⁺*. A computer search of the deduced amino acid sequence showed that the identity percentage observed between Rgf1p and Rgf2p was ~39.4% and this rose to 63.4% upon comparing the GEF domains, whereas the identity between Rgf1p and Rgf3p (whole proteins) was 16 and 22.6% in the GEF domain.

rgf1Δ Null Cells Show Defects in Cell Integrity Similar to the Depletion of Rho1p

To characterize the relationship between Rgf1 and Rho proteins, we carried out a series of experiments to determine whether Rgf1p was acting upstream from any of the Rho proteins. First, we created a strain defective in *rgf1* by replacing the *rgf1* ORF with the *his3⁺* marker, as detailed in *Materials and Methods.* The resulting strain, *rgf1Δ*, showed a slow growth pattern at 28°C (Figure 2A), and the viability of the *rgf1Δ* cells was 55% compared with the wild-type isogenic strain. Curiously, the growth defect was less severe when *rgf1Δ* cells were grown at 37°C (unpublished data). We observed the morphology in the 25–28 to 32°C temperature range and found that regardless of the growth temperature 30–35% of the cells were lysed, whereas the rest of the cells exhibited the wild-type morphology (Figure 2B). The lysed cell phenotype of the *rgf1Δ* cells was similar to that observed in the *ehs2-1* mutant (affected in the *rgf3⁺* gene; Tajadura *et al.*, 2004) and in cells depleted for Rho1p (Arellano *et al.*, 1997). The same phenotype has also been reported in cells expressing the Rho1T20N dominant-negative mutant (Nakano *et al.*, 1997). Lysis of the *rgf1Δ* mutants cells was suppressed by the addition of 1.2 M sorbitol (Figure 2B). These phenotypes prompted us to investigate whether the mutants had a defect in cell wall architecture. We examined the sensitivity of *rgf1Δ* null mutants to different concentrations of Csp (CANCIDAS, Merck), a lipopeptide antibiotic that inhibits β(1,3)-glucan biosynthesis (Deresinski and Stevens, 2003). As shown in Figure 2C, *rgf1Δ* cells were unable to grow on plates supplemented with Csp (0.1 μg/ml), whereas the wild-type cells were able to withstand concentrations of up to 5 μg/ml (unpublished data). These results suggest that the *rgf1* null mutant cells lose their integrity, probably because of defects in cell wall biosynthesis.

The rgf1 Mutation Causes Defect in Bipolar Growth

Activation of Rho-family GTPases leads to the assembly of contractile actin-myosin filaments (Jaffe and Hall, 2005). In fission yeast, actin is organized as longitudinal F-actin cables and cortical F-actin patches at the growing ends of interphase cells, where the cell wall is newly synthesized (Marks and Hyams, 1985). To determine whether Rgf1p plays a role in any of these events, we used Crn1p-GFP (coronin), a marker for actin patches (Pelham and Chang, 2001), and Atb2p-GFP (alpha-tubulin 2) for microtubule observation (Ding *et al.*, 1998). As shown in Figure 3A, the *rgf1* mutants showed a defect in actin organization in that they organized actin patches mostly at one end of the cell only (Figure 3A, photos and graphic). This cell end corresponded to the growing end in these monopolar cells as also assessed by Calcofluor staining. Actin organization at the cell division site and F-actin cable formation was not affected in *rgf1Δ* cells (Figure 3A). We also failed to detect any significant interphase MTs defects (unpublished data).

We next investigated the behavior of *rgf1Δ* cells in the G2 phase of growth and wondered whether the lysed cell phenotype of *rgf1Δ* null mutants was due to a defect in tip elongation. To this end, we constructed the double mutant *cdc10-129 rgf1Δ* (see *Materials and Methods*) and synchronized cells in G1 by arrest at 37°C. The areas in which new cell wall deposition, and hence growth, was occurring were visualized using the fluorescent dye Calcofluor white (Cfw). Eighty minutes after being shifted to the permissive temperature 55% of *cdc10-129* cells displayed bipolar growth, whereas only 4% of *cdc10-129 rgf1Δ* cells were bipolar (Figure 3B). At 150 min after shifting, only 14% *cdc10-129 rgf1Δ* cells were bipolar. However in both strains, septation started and proceeded almost at the same time (Figure 3B, bipolar and septa plots). In this situation, the percentage of lysis in the *cdc10-129 rgf1Δ* mutant remained high (45–30%) during the first part of the time course, when bipolar growth takes place, and declined slightly in septating cells (25–15%; Figure 3B). In sum, *rgf1Δ* cells showed a defect in the activation of bipolar growth that coincided with the highest percentage of lysis.

To examine septation in *rgf1Δ* cells, we constructed the double mutant *cdc25-22 rgf1Δ* and synchronized cells in G2 by *cdc25-22* arrest at 37°C. Cells were grown at 25°C to log phase, changed to 37°C for 4 h, and then returned to 25°C. Aliquots were taken at different times to count cells with septa and lysed cells. On shifting the cells to the permissive temperature, septation was initiated at the same time in *cdc25-22* and *cdc25-22 rgf1Δ* cells. However, the second round of septation was slightly ahead in the *rgf1Δ* strain

Figure 3 (cont). Micrographs show Cfw stained *cdc10-129* and *cdc10-129 rgf1Δ* cells 60 min after the shift to 25°C. (C) *cdc25-22* (NG669) and *cdc25-22 rgf1Δ* (PG43) cells grown at 25°C to an OD_{600} 0.15, shifted to 37°C for 4 h, and then grown at 25°C for 300 min. Aliquots of cells were harvested before and every 20 min after the shift to 25°C. On the left, micrographs showing *cdc25-22* and *cdc25-22 rgf1Δ* cells 100 min (top panels) and 200 min (bottom panels) after the shift to 25°C. The graphic on the right represents the percentage of lysis of *cdc25-22*-synchronized cells (□) or *cdc25-22 rgf1Δ*-synchronized cells (■) at each time point. The septation index of *cdc25-22 rgf1Δ* cells is shown by the dashed line (– – –).

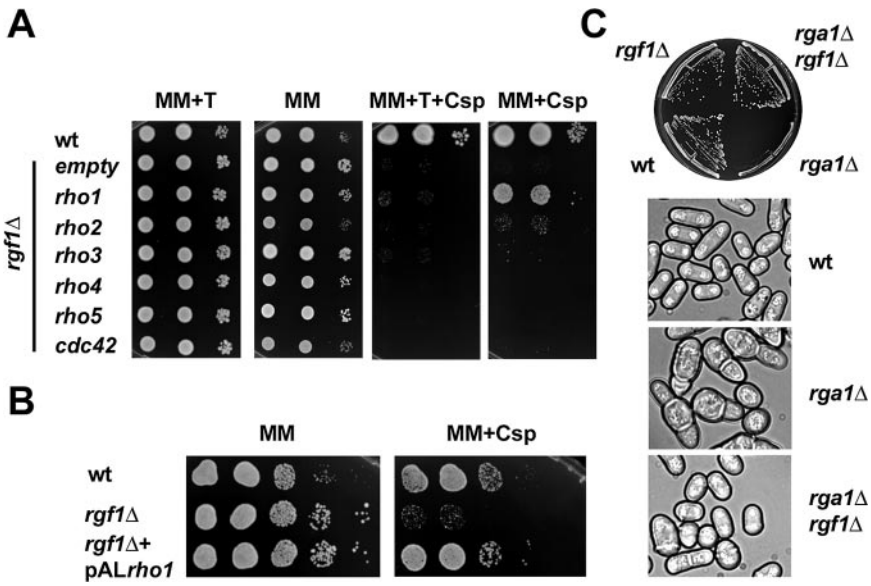

Figure 4. Rgf1p acts as a positive regulator of Rho1p. (A and B) The Caspofungin-hypersensitive growth phenotype of *rgf1*Δ mutants is suppressed by overexpression of *rho1*$^+$. HVP54 (*rgf1*$^+$) was transformed with pREP3X (empty vector), and VT14 (*rgf1*Δ) was transformed with pREP3X-*rho1* (*rho1*$^+$), pREP3X-*rho2* (*rho2*$^+$), pREP3X-*rho3* (*rho3*$^+$), pREP3X-*rho4* (*rho4*$^+$), pREP3X-*rho5* (*rho5*$^+$), pREP3X-*cdc42* (*cdc42*$^+$), and pREP3X (empty vector). Transformants were spotted onto MM, MM plus thiamine, MM plus 2 μg/ml Caspofungin (Csp), and MM plus thiamine and 2 μg/ml Csp plates as serial dilutions (4×10^4 cells in the left row and then 2×10^4 cells and 2×10^2 in each subsequent spot) and incubated at 28°C for 3 d. (B) HVP54 (*rgf1*$^+$) was transformed with pAL (empty vector) and VT14 (*rgf1*Δ) was transformed with pAL-*rho1* (*rho1*$^+$) and pAL (empty vector). Serial dilutions of the transformant cultures were spotted onto MM and MM plus 2 μg/ml Csp (from left to right 4×10^4, 2×10^4, 2×10^3, 2×10^2, 2×10) and incubated as above. (C) Deletion of *rgf1*$^+$ suppresses the growth defect in *rga1*Δ cells. Wild-type (wt) (PG75-4c), *rgf1*Δ (PG76-5a), *rga1*Δ (PG74-2b), and *rgf1rga1*Δ (PG73-1c) segregants were streaked onto YES medium and incubated at 28°C for 3 d. *rgf1rga1*Δ, but not *rga1*Δ, cells grew as wild-type cells. DIC (differential interference contrast) images of (wt) (PG75-4c), *rga1*Δ (PG74-2b), *rgf1rga1*Δ (PG73-1c)—from micromanipulation plates—grown in YES liquid medium at 28°C for 8 h are shown in the bottom panel.

(unpublished data). The appearance and number of septa were similar in both strains (Figure 3C, top panels). This result suggested that septum formation and cell separation proceeded normally in the absence of Rgf1p. However, regarding cell lysis we found a peak of broken cells in the *cdc25-22 rgf1*Δ strain just before the second round of septation, corresponding to cells in the G2 phase (Figure 3C, lower panels and graph). Thus, *rgf1*Δ cells display several phenotypes that are consistent with a role of Rgf1p in actin reorganization during activation of bipolar growth, one of the major changes in polarized growth during the *S. pombe* morphogenetic cycle.

Rgf1p Acts as a Positive Regulator of Rho1p

If *rgf1*$^+$ functions as a regulator of *rho1*$^+$, it could be expected that overexpression of *rho1*$^+$ would partially suppress the hypersensitivity to Csp as well as the lytic growth phenotype of the *rgf1* null mutant. The VT14 strain (*rgf1*Δ, *leu1-32*) was transformed with plasmids bearing *rho1*$^+$, *rho2*$^+$, *rho3*$^+$, *rho4*$^+$, *rho5*$^+$, and *cdc42*$^+$ under the control of the *nmt1* promoter or with an empty vector (pREP3X) as a control. As shown in Figure 4A, the Csp hypersensitivity of the *rgf1*Δ mutant was suppressed by *rho1*$^+$ in minimal medium without thiamine (promoter on). In minimal medium with thiamine (promoter off), no suppression was observed. None of the other genes was able to suppress the hypersensitivity of *rgf1*Δ; this being consistent with the idea that *rgf1*$^+$ could act in the same pathway as *rho1*$^+$ (Figure 4A). Overexpression of *rho2*$^+$ in wild-type cells is lethal (Hirata *et al.*, 1998), whereas overexpression of *rho1*$^+$ alone rendered the wild-type cells more sensitive to Papulacandin B (Arellano *et al.*, 1996). To avoid problems related to overexpression, we repeated the complementation experiment with the GTPases driven by the *41nmt* promoter (medium level). In this situation *rho1*$^+$ and *rho2*$^+$ constructs produced cells that exhibited wild-type morphology, however, except *for rho1*$^+$, no complementation of the hypersensitivity to Csp was found either (unpublished data). Cells that overexpressed *rho1*$^+$ from a multicopy plasmid, under the control of its own promoter behaved as wild-type cells regarding growth either with or without Csp (unpublished data). Interestingly, this construct, fully suppressed the *rgf1*Δ mutant hypersensitivity to Csp (Figure 4B) and cell lysis.

To gain further evidence that Rgf1p was a GEF for Rho1p, we tested whether an *rgf1* null mutation was able to counteract a mutation in Rga1p, a protein with GAP activity toward Rho1p and hence a negative regulator (Nakano *et al.*, 2001). Lack of Rga1p produces small colonies and the cells show a swollen, multiseptated or branched shape; a phenotype similar to that seen in cells in which Rho1p is excessively activated (Figure 4C; Nakano *et al.*, 2001). We replaced the *rgf1*$^+$ gene with the *his3*$^+$ marker in a diploid strain, *rga1*Δ/*rga1*$^+$, and the *rgf1*Δ, *rga1*Δ, *rgf1rga1*Δ segregants from 16 tetrads were analyzed. We found that the *rgf1*Δ*rga1*Δ cells formed regularly sized colonies, like *rgf1*Δ cells (unpublished data). When *rgf1*Δ, *rga1*Δ, *rgf1*Δ*rga1*Δ strains (respectively) were streaked out on rich medium (YES) at 28°C, the *rga1*Δ cells were severely impaired for growth, whereas *rgf1*Δ*rga1*Δ exhibited a better growth pattern and resembled *rgf1*Δ cells. The rounded and branched shape seen in the *rga1*Δ mutant cells returned to the wild-type morphology in the double mutant *rgf1*Δ*rga1*Δ cells (Figure 4C). Thus, *rgf1*$^+$ and *rga1*$^+$ indeed appear to antagonize each other, presumably acting on the same Rho-like GTPase.

Rgf1 Associates with Rho1p in S. pombe Cells and Promotes the GDP-GTP Exchange

To examine whether there was a direct interaction between Rgf1p and Rho1p in *S. pombe* cells, we performed coprecipitation experiments. We coexpressed HA-epitope–tagged Rgf1 protein (Rgf1-HA) together with either GST-Rho1 or GST in *S. pombe* cells. Cells were lysed, and the supernatant fractions of the lysates were incubated with glutathione-Sepharose beads to isolate GST complexes, which were analyzed by immunoblotting. As shown in Figure 5A,

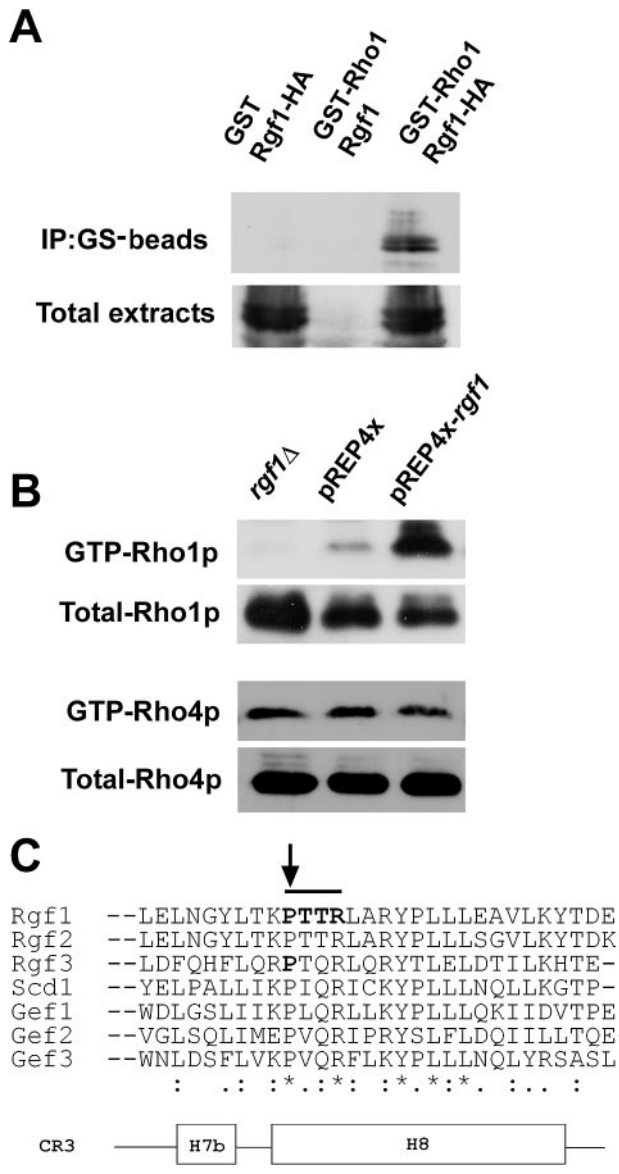

Figure 5. Rgf1p is a specific Rho1-GEF. (A) Coprecipitation of Rgf1p and Rho1p. Cell extracts from cells expressing, GST and Rgf1p-HA; GST-Rho1p and Rgf1p, and GST-Rho1p and Rgf1p-HA were precipitated with glutathione beads and blotted against 12CA5 monoclonal anti-HA antibody (top). Western blot with anti-HA antibody was performed on total extracts to visualize total Rgf1p-HA levels (bottom). (B) In vivo, the Rgf1p level modulates the amount of GTP-bound Rho1p. Wild-type (YS64) cells expressing pREP4X or pREP4X-*rgf1*, and *rgf1*Δ (VT14) mutant cells were transformed with either pREP3X-HA-*rho1* or pREP3X-HA-*rho4*. GTP-Rho1p or GTP-Rho2p were pulled down from the cell extracts with GST-C21RBD and blotted against 12CA5, anti-HA mAb. Total HA-Rho1p and HA-Rho4p was visualized by Western blot. (C) Alignment of predicted amino acid sequence at the CR3 region of Rgf1p with the corresponding region of proteins with a Rho-GEF domain found in *S. pombe*. Multiple sequence alignments were performed using the ClustalW program. The amino acids deleted in the *rgf1-PTTR*Δ mutant are marked with "black caps" over the predicted amino-acid sequence of Rgf1p. The proline (P) mutated in the *rgf3* mutant (*ehs2-1*) is also highlighted and signaled by an arrow. Asterisks indicate identical amino acids among all identified gene products. (.) and (:) indicate well- and highly-conserved amino acids, respectively.

Rgf1-HA was found to be associated with GST-Rho1, but not with GST.

To further investigate the possible role of Rgf1p as a Rho1p activator, we analyzed the in vivo amount of GTP-bound Rho1p in cells with different amounts of Rgf1p. *rgf1*Δ mutant cells carrying the control plasmid pREP4X and wild-type cells carrying either pREP4X or pREP4X-*rgf1*$^+$ were transformed with pREP3X-*HA-rho1*$^+$. After induction of the *nmt1* promoter for 18 h, the amount of Rho1p bound to GTP was analyzed by precipitation with GST-C21RBD, the rhotekin-binding domain (which had previously been obtained and purified from bacteria) and blotting with anti-HA antibody (Figure 5B). Western blots of whole extracts (25 μg protein) showed that the total amount of Rho1p was similar in all three strains with different amounts of Rgf1p (Figure 5B). The amount of active Rho1p increased considerably in the strain overexpressing Rgf1p compared with the wild-type strain. Moreover, only a minor amount of GTP-Rho1p was detected in the strain lacking Rgf1p. As a control, we also analyzed the amount of GTP-bound Rho4p in *rgf1*Δ, wild-type, and cells overexpressing *rgf1*$^+$ (Figure 5B, bottom panel). These cells were transformed with the plasmid pREP3X-HA-*rho4*$^+$ and GTP-bound Rho4p was pulled down from the extract by binding to GST-C12RBD. No changes in the level of Rho4p bound to GTP were observed among the three strains (Figure 5B). These results provide evidence that Rgf1p interacts with Rho1p and acts as a specific Rho1p activator in *S. pombe*. To examine whether the GEF domain was essential for Rgf1p function, we created a deletion mutant in the RhoGEF domain of Rgf1p (*rgf1-PTTR*Δ; Figure 5C). The DH domain contains three conserved blocks of sequences that have previously been referred to as conserved regions 1–3, or CR1–3. These three conserved regions form three long helices, H1a, H2b, and H8, which pack together to form the core of the DH domain. The four amino acids that were deleted in the *rgf1-PTTR*Δ mutant (proline-threonine-threonine-arginine, PTTR) have been predicted to be located on helix H8 (CR3), which is the most highly conserved region of the DH domain and where many mutations that decrease nucleotide exchange activity map (Liu *et al.*, 1998; Soisson *et al.*, 1998). Moreover, a single change from a proline to a serine in that conserved region of Rgf3p is responsible for the thermosensitive lytic phenotype in the *ehs2-1* mutant (Tajadura *et al.*, 2004). We found that the *rgf1-PTTR*Δ mutant integrated in a single copy in *rgf1*Δ strain maintained the lytic and the Csp-hypersensitive phenotype of the *rgf1*Δ null mutants, thus supporting the hypothesis that Rgf1p acts as a GEF.

rgf1$^+$ *Overexpression Causes Aberrant Morphology and Increases β(1,3)-glucan Synthase Activity*

It has previously been reported that overexpression of *rho1*$^+$ or constitutively active *rho1* mutants from the strong *nmt1* promoter causes an aberrant morphology in *S. pombe* cells (Arellano *et al.*, 1996; Nakano *et al.*, 1997). If *rgf1*$^+$ functions as a positive regulator of Rho1p, overexpression of *rgf1*$^+$ would be expected to produce phenotypes similar to that of Rho1-overexpressing cells. The *rgf1*$^+$ gene was cloned under the thiamine-repressible *nmt1* promoter in the pREP3X vector. When thiamine was eliminated to enhance *rgf1*$^+$ expression, the cells were unable to grow on plates (unpublished data). After 18 h of induction, cells were larger than the wild-type, round, or misshapen, with abnormal septa. These cells also showed a general increase in Cfw fluorescence, some containing aberrant depositions of Cfw-stainable material (see cells marked with an arrow and enlarged cells in Figure 6A).

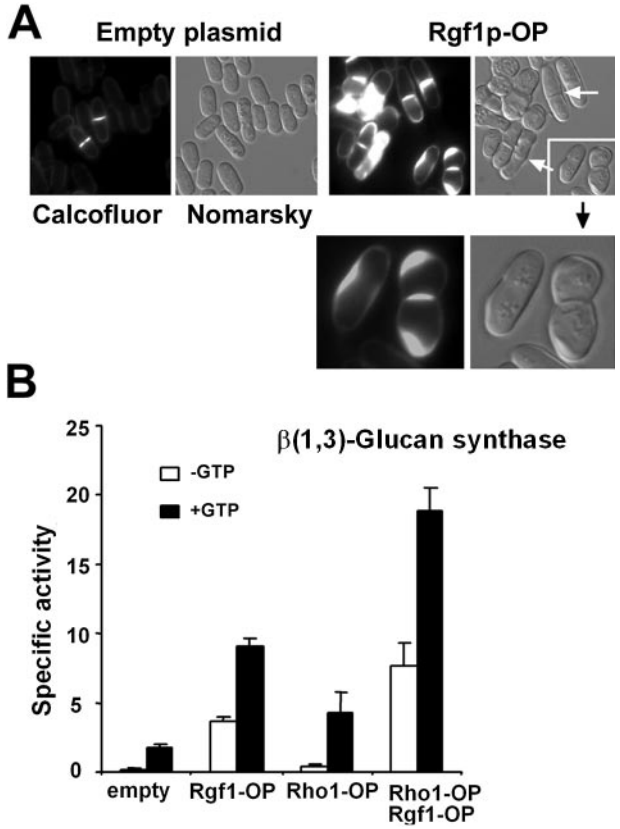

Figure 6. Phenotypes of Rgf1p overexpression (OP). (A) Overexpression of *rgf1*$^+$ causes cell growth arrest and an abnormal accumulation of cell wall material. DIC and Calcofluor-stained UV micrographs of wild-type cells transformed with pREP3X (empty plasmid) or pREP3X-*rgf1* (Rgf1-OP) grown without thiamine for 18 h. (B) In vitro glucan synthase (GS) activity assayed with the membrane fraction of wild-type cells (HVP54) transformed with pREP3X (empty plasmid), pREP3X-*rgf1* (Rgf1-OP), pREP3X-*rho1* (Rho1-OP), or both pREP4X-*rgf1* and pREP3X-*rho1* (Rgf1-OP and Rho1-OP). Extracts were prepared from cells grown in MM without thiamine at 32°C for 18 h. Specific activity is expressed as milliunits mg^{-1} protein. Values are means of at least three independent experiments with duplicated samples, and error bars represent SDs.

As expected, GS activity increased during *rgf1*$^+$ overexpression. This activity was fourfold higher than that observed in the wild-type strain (Figure 6B). To corroborate these results, we also studied the activity in cells that overexpressed *rho1*$^+$ and *rgf1*$^+$ at the same time (transformed with pREP3X-*rho1* and pREP4X-*rgf1* plasmid). As described previously, cells overexpressing *rho1*$^+$ showed an increase in GS activity (Figure 6B; Arellano *et al.*, 1996). This increase was considerably (10-fold) higher in cells that overexpressed *rgf1*$^+$ at the same time (Figure 6B). These results clearly indicate that Rgf1p is involved in the regulation of β(1,3)-glucan biosynthesis.

Genetic Evidence that Rgf1p Interacts Functionally with Bgs4p and Pck1p

It is known that Rho1p functions by activating β-glucan biosynthesis, but the issues of which of the GS catalytic subunits it activates, remain unclear. In a previous work, we reported that a mutation in *rgf3*$^+$ (*ehs2-1* mutation) was suppressed by *bgs3*$^+$, one of the putative β(1,3)-GS subunits. Multiple copies of *bgs3*$^+$ complemented the hypersensitivity to Ech and Cfw but not the temperature-sensitive phenotype (Martin *et al.*, 2003). To define a possible relationship between Rgf1p and known Rho1p effectors, we first tested whether overexpression of any of the β-GS subunits could suppress the hypersensitive phenotype of the *rgf1*Δ mutants. The *rgf1* strain VT14 was transformed with the high-copy number plasmids pAL-*bgs1*$^+$, pAL-*bgs2*$^+$, pAL-*bgs3*$^+$, and pAL-*bgs4*$^+$, and transformants were monitored for growth in Csp. As shown in Figure 7A, only a moderate expression of *bgs4*$^+$ restored growth of an *rgf1*Δ mutant in the presence of the antifungal agent. We also examined the consequences of overexpressing *rgf1*$^+$ in *cwg1-1* cells, which hold a nonlethal thermosensitive mutation in the essential *bgs4*$^+$ gene (Cortes *et al.*, 2005). When the *cwg1-1* strain was transformed with the *rgf1*$^+$ gene driven by the *nmt1* promoter, neither the pREP3X-*rgf1* with thiamine (promoter off) nor the same without thiamine (promoter on) suppressed lysis at 37°C of *cwg1-1* cells (unpublished data). In addition, *rgf1*Δ *cwg1-1* cells were phenotypically similar to *cwg1-1* (*bgs4*) cells at 37°C (Figure 7B). This finding, combined with the above observations, suggests that Rgf1p specifically activates the Rho1p-Bgs4p GS complex.

Another target of Rho1p in *S. pombe* are the PKC homologues Pck1p and Pck2p; Both genes—*pck1*$^+$ and *pck2*$^+$—share overlapping roles in cell viability and partially complement each other (Toda *et al.*, 1993); Pck2p also plays a role in the regulation of the β(1,3)-GS membrane component (Arellano *et al.*, 1999b). Because overexpression of Rho1p suppresses hypersensitivity to Csp in *rgf1*Δ mutants, we asked ourselves whether the overexpression of either Pck1p or Pck2p might function in a similar way. The *rgf1*Δ strain VT14 was transformed with the high-copy number plasmids pDB248-*pck1*$^+$and pDB248-*pck2*$^+$ (Toda *et al.*, 1993), and transformants were monitored for growth on Csp. As shown in Figure 7C, only a moderate expression of *pck1*$^+$ restored growth of an *rgf1*Δ mutant in the presence of the antifungal agent.

Rgf1p Localizes to One or Both Poles during Cell Growth and to the Contractile Ring and Septum during Cytokinesis

To determine the subcellular localization of Rgf1p, the coding sequence of the green fluorescence protein (GFP) was fused in-frame before the *rgf1*$^+$ stop codon. The GFP-*rgf1*$^+$ *rgf1*Δ strain (GFP at amino acid 1334, integrated in single copy, with its own promoter and in the absence of original *rgf1*$^+$ gene) completely restored the wild-type phenotype to *rgf1*Δ mutant cells. The cells were visualized using GFP fluorescence in order to detect Rgf1p and by Cfw staining. Rgf1p was found to localize to the growing ends and septum along the mitotic cycle, overlapping with Cfw staining (Figure 8A). When cell growth began, Rgf1p accumulated at the old growing end. During bipolar growth, Rgf1p also localized to the opposite pole. Finally, the GFP disappeared from both poles and localized only to the middle of the cell, concentrating as two faint dots on either side of the emerging septum. The GFP then moved to the inner border of the growing septum, forming a ring that moved centripetally with the edge of the growing septum (Figure 8B). After the septum wall had been completed, the GFP appeared in two separate bands (unpublished data). During cell division, Rgf1p remained on both sides of the septum until the two daughter cells were ready to separate or had already done so. To confirm these observations, confocal microscopy was used. The results of the 3-D reconstruction of the green fluorescence indicated that during septum formation Rgf1p-GFP was localized to a platelike structure; fluorescence was

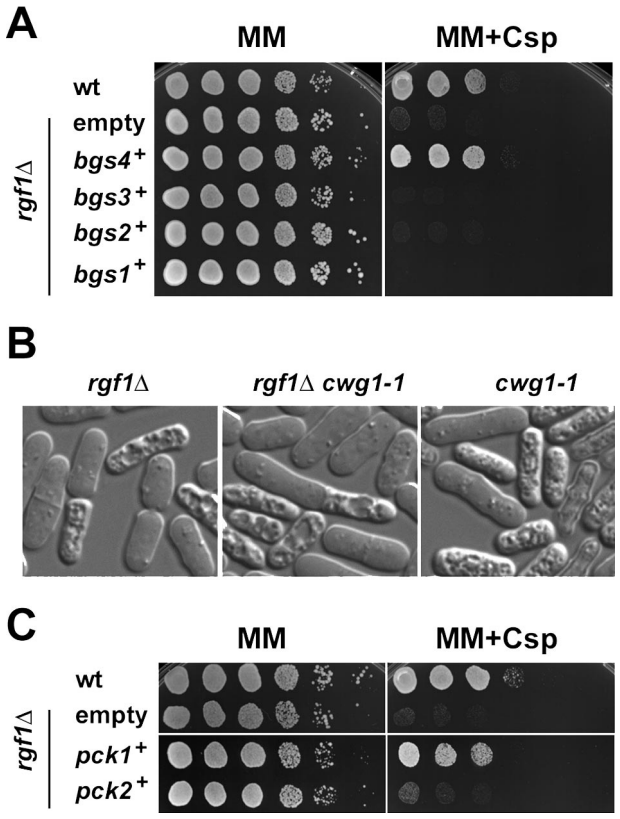

Figure 7. The Caspofungin-hypersensitive growth phenotype of *rgf1*Δ mutants is suppressed by overexpression of *bgs4*$^+$ and *pck1*$^+$. (A) HVP54 (*rgf1*$^+$) was transformed with pAL (empty vector) and VT14 (*rgf1*Δ) was transformed with pAL (empty vector), pAL-*bgs4* (*bgs4*$^+$), pAL-*bgs3* (*bgs3*$^+$), pAL-*bgs2* (*bgs2*$^+$), and pAL-*bgs1* (*bgs1*$^+$). Transformants were spotted onto MM and MM plus 2 μg/ml Csp plates as serial dilutions (8×10^4 cells in the left row and then 4×10^4, 2×10^4, 2×10^3, 2×10^2, and 2×10^1 in each subsequent spot) and incubated at 28°C for 3 d. (B) PG76 (*rgf1*Δ), JCR132 (*cwg1-1*), and PG82 (*rgf1*Δ *cwg1-1*) cells derived from a cross between strains PG76 (*rgf1*Δ) and JCR132 (*cwg1-1*) were grown to log phase in rich medium at 28°C, shifted for 4 h to 37°C, and then visualized under differential interference contrast (DIC) microscopy. The relevant genotype of each strain is indicated at top of each panel. (C) HVP54 (*rgf1*$^+$) was transformed with pDB248 (empty vector) and VT14 (*rgf1*Δ) was transformed with pDB248 (empty vector), pDB248-*pck1* (*pck1*$^+$), and pDB248-*pck2* (*pck2*$^+$). Transformants were spotted onto MM and MM plus 2 μg/ml Casp plates and processed as in A.

ring-shaped in the first stages, and as the ring was closing the fluorescence remained behind the edge and ended up distributed as a division plate (Figure 8C).

Rgf1p localized to growing areas (septum and poles) and played an important function during bipolar growth; Rgf3p localizes and functions specifically during cytokinesis (Tajadura *et al.*, 2004). We therefore investigated whether the role of Rgf1p in the regulation of Rho1p was overlapping that of Rgf3p. Previous work had shown that moderate expression of *rgf1*$^+$ did not suppress lysis at 37°C of the *rgf3* mutant (*ehs2-1*; Tajadura *et al.*, 2004). We wondered if the opposite was also true; whether overexpression of *rgf3*$^+$ was able to suppress the hypersensitive phenotype or the lysis of the *rgf1*Δ strain. *rgf3*$^+$, driven either by its own promoter or by the *nmt1* promoter, was not able to suppress the hypersensitivity in the presence of Csp nor the lysis of *rgf1*Δ cells. Moreover, disruption of *rgf1*$^+$ in an *rgf3* mutant (*ehs2-1*) produced viable cells at 28°C but not at 37°C, the temperature at which both mutants were able to grow on plates (unpublished data). These result support the hypothesis that Rgf1p and Rgf3p are not functionally interchangeable. Previous studies (Iwaki *et al.*, 2003; Tajadura *et al.*, 2004) and our own results suggest that both Rgf3p and Rgf1p are GEFs of Rho1p. The experiments reported here indicate that Rgf1p activates a Rho1p pathway (Rho1p-Bgs4p) other than that activated by Rgf3p.

DISCUSSION

Guanine nucleotide exchange factors (GEFs) are directly responsible for the activation of Rho-family GTPases in response to diverse stimuli and ultimately regulate many cellular responses such as proliferation, differentiation, and movement (Rossman *et al.*, 2005). Seven Rho-GEFs belonging to the Dbl family of proteins have been identified in *S. pombe* (Iwaki *et al.*, 2003). Here we studied the Rho-GEF, designated Rgf1, which like other Rho-GEFs contains the DH-PH tandem motifs required to activate Rho proteins (Schmidt and Hall, 2002).

Here we have shown that Rgf1p is likely to be a GEF for Rho1p. *rgf1*Δ cells are defective in cell integrity and lyse with a phenotype similar to cells devoid of Rho1 or Pck1/2 activity. Moreover, mutants lacking *rgf1* display a defect in actin organization and in β-glucan biosynthesis. The fact that both processes are controlled by Rho1p suggests that the main function of Rgf1p would be to regulate this GTPase. Consistent with this idea, the hypersensitivity to Csp and the lytic phenotype were suppressed by overexpression of *rho1*$^+$ but not other *rho* genes. Additionally, we provide genetic and biochemical evidence to support the view that Rgf1p interacts functionally with and acts as a positive regulator of Rho1p: 1) Deletion of *rgf1*$^+$ suppresses the slow growth defect of a null mutant in the *rga1*$^+$ gene, encoding a GTPase-activating protein for Rho1 (Nakano *et al.*, 2001). This finding suggests that Rgf1 may play a role antagonistic to that of Rga1p GAP. 2) Rgf1p specifically coprecipitated with Rho1p, and the level of Rgf1p modulated the level of GTP-Rho1p in vivo. 3) Overexpression of *rgf1*$^+$ was lethal and caused a phenotype similar to that of the constitutively active allele *Rho1G15V* in wild-type *S. pombe* cells, whereas it was not deleterious when overexpressed in a GTPase-deficient Rho1p strain (Rho1F85I; S. Rincón and P. Pérez, unpublished data). Furthermore, we found that the GEF domain of Rgf1p was essential for its function; a deletion mutation in a highly conserved region of the Rgf1p-DH-domain produced a lack of function phenotype. We also found that a functional GEF domain was not necessary for its localization, because the mutated protein tagged with GFP localized correctly (unpublished data).

In *S. pombe*, Rho1p signaling is required to maintain cell integrity, regulating the biosynthesis of β(1,3)-glucan and the cell wall in general, and it is also required for actin polymerization. The experiments reported in this study indicate that *rgf1*$^+$ is involved in the regulation of cell wall biosynthesis. *rgf1*Δ mutant cells were unable to grow at 50-fold lower concentrations of the antifungal drug Csp than wild-type cells and showed a lytic phenotype that could be rescued by the presence of 1.2 M sorbitol. Cells that overexpressed *rgf1*$^+$ showed aberrant depositions of Cfw-stainable material, accompanied by a GS activity that was 5-fold that of wild-type cells. Furthermore, cells overexpressing *rgf1*$^+$ together with *rho1*$^+$ showed a huge increase in GS activity (approximately 7- to 10-fold) compared with the wild-type level. Even without GTP added to the reaction, the GS

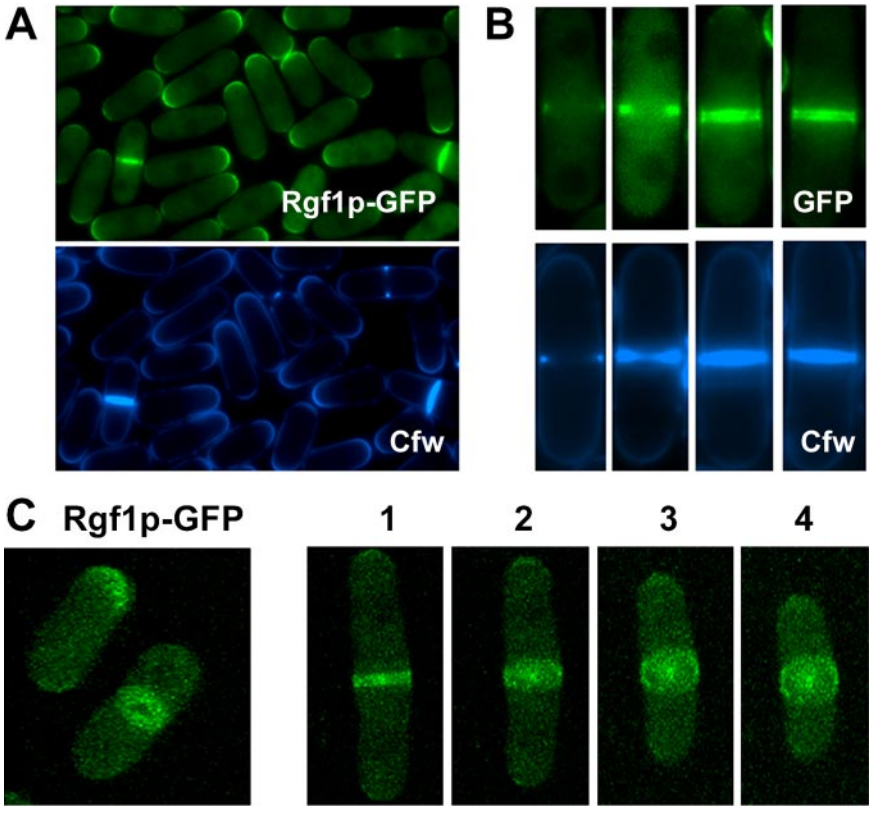

Figure 8. Rgf1p localizes to the growing regions: one or both poles, the medial ring, and the septum. (A) Rgf1p-GFP localization at different stages of the cell cycle. Early log phase cells containing the *rgf1*$^+$*-GFP* fusion allele were visualized for GFP fluorescence and Cfw staining. Cfw was added at 20 μg/ml, followed by immediate examination of the cells (bottom panel). (B) Magnification of Rgf1p-GFP localization to the medial ring and along the plasma membrane during septum formation. The cells shown were chosen from among a population of >50 dividing cells. (C) Three-dimensional reconstruction of Rgf1p localization. Cells containing the *rgf1*$^+$*-GFP* fusion allele observed under a confocal microscope and z-sections of 0.3 mm were taken. The image was reconstructed using LMS510 software.

activity of cells that overproduced *rgf1*$^+$ was 20 times higher than in the wild type, indicating that an excess of Rgf1p had raised the intracellular pool of GTP-bound Rho1p (already activated). Furthermore, our results suggest that Rgf1p would activate the β-GS complex containing the catalytic subunit Bgs4p. Rgf1p, Rho1p, and Bgs4p localize to growth areas, the septum and the poles (Arellano *et al.*, 1997; Cortes *et al.*, 2005). Individual mutants (in *rgf1*$^+$ and *bgs4*$^+$) showed similar cell wall–related phenotypes (lysis and hypersensitivity to antifungal drugs), and the double mutant *rgf1*Δ *cwg1-1* was very similar to *bgs4* (*cwg1-1*) single mutant. Moreover, overexpression of *bgs4*$^+$ suppressed the *rgf1*Δ hypersensitive phenotype.

The interaction observed between Rgf1p and Pck1p is more intriguing because the role of Pck1p in cell wall integrity remains to be established. The patterns of cell wall regulation by Pck1p and Pck2p seem to be different. *pck1*Δ, but not *pck2*Δ, cells are hypersensitive to Ech and additional copies of Pck2p cannot suppress this phenotype (Arellano *et al.*, 1999b), suggesting that in the absence of Pck1p the genes specifically involved in protection against antifungal drugs cannot be turned on. The fact that multiple copies of *pck1*$^+$ (with its own promoter) are able to suppress Csp hypersensitivity in *rgf1*Δ mutants is in agreement with the notion of Pck1p kinase being an effector of Rho1p and suggests that Pck1p would be necessary for the activation of genes (probably *bgs4*$^+$ or other GS) in response to signaling after cell wall damage. In fact, mild overexpression of either *bgs4*$^+$ (our unpublished observations), *bgs1*$^+$ or *bgs2*$^+$ (Arellano *et al.*, 1999b) was able to suppress the hypersensitive phenotype of *pck1*Δ mutants.

Activation of Rho family GTPases leads to the assembly of contractile actin:myosin filaments (Jaffe and Hall, 2005). In *S. pombe*, actin patch disassembly is one of the effects of Rho1p depletion. Interestingly, *rgf1*Δ cells showed a defect in the actin reorganization required for the transition from monopolar to bipolar growth. Among the genes required for NETO, *tea1*$^+$ plays a critical function; the most penetrant phenotype of *tea1*Δ mutants is their failure to initiate growth at the new cell tip, such that these cells only grow in a monopolar manner (Verde *et al.*, 1995; Mata and Nurse, 1997). Tea1p has been found to form a large protein complex; during NETO, the tea1p-complex at the cortex interacts with formins (and probably other polarity factors), triggering actin cable assembly and polarized cell growth (Martin *et al.*, 2005). In a *tea1*Δ mutant, Rgf1p was not maintained at one of the new cell ends, and the cells did not grow at that end (unpublished data). Rgf1p may function downstream from Tea1p, because Tea1p is required to recruit Rgf1p to a new end. The identification of proteins that directly interact with Rgf1p will be necessary to understand how Rho1p participates in the transition in which monopolar cells initiate bipolar growth.

Mutants defective in monopolar growth, *tea1*Δ, *tea4*Δ, and *bud6*Δ, grew at wild-type rates. However, a novel aspect of the *rgf1*Δ mutants is that their failure to initiate bipolar growth was accompanied by cell lysis. In a *cdc10-129 rgf1*Δ mutant, which at the restrictive temperature arrested in G1, before the activation of bipolar growth, 45% of the cells lysed 30 min after release from the restrictive temperature, whereas in a *cdc25-22 rgf1*Δ mutant, which at high temperature arrested in G2 with both ends growing, the highest percentage of lysis (55%) after release was seen after the first round of septation, coinciding in time with bipolar growth activation. Our current model is that activation of Rho1p, and in consequence β-GS activation, during bipolar growth is not achieved properly in *rgf1*Δ mutants, producing cell wall weakness. To our knowledge, *rgf1*$^+$ is the first gene that

has been implicated in cell wall biogenesis and NETO and might well provide a link between these two processes.

Rgf1p is the second exchange factor identified for Rho1p. Why does Rho1p have multiple GEFs? A similar situation has been described for mammalian cells, where the number of Rho-GEFs (~69 members) exceeds the number of Rho-type GTPases (so far 22 members; Rossman *et al.*, 2005). An attractive hypothesis is that the GEF could determine the downstream signaling specificity of Rho GTPases. This has been suggested for Ras signaling in fission yeast, where two GEFs, Ste6p and Efc25p, differentially regulate two Ras pathways (Papadaki *et al.*, 2002). In agreement with such a hypothesis, we propose that Rgf1p would specifically activate the Rho1-GS complex during the transition from monopolar to bipolar growth, whereas Rgf3p, the former Rho1-GEF, would accumulate at the contractile ring, probably activating the Rho functions that coordinate cell-wall biosynthesis to maintain cell integrity during septation (Tajadura *et al.*, 2004). Moreover, our results suggest that Rho1-GEFs, Rgf1p and Rgf3p, are not functionally interchangeable; each single *rgf1*Δ and *ehs2-1* mutant was able to grow on plates at 37°C, whereas the double *rgf1*Δ *ehs2-1* mutant was not.

In conclusion, here we provide evidence that Rgf1p is a new Rho1-GEF that participates in the regulation of bipolar growth and we propose that Rgf1p may coordinate a growth polarity transition with cell wall biosynthesis to prevent losses of cell integrity and allow cell expansion.

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REFERENCES

Arellano, M., Duran, A., and Perez, P. (1997). Localization of the *Schizosaccharomyces pombe* Rho1 GTPase and its involvement in the organization of the actin cytoskeleton. J. Cell Sci. *110*, 2547–2555.

Arellano, M., Durán, A., and Pérez, P. (1996). Rho1 GTPase activates the (1–3)b-D-glucan synthase and is involved in *Schizosaccharomyces pombe* morphogenesis. EMBO J. *15*, 4584–4591.

Arellano, M., Valdivieso, M. H., Calonge, T. M., Coll, P. M., Durán, A., and Pérez, P. (1999b). *Schizosaccharomyces pombe* PKC homologues, pck1p and pck2p, are targets of rho1p and rho2p and differentially regulate cell integrity. J. Cell Sci. *112*, 3569–3578.

Bähler, J., Wu, J.-Q., Longtine, M. S., Shah, N. G., McKenzie, A., III, Steever, A. B., Wach, A., Philippsen, P., and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe.* Yeast *14*, 943–951.

Calonge, T. M., Arellano, M., Coll, P. M., and Perez, P. (2003). Rga5p is a specific Rho1p GTPase-activating protein that regulates cell integrity in *Schizosaccharomyces pombe.* Mol. Microbiol. *47*, 507–518.

Chang, F., and Verde, F. (2004). Control of cell polarity and morphogenesis in fission yeast. In: The Molecular Biology of *Schizosaccharomyces pombe.* Genetics, Genomics and Beyond., ed. R. Egel:, New York: Springer, 255–268.

Coll, P. M., Trillo, Y., Ametzazurra, A., and Perez, P. (2003). Gef1p, a new guanine nucleotide exchange factor for Cdc42p, regulates polarity in *Schizosaccharomyces pombe.* Mol. Biol. Cell *14*, 313–323.

Cortes, J. C., Carnero, E., Ishiguro, J., Sanchez, Y., Duran, A., and Ribas, J. C. (2005). The novel (1,3)b-D-glucan synthase catalytic subunit Bgs4p from fission yeast is essential during both cytokinesis and polarized growth. J. Cell Sci. *118*, 157–174.

Cortes, J. C., Ishiguro, J., Duran, A., and Ribas, J. C. (2002). Localization of the (1,3)beta-D-glucan synthase catalytic subunit homologue Bgs1p/Cps1p from fission yeast suggests that it is involved in septation, polarized growth, mating, spore wall formation and spore germination. J. Cell Sci. *115*, 4081–4096.

Craven, R. A., Griffiths, D. J., Sheldrick, K. S., Randall, R. E., Hagan, I. M., and Carr, A. M. (1998). Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe.* Gene *221*, 59–68.

Deresinski, S. C., and Stevens, D. A. (2003). Caspofungin. Clin. Infect. Dis. *36*, 1445–1457.

Ding, D. Q., Chikashige, Y., Haraguchi, T., and Hiraoka, Y. (1998). Oscillatory nuclear movement in fission yeast meiotic prophase is driven by astral microtubules, as revealed by continuous observation of chromosomes and microtubules in living cells. J. Cell Sci. *111*, 701–712.

Duran, A., and Perez, P. (2004). Cell wall synthesis. In: The Molecular Biology of *Schizosaccharomyces pombe.* Genetics, Genomics and Beyond, ed. R. Ege, New York: Springer, 269–276.

Forsburg, S. L., and Sherman, D. A. (1997). General purpose tagging vectors for fission yeast. Gene *191*, 191–195.

Gachet, Y., Mulvihill, D. P., and Hyams, J. S. (2004). The fission yeast actomyosin cytoskeleton. In: The Molecular Biology of *Schizosaccharomyces pombe.* Genetics, Genomics and Beyond., ed. R. Egel, New York: Springer, 225–242.

Gulli, M. P., and Peter, M. (2001). Temporal and spatial regulation of Rho-type guanine-nucleotide exchange factors: the yeast perspective. Genes Dev. *4*, 365–379.

Hayles, J. A., and Nurse, P. (2001). A journey into space. Nat. Rev. Mol. Cell Biol. *2*, 647–656.

Hirata, D., Nakano, K., Fukui, M., Takenaka, H., Miyakawa, T., and Mabuchi, I. (1998). Genes that cause aberrant cell morphology by overexpression in fission yeast: a role for a small GTP-binding protein Rho2 in cell moephogenesis. J. Cell Sci. *111*, 149–159.

Hirota, K., Tanaka, K., Ohta, K., and Yamamoto, M. (2003). Gef1p and Scd1p, the two GDP-GTP exchange factors for Cdc42p, form a ring structure that shrinks during cytokinesis in *Schizosaccharomyces pombe.* Mol. Biol. Cell *14*, 3617–3627.

Iwaki, N., Karatsu, K., and Miyamoto, M. (2003). Role of guanine nucleotide exchange factors for Rho family GTPases in the regulation of cell morphology and actin cytoskeleton in fission yeast. Biochem. Biophys. Res. Commun. *312*, 414–420.

Jaffe, A. B., and Hall, A. (2005). Rho GTPases: biochemistry and biology. Annu. Rev. Cell Dev. Biol. *21*, 247–269.

Kobori, H., Yamaka, N., Taki, A., and Osumi, M. (1989). Actin is associated with the formation of the cell wall in reverting protoplasts of the fission yeast *Schizosaccharomyces pombe.* J. Cell Sci. *94*, 635–646.

Le Goff, X., Woollard, A., and Simanis, V. (1999). Analysis of the *cps1* gene provides evidence for a septation checkpoint in *Schizosaccharomyces pombe.* Mol. Gen. Genet. *262*, 163–172.

Letunic, I., Goodstadt, L., Dickens, N. J., Doerks, T., Schultz, J., Mott, R., Ciccarelli, F., Copley, R., Ponting, C., and Bork, P. (2002). Recent improvements to the SMART domain-based sequence annotation resource. Nucleic Acids Res. *30*, 242–244.

Liu, J., Tang, X., Wang, H., and Balasubramanian, M. K. (2000a). Bgs2p, a 1,3-b-glucan synthase subunit is essential for maturation of ascospore wall in *Schizosaccharomyces pombe.* FEBS Lett. *478*, 105–108.

Liu, J., Tang, X., Wang, H., Oliferenko, S., and Balasubramanian, M. K. (2002). The localization of the integral membrane protein Cps1p to the cell division site is dependent on the actomyosin ring and the septation-inducing network in *Schizosaccharomyces pombe.* Mol. Biol. Cell *13*, 989–1000.

Liu, J., Wang, H., and Balasubramanian, M. K. (2000b). A checkpoint that monitors cytokinesis in *Schizosaccharomyces pombe.* J. Cell Sci. *113*, 1223–1230.

Liu, X., Wang, H., Eberstadt, M., Schnuchel, A., Olejniczak, E. T., Meadows, R., and Fesik, S. W. (1998). NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor Trio. Cell *95*, 269–277.

Manners, D. J., and Meyer, M. T. (1977). The molecular structures of some glucans from the cell wall of *S. pombe.* Carbohydr. Res. *57*, 189–203.

Marks, J., and Hyams, J. S. (1985). Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe.* Eur. J. Cell Biol. *39*, 27–32.

Martin, S. G., McDonald, W. H., Yates, J., III, and Chang, F. (2005). Tea4p links microtubule plus ends with the formin For3p in the establishment of cell polarity. Dev. Cell *8*, 479–491.

Martin, V., García, B., Carnero, E., Duran, A., and Sanchez, Y. (2003). Bgs3p, a putative 1,3-b-glucan synthase subunit, is required for cell wall assembly in *Schizosaccharomyces pombe.* Eukaryot. Cell *2*, 159–169.

Martin, V., Ribas, J. C., Carnero, E., Durán, A., and Sánchez, Y. (2000). Bgs2+, a sporulation-specific glucan synthase homologue is required for proper ascospore wall maturation in fissión yeast. Mol. Microbiol. *38*, 308–321.

Mata, J., and Nurse, P. (1997). tea1 and the microtubular cytoskeleton are important for generating global spatial order within the fission yeast. Cell *89*, 939–950.

Mitchison, J. M., and Nurse, P. (1985). Growth in cell length in the fission yeast *Schizosaccharomyces pombe.* J. Cell Sci. *75*, 357–376.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe.* Methods Enzymol. *194*, 795–823.

Nakano, K., Arai, R., and Mabuchi, I. (1997). The small GTP binding protein Rho1 is a multifunctional protein that regulates actin localization, cell polarity, and septum formation in the fission yeast *Schizosaccharomyces pombe.* Genes Cells *2*, 679–694.

Nakano, K., Mutoh, T., and Mabuchi, I. (2001). Characterization of GTPase-activating proteins for the function of the Rho-family small GTPases in the fission yeast *Schizosaccharomyces pombe.* Genes Cells *6*, 1031–1042.

Osumi, M., Yamada, N., Kobori, H., Taki, A., Naito, N., Baba, M., and Nagatani, T. (1989). Cell wall formation in regenerating protoplast of *Schizosaccharomyces pombe*: study by high resolution, low voltage scanning electron microscopy. J. Electron. Microsc. *38*, 457–468.

Papadaki, P., Pizon, V., Onken, B., and Chang, E. (2002). Two ras pathways in fission yeast are differentially regulated by two ras guanine nucleotide exchange factors. Mol. Cell Biol. *22*, 4598–4606.

Pelham, R.J.J., and Chang, F. (2001). Role of actin polymerization and actin cables in actin-patch movement in *Schizosaccharomyces pombe*. Nat. Cell Biol. *3*, 235–244.

Reid, T., Furiyashiki, T., Ishizaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madaule, P., and Nayumira, S. (1996). Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain. J. Biol. Chem. *271*, 13556–13560.

Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J. *18*, 578–585.

Rossman, K. L., Channing, J. D., and Sondek, J. (2005). GEF means go: turning on Rho GTPases with guanine nucleotide-exchange factors. Nat. Rev. Mol. Cell Biol. *6*, 167–180.

Sayers, L. G., Katayama, S., Nakano, K., Mellor, H., Mabuchi, I., Toda, T., and Parker, P. J. (2000). Rho-dependence of *Schizosaccharomyces pombe* Pck2. Genes Cells *5*, 17–27.

Schmidt, A., and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev. *16*, 1587–1609.

Soisson, S. M., Nimnual, A. S., Uy, M., Bar-Sagi, D., and Kuriyan, J. (1998). crystal structure of the Dbl and pleckstrin homology domains from the human son of sevenless protein. Cell *95*, 259–268.

Tajadura, V., Garcia, B., Garcia, I., Garcia, P., and Sanchez, Y. (2004). *Schizosaccharomyces pombe* Rgf3p is a specific Rho1 GEF that regulates cell wall b-glucan biosynthesis through the GTPase Rho1p. J. Cell Sci. *117*, 6163–6174.

Toda, T., Shimanuki, M., and Yanagida, M. (1993). Two novel PKC-related genes of fission yeast are essential for cell viability and implicated in cell shape control. EMBO J. *12*, 1987–1995.

Verde, F., Mata, J., and Nurse, P. (1995). Fission yeast cell morphogenesis: identification of new genes and analysis of their role during the cell cycle. J. Cell Biol. *131*, 1529–1538.

Win, T. Z., Gachet, Y., Mulvihill, D. P., May, K. M., and Hyams, J. S. (2001). Two type V myosins with non-overlapping functions in the fission yeast *Schizosaccharomyces pombe*: Myo52 is concerned with growth polarity and cytokinesis, Myo 51 is a component of the cytokinetic actin ring. J. Cell Sci. *114*, 69–79.

Yarm, F., Sagot, I., and Pellman, D. (2001). The social life of actin and microtubules: interaction versus co-operation. Curr. Opi. Microbiol. *4*, 696–702.

CAPÍTULO II

La proteína Rgf1p participa en el establecimiento del crecimiento bipolar en S. pombe

En el capítulo anterior de esta memoria describimos la función de Rgf1p en el establecimiento del crecimiento bipolar durante NETO. Nuestros resultados sugieren que Rgf1p participa en la coordinación entre el crecimiento polarizado y la síntesis de la pared celular. Cuando falta Rgf1p no se produce crecimiento bipolar en un gran porcentaje de células, por lo que en esas condiciones las células podrían ser más susceptibles a la lisis y perder la integridad.

Para estudiar con más detalle la participación de Rgf1p en este proceso, realizamos una serie de experimentos que se recogen en este segundo capítulo.

2.1. Patrón de crecimiento de las células *rgf1*Δ

El *Calcofluor-white* se une preferentemente a los lugares de crecimiento activo de la célula, por lo que lo utilizamos normalmente para saber por dónde crecen las células en un momento determinado del ciclo. En el caso del mutante *rgf1*Δ la tinción con Calcoflúor muestra un 80% de células monopolares, pero no nos permite saber cuál de los dos polos de la célula está inactivo, si es el antiguo que ya estaba en la célula madre, o es el nuevo que acaba de formarse en el momento de la división. Para conocer el patrón de crecimiento de las células *rgf1*Δ, realizamos un ensayo de "*time lapse*", que consiste en fotografiar las células secuencialmente con un microscopio óptico cada cierto tiempo, de forma que se puedan superponer las imágenes obtenidas y ver el crecimiento. Hicimos fotos cada 5 min. durante 2-4 horas y observamos que en la cepa silvestre el 85% de las células crecen primero sólo por el polo viejo y después activan el polo nuevo (crecimiento bipolar). El otro 15% no activa el polo nuevo en ningún momento (**Figura 14**).

En la cepa *rgf1*Δ, las dos células hijas resultantes después de la división sólo crecen por el polo viejo y nunca activan el crecimiento del polo nuevo (66% en n=50 células). Este resultó ser el patrón mayoritario, sin embargo, en otro grupo de células (23%), una de ellas crecía por el polo antiguo, mientras que la otra lo hacía por el nuevo (**Figura 14**). Este tipo de crecimiento aberrante también se ha observado en otros mutantes afectados en NETO, como *tea1*Δ, *bud6* Δ y *for3*Δ (Chang and Verde, 2004). En el caso de *bud6*Δ la mayoría de las células muestran un patrón de crecimiento similar al de *rgf1*Δ, ya que crecen sólo por el polo antiguo. Sin embargo, en los mutantes *tea1*Δ de las dos células hijas una crece por el polo viejo y la otra por el nuevo. Además, existe un tercer patrón aberrante observado en el mutante nulo *for3*Δ, en el que una de las células resultantes después de la división crece sólo por el polo viejo, mientras que la otra presenta un crecimiento bipolar desde el principio (Chang and Verde, 2004).

2.2. La localización de Rgf1p en los polos depende de la actina

Como ya vimos en el Capítulo I, Rgf1p se localiza en el septo y en los polos celulares. Su localización en los polos parece coincidir con la existencia de crecimiento en esos lugares. Para comprobarlo, utilizamos dos mutantes termosensibles (*cdc10-129* y *cdc25-22*) que a 37ºC detienen su crecimiento en G1 y G2, respectivamente. Construimos cepas con cada una de estas mutaciones y el gen *rgf1*$^{+}$ marcado con GFP (integrado en el genoma) y observamos la localización de la proteína

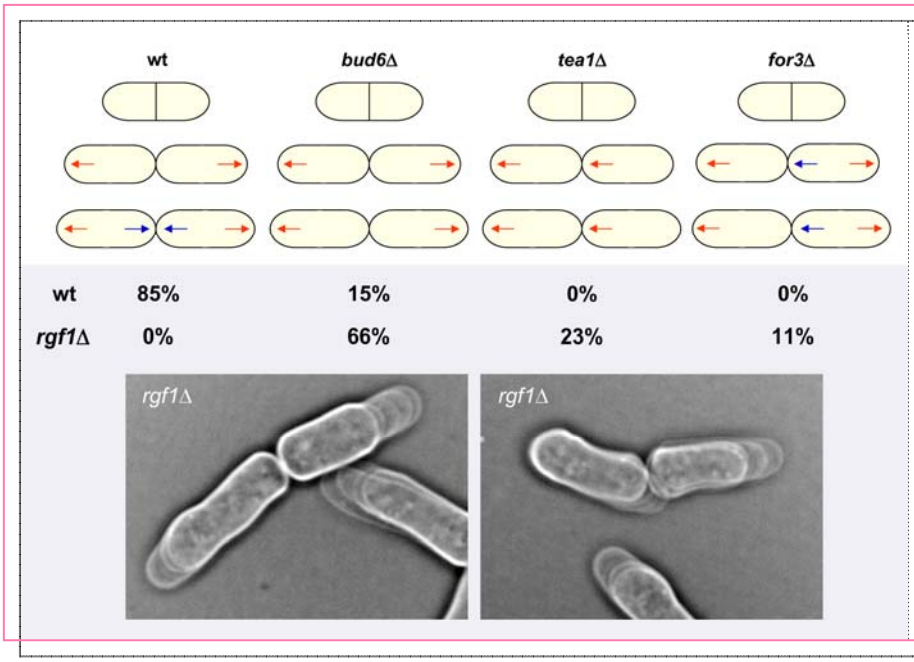


Figura 14:
Patrón de crecimiento de las células *rgf1*Δ. En la parte superior se muestran los esquemas de los patrones de crecimiento de la cepa silvestre (wt) y de tres mutantes nulos afectados en NETO, *bud6*Δ, *tea1*Δ y *for3*Δ La flecha roja indica el crecimiento que se da en primer lugar y la azul secundariamente. En la parte inferior se detallan los porcentajes de células que presentan cada uno de los patrones de crecimiento, en las cepas wt y *rgf1*Δ. También se muestran dos imágenes resultantes de la superposición de varias fotografías secuenciales de células *rgf1*Δ, en las que se puede observar los dos patrones de crecimiento mayoritarios en este mutante.

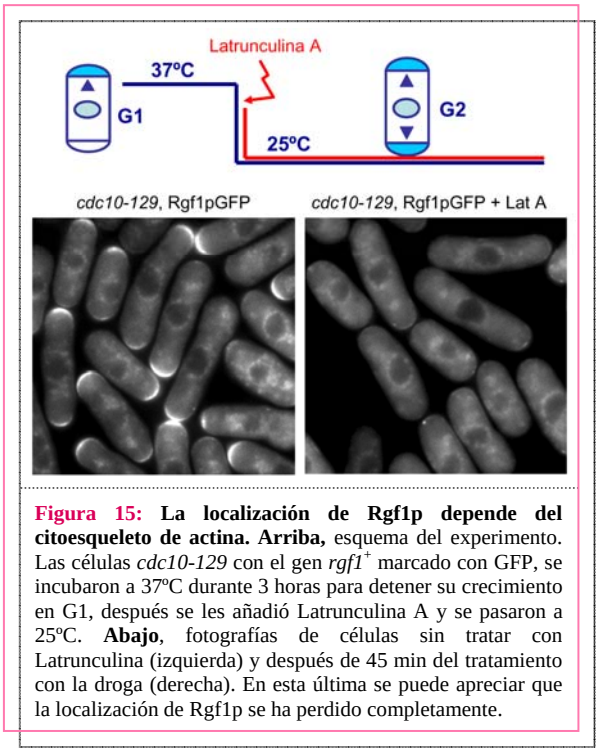


Figura 15: La localización de Rgf1p depende del citoesqueleto de actina. Arriba, esquema del experimento. Las células *cdc10-129* con el gen *rgf1*$^{+}$ marcado con GFP, se incubaron a 37ºC durante 3 horas para detener su crecimiento en G1, después se les añadió Latrunculina A y se pasaron a 25ºC. **Abajo**, fotografías de células sin tratar con Latrunculina (izquierda) y después de 45 min del tratamiento con la droga (derecha). En esta última se puede apreciar que la localización de Rgf1p se ha perdido completamente.

Rgf1p. En células *cdc10-129* a 37ºC (crecimiento parado en G1), vimos que Rgf1p se localiza exclusivamente en un polo, el que presenta crecimiento. Sin embargo, en las células *cdc25-22* detenidas en G2, Rgf1p se observa en ambos polos, ya que ambos están creciendo (datos no mostrados).

Nos preguntamos si la localización de Rgf1p depende del citoesqueleto de actina, y si éste es necesario para que Rgf1p se trasladase al polo nuevo. Para ello, tratamos las células del mutante *cdc10-129* (con Rgf1p marcada con GFP) con Latrunculina A. Esta droga secuestra los monómeros de actina, de forma que podemos ver si existe alguna diferencia en la localización de Rgf1p, en presencia o ausencia de un citoesqueleto de actina correctamente polimerizado. Sincronizamos las células, incubándolas a 37ºC durante 3 horas (parada en G1), después añadimos Latrunculina A (50µM) y las incubamos a 25ºC para permitir que continuaran su ciclo de vida. En las células sin tratar, Rgf1p-GFP es bipolar después de 45 min. a 25ºC. Sin embargo, en las células tratadas no hay fluorescencia en el polo nuevo, y curiosamente la localización de Rgf1p en el polo antiguo desaparece (**Figura 15**). También hemos visto que la localización de Rgf1p en el septo no depende tanto del citoesqueleto de actina. En las células tratadas con Latrunculina A (45 min.), la proteína Rgf1p aún permanece en el septo, aunque la fluorescencia que se observa es menos intensa que en células sin tratar (datos no mostrados). Estos resultados indican que la localización de Rgf1p en los polos depende del citoesqueleto de actina, mientras que en la zona del septo podría anclarse de forma diferente.

2.3. El crecimiento bipolar depende de la actividad GEF de Rgf1p

Como ya se ha descrito en el Capítulo I y se demostrará con nuevas evidencias en el Capítulo III, la actividad intercambiadora de nucleótidos de guanina de Rgf1p reside en su dominio DH (RhoGEF) y de ella depende también el mantenimiento de la integridad celular y la resistencia a antifúngicos.

Nos preguntamos si la actividad GEF de Rgf1p también era necesaria para la activación del crecimiento bipolar. Para comprobar esto, utilizamos el mutante en el dominio DH descrito anteriormente (Capítulo I), *rgf1-PTTR*Δ y analizamos el patrón de crecimiento en una población asincrónica, mediante tinción con Calcoflúor (**Figura 16**). Las células *rgf1-PTTR*Δ presentan un defecto en el crecimiento bipolar, de manera que sólo el 27% de las células crece por los dos polos, frente a un 80% de las células silvestres. Este resultado indica que Rgf1p promueve el crecimiento de la célula por los dos polos a través de su actividad GEF.

La mutación en el dominio RhoGEF podría estar afectando a la estabilidad o la localización de la proteína y ésta podría ser la causa de su pérdida de actividad. Para comprobarlo, obtuvimos una proteína Rgf1p mutante (Rgf1PTTRΔ) marcada con GFP en su extremo carboxilo-terminal. Tanto la proteína mutante como la silvestre marcadas con GFP presentan una movilidad similar en Western blot (datos no mostrados), sin embargo aunque la proteína mutada se localiza correctamente en el septo, sólo aparece en uno de los polos (datos no mostrados).

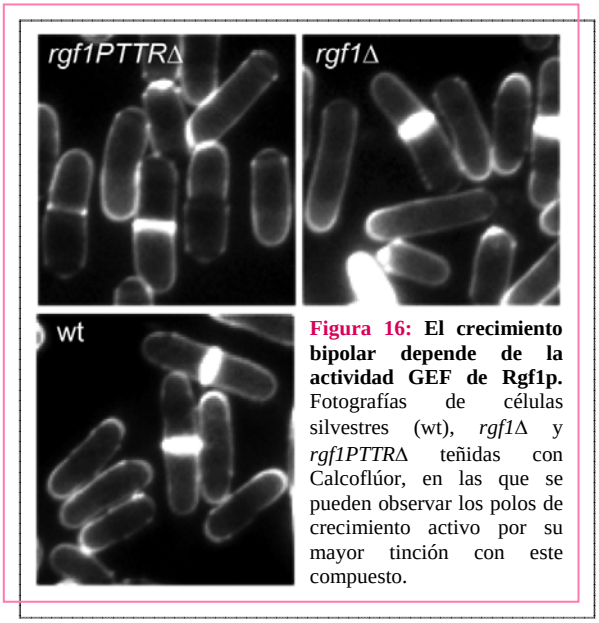


Figura 16: El crecimiento bipolar depende de la actividad GEF de Rgf1p. Fotografías de células silvestres (wt), *rgf1*Δ y *rgf1PTTR*Δ teñidas con Calcoflúor, en las que se pueden observar los polos de crecimiento activo por su mayor tinción con este compuesto.

El crecimiento monopolar de las células con la proteína mutada (Rgf1PTTRΔ) podría ser debido a la ausencia de la proteína en el segundo polo, o justamente al contrario, que la ausencia de Rgf1p en ese polo fuera consecuencia del crecimiento monopolar de las células. Para distinguir entre estas dos posibilidades, analizamos la localización de la proteína Rgf1PTTRΔ-GFP en una cepa silvestre (*rgf1*$^{+}$). En este caso la proteína mutada se localiza en los dos polos en la mayoría de las células (datos no mostrados). Este resultado indica que la ausencia de la proteína mutante en el segundo polo, podría ser una consecuencia de la falta de actividad de Rgf1p y no al contrario.

2.4. Rgf1p no funciona como marcador de polaridad

Por lo expuesto hasta el momento, parece claro que la actividad de la proteína Rgf1p es esencial para el funcionamiento de NETO. Sin embargo, sólo algunas de las proteínas implicadas en NETO actúan como marcadores de polaridad, entre ellas está Tea1p que funciona señalando los sitios por donde debe crecer la célula (Mata and Nurse, 1997) y Sla2p que también es necesaria para establecer zonas nuevas de crecimiento y que actúa por debajo de Tea1p (Castagnetti et al., 2005).

Rgf1p podría actuar bien como identificador del polo nuevo o como desencadenante o regulador de la remodelación de la actina durante NETO. Para comprobarlo nos preguntamos si es posible desencadenar NETO en células del mutante *rgf1*Δ provocando la despolimerización de la actina y surtiendo así de monoméros de actina a las células.

Se sabe, que una despolimerización momentánea del citoesqueleto de actina, es capaz de desencadenar NETO incluso en células paradas en G1 (Rupes et al., 1999), como por ejemplo en el mutante *cdc10-129* a 37ºC. El experimento que realizamos consistió en mantener las células *cdc10-129* bloqueadas en G1 a 37ºC (con la actina monopolar), tratarlas durante 5 min con Latrunculina A y a continuación eliminar la droga, de forma que la actina pueda volver a repolimerizarse.

Cuando se realiza este experimento con el mutante *tea1*Δ, la actina se mantiene monopolar después del pulso con Latrunculina A y de lavar la droga (Rupes et al., 1999). En nuestro caso, observamos que después del pulso de Latrunculina, la actina se distribuyó de forma bipolar tanto en el mutante *cdc10-129*, como en la cepa *cdc10-129 rgf1*Δ (**Figura 17**), lo cual indica que Rgf1p no es necesario para marcar una nueva zona de crecimiento.

Otra manera de probar si Rgf1p actúa como marcador de polaridad es observar el comportamiento de un mutante *cdc11-123 rgf1*Δ. Los mutantes *cdc11-123* son termosensibles y acumulan células alargadas con muchos núcleos, esto quiere decir que hacen múltiples ciclos de división y en cada ciclo tienen que pasar por NETO. Si la mutación *cdc11-123* se combina con la mutación en una proteína marcadora de la polaridad, se producen células áltamente ramificadas, que tienen dificultad al elegir el nuevo sitio de crecimiento polarizado. Nosotros no observamos células ramificadas en el mutante doble *cdc11-123 rgf1* Δ, lo que indica de nuevo que Rgf1p no es necesario para determinar los sitios de crecimiento.

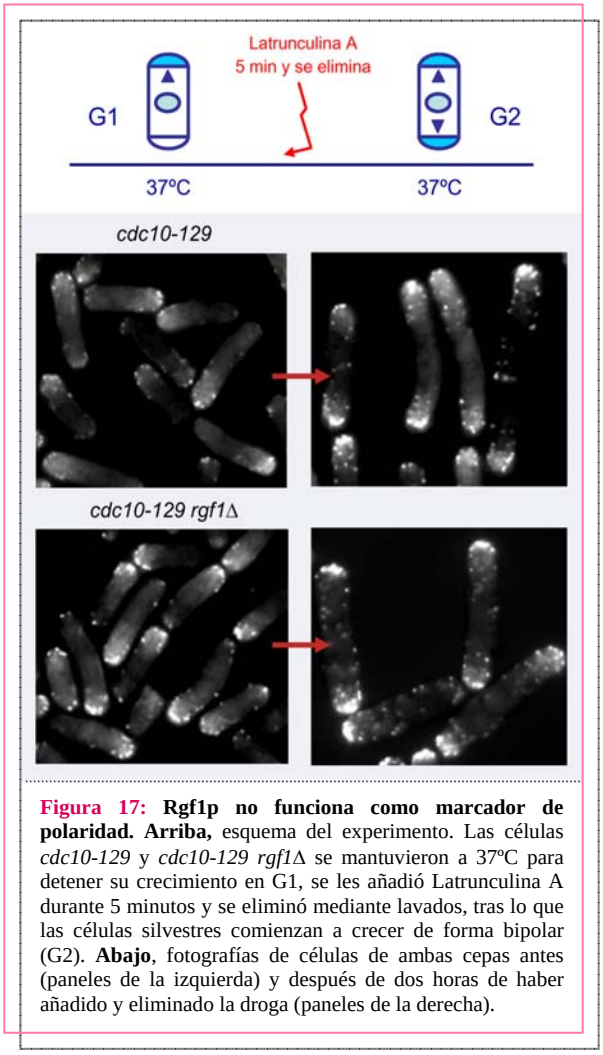


Figura 17: Rgf1p no funciona como marcador de polaridad. Arriba, esquema del experimento. Las células *cdc10-129* y *cdc10-129 rgf1*Δ se mantuvieron a 37ºC para detener su crecimiento en G1, se les añadió Latrunculina A durante 5 minutos y se eliminó mediante lavados, tras lo que las células silvestres comienzan a crecer de forma bipolar (G2). **Abajo**, fotografías de células de ambas cepas antes (paneles de la izquierda) y después de dos horas de haber añadido y eliminado la droga (paneles de la derecha).

2.5. Localización de factores de polaridad en el mutante *rgf1Δ*

El desencadenamiento y mantenimiento del crecimiento bipolar en *S. pombe* es un proceso complejo en el que intervienen un gran número de proteínas. Algunas de ellas, como Tea1p y Pom1p, actúan como marcadores de polaridad indicándole a la célula dónde se encuentran sus polos de crecimiento. Estas dos proteínas se localizan en ambos polos,

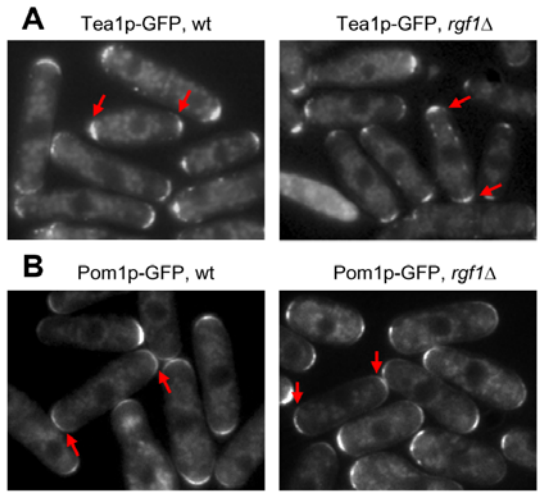


Figura 18: Localización de Tea1p-GFP y Pom1p-GFP en *rgf1* Δ. A Localización de Tea1p-GFP en las cepas silvestre (wt) y *rgf1*Δ. En ambas se localiza en los dos polos celulares. **B** Localización de Pom1p-GFP en las cepas silvestre y *rgf1*Δ. En ambas se localiza en los dos polos celulares.

incluso cuando las células están creciendo sólo por uno (Bähler and Pringle, 1998; Mata and Nurse, 1997).

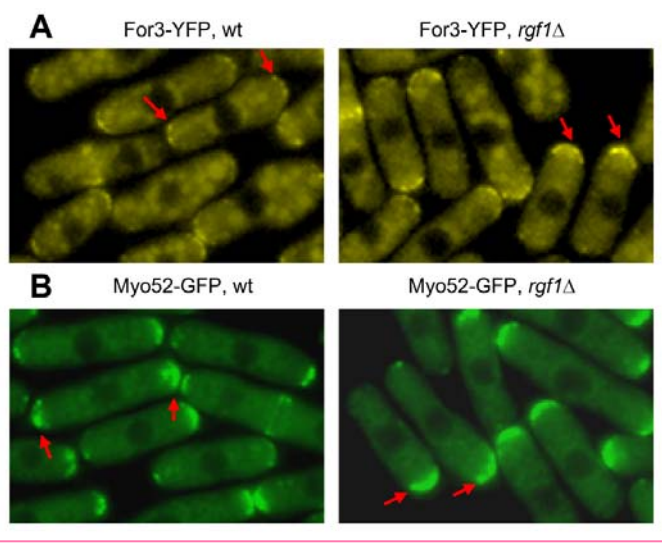
Tea1p y Tea4p son transportadas a los polos por los extremos de los microtúbulos. Tea4p recluta a Bud6p y a For3p, formando un complejo llamado polarisoma, que es necesario y suficiente para el establecimiento de la polaridad y el ensamblaje de la actina en el polo de crecimiento (Martin et al., 2005).

Otras proteínas, como las miosinas y los componentes del complejo Arp2/3 (relacionados con la formación de los parches de actina) se localizan siempre en el polo que está creciendo activamente (Arai et al., 1998; Sirotkin et al., 2005; Win et al., 2001). Finalmente, algunos de los últimos responsables del crecimiento del polo nuevo, como las distintas subunidades de la β- y α-glucán sintasa, también se localizan de manera polarizada (Cortés et al., 2005; Cortés et al., 2002; Liu et al., 2002; Martín et al., 2003).

Cuando intentamos definir en qué paso del proceso de activación de NETO estaba interviniendo Rgf1p, nos preguntamos si la localización correcta de Tea1p, Pom1p y For3p dependía de la presencia o ausencia de Rgf1p y viceversa, es decir cómo se localizaba Rgf1p en estos mutantes.

Comenzamos estudiando la localización de las proteínas Tea1p-GFP y Pom1p-GFP en el mutante *rgf1* Δ. Observamos que tanto Tea1p como Pom1p se localizan en ambos polos en los mutantes *rgf1*Δ, igual que en la cepa silvestre (**Figura 18**). La ausencia de Rgf1p no parece afectar a la localización de estas dos proteínas. Por el contrario, Rgf1p se localiza de forma monopolar en los mutantes *tea1*Δ y *pom1*Δ (datos no mostrados).

A continuación, quisimos ver qué ocurría con la formina For3p marcada con la proteína fluorescente amarilla (YFP). For3p-YFP se localiza normalmente con una distribución punteada en la zona del septo y los polos que están creciendo. En el mutante *rgf1*Δ, se observa un aumento en la distribución monopolar de For3p, presentándose de forma bipolar en menos del 20% de las células, mientras que en una cepa silvestre este porcentaje es del 75% (**Figura 19A**). Esto nos indicó que Rgf1p es importante para el desplazamiento de la formina al polo nuevo, lo cual está de acuerdo con la ausencia de nucleación de actina en ese polo. Cuando analizamos la localización de Rgf1p-GFP en la cepa *for3*Δ, observamos que Rgf1p se localiza en los polos de crecimiento activo en ese mutante (datos no mostrados).



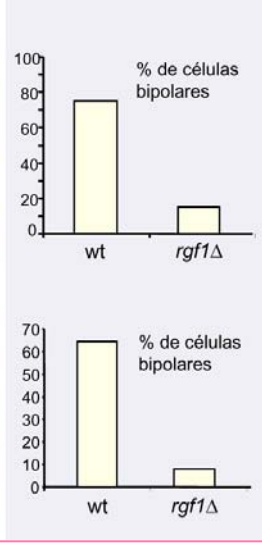
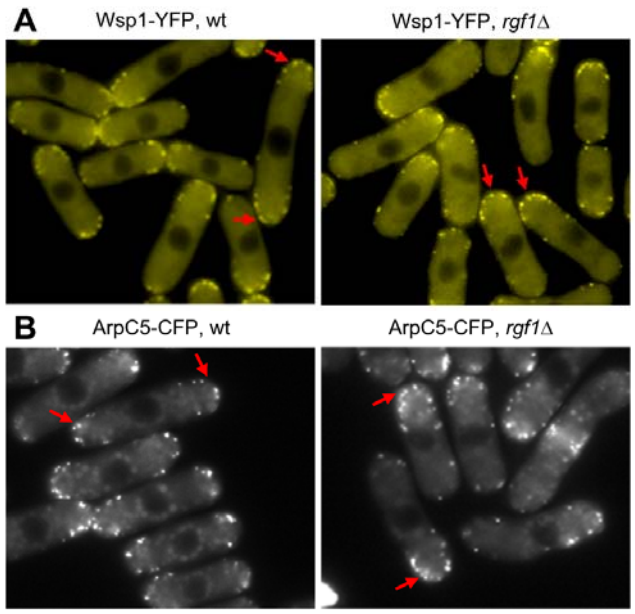


Figura 19: Localización de For3p-YFP y Myo52p-GFP en *rgf1*Δ. A Localización de For3p-YFP en las cepas silvestre (wt) y *rgf1*Δ. En la gráfica se representa el porcentaje de células con localización bipolar en ambas cepas. En el mutante *rgf1*Δ este porcentaje es mucho menor que en las células silvestres. **B** Localización de Myo52p-GFP en las cepas silvestre y *rgf1*Δ. En la gráfica se representa lo mismo que en A. En este caso también hay una disminución de la localización bipolar en las células *rgf1*Δ.



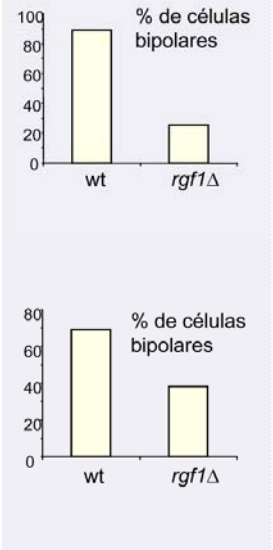


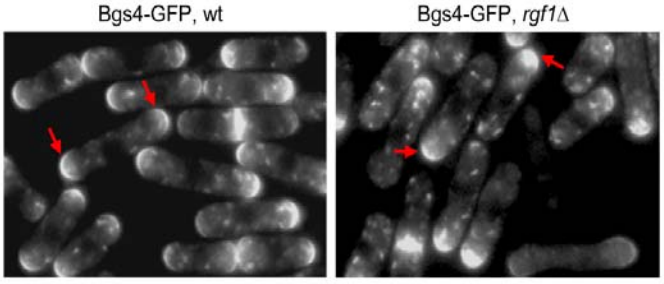
Figura 20:
Localización de Wsp1p-YFP y ArpC5-CFP en *rgf1Δ*. **A** Localización de Wsp1p-YFP en las cepas silvestre (wt) y *rgf1*Δ. En la gráfica se representa el porcentaje de células con localización bipolar en ambas cepas. En el mutante *rgf1*Δ este porcentaje es mucho menor que en las células silvestres. **B** Localización de ArpC5p-CFP en las cepas silvestre y *rgf1*Δ. En la gráfica se representa lo mismo que en A. En este caso también hay una disminución de la localización bipolar en las células *rgf1*Δ.

Exactamente lo mismo ocurre con la miosina de tipo V, Myo52p. Este tipo de miosinas son importantes para el transporte de las vesículas secretoras a lo largo de los cables de actina. Al igual que las proteínas anteriores, se puede detectar en el septo y los polos en crecimiento. Se sabe, que en el mutante *for3*Δ esta miosina se deslocaliza completamente (Feierbach and Chang, 2001). Curiosamente, en el mutante *rgf1*Δ esto no ocurre, aunque su localización es claramente monopolar (**Figura 19B**), sólo se distribuye de forma bipolar en el 10% de las células, frente a un 60% en el silvestre.

Las proteínas del complejo Arp2/3, Wsp1p y ArpC5p (marcadas con YFP y CFP, respectivamente) se comportan de una manera muy similar a For3p y Myo52p: en el silvestre muestran una distribución bipolar generalizada, mientras que en el mutante *rgf1*Δ son monopolares (**Figura 20**).

Finalmente, observamos la localización de uno de los últimos factores responsables del crecimiento del polo, la subunidad catalítica de la βGS, Bgs4p. Como en los casos anteriores, esta proteína se localiza en el septo y los polos en células silvestres, sin embargo en ausencia de Rgf1p presenta una localización monopolar en la gran mayoría de las células observadas (**Figura 21**).

Todos estos datos sugieren que una vez determinado el polo nuevo como lugar de crecimiento, Rgf1p podría ser un elemento clave en la ruta de señalización desencadenante del crecimiento en ese polo. Cuando Rgf1p no está presente, las células no recibirían esta señal y las proteínas encargadas de que se lleve a cabo el crecimiento efectivo en ese punto, no se desplazarían hasta él o si se desplazaran no llegarían a formar una masa estable y por consiguiente no habría crecimiento.



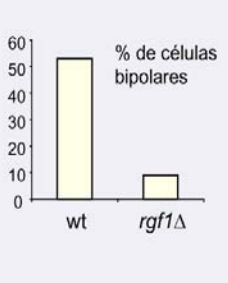


Figura 21:
Localización de Bgs4p-GFP en *rgf1Δ*. Localización de Bgs4p-GFP en las cepas silvestre (wt) y *rgf1*Δ. En la gráfica se representa el porcentaje de células con localización bipolar en ambas cepas. En el mutante *rgf1*Δ este porcentaje es mucho menor que en las células silvestres.

CAPÍTULO III

El GEF de Rho1p, Rgf1p, señaliza por encima de la cascada de proteínas quinasas activadas por mitógeno de Pmk1p en la levadura de fisión

Antecedentes

Las rutas de MAPKs (*mitogen-activated protein kinase*) son mecanismos de transducción de señales que regulan muchos procesos importantes en los organismos eucariotas.

El mecanismo básico de funcionamiento de estas cascadas consiste en la activación secuencial de tres proteínas quinasas en respuesta a determinados estímulos. En primer lugar, una MAPKKK (MAPK *kinase kinase*) fosforila y activa a una MAPKK (MAPK *kinase*), que a su vez fosforila a una MAPK (Marshall, 1995; Waskiewicz and Cooper 1995).

Mientras que en la levadura de gemación hay seis o más cascadas de MAPKs (Hohmann, 2002), en *S. pombe* sólo se conocen tres: una de respuesta a feromonas, otra de respuesta a estrés, cuyos elementos centrales son las MAPKs Spk1p y Sty1p/Spc1p respectivamente (Shiozaki and Russell, 1995b; Toda et al., 1991) y finalmente, la encargada del mantenimiento de la integridad celular.

La ruta de integridad está compuesta por un módulo de quinasas formado por una MAPKKK denominada Mkh1p, una MAPKK Pek1p/Shk1p y una MAPK Pmk1p/Spm1p (Loewith et al., 2000; Sengar et al., 1997; Sugiura et al., 1999; Toda et al., 1996b; Zaitsevskaya-Carter and Cooper, 1997). Se ha descrito al menos una fosfatasa capaz de desfosforilar a la MAPK Pmp1p y que regula negativamente esta ruta de señalización (Sugiura et al., 1998).

La disrupción de cualquiera de los tres genes que forman el módulo de MAPKs causa hipersensibilidad a β(1,3)-glucanasas y sensibilidad a estrés hiperosmótico con células ramificadas y multiseptadas. Estos datos fueron los primeros que relacionaron esta ruta con los procesos de construcción de la pared celular, citoquinesis y homeostasis iónica en *S. pombe*. Más tarde, se descubrió que la ruta de integridad es capaz de responder a una gran variedad de estreses, incluyendo estrés hiper e hipotónico, ausencia de glucosa, presencia de compuestos que dañan la pared celular, altas temperaturas y estrés oxidativo, entre otros (Madrid et al., 2006).

Una característica que presentan los mutantes de los genes implicados en esta ruta, es su capacidad de contrarrestar los efectos de la eliminación de la calcineurina. En *S. pombe* se sabe que la eliminación del gen de la calcineurina, *ppb1*$^+$, o la inhibición de su actividad mediante inmunosupresores (como FK506), produce hipersensibilidad al Cl^-, mientras que una mutación adicional en los miembros de la ruta de MAPKs suprime este fenotipo.

Basándose en esta interacción se ha descrito el denominado fenotipo *vic* (*viable in the presence of immunosuppressant and choride ion*), y se ha visto que todos los mutantes nulos de los componentes conocidos de la cascada de integridad presentan este fenotipo, es decir, son viables en presencia del inmunosupresor FK506 y altas concentraciones de $MgCl_2$ (Sugiura et al., 1999; Sugiura et al., 1998).

El gen *cpp1*$^+$ se identificó a partir de un mutante con fenotipo *vic* y codifica una farnesiltransferasa cuya función es favorecer la localización en la membrana de la GTPasa Rho2p (Ma et al., 2006). A partir de ahí, se han descrito otras dos proteínas que actúan por encima de este módulo de MAPKs. Una de ellas es Pck2p que actúa por encima de la MAPKKK Mkh1p e interacciona físicamente con ella y la otra es Rho2p que también interacciona con Pck2p (Calonge et al., 2000; Ma et al., 2006). La sobreexpresión de *rho2*$^+$ es viable en el mutante *pck2*Δ, y la sobreexpresión de ambos genes, *pck2*$^+$ y *rho2*$^+$ por separado, es letal en células silvestres, pero no lo es en los mutantes *mkh1*Δ, *pek1*Δ y *pmk1*Δ. Además, se ha visto que la sobreexpresión y la disrupción de estos dos genes, *pck2*$^+$ y *rho2*$^+$, hace que aumente o disminuya sustancialmente el grado de fosforilación de Pmk1p (Barba et al., 2008; Ma et al., 2006).

No todos los estreses parecen activar la cascada de la misma manera, ni a través de los mismos activadores, Rho2p y Pck2p, sino que existe una compleja red de señalización que modula la activación de Pmk1p en respuesta a diferentes tipos de estrés (Barba et al., 2008).

Trabajo Experimental

1. Pck2p y Pmk1p actúan por debajo de Rgf1p

Como ya se ha descrito en el Capítulo I, Rgf1p actúa como GEF de la GTPasa Rho1p y dos de los principales efectores de Rho1p son las proteínas quinasas Pck1p y Pck2p. Estas dos proteínas comparten funciones esenciales en la viabilidad celular y se complementan mutuamente de forma parcial (Toda et al., 1993). Ya habíamos visto que la expresión moderada de *pck1*$^+$ (multicopia y con su propio promotor) suprime la hipersensibilidad a Caspofungina (Csp) del mutante nulo *rgf1*Δ, pero posteriormente descubrimos que también la sobreexpresión de *pck2*$^+$ era capaz de suprimir este fenotipo.

Pck2p forma parte de la cascada de MAPKs de Pmk1p (Ma et al., 2006) por lo que nos propusimos comprobar si Rgf1p podría interaccionar funcionalmente con la MAPK de la ruta. El resultado fue positivo, ya que la sobreexpresión de *pmk1*$^+$ también suprimió la hipersensibilidad a Csp de la cepa *rgf1*Δ.

Estos fueron los primeros datos que nos

indicaron que Rgf1p podría estar participando en la ruta de MAPKs encargada del mantenimiento de la integridad de *S. pombe*.

2. Las células *rgf1*Δ presentan el fenotipo *vic*

Los mutantes *rgf1*Δ y los mutantes de la cascada de integridad muestran el denominado fenotipo *vic*, es decir, son viables en presencia de FK506 y altas concentraciones de $MgCl_2$ (Sugiura et al., 1999; Sugiura et al., 1998). Además, también vimos que la cepa *rgf1*Δ y los mutantes en los genes de la cascada (*mkh1*Δ, *pek1*Δ/*mkh1*Δ y *pmk1*Δ) compartían otros defectos. Todos ellos son sensibles a β-glucanasas, incapaces de crecer en medio con alta concentración de sal y en presencia de Caspofungina.

Estos datos podrían indicar que estas proteínas participan en la misma vía de señalización, pero además comprobamos que los mutantes dobles *rgf1*Δ *mkh1*Δ, *rgf1*Δ*skh1*Δ y *rgf1*Δ*pmk1*Δ, son viables y que no muestran sinergia en cuanto a la hipersensibilidad a Csp.

3. Rgf1p participa en la activación de Pmk1p

Para confirmar si Rgf1p activa y transmite la señal a través de Pmk1p, examinamos los niveles de fosforilación de esta MAPK en respuesta a diferentes tipos de estrés, en un mutante *rgf1*Δ. El resultado fue muy claro, en ausencia de $rgf1^+$ apenas se produjo fosforilación de Pmk1p en respuesta a cambios en la osmolaridad y daños en la pared celular. Sin embargo, Rgf1p no parece ser importante en la respuesta a otros estreses que también activan a Pmk1p, como estrés oxidativo, ausencia de glucosa o calor.

Rgf1p es pues un componente de la cascada de integridad, esencial para transmitir la señal sólo bajo determinadas circunstancias. Además activa específicamente esta cascada de MAPKs, puesto que no observamos diferencias entre el mutante *rgf1*Δ y la cepa silvestres en la fosforilación de la MAPK de la ruta de estrés Spc1p, en respuesta a estrés oxidativo u osmótico.

4. Rho1p participa en la activación de Pmk1p

Nos preguntamos si la actividad GEF (intercambiador de nucleótidos de guanina) de Rgf1p, era importante para llevar a cabo la transmisión de la señal hacia el módulo de MAPKs de esta cascada. Para ello, utilizamos un mutante puntual en una región muy conservada del dominio RhoGEF de Rgf1p. La proteína mutada en el dominio Rho-GEF es estable y presenta unos niveles muy bajos de Rho1p-GTP (activo). En cuanto a la fosforilación de la MAPK Pmk1p en respuesta a estrés osmótico, el mutante puntual se comportó igual que la cepa delecionada, indicando que la actividad GEF de Rgf1p es imprescindible para llevar a cabo su función dentro de la ruta de integridad.

Rgf1p es un regulador positivo de Rho1p, por lo que también analizamos si esta GTPasa podría estar involucrada en la activación de Pmk1p, en respuesta a determinados tipos de estrés. La respuesta es afirmativa, tanto la sobreexpresión de $rho1^+$ como de *rho1G15V* (alelo constitutivamente activo) aumentan los niveles de Pmk1p fosforilado, y lo hacen de una forma dependiente de Pck2p y de Mkh1p.

Todos estos datos puestos en conjunto, sugieren que la cascada Rgf1p-Rho1p-Pck2p regula la activación de Pmk1p en *S. pombe*, y que lo hace a través del módulo de MAPKs.

5. Rgf2p y Rgf3p no participan en la activación de Pmk1p

Por último, examinamos la posible contribución de otros GEFs conocidos de Rho1p (Rgf2p y Rgf3p) en la activación de la cascada de integridad.

Tanto la sobreexpresion de $rgf2^+$ como la de $rgf3^+$ produjeron un aumento de la fosforilación de Pmk1p. Esto podría ser consecuencia del aumento en la cantidad de Rho1p activo en la célula (que ya sabemos que activa la cascada) o debido a un papel más específico de estas proteínas dentro de la cascada de integridad. Para diferenciar entre estas dos posibilidades, comprobamos si los mutantes *rgf2*Δ y *ehs2-1* (afectado en el gen $rgf3^+$) presentaban algún defecto en la activación de Pmk1p en respuesta a una amplia variedad de estreses. Los resultados fueron negativos en todos los casos, por lo que podemos concluir, que Rgf1p es el único GEF conocido de Rho1p que participa en la transmisión de la señal por la cascada de MAPKs de Pmk1p en *S. pombe*.

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The Rho1p Exchange Factor Rgf1p Signals Upstream from the Pmk1 Mitogen-activated Protein Kinase Pathway in Fission Yeast

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The *Schizosaccharomyces pombe* exchange factor Rgf1p specifically regulates Rho1p during polarized growth. Rgf1p activates the β-glucan synthase (GS) complex containing the catalytic subunit Bgs4p and is involved in the activation of growth at the second end, a transition that requires actin reorganization. In this work, we investigated Rgf1p signaling and observed that Rgf1p acted upstream from the Pck2p-Pmk1p MAPK signaling pathway. We noted that Rgf1p and calcineurin play antagonistic roles in Cl^- homeostasis; *rgf1*Δ cells showed the *vic* phenotype (viable in the presence of immunosuppressant and chlorine ion) and were unable to grow in the presence of high salt concentrations, both phenotypes being characteristic of knockouts of the MAPK components. In addition, mutations that perturb signaling through the MAPK pathway resulted in defective cell integrity (hypersensitivity to caspofungin and β-glucanase). Rgf1p acts by positively regulating a subset of stimuli toward the Pmk1p-cell integrity pathway. After osmotic shock and cell wall damage HA-tagged Pmk1p was phosphorylated in wild-type cells but not in *rgf1*Δ cells. Finally, we provide evidence to show that Rgf1p regulates Pmk1p activation in a process that involves the activation of Rho1p and Pck2p, and we demonstrate that Rgf1p is unique in this signaling process, because Pmk1p activation was largely independent of the other two Rho1p-specific GEFs, Rgf2p and Rgf3p.

INTRODUCTION

Fission yeast cells display a simple rod shape; after cytokinesis, growth is initiated monopolarly and occurs exclusively at the old end. After a point in G2, cells initiate growth from the new end in a process known as new end take-off (NETO), such that they grow in bipolar mode up to mitosis (Mitchison and Nurse, 1985). The spatial control of cell growth in the fission yeast *Schizosaccharomyces pombe* involves organization of the microtubule and actin cytoskeletons as well as that of the cell wall synthesis (Chang, 2001; Hayles and Nurse, 2001). A polarized actin cytoskeleton targets secretion to the growth sites, where probably specialized multienzyme complexes, consisting of both synthases and hydrolases, assemble the cell wall during the cell cycle (Motegi *et al.,* 2001; Win *et al.,* 2001; Mulvihill *et al.,* 2006). Both the cytoskeleton and the cell wall are dynamic structures that are constantly remodelled and reorganized in response to growth signals and environmental stresses in order to ensure the integrity of the yeast cell (Levin, 2005; Madrid *et al.,* 2006).

In mammals, the polarized assembly of the actin and microtubule cytoskeletons is regulated by site-specific activation of Rho-type GTPases (Jaffe and Hall, 2005; Ridley, 2006). In fission yeast cells, Rho1p is involved in cell wall synthesis (Arellano *et al.,* 1996), actin organization (Arellano *et al.,* 1997; Nakano *et al.,* 1997), stress responses, and exocytosis. In both systems, one of the challenges is to figure out how the activity of Rho1p is regulated to control these different processes. Guanine nucleotide exchange factors (GEFs) activate signaling by promoting the exchange of GDP by GTP, and GTPase-activating proteins (GAPs) arrest signaling by stimulating GTP hydrolysis to GDP. This network (Rho GTPases, GEFs, and GAPs) relays a surprisingly large number of diverse extracellular signals to many morphological and functional responses. The regulation of Rho1p in fission yeast involves at least three GEFs (Rgf1p, Rgf2p, and Rgf3p) and several putative GAPs (Rga1p, Rga5p, and Rga8p). *rgf3*$^+$ is an essential gene and the protein specifically activates Rho1p during cytokinesis (Tajadura *et al.,* 2004; Morrell-Falvey *et al.,* 2005; Mutoh *et al.,* 2005). Rgf1p and Rgf2p are the closest relatives, and they provide a redundant function for the activation of Rho1p (Mutoh *et al.,* 2005). Loss of Rgf1p function produces cell lysis, whereas loss of both Rgf1p and Rgf2p is lethal. In addition, Rgf2p may perform an essential function during the sporulation process (García *et al.,* 2006b). Among the negative regulators, none of them is essential for cell viability, although deletion of *rga1*$^+$ causes a slow-growth defect and severe morphological abnormalities. Rga5p is involved in the regulation of GS activity and cell integrity, and Rga8p is a Shk1p (Cdc42/p21-activated kinase) substrate that negatively regulates Shk1p-dependent growth control pathways (Calonge *et al.,* 2003; Yang *et al.,* 2003).

In this study we focused on Rgf1p. Like most Rho-GEFs, Rgf1p contains a domain with strong similarity to the Dbl-family of exchange factors (residues aa 625–807, Dbl-homology domain[DH]) and a nearby pleckstrin-homology (PH) domain (residues 844–973). The DH-PH tandem is responsible for the activation of Rho-family GTPases in response to

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diverse extracellular stimuli (reviewed in Rossman *et al.*, 2005; Rossman and Sondek, 2005). Rgf1p also contains a *Dishevelled*, Egl-10, and pleckstrin (DEP) domain at its amino-terminus (residues 424–497), which in some G-protein receptors has been implicated in mediating the nature and sustainability of the response (Chen and Hamm, 2006). Finally, Rgf1p has a Citron and NIK1-like kinase homology-domain (CNH) at its carboxy-terminus (residues 997-1293). Its function is not clear, but in most cases it acts as a regulatory domain involved in macromolecular interactions (http://www.genedb.org/genedb/pombe/index.jsp).

We previously demonstrated that Rgf1p plays an important role in regulating the growth pattern of fission yeast cells. After cell division, cells initially grow in a monopolar manner, after which they initiate polarized growth at the second end in the G2 phase of the cell cycle. This transition to bipolar growth, termed NETO, relies on the localization of Rgf1p to the new end (García *et al.*, 2006a). In this process, the position of Rgf1p depends on Tea1p, which is necessary for NETO. Interestingly, although other mutants defective in bipolar growth, *tea1*Δ, *tea4*Δ, and *bud6*Δ, grow at wild-type rates, a novel aspect of the *rgf1*Δ is that its growth rate and viability are compromised. In *rgf1*Δ cells, failure to initiate bipolar growth coincides with cell lysis, thus coupling a growth polarity transition with cell wall biosynthesis. Our current model is that activation of Rho1p during bipolar growth is not achieved properly in *rgf1*Δ mutants, producing cell wall weakness. Rho1p localizes to sites of polarized growth and participates directly in the production of new cell wall by functioning as the regulatory subunit of the β-1,3-glucan synthase (GS) that synthesizes glucan, the main component of the cell wall (Arellano *et al.*, 1996, 1997; Nakano *et al.*, 1997). However, it has not been tested whether Rho1p also regulates the expression of cell integrity-related genes or actin genes via the Pmk1p mitogen-activated protein kinase (MAPK) cell integrity signaling pathway.

The Pmk1p MAPK from fission yeast is very similar to the budding yeast Mpk1p/Slt2p, which plays a central role in cell integrity signaling (Levin, 2005), and to the extracellular signal-regulated kinases, ERK1/2 (p42/p44), from animal cells that are activated by phorbol esters, cytokines, or osmotic stress (Roux and Blenis, 2004). In fission yeast, the Pmk1p MAPK pathway also regulates morphogenesis and ion homeostasis and becomes activated under multiple stresses, including hyper- or hypotonic conditions, the presence of cell wall–damaging compounds, glucose deprivation, and oxidative stress (Madrid *et al.*, 2006). The MAPK module is composed of MAPKKK Mkh1p (Sengar *et al.*, 1997), MAPKK Pek1p/Shk1p (Sugiura *et al.*, 1999; Loewith *et al.*, 2000), and MAPK Pmk1p/Spm1p (Toda *et al.*, 1996b; Zaitsevskaya-Carter and Cooper, 1997). Although this module was identified several years ago, little is known about its upstream components or the phosphorylation substrates activated by the cell integrity signaling pathway (Ma *et al.*, 2006; Takada *et al.*, 2007). The MAPKKKs at the head of the module are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli (Roux and Blenis, 2004). In fission yeast, Rho1p binds directly to the Pck1p and Pck2p protein kinases of the PKC family, and it functions as a positive regulator for these kinases (Arellano *et al.*, 1999b; Sayers *et al.*, 2000). Recently it has been shown that Pck2p interacts with Mkh1p (MAPKKK) and activates the Pmk1p signaling pathway (Ma *et al.*, 2006). Here, we show that Rgf1p, a Rho1p-specific GEF, acts upstream from the Pck2-Pmk1p MAPK cell integrity signaling pathway. More importantly, our results suggest that Rgf1p is necessary for the signal transduction of a subset of stimuli, in particular those related to changes in osmolarity and cell wall damage.

MATERIALS AND METHODS

Media, Reagents, and Genetics

The genotypes of the *S. pombe* strains used in this study are listed in Table 1. The complete yeast growth medium (YES), selective medium (MM) supplemented with the appropriate requirements, and sporulation medium (MEA) have been described elsewhere (Moreno *et al.*, 1991). Caspofungin (Csp; Vicente *et al.*, 2003) was stored at [minusS]20°C in a stock solution (2.5 mg/ml) in H_2O and was added to the media at the corresponding final concentration after autoclaving. Crosses were performed by mixing appropriate strains directly on MEA plates. Recombinant strains were obtained by tetrad analysis or the "random spore" method. For overexpression experiments using the *nmt1* promoter, cells were grown in EMM containing 15 μM thiamine up to logarithmic phase. Then, the cells were harvested, washed three times with water, and inoculated in fresh medium (without thiamine) at an OD_{600} = 0.01 for 14, 16, or 18 h, depending on the experiment.

Plasmid and DNA Manipulations

pREP3X contains a thiamine-repressible *nmt1* promoter (full-strength, induction ratio, 300×), *Saccharomyces cerevisiae LEU2*, and *ars1*$^+$ (Forsburg, 1993); to overexpress *rgf1*$^+$, an Xho1I-SmaI fragment containing the *rgf1*$^+$ gene was ligated into the XhoI-SmaI sites of plasmid pREP3X (García *et al.*, 2006a). pREP3X-*pck2*$^+$ (*nmt1-pck2*$^+$) was made by inserting the entire ORF of *pck2*$^+$ cloned by PCR into pREP3X. pREP3X-*pck1* (*nmt1-pck1*$^+$) was made by inserting the entire ORF of *pck1*$^+$ cloned by PCR into pREP3X. pREP41X-*pck2* (*nmt41x-pck2*) was made by inserting the entire ORF of *pck2*$^+$ cloned by PCR into pREP41X (induction ratio, 25×; Arellano *et al.*, 1999b). For pREP3X-*rho1*, the ORF of Rho1p was amplified by PCR from a cDNA library and cloned into pREP3X; for pREP3X-*rho1*-G15V, the *rho1* ORF was mutagenized by site-directed mutagenesis and subcloned as a SalI-BamHI fragment into the same sites of pREP3X (Arellano *et al.*, 1997). *pck2*$^+$ and *rho1*$^+$ overexpression plasmids were kindly provided by P. Pérez (IMB, Salamanca, Spain). To make pART-*spm1*$^+$, the Spm1p ORF was cloned by PCR behind the *adh* promoter in pART1. pART-*spm1*$^+$ was kindly provided by J. Cooper (Washington University, St. Louis; Zaitsevskaya-Carter and Cooper, 1997).

Glucanase Sensitivity

The glucanase sensitivities of several mutant strains were determined as indicated in Carnero *et al.* (2000). Cells were grown in MM to an optical density at 600 nm (OD_{600}) of 1.0, washed in 10 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA, and 1 mM β-mercaptoethanol, and incubated in the same buffer containing 20 μg/ml β-glucanase (Zymolyase 100T; Seikagaku, Tokyo, Japan) per ml at 28°C with vigorous shaking. The OD_{600} was monitored at the indicated times and was normalized relative to the absorbance of a control sample of each strain without enzyme at each time point.

Stress Treatments

Experiments designed to investigate Pmk1p activation under stress were performed using log-phase cell cultures (OD_{600} of 0.5) grown at 28°C in YES medium and the appropriate stress treatment. In overexpression experiments, cells were first grown in MM medium plus thiamine, washed three times, and reinoculated into fresh medium (with or without thiamine) for 14, 16, or 18 h at 28°C, depending on the protein to be overexpressed. In glucose-deprivation experiments, cells were grown in YES medium with 7% glucose to an OD_{600} of 0.5, recovered by filtration, and resuspended in the same medium without glucose but equilibrated osmotically with 3% glycerol. Hypotonic treatment was achieved by growing cells in YES medium plus 0.8 M sorbitol and then transferring them to the same medium without polyol. In all cases, 30 ml of culture was harvested by filtration and immediately frozen in liquid nitrogen for analysis.

Purification and Detection of Activated Pmk1p-HA6H and Sty1p-HA6H after Different Stresses

Cell homogenates were prepared under native conditions employing chilled acid-washed glass beads and lysis buffer (10% glycerol, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Nonidet P-40, plus a specific protease inhibitor cocktail: 100 μM *p*-aminophenyl methanesulfonyl fluoride, leupeptin, and aprotinin). The lysates were cleared by centrifugation at 13,000 × *g* for 10 min and Pmk-HA6H was purified with Ni^{2+}-NTA-agarose beads (Novagen, Madison, WI). The purified proteins were loaded on 10% SDS-PAGE gels, transferred to an Immobilon-P membrane (Millipore, Bedford, MA), and blotted to detect Pmk1-HA with 1:5000 diluted 12CA5 mAb as primary antibody (Roche, Indianapolis, IN), with polyclonal rabbit anti-phospho-p42/44 antibodies (1:2500; Cell Signaling, Beverly, MA), or with polyclonal rabbit anti-phospho-p38 antibodies (1:8000; Cell Signaling). The immunoreactive bands

Table 1. *S. pombe* strains used in this work

Strains	Genotypes
YSM180	h$^-$ 972
YS64	h$^-$ leu1-32 ade6M210 ura4D-18 his3D1
HVP54[a]	h$^-$ leu1-32 ade6M210 ura4D-18
VT14	h$^-$ leu1-32 ade6M210 ura4D-18 his3D1rgf1::his3$^+$
VT18	h$^+$ leu1-32 ade6M210 ura4D-18 his3D1rgf1::his3$^+$
PG65	h$^-$ leu1-32 ade6M210 ura4D-18 his3D1rgf1::kanMX6
MI200[b]	h$^+$ pmk1-HA6his: ura4$^+$ ade6M216 leu1-32 ura4d18
PG285	h$^-$ rgf1::kan pmk1-HA6his:ura$^+$ ura4D18 leu1-32 ade6M210
PG318	h$^-$ mkh1::ura4$^+$ pmk1-HA6his:ura$^+$ ura4D18 leu1-32 ade6M210
PG332	h$^-$ pck2::kan pmk1-HA6his:ura$^+$ ura4D18 leu1-32
MS192[c]	h$^-$ spm1::LEU2 leu1-32 ura4D18
MS189[d]	h$^+$ skh1::ura4$^+$ ura4D18 leu1-32 ade6M210
MS196[d]	h$^+$ mkh1::ura4$^+$ ura4D18 ade6M210
YKOB01[e]	h$^-$ spm1::ura4$^+$ ura4D18 leu1-32
PG335	h$^-$ spm1::ura4$^+$ rgf1::kan ura4d18
PG337	h$^-$ mkh1::ura4$^+$ rgf1::kan ura4d18
PG339	h$^-$ skh1::ura4$^+$ rgf1::kan ura4d18
PG347	h$^-$ pmk1::ura4$^+$ ura4d18
PG348	h$^-$ mkh1::ura4$^+$ ura4d18
PG349	h$^-$ skh1::ura4$^+$ ura4d18
PG77	h$^+$ rgf1::kan ade6M210
PG272[f]	h$^-$ pck2::kan leu1-32 ura4D18
VT20[f]	h$^-$ rho2::ura4$^+$ ura4D18 leu1-32 ade6M210
PG1	h$^-$ rgf2::ura4$^+$ ura4D18 leu1-32 ade6M210 his3D1
MS190[h]	h$^-$ wis1::his3$^+$ his3D1
MS194[h]	h$^-$ spk1::ura4$^+$ ura4D18 leu1-32
YS1295[f]	h$^+$ pmp1::kan leu1-32 ade6M210 ura4D-18
PG351	h$^-$ pmp1::kan rgf1::kan leu1-32 ade6M210 ura4D-18
KS1489 [g]	h$^+$ spc1-HA6his:ura4$^+$ leu1-32 ura4D-18
PG362	h$^-$ spc1-HA6his:ura4$^+$ rgf1::kan leu1-32 ura4D-18
PG40	h$^-$ rgf1::his3 his3DI leu1-32 ade6M210 ura4D-18 leu1: rgf1$^+$-GFP
PG52	h$^-$ rgf1::his3 his3DI leu1-32 ade6M210 ura4D-18 leu1: rgf1$^+$ (PTTRΔ)
PG199	h$^-$ rgf1::his3 his3DI leu1-32 ade6M210 ura4D-18 leu1: rgf1$^+$ (PTTRΔ)-GFP
PPG160[f]	h$^-$ rho1:HA leu1-32
PG378	h$^-$ rho1:HA leu1-32rgf1::kanMX6
PG380	h$^-$ rho1:HA leu1-32rgf1::kanMX6 leu1: rgf1$^+$ (PTTRΔ)
PG287	h$^-$ pmk1-HA6his: ura4$^+$ ade6M216 leu1-32 ura4d18 rgf2::ura4$^+$
PG359	h$^-$ ehs2-1 pmk1-HA6his: ura4$^+$ leu1-32 ura4d18
PG331	h$^-$ pmk1-HA6his: ura4$^+$ leu1-32 ura4d18 his3D1rgf1::his3$^+$ leu1: rgf1$^+$ (PTTRΔ)

All strains were generated in this study except for the following strains: [a] from H. Valdivieso (IMB, University of Salamanca), [b] from J. Cansado (University of Murcia), [c] from J. Cooper (Washington University, St. Louis), [d] from D. Young (University of Calgary, Canada), [e] from C. R. Vazquez de Aldana (IMB, University of Salamanca), [f] from P. Perez (IMB, University of Salamanca), [g] from M. A. Rodriguez-Gabriel (UCM, University Complutense Madrid), and [h] from S. Moreno (CIC, University of Salamanca).

were revealed with anti-mouse or anti-rabbit HRP secondary antibodies (Bio-Rad, Hercules, CA) and the enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ).

Pulldown Assays for GTP-bound Rho Proteins

The expression vector pGEX-C21RBD (rhotekin-binding domain; Reid *et al.*, 1996) was used to transform *Escherichia coli* cells. The fusion protein was produced according to the manufacturer's instructions and immobilized on glutathione-Sepharose 4B beads (Amersham). After incubation, the beads were washed several times, and the bound proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. The amount of GTP-bound Rho proteins was analyzed using the Rho-GTP pulldown assay modified from Ren *et al.* (1999). Briefly, extracts from 50-ml cultures of wild-type, *rgf1*Δ, and *rgf1-PTTR*Δ cells containing HA-*rho1*$^+$ expressed from its own promoter were obtained using 200 μl of lysis buffer (50 mM Tris, pH 7.5, 20 mM NaCl, 0.5% NP-40, 10% glycerol, 0.1 mM dithiothreitol, 1 mM NaCl, 2 mM $MgCl_2$, containing 100 μM *p*-aminophenyl methanesulfonyl fluoride, leupeptin, and aprotinin). GST-RBD fusion protein, 100 μg, coupled to glutathione-agarose beads was used to immunoprecipitate 1.5 mg of the cell lysates. The extracts were incubated with GST-RBD beads for 2 h. The beads were washed with lysis buffer four times, and bound proteins were blotted against 1:5000-diluted 12CA5 mAb as primary antibody to detect HA-Rho1p. The total amount of HA-Rho1p was monitored in whole-cell extracts (25 μg of total protein), which were used directly for Western blotting and were developed with the 12CA5 mAb. Immunodetection was accomplished using the ECL detection kit (Amersham Biosciences).

Microscopy Techniques

The localization of Rgf1p-green fluorescent protein (GFP) was visualized in living cells under a DMRXA microscope (Leica, Wetzlar, Germany).

RESULTS

Pck2p Acts Downstream from Rgf1p

Previous studies have shown that Rgf1p is a Rho1p GEF (García *et al.*, 2006a). The Rgf1p deletion causes cell lysis, hypersensitivity to the antifungal drug Csp, and defects in the establishment of bipolar growth (García *et al.*, 2006a). Regarding the downstream targets of Rgf1p, we wondered whether the overexpression of either Pck1p or Pck2p might suppress hypersensitivity to Csp in the *rgf1*Δ mutant. In fission yeast, the protein kinase C homologues Pck1p and Pck2p are targets for Rho1p; both genes—*pck1*$^+$ and *pck2*$^+$—share overlapping roles in cell viability and partially com-

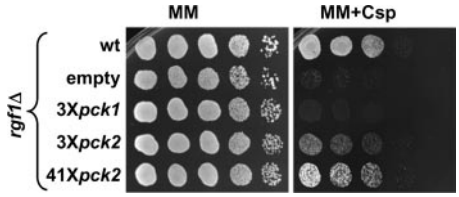

Figure 1. Pck2p acts downstream from Rgf1p. The Csp-hypersensitive growth phenotype of *rgf1*Δ mutants is suppressed by overexpression of *pck2*$^+$. HVP54 (*rgf1*$^+$) was transformed with pREP3X (empty vector) and VT14 (*rgf1*Δ) was transformed with pREP3X (empty vector), pREP3X-*pck1* (3X*pck1*$^+$), pREP3X-*pck2* (3X*pck2*$^+$), and pREP41x-*pck2* (41X*pck2*$^+$). Transformants were spotted onto MM and MM plus 2 μg/ml caspofungin (Csp) plates as serial dilutions (8×10^4 cells in the left row and then 4×10^4, 2×10^4, 2×10^3, and 2×10^2 in each subsequent spot) and incubated at 28°C for 3 d.

plement each other (Toda *et al.*, 1993). Pck2p plays a role in the regulation of the two main polymers of the cell wall, β- and α-glucans, whereas the function of Pck1p in cell integrity is not so well known (Arellano *et al.*, 1999b; Calonge *et al.*, 2000). To this end, the *rgf1*Δ strain (VT14) was transformed with the overexpression plasmids pREP3X-*pck1*$^+$ and pREP3X-*pck2*$^+$ (Arellano *et al.*, 1999b), and the transformants were monitored for growth on Csp. Figure 1 shows that *pck2*$^+$, but not *pck1*$^+$, expressed from the plasmid containing the high-strength *nmt1* promoter (pREP3X) partially rescued the lysis and Csp hypersensitivity of the *rgf1*Δ cells. We realized that the strong overexpression of *pck2*$^+$ in *rgf1*Δ mutant cells could be deleterious (Mazzei *et al.*, 1993), so to avoid this problem we used *pck2*$^+$ driven by the *P41nmt* promoter (medium level); we found a much better complementation of the hypersensitivity to Csp (Figure 1).

Cells Lacking rgf1 Show the vic Phenotype

It has recently been shown that Rho2p and Pck2p act upstream from the Pmk1p MAPK signaling pathway, thereby resulting in the *vic* (viable in the presence of immunosuppressant FK506 and chloride ion) phenotype upon mutation (Ma *et al.*, 2006). Because Rgf1p was linked to Pck2p, which in turn has been linked to the MAPK pathway, we decided to examine the functional relationship between Rgf1p and Pmk1p signaling by analyzing whether the *rgf1*Δ mutants showed the *vic* phenotype. The results clearly showed that *rgf1*Δ, as well as *pmk1*Δ, and *pek1*Δ, cells grew in the presence of FK506 and 0.2 M $MgCl_2$, whereas the wild-type cells were unable to grow in the same conditions (Figure 2A). It is known that calcineurin mutants (*ppb1*Δ) cannot grow in the presence of $MgCl_2$, whereas an additional mutation in a member of the MAPK pathway suppresses that phenotype. The addition of FK506 (a calcineurin-specific inhibitor) to wild-type cells mimics the calcineurin deletion and prevents growth in the presence of $MgCl_2$. The fact that the *rgf1*Δ mutants were able to grow in these conditions indicates that Rgf1p could play an antagonistic role for calcineurin mutants and is thus a strong candidate component of the Pmk1p-MAPK signaling pathway.

We further investigated the relationship between Rgf1p and the MAPK integrity pathway and we found that *rgf1*Δ cells shared other phenotypes with *pmk1*Δ mutants. *rgf1*Δ cells, like *pmk1*Δ cells, were hypersensitive to β-glucanase treatment (Figure 2B; Toda *et al.*, 1996b; Sengar *et al.*, 1997), and their growth was inhibited by high salt concentrations, a phenotype characteristic of the knockouts of the *pmk1*$^+$, *mkh1*$^+$, and *pek1*$^+$ genes (Figure 2C). Moreover, we found that *rgf1*Δ cells required a significantly longer period of time to reenter the cell cycle upon reinoculation into fresh medium after prolonged stationary phase arrest compared with

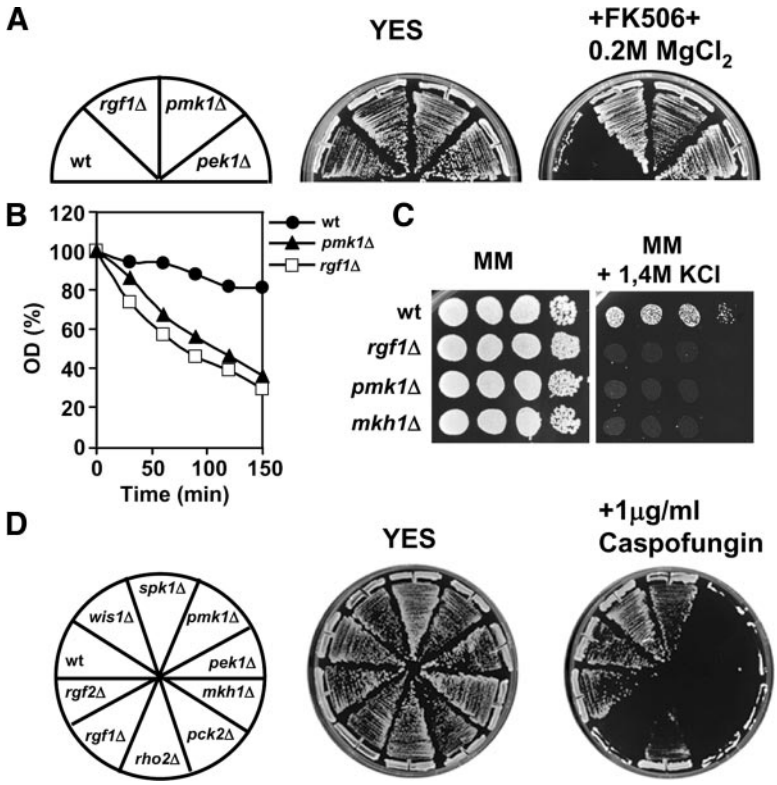

Figure 2. Knockout of *rgf1*$^+$ and the components of the Pmk1 MAPK pathway exhibits the *vic* phenotype, sensitivity to high osmolarity, and caspofungin (Csp) hypersensitivity. (A) *rgf1*Δ cells show the *vic* phenotype. Wild-type (YS64), *rgf1*Δ (VT14), *pmk1*Δ (MS192), and *pek1*Δ (MS189) cells were streaked onto YES or YES plus 0.5 μg/ml FK506 and 0.2 M $MgCl_2$ and incubated at 32°C for 3 d. (B) Sensitivity of *rgf1*Δ and *pmk1*Δ to β-glucanase. Cell lysis was measured at different times during treatment with β-glucanase by determining the OD_{600} (see *Materials and Methods*). The strains examined were wt (YS64), *rgf1*Δ (VT14), and *pmk1*Δ (MS192). (C) Growth inhibition of *rgf1*Δ, *pmk1*Δ, and *mkh1*Δ mutants by KCl. Wild-type (YS64), *rgf1*Δ (VT14), *pmk1*Δ (MS192), and *mkh1*Δ (MS196) strains were grown in MM or in MM plus 1.4 M KCl for 5 d at 32°C. (D) Knockout of the components of the Pmk1 MAPK pathway (*pmk1*$^+$, *mkh1*$^+$, *pek1*$^+$, *pck2*$^+$, and *rho2*$^+$) elicited Csp hypersensitivity. Wild-type (YS64), *wis1*Δ (MS190), *spk1*Δ (MS194), *pmk1*Δ (MS192), *pek1*Δ (MS189), *mkh1*Δ (MS196), *pck2*Δ (PG272), *rho2*Δ (VT20), *rgf1*Δ (VT14), and *rgf2*Δ (PG1) were streaked onto YES or YES plus 1 μg/ml Csp (CANCIDAS) and incubated at 32°C for 3 d.

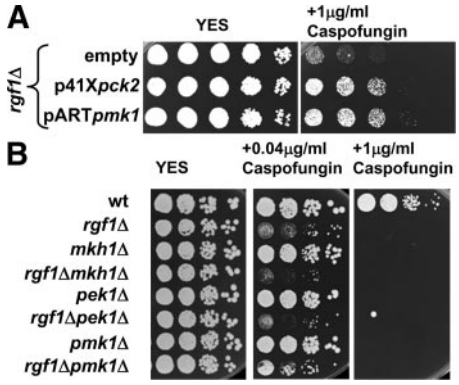

Figure 3. The Csp-hypersensitive growth phenotype of *rgf1*Δ mutants is suppressed by overexpression of *pmk1*$^+$. (A) VT14 (*rgf1*Δ) was transformed with pART1 (empty vector), p41X*pck2* (*pck2*$^+$), and pART*spm1* (*pmk1*$^+$; Zaitsevskaya-Carter and Cooper, 1997). Transformants were spotted onto MM and MM plus 1 μg/ml Csp plates as serial dilutions (8×10^4 cells in the left row and then 4×10^4, 2×10^4, 2×10^3, and 2×10^2 in each subsequent spot) and incubated at 28°C for 3 d. (B) *rgf1*Δ*mkh1*Δ, *rgf1*Δ*pek1*Δ, or *rgf1*Δ*pmk1*Δ did not show synergism in sensitivity to Csp. Equal numbers of wild-type (YSM180) and *rgf1*Δ (PG77), *mkh1*Δ (PG348), *rgf1*Δ*mkh1*Δ (PG337), *pek1*Δ (PG349), *rgf1*Δ*pek1*Δ (PG339), *pmk1* (PG347), and *rgf1*Δ*pmk1*Δ (PG335) mutant strains were diluted (2×10^4 cells in the left row and then 2×10^3, 2×10^2 and 2×10^1 in each subsequent spot) and were spotted onto YES plates with 0, 0.04, and 1 μg/ml Csp. Colony formation was analyzed after 4 d at 28°C.

the wild-type cells (not shown). This phenotype, also seen in *pmk1*Δ and *mkh1*Δ mutants, might represent a change in the response to stress conditions rather than a defect in cell integrity per se. Stationary-phase *rgf1*Δ cells spotted onto plates were protected osmotically (without lysis); however, they became visible 1 d after the wild-type colonies, and once this had occurred, they displayed a smaller colony size than the wild-type cells in the same conditions (not shown). We then examined whether knockout of the components of the protein kinase C-Pmk1p MAPK signaling pathway displayed hypersensitivity to Csp, an inhibitor of β-(1,3)-glucan synthase (Vicente *et al.*, 2003). As shown in Figure 2D (+1 μg/ml Csp), all knockouts of the components of the MAPK pathway were hypersensitive to Csp, whereas the growth of the wild type, *rgf2*Δ (deleted for another Rho1p GEF), *wis1*Δ (deleted for the stress-activated MAPKK), and *spk1*Δ (deleted for the MAPK involved in meiosis) was not inhibited. We also noticed that *rho2*Δ cells were not as sensitive to the antifungal agent as the other knockouts.

*Overproduction of MAPK Pmk1p Suppresses the Hypersensitivity of rgf1*Δ Cells to Caspofungin

We found that Pck2p overexpression suppressed the hypersensitive phenotype of Csp in *rgf1*Δ mutants. Accordingly, we examined whether up-regulation of the Pck2p effector, Pmk1p, might suppress the growth defect of the *rgf1*Δ cells in the presence of the antimycotic agent. As shown in Figure 3A, overexpression of Pmk1p under the control of the strong and constitutively active ADH promoter (Zaitsevskaya-Carter and Cooper, 1997) partially suppressed the growth defect of *rgf1*Δ cells in the presence of Csp.

We also examined the effect of an *rgf1*Δ mutation in combination with mutations affecting components of the MAPK integrity pathway, such as Mkh1p, Pek1p, and Pmk1p. The double mutants grew as well as the single mutants at 28°C (Figure 3B). We also compared the sensitivity to Csp of the single and double mutants. The knockout of *mkh1*$^+$, *pek1*$^+$, and *pmk1*$^+$ elicited a weaker sensitivity to Csp than *rgf1*Δ, because the *mkh1*Δ, *pek1*Δ, and *pmk1*Δ cells grew on YES plates supplemented with 0.3 μg/ml Csp, whereas the *rgf1*Δ cells failed to grow in the presence of 0.1, 0.08, and even 0.04 μg/ml Csp (Figure 3B). However, the *rgf1*Δ*mkh1*Δ, *rgf1*Δ*pek1*Δ, and *rgf1*Δ*pmk1*Δ double knockout mutants failed to grow in the presence of 0.04 μg/ml Csp and did not show synergism in their sensitivity to Csp compared with the parental *rgf1*Δ single knockout (Figure 3B).

Rgf1p Is Involved in Pmk1p Activation Due to Hypertonic and Hypotonic Stress and Cell Wall Damage

To confirm that Rgf1p activates and transmits signaling through Pmk1p, we examined the level of Pmk1p phosphorylation upon different stresses in *rgf1*Δ mutants. It has been reported that MAP kinase Pmk1p activation is induced by multiple stressing situations, including hyper- and hypotonic conditions, the presence of cell wall damaging compounds, heat shock, glucose deprivation, and oxidative stress (Madrid *et al.*, 2006). The catalytic activity of this family of kinases depends on the phosphorylation of both the Thr-186 and the Tyr-188 residues, and can be detected by Western blotting with polyclonal anti-phospho-p42/44 antibody (see *Materials and Methods*; Zaitsevskaya-Carter and Cooper, 1997; Loewith *et al.*, 2000). First, we looked at Pmk1p activation after osmotic stress caused by KCl or sorbitol. To this end, cells from wild-type (MI200) and mutant *rgf1*Δ (PG285) strains with the genomic copy of *pmk1*$^+$ tagged with HA6H (Madrid *et al.*, 2006) were grown at 28°C to the early log-phase. Extracts were obtained from samples taken before and after the addition of 0.6 M KCl or 1 M sorbitol for the times indicated. As shown in Figure 4A (KCl), whereas extracts from the wild-type showed a strong increase in Pmk1p signal intensity after treatment, this band was almost absent in the *rgf1*Δ mutant. Similarly, the Pmk1p phosphorylation seen after sorbitol treatment in *rgf1*$^+$ cells was severely impaired in the *rgf1*Δ mutant (Figure 4A). In both situations, the induction of Pmk1 activity was not the result of an increase or decrease in Pmk1p protein levels, as observed after probing the same extracts with anti-hemagglutinin (HA) monoclonal antibodies (Figure 4A). We performed quantitative analysis by calculating the value termed induction-fold as the ratio between quantitative levels of Pmk1p phosphorylation at 15 min (treated cells) and Pmk1p phosphorylation at time 0 (untreated cells). All the single quantified values were also normalized with respect to their respective loading controls, and the results are shown below each panel (Figure 4). We also analyzed Pmk1p activation in control and *rgf1*Δ cells subjected to hypotonic stress, which induces a very rapid and transient phosphorylation of the MAPK in control cells (Madrid *et al.*, 2006; Figure 4B). As under osmostress, the Rgf1p deletion strongly decreased Pmk1p activation under hypotonic conditions (Figure 4B). These results therefore indicate a role for Rgf1p in osmotic stress sensing.

Hyper- and hypotonic stress initiates a variety of compensatory and adaptive responses, which serve to restore near-normal cell volumes and to reinforce the cell membrane and cell wall structure to withstand the physical challenge. The above results prompted us to test whether Rgf1p was also involved in the MAPK response to cell wall damage. As expected, we found that the phosphorylation of Pmk1p was markedly induced upon Csp treatment in wild-type cells, whereas in the absence of Rgf1p this activation had decreased considerably (Figure 4C).

We next wondered whether the role of Rgf1p was specific for stress conditions related to changes in the cellular volume that could involve the reorganization of the actin cy-

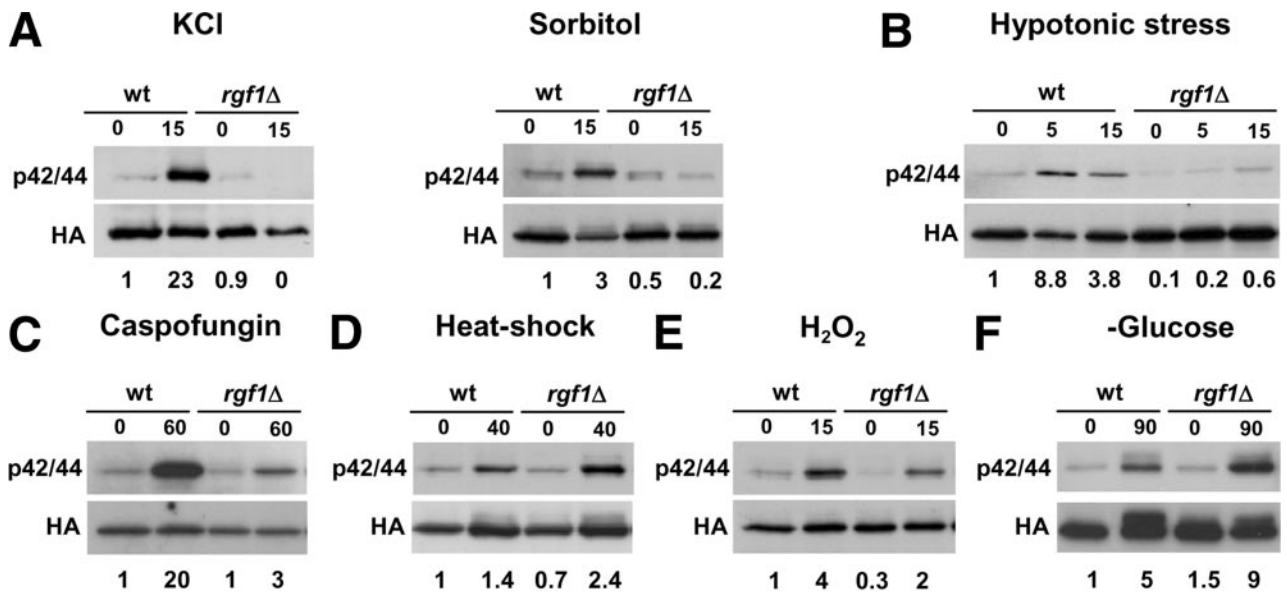

Figure 4. Rgf1p regulates Pmk1p activation induced by hyper- and hypotonic stress and by cell wall stress. The wild-type strain (*rgf1*$^+$, MI200) or a mutant (*rgf1*Δ, PG285), both carrying a *HA6H*-tagged chromosomal version of *pmk1*$^+$, were grown in YES medium to mid-log phase and subjected to different treatments for the indicated times. Pmk1-HA6H was purified by affinity chromatography under native conditions. Activated and total Pmk1p were detected by immunoblotting with anti-phospho-p42/44 or anti-HA antibodies, respectively. (A) For hypertonic stress, cells were subjected to 0.6 M KCl or 1 M sorbitol for 15 min. (B) For hypotonic stress, cells growing in YES medium supplemented with 0.8 M sorbitol were transferred to YES medium without sorbitol and collected at 0, 5, and 15 min. (C) For cell wall stress, cultures were supplemented with 1 μg/ml Csp and grown for 60 min. (D) For thermal shock, cultures growing at 28°C were split and incubated at either 28 or 40°C for 40 min. (E) For oxidative stress, cultures were treated for 15 min with 6 mM H_2O_2. (F) For glucose starvation, cells growing in YES medium with 7% of glucose were shifted to the same medium without glucose but containing an equivalent osmotic concentration of glycerol. Each treatment was repeated at least three times. Relative units comparing the induction-fold of wild-type and mutant *rgf1*Δ cells in each individual experiment are shown below.

toskeleton or whether it was also involved in repairing other types of cell damage, such as oxidative stress or heat shock. As shown in Figure 4D, the intensity of Pmk1p phosphorylation after transferring the cultures from 28 to 40°C was not affected very much by deletion of the *rgf1*$^+$ gene. Similarly, the MAPK activation achieved with hydrogen peroxide in control cells was still evident in *rgf1*Δ cells (Figure 4E), and the induction-fold value upon comparing the stressed *rgf1*Δ cells with their controls (2/0.3) was higher than the induction seen in wild-type cells (4/1; Figure 4E). Thus, the heat-shock– and the oxidative stress–induced activations of Pmk1p in the fission yeast were largely independent of the presence or absence of Rgf1p. Next, we wondered whether Rgf1p was also involved in sensing nutrient limitation, a process that in the long-term also enhances the turnover of excess mass. Yeast cells starved for glucose dramatically down-regulate general protein synthesis and activate stress responses (Ashe *et al.*, 2000). In fission yeast cells, previous work has shown that stress caused by glucose depletion elicits a clearly delayed Pmk1p activation (Madrid *et al.*, 2006). We found that cells responded to glucose starvation by activating Pmk1p in a way totally independent of the presence or absence of Rgf1p (Figure 4F). Taken together, these data indicate that Rgf1p plays a substantial role in the osmotic and cell wall stress response by positively regulating the Pmk1p-cell integrity pathway.

Rgf1p Is Not Involved in Spc1p Activation Due to Hypertonic or Oxidative Stress

The above results led us to the hypothesis that Rgf1p could also be regulating the osmotic stress response through activation of the Spc1p/Sty1p MAPK signaling pathway (Shiozaki and Russell, 1995b). The Spc1p kinase is activated by increases in osmolarity and by a wide range of environmental stresses. This activation involves the relocalization of Spc1p to the nucleus and phosphorylation of the transcription factor Atf1p, which results in changes in the expression of genes associated with the stress response (Takeda *et al.*, 1995). We investigated the effect of loss of the *rgf1*$^+$ gene on Spc1p activation after osmotic stress (KCl). We used cells from wild-type (KS1489) and mutant *rgf1*Δ (PG362) strains with the genomic copy of *spc1*$^+$ tagged with HA6H, allowing Spc1p purification with Ni^{2+}-NTA and detection with anti-HA antibody (Shiozaki and Russell, 1995b). As previously reported (Degols *et al.*, 1996), exposure of the cells to high-osmolarity conditions, in this case YES medium containing 0.6 M KCl, led to a rapid increase in the tyrosine-phosphorylation of Spc1p (Figure 5). In *rgf1*Δ cells, the same treatment produced a similar increase in the Spc1p signal, indicating that Spc1p induction upon KCl application was completely independent of Rgf1p (Figure 5). We also examined whether the activation of the MAPK after oxidative stress was dependent on the presence of Rgf1p. As shown in Figure 5, bottom panel, the intensity of Spc1p phosphorylation after 0.3 mM H_2O_2 treatment was not affected very much by deletion of the *rgf1*$^+$ gene.

Rho1p Participates in the Activation of Pmk1p

GEFs are multidomain proteins, and previous studies have suggested that many of these domains are protein- or lipid-interaction domains, indicating that they serve as localization signals and/or as scaffolds for the formation of protein complexes (Yeh *et al.*, 2007). To determine whether Rgf1p function in Pmk1p signaling requires GEF activity, we used

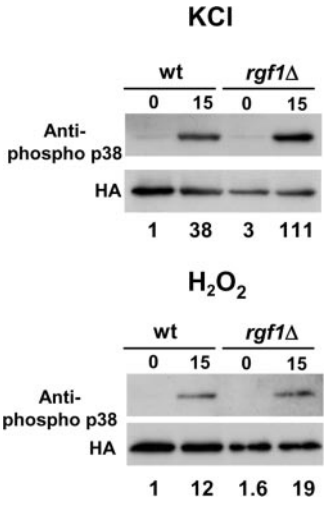

Figure 5. Rgf1p-independent tyrosine phosphorylation of Spc1p by osmotic- and oxidative-stress signals. Cells expressing epitope-tagged Spc1p, wild-type (KS1489) and *rgf1*Δ (PG362) were grown to log phase in YES medium and then exposed to osmotic stress (15 min of exposure to YES plus 0.6 M KCl, top panel, or oxidative stress (15 min of growth in YES plus 0.3 mM H_2O_2, bottom panel). Spc1p was isolated by Ni^{2+}-NTA-agarose affinity precipitation and then analyzed by immunoblotting with anti-phospho-p38 and anti-HA antibodies. Spc1p tyrosine phosphorylation increased in both strains after the two different types of stress.

a deletion mutant in the RhoGEF domain of Rgf1p (*rgf1-PTTR*Δ; García *et al.,* 2006a). The four amino acids deleted in the *rgf1-PTTR*Δ mutant (proline-threonine-threonine-arginine) have been predicted to be located on helix H8 (CR3), which is the most highly conserved region of the DH domain and is where many mutations that decrease nucleotide exchange activity map (Liu *et al.,* 1998; Soisson *et al.,* 1998). As expected, the *rgf1-PTTR*Δ mutant integrated in a single copy in *rgf1*Δ strain displayed a significantly reduced GEF activity toward Rho1p. In a pulldown binding assay, only a minor amount of GTP-Rho1p (active-Rho1p) was detected in the mutant strain *rgf1-PTTR*Δ (PG52) as compared with the levels of GTP-Rho1p in the wild-type strain (Fig. 6A). We then tagged the full-length wild-type and the mutant *rgf1-PTTR*Δ gene with GFP at the carboxy-end. These proteins were expressed at comparable levels and the mutated *rgf1-PTTR*Δ-GFP localized to the cell ends in the wild-type strain (Figure 6B). However, the *rgf1-PTTR*Δ mutant was completely nonfunctional in terms of Pmk1p activation after osmotic stress (Figure 6C). Thus, the role of Rgf1p in Pmk1p MAPK activation must depend on its GEF activity.

This result raised the possibility that either low or high GTP-Rho1p levels could be regulating MAPK activation in response to stress. To examine this option, we first tested whether overexpression of Rho1p or a dominant-active Rho1p mutant (Rho1G15V) might result in an increased phospho-Pmk1p signal in the absence of environmental stress. To this end, we transformed the Pmk1-HA6H-tagged strain MI200 with plasmids pREP3X, pREP3X-*rho1*$^+$, and pREP3X-*rho1G15V*. These plasmids expressed the wild-type *rho1*$^+$ and the hyperactive allele of *rho1*$^+$ under the control of the high-strength thiamine-repressible promoter *nmt*1 (Arellano *et al.,* 1996; Forsburg and Sherman, 1997). As shown in Figure 6D (left), the overexpression of both Rho1p and Rho1G15Vp (constitutively active) increased the phophorylation levels of Pmk1p, similar to the increase obtained with the overexpression of Pck2p. As expected, cells overexpressing the constitutively active allele of Rho1p activated the cascade earlier on in derepression than cells bearing the wild-type allele of Rho1p. We also found that a high level of Rgf1p, expressed from pREP3X-*rgf1*$^+$, elicited the activation of Pmk1p (Figure 6D).

We next examined whether ectopic expression of Rho1p and Rgf1p caused direct activation of Pmk1p or whether it was being funnelled through the Mkh1p-Pek1p module. We constructed strains that expressed the Pmk1-HA6H fusion in an *mkh1*Δ background and transformed them with plasmids pREP3X-*rho1G15V* and pREP3X-*rgf1*$^+$. Deletion of *mkh1*$^+$ completely abolished the Pmk1p activation observed after overexpression of Rho1G15Vp or Rgf1p (Figure 6D). Moreover, we tested whether the overexpression of Rho1G15Vp and Rgf1p caused Pmk1p activation in a *pck2*Δ background and found that the lack of *pck2*$^+$ completely abolished Pmk1p activation (Figure 6D, right). Taken together, these results strongly suggest that the Rgf1p-Rho1p-Pck2p cascade does regulate the activation of Pmk1p in *S. pombe*.

Involvement of Rgf2p and Rgf3p in Pmk1p Activation

Finally, we investigated what the contribution of the other Rho1p GEFs to Pmk1p activation might be. We have previously shown that overexpression of *rgf1*$^+$, *rgf2*$^+$ or *rgf3*$^+$, driven by the *nmt1* promoter, produces a strong induction of the amount of GTP-bound Rho1p (active Rho1p; Tajadura *et al.,* 2004; García *et al.,* 2006a), and our unpublished results). However, in vivo the contribution of each GEF to the activation or Rho1p must be very different. The level of Rho1p-GTP is very diminished in *rgf1*Δ *cells* (Figure 6A (*García et al.,* 2006a), whereas in the *rgf3* mutant the amount of Rho1p-GTP (activated) is almost the same as that of the wild type (Tajadura *et al.,* 2004).

We found that overexpression of either Rgf2p or Rgf3p, expressed from pREP3X-*rgf2*$^+$ or pREP3X-*rgf3*$^+$, respectively, elicited the activation of Pmk1p (Figure 7A). This result led us to wonder whether this was due to a real contribution of each GEF or whether it was merely a consequence of a high level of active Rho1p. To this end, we examined the effect of the loss of Rgf2p or the Rgf3p alone on the Pmk1p activation induced by different types of stress. Rgf3p is essential, so we used a strain with a TS mutation in *rgf3* (the *ehs2-1* mutant stands for echinocandin-hypersensitive). The *ehs2-1* cells showed a lytic thermosensitive phenotype at 37°C, which was suppressed when an osmotic stabilizer (1.2 M sorbitol) was added to the medium (Tajadura *et al.,* 2004). We *first* tested whether the *ehs2-1* mutant was impaired for *the* Pmk1p activation induced by osmotic stress or cell wall damage. As shown in Figure 7B, Pmk1p phosphorylation levels were similar in *ehs2-1* cells and in wild-type cells grown for 2 h at 37°C and then exposed to KCl (15 min.) or Csp (1 h) at the same temperature. Moreover, the *ehs2-1* mutant was not involved in repairing other types of cell damage, such as oxidative or heat shock. As shown in Figure 7B, the intensity of Pmk1p phosphorylation after treatment with H_2O_2 (6 mM) or heat shock at 40°C was not significantly affected in the *ehs2-1* mutant. We also tested Pmk1p activation in the presence of sorbitol (1 M) and under glucose starvation. The *ehs2-1* cells did not behave differently from the wild-type cells in either situation (not shown).

Our investigations with the *rgf2*Δ null mutant revealed that the intensity of Pmk1p phosphorylation after exposure to osmotic shock (1M sorbitol), cell wall damage (1 μg/ml Csp), oxidative stress (6 mM H_2O_2), or heat shock (40°C), and glucose starvation was not affected very much by deletion of the *rgf2*$^+$ gene (Figure 7C).

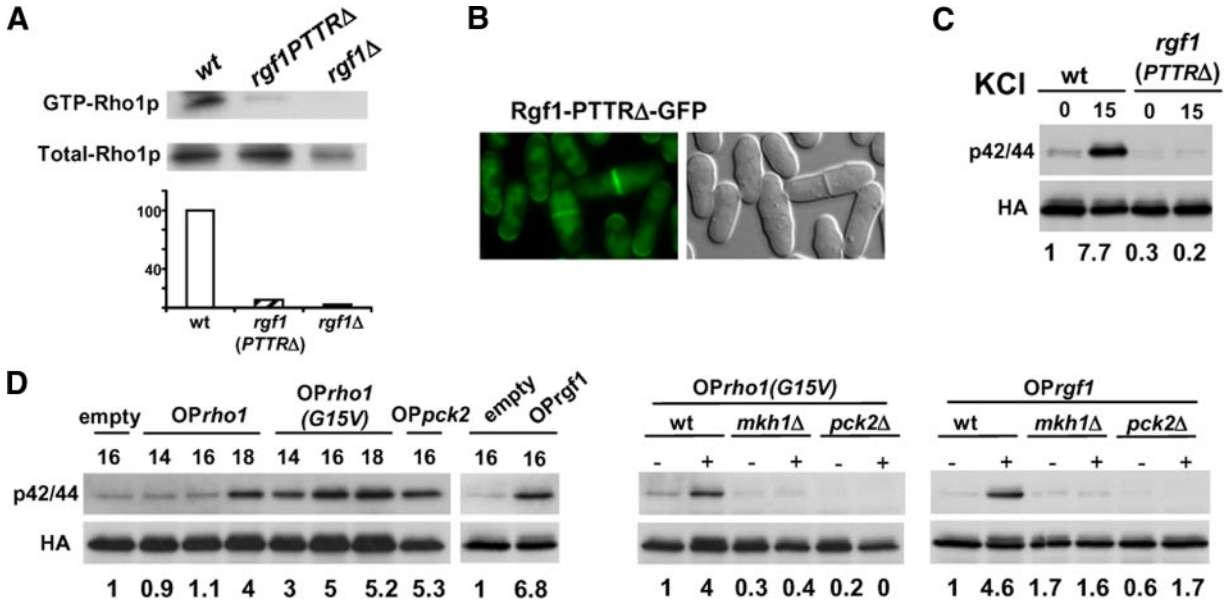

Figure 6. Rho1p is involved in Pmk1p activation. (A) The *rgf1-PTTR*Δ mutant displayed significantly reduced GEF activity toward Rho1p. Wild-type (PPG160), *rgf1*Δ (PG378), and *rgf1-PTTR*Δ (PG380) cells expressing HA-*rho1*$^+$ from its own promoter were precipitated with GST-RBD and blotted with anti-HA antibodies (12CA5). Total HA-Rho1p in cell lysates was visualized by Western blotting. Data are quantified and presented as percentages relative to the wild-type extracts run in the same experiment. (B) Early log-phase cells containing the *rgf1-PTTR*Δ allele fused to *GFP* (PG52) were visualized for GFP fluorescence and Nomarsky optics. The Rgf1-PTTR protein (mutated in the RhoGEF domain) localizes to the growing regions: one or both poles and the medial zone. (C) Rgf1p function in Pmk1p signaling requires GEF activity. The *rgf1*$^+$ wild-type strain (MI200) or the *rgf1-PTTR*Δ mutant (PG331), both carrying an *HA6H*-tagged chromosomal version of *pmk1*$^+$, were grown in YES medium to mid-log phase and treated with 0.6 M KCl, for 15 min. Pmk1-HA6H was purified by affinity chromatography under native conditions. Activated and total Pmk1p was detected by immunoblotting with anti-phospho-p42/44 or anti-HA antibodies, respectively. (D) High levels of Rho1p resulted in an increased phospho-Pmk1p signal. Left panels, the wild-type strain (*rgf1*$^+$, MI200) carrying a *HA6H*-tagged chromosomal version of *pmk1*$^+$ was transformed with plasmids for *rho1*$^+$ (pREP3X-*rho1*$^+$, pREP3X-*rho1G15V*), *pck2*$^+$ (pREP-*pck2*$^+$), *rgf1*$^+$ (pREP3X-*rgf1*$^+$), and with the empty plasmid (pREP3X). Transformants were grown in MM without thiamine for the indicated times. Purification and detection of active or total Pmk1 was performed as described above. Right panels, basal activation of Pmk1p produced by Rho1p and Rgf1p is channelled exclusively through Mkh1p and Pck2p. The wild-type, *mkh1*Δ (PG318) and *pck2*Δ (PG332) strains carrying a *HA6H*-tagged chromosomal version of *pmk1*$^+$ were transformed with plasmids pREP3X, pREP3X-*rho1G15V* and pREP3X-*rgf1*$^+$. For promoter derepression, transformants were grown in MM without thiamine for 16 h. Lines are labeled (−) for the empty plasmid pREP3X and (+) for the OP-plasmid, either pREP3X-*rho1G15V* or pREP3X-*rgf1*$^+$. Purification and detection of active and total Pmk1p were performed as described above.

Thus, together these results indicate that among the Rho1p GEFs, Rgf1p contributes the greatest part by modulating the activity of the pathway after physical destabilization of the cell surface.

DISCUSSION

The main conclusion that can be drawn from the present work is that Rgf1p, a Rho1p-specific GEF, is a new member of the Pmk1p MAPK pathway in fission yeast. We provide genetic and biochemical evidence to support this view. First, mutants lacking *rgf1*$^+$ exhibited the *vic* phenotype, which is strong indication of the involvement of the components of the Pmk1p signaling pathway (Ma *et al.*, 2006). Moreover, *rgf1*Δ cells were hypersensitive to β-glucanase treatment, and their growth was inhibited by high salt concentrations, both phenotypes being characteristic of knockouts in the *pmk1*$^+$, *mkh1*$^+$, and *pek1*$^+$ genes. Second, knockout mutants of the components of the Pmk1p MAPK signaling pathway displayed hypersensitivity to Csp, a cell wall–damaging agent that specifically inhibits β-glucan biosynthesis. As expected, deletion of the components of the Pmk1p pathway did not exacerbate the hypersensitivity to Csp of strains lacking *rgf1*$^+$. This observation supports the view that Rgf1p plays a role in the Pmk1p pathway. Third, Pmk1p MAPK phosphorylation/activation in response to osmotic stress or cell wall damage depends on the Rgf1 protein. Our data provide new evidence to clarify the complex regulatory network modulating the level of activation of the Pmk1p. Although it seems likely that hypertonic and hypotonic stress and cell wall damage would be transduced by Rgf1p via Rho1p and Pck2p, other stimuli such as treatment with H_2O_2 and heat shock are largely independent of Rgf1p function.

MAP kinase cascades serve to amplify a small signal initiated at the cell surface and to convert a graded input into a highly sensitive, switch-like response (Ferrel, 1996). In budding yeast, the Rho1p effector pathway most studied is the Pkc1p-activated MAPK cascade. This is principally because mutants in this pathway display conditional cell lysis defects that render them genetically tractable. In this pathway, a linear series of protein kinases, known as an MAPK cascade, is responsible for amplification of the cell wall integrity (CWI) signal from Rho1p (Levin, 2005). In fission yeast, the cell integrity pathway contains a module of three kinases (Mkh1p, Skh1p/Pek1p, and Pmk1p/Spm1p) that regulate cell integrity and that, with calcineurin phosphatase, antagonize chlorine homeostasis. However, the involvement of this pathway in the gene expression related to cell wall remodelling or to the organization of the actin

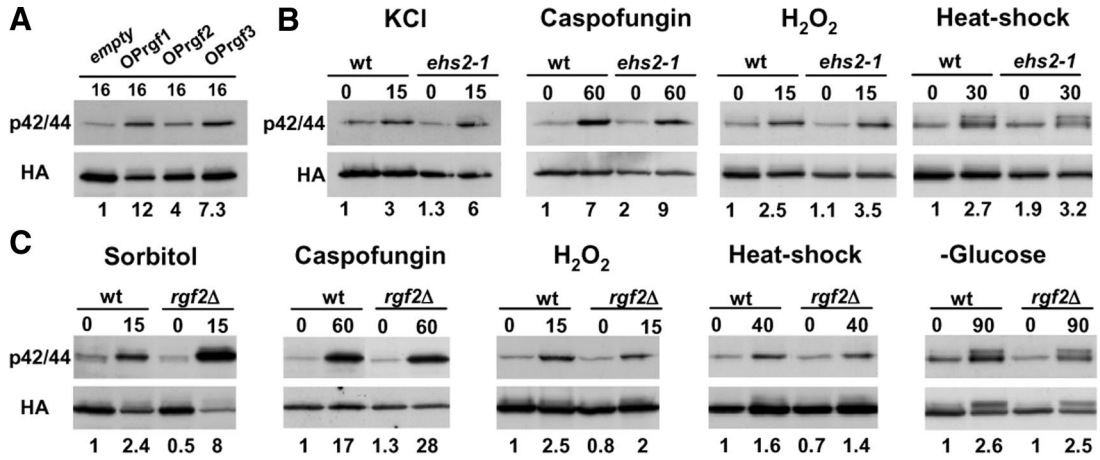

Figure 7. Rgf2p and Rgf3p involvement in Pmk1p activation. (A) High levels of Rgf2p and Rgf3p resulted in an increased phospho-Pmk1p signal. The wild-type strain (*rgf1*$^+$, MI200) carrying a *HA6H*-tagged chromosomal version of *pmk1*$^+$ was transformed with plasmids for *rgf1*$^+$, *rgf2*$^+$, or *rgf3*$^+$ overexpression (pREP3X-*rgf1*$^+$, pREP3X-*rgf2*$^+$, and pREP3X-*rgf3*$^+$, respectively) and with the empty plasmid (pREP3X). For promoter derepression, transformants were grown in MM without thiamine for 16 h. (B) Rgf3p is not required for induction of Pmk1p phosphorylation in response to hypertonic stress or cell wall stress. Strains (*rgf3*$^+$, MI200) and (*ehs2-1*, PG359) were grown to mid-log phase in YES medium and then incubated for 2 h at 37°C. For osmotic stress treatment, the cells were incubated for an additional 15 min in YES plus 0.6 M KCl. For cell wall stress, the cells were incubated for an additional hour in YES plus 1 μg/ml Csp, in both cases at 37°C. For oxidative stress, cultures were treated for 15 min with 6 mM H_2O_2. For thermal shock, after 2 h at 37°C the cells were incubated at 40°C for 30 min. (C) Rgf2p is not required for induction of Pmk1p phosphorylation in response to hypertonic stress or cell wall stress. The wild-type strain (*rgf2*$^+$, MI200) or a mutant (*rgf2*Δ, PG287), both carrying a *HA6H*-tagged chromosomal version of *pmk1*$^+$, were grown in YES medium to mid-log phase and then exposed to osmotic stress (15 min in YES plus 1 M sorbitol), cell wall damage (1 h of growth in YES plus 1 μg/ml Csp), oxidative stress (15 min growth plus 6 mM H_2O_2), heat shock (40°C for 40 min), or glucose starvation. Pmk1-HA6H was purified by affinity chromatography under native conditions. Activated and total Pmk1p were detected by immunoblotting with anti-phospho-p42/44 or anti-HA antibodies, respectively.

cytoskeleton is not very well understood. Deletion of *mkh1*, *pek1*, or *pmk1* did not significantly affect cell growth under standard conditions. However, the mutants are sensitive to β-glucanase treatment and to antifungal agents that interfere with cell wall biosynthesis (Toda *et al.*, 1996b; Sengar *et al.*, 1997). In the presence of hyperosmotic medium, high temperatures or nutrient starvation, a number of cells (30%) exhibit filamentous, multiseptated growth, and the cells are swollen (Sengar *et al.*, 1997; Zaitsevskaya-Carter and Cooper, 1997; Sugiura *et al.*, 1999). This phenotype is accompanied by thickened cell walls and prominent septa, and this might indicate that these cells are defective in cell wall biosynthesis or degradation under stressful conditions.

The finding that Rho2p and Pck2p are upstream components of the Pmk1p MAPK pathway supports the notion of the participation of this module of kinases in the cell remodelling necessary to reinforce the cell wall structure or to reorganize the cytoskeleton in order to withstand osmotic stress or other physical challenges (Ma *et al.*, 2006). Rho2p is involved in the control of cell morphogenesis (Hirata *et al.*, 1998) and regulates cell wall α-glucan biosynthesis through the protein kinase Pck2p (Calonge *et al.*, 2000). Pck2p is also related to cell wall β-glucan; although *pck2*Δ mutants have less cell wall, the mutants that overexpress Pck2p show an increase in the β-glucan content and a higher β-glucan synthase activity (Arellano *et al.*, 1999b).

Rho2p acts upstream from Pck2p, regulating the Pmk1p MAPK pathway (Ma *et al.*, 2006). Moreover, it has been recently shown that Rho2p is critical for Pmk1p activation in the presence of KCl, sorbitol and hypotonic stress, whereas it is dispensable for heat shock, H_2O_2, or glucose starvation (Barba *et al.*, 2008). It is possible that one or more elements could contribute to Pmk1p activation through Pck2p, alternatively to Rho2p (Barba *et al.*, 2008). A good candidate for Pmk1p activation via Pck2p is the essential GTPase Rho1p. Rho1p interacts with Pck1p and with Pck2p, and this interaction stabilizes these kinases, raising their concentrations precisely in the areas of growth (Arellano *et al.*, 1999b; Sayers *et al.*, 2000). Our results indicate that Rgf1p contributes to Pmk1p signaling only when Rho1p is working properly. In this sense, we found that both low and high levels of GTP-bound-Rho1p had an impact on the proper functioning of the cascade.

We observed that a deletion mutation in a highly conserved region of the Rgf1p-DH-domain produced a lack of function phenotype in terms of the induction of Pmk1p in response to osmotic stress. The amount of GTP-Rho1p in the *rgf1-PTTR*Δ mutant was minute compared with the wild-type *rgf1*$^+$, suggesting that wild-type levels of activated Rho1p are important for Pmk1p activation. Although it seems evident that Rgf1p could act as a GEF for Rho1p, we cannot rule out the possibility of its acting as a GEF for Rho2p.

However, our results do not favor this hypothesis because no changes in the levels of Rho2p bound to GTP were observed in strains with different levels of Rgf1p, either with or without osmotic stress (not shown). In addition, Rho2p overexpression did not suppress the hypersensitivity to Csp or sensitivity to KCl in the *rgf1*Δ mutant cells (García *et al.*, 2006a and data not shown, respectively). We also found that high levels of Rho1p increased the phosphorylation level of Pmk1p, similarly to that obtained by overexpression of Pck2p. As expected, overexpression of the constitutively active allele of Rho1p activated the cascade earlier on in derepression than cells that carried the wild-type allele of Rho1p. In both situations, Pck2p and Mkh1p activity was necessary for MAPK activation, suggesting that the stimulus was being funnelled through Pck2p and the three kinases.

We have previously shown that overexpression of both Rgf1p and Rgf3p raises the amount of GTP-bound Rho1p

and β-glucan synthase activity (Tajadura *et al.*, 2004; García *et al.*, 2006a). We reasoned that if Rho1p was activating Pmk1p, then it would be expected that ectopic activation of Rho1p by any of its GEFs would also activate Pmk1p. This is in fact the case, because the overproduction of Rgf2p or Rgf3p activated Pmk1p to a similar extent to Rgf1p. Rgf2p is required for spore wall maturation, and the protein shows a low expression profile in vegetatively growing cells (our unpublished observation). The *rgf3*$^+$ gene is essential, and the protein has been proposed to specifically activate Rho1p function during cytokinesis (Tajadura *et al*., 2004). Nevertheless, there exists the possibility that these GEFs could be activating the pathway by themselves. It seems very unlikely that Rgf2p would be involved in the signal transduction of the Pmk1p cascade. The knockout of Rgf2p did not elicit the characteristic *vic* phenotype and was not hypersensitive to Csp treatment or sensitive to high-salt concentrations either (not shown); even more conclusively, Pmk1p activation after osmotic stress, oxidative stress, heat shock, or Csp treatment was not impaired in the *rgf2*Δ mutant. Regarding Rgf3p, we observed that the activation of Pmk1p in response to a number of different stresses was not affected in the *ehs2-1* (mutated in *rgf3*$^+$) at the restrictive temperature. In sum, the Pmk1p activation seen after overexpression of Rgf2p and Rgf3p is probably due to a large increase in activated Rho1p levels. Our data also show that Rgf1p, but not Rgf2p or Rgf3p, activity is involved in Pmk1p activation during osmotic or cell wall stress, strongly suggesting that the expression, localization, and/or activity of different GEFs are regulated in vivo to mediate the function of Rho1p in different cellular processes.

In *S. pombe*, Rho1p signaling is required to maintain cell integrity. Here we show that the Rgf1p-Rho1p duo is involved in osmotic and cell wall stress signaling through the Pmk1p cell integrity cascade. The finding that the up-regulation of Pck2p and Pmk1p suppressed, at least partially, the Csp defect of *rgf1*Δ mutants suggests that Pck2p signals to the cell wall integrity pathway via the MAPK cascade. It has been shown that Atf1p is phosphorylated by Pmk1p under cell wall stress, but the consequences of such activation are unknown (Takada *et al.*, 2007). Besides its role in the cell integrity pathway, Rho1p acts by regulating the biosynthesis of β(1,3)-glucan and the cell wall in general, and it is also required for actin polymerization. Its role as a GS activator seems to be at least partially independent of its role in the MAPK cascade. The cells that overexpressed either Rgf1p or Rho1G15V (dominant-active allele of Rho1p) showed aberrant depositions of Cfw-stainable material and a huge increase in GS activity, and finally the cells' death. We found that deletion of Pmk1p did not release the lethal phenotype seen after the overproduction of Rgf1p or Rho1G115V; moreover, the GS level was not diminished in the *pmk1*$^+$ deletion mutants. It is likely that Rho1p would regulate GS at two levels: direct regulation of the enzyme itself and, through the MAPK cascade, regulation of the expression of the enzyme.

Our work demonstrates that a specific GEF regulates a subset of Rho1p functions, specifically linking the stimulus-induced signaling to cell wall and/or cytoskeletal remodelling. This supports the hypothesis that GEFs play specialized roles and is consistent with the hypothesis that the excess of regulators for GTPases would have evolved to exert spatiotemporal regulation or adaptive responses that enable GTPases to accomplish diverse cellular roles.

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REFERENCES

Arellano, M., Duran, A., and Perez, P. (1997). Localization of the *Schizosaccharomyces pombe* Rho1 GTPase and its involvement in the organization of the actin cytoskeleton. J. Cell Sci. *110*, 2547–2555.

Arellano, M., Durán, A., and Pérez, P. (1996). Rho1 GTPase activates the (1–3)β-D-glucan synthase and is involved in *Schizosaccharomyces pombe* morphogenesis. EMBO J. *15*, 4584–4591.

Arellano, M., Valdivieso, M. H., Calonge, T. M., Coll, P. M., Durán, A., and Pérez, P. (1999b). *Schizosaccharomyces pombe* protein kinase C homologues, pck1p and pck2p, are targets of rho1p and rho2p and differentially regulate cell integrity. J. Cell Sci. *112*, 3569–3578.

Ashe, M. P., De Long, S. K., and Sachs, A. L. (2000). Glucose depletion rapidly inhibits translation initiation in yeast. Mol. Biol. Cell *11*, 833–848.

Barba, G., Soto, T., Madrid, M., Núñez, A., Vicente, J., Gacto, M., and Cansado, J. (2008). Activation of the cell integrity pathway is channelled through diverse signalling elements in fission yeast. Cell Signal. *20*, 748–757.

Calonge, T. M., Arellano, M., Coll, P. M., and Perez, P. (2003). Rga5p is a specific Rho1p GTPase-activating protein that regulates cell integrity in *Schizosaccharomyces pombe*. Mol. Microbiol. *47*, 507–518.

Calonge, T. M., Nakano, K., Arellano, M., Arai, R., Katayama, S., Toda, T., Mabuchi, I., and Perez, P. (2000). *Schizosaccharomyces pombe* Rho2 GTPase regulates the cell wall a-glucan biosynthesis, through the protein kinase Pck2. Mol. Biol. Cell *11*, 4393–4401.

Carnero, E., Ribas, J. C., Garcia, B., Duran, A., and Sanchez, Y. (2000). *Schizosaccharomyces pombe* Ehs1p is involved in maintaining cell wall integrity and in calcium uptake. Mol. Gen. Genet. *264*, 173–183.

Chang, F. (2001). Establishment of a cellular axis in fission yeast. Trends Genet. *15*, 273–278.

Chen, S., and Hamm, H. E. (2006). DEP domains: more than just membrane anchors. Dev. Cell *11*, 436–438.

Degols, G., Shiozaki, K., and Russell, P. (1996). Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. Mol. Cell. Biol. *16*, 2870–2877.

Ferrel, J.E.J. (1996). Tripping the switch fantastic: how a protein kinase cascade can convert graded imputs into switch-like outputs. Trends Biochem. Sci. *21*, 460–466.

Forsburg, S. L. (1993). Comparison of *Schizosaccharomyces pombe* expression systems. Nucleic Acids Res. *21*, 2955–2956.

Forsburg, S. L., and Sherman, D. A. (1997). General purpose tagging vectors for fission yeast. Gene *191*, 191–195.

García, P., Tajadura, V., García, I., and Sánchez, Y. (2006a). Rgf1p is a specific Rho1-GEF that coordinates cell polarization with cell wall biogenesis in fission yeast. Mol. Biol. Cell *17*, 1620–1631.

García, P., Tajadura, V., García, I., and Sánchez, Y. (2006b). Role of Rho GTPses and Rho-GEFs in the regulation of cell shape and integrity in fission yeast. Yeast *23*, 1031–1043.

Hayles, J. A., and Nurse, P. (2001). A journey into space. Nat. Rev. Mol. Cell Biol. *2*, 647–656.

Hirata, D., Nakano, K., Fukui, M., Takenaka, H., Miyakawa, T., and Mabuchi, I. (1998). Genes that cause aberrant cell morphology by overexpression in fission yeast: a role for a small GTP-binding protein Rho2 in cell moephogenesis. J. Cell Sci. *111*, 149–159.

Jaffe, A. B., and Hall, A. (2005). Rho GTPases: biochemistry and biology. Annu. Rev. Cell Dev. Biol. *21*, 247–269.

Levin, D. (2005). Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. *69*, 262–291.

Liu, X., Wang, H., Eberstadt, M., Schnuchel, A., Olejniczak, E. T., Meadows, R., and Fesik, S. W. (1998). NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor Trio. Cell *95*, 269–277.

Loewith, R., Hubberstey, A., and Young, D. (2000). Skh1, the MEK component of the Mkh1 signaling pathway in *Schizosaccharomyces pombe*. J. Cell Sci. *113*, 153–160.

Ma, Y., Kuno, T., Kita, A., Asayama, Y., and Sugiura, R. (2006). Rho2 is a target of the farnesiltransferase Cpp1 and acts upstream of Pmk1 mitogen-activated protein kinase signaling in fission yeast. Mol. Biol. Cell *17*, 5028–5037.

Madrid, M., Soto, T., Khong, H. K., Franco, A., Vicente, J., Pérez, P., Gacto, M., and Cansado, J. (2006). Stress-induced response, localization, and regulation of the Pmk1 cell integrity pathway in *Schizosaccharomyces pombe*. J. Biol. Chem. *281*, 2033–2043.

Mazzei, G.J.E.M.S., Knowles, J.K.C., Payton, M. A., and Maundrell, K. (1993). A Ca^{2+}-independent protein kinase C from fission yeast. J. Biol. Chem. *268*, 7401–7406.

Mitchison, J. M., and Nurse, P. (1985). Growth in cell length in the fission yeast *Schizosaccharomyces pombe*. J. Cell Sci. *75*, 357–376.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. *194*, 795–823.

Morrell-Falvey, J. L., Ren, L., Feoktistova, A., Haese, G. D., and Gould, K. L. (2005). Cell wall remodeling at the fission yeast cell division site requires the Rho-GEF Rgf3p. J. Cell Sci. *118*, 5563–5573.

Motegi, F., Arai, R., and Mabuchi, I. (2001). Identification of two type V myosins in fission yeast, one of which functions in polarized cell growth and moves rapidly in the cell. Mol. Biol. Cell *12*, 1367–1380.

Mulvihill, D. P., Edward, S. R., and Hyams, J. S. (2006). A critical role for the type V myosin, Myo52, in septum deposition and cell fission during cytokinesis in *Schizosaccharomyces pombe*. Cell Motil. Cytoskelet. *63*, 149–161.

Mutoh, T., Nakano, K., and Mabuchi, I. (2005). Rho1-GEFs Rgf1 and Rgf2 re involved in formation of cell wall and septum, while Rgf3 is involved in cytokinesis in fission yeast. Genes Cells *10*, 1189–1202.

Nakano, K., Arai, R., and Mabuchi, I. (1997). The small GTP binding protein Rho1 is a multifunctional protein that regulates actin localization, cell polarity, and septum formation in the fission yeast *Schizosaccharomyces pombe*. Genes Cells *2*, 679–694.

Reid, T., Furiyashiki, T., Ishizaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madaule, P., and Nayumira, S. (1996). Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain. J. Biol. Chem. *271*, 13556–13560.

Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J. *18*, 578–585.

Ridley, A. J. (2006). Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends Cell Biol. *16*, 522–529.

Rossman, K. L., Channing, J. D., and Sondek, J. (2005). GEF means go: turning on Rho GTPases with guanine nucleotide-exchange factors. Nat. Rev. Mol. Cell Biol. *6*, 167–180.

Rossman, K. L., and Sondek, J. (2005). Larger than Dbl: new structural insights into RhoA activation. Trends Biochem. Sci. *30*, 163–165.

Roux, P. P., and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol. Mol. Biol. Rev. *68*, 320–344.

Sayers, L. G., Katayama, S., Nakano, K., Mellor, H., Mabuchi, I., Toda, T., and Parker, P. J. (2000). Rho-dependence of *Schizosaccharomyces pombe* Pck2. Genes Cells *5*, 17–27.

Sengar, A. S., Markley, N. A., Marini, N. J., and Young, D. (1997). Mkh1, a MEK kinase required for cell wall integrity and proper response to osmotic and temperature stress in *Schizosaccharomyces pombe*. Mol. Cell. Biol. *17*, 3508–3519.

Shiozaki, K., and Russell, P. (1995b). Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature *378*, 739–743.

Soisson, S. M., Nimnual, A. S., Uy, M., Bar-Sagi, D., and Kuriyan, J. (1998). Crystal structure of the Dbl and pleckstrin homology domains from the human son of sevenless protein. Cell *95*, 259–268.

Sugiura, R.T.T., Dhut, S., Shuntoh, H., and Kuno, T. (1999). The MAPK kinase Pek1 acts as a phosphorylation-dependent molecular switch. Nature *399*, 479–483.

Tajadura, V., Garcia, B., Garcia, I., Garcia, P., and Sanchez, Y. (2004). *Schizosaccharomyces pombe* Rgf3p is a specific Rho1 GEF that regulates cell wall β-glucan biosynthesis through the GTPase Rho1p. J. Cell Sci. *117*, 6163–6174.

Takada, H. *et al*. (2007). Atf1 is a target of the MAP kinase Pmk1 and regulates cell integrity in fission yeast. Mol. Biol. Cell *18*, 4794–4802.

Takeda, T., Toda, T., Kominami, K., Kohnosu, A., Yanagida, M., and Jones, N. (1995). *Schizosaccharomyces pombe* $atf1^+$ encodes a transcription factor required for sexual development and entry into stationary phase. EMBO J. *14*, 6193–6208.

Toda, T., Dhut, S., Superti-Furga, G., Gotoh, Y., Nishida, E., Sugiura, R., and Kuno, T. (1996b). The fission yeast $pmk1^+$ gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. Mol. Cell. Biol. *16*, 6752–6764.

Toda, T., Shimanuki, M., and Yanagida, M. (1993). Two novel protein kinase C-related genes of fission yeast are essential for cell viability and implicated in cell shape control. EMBO J. *12*, 1987–1995.

Vicente, M. F., Basilio, A., Cabello, A., and Peláez, F. (2003). Microbial natural products as a source of antifungals. Clin. Microbiol. Infect. *9*, 15–32.

Win, T. Z., Gachet, Y., Mulvihill, D. P., May, K. M., and Hyams, J. S. (2001). Two type V myosins with non-overlapping functions in the fission yeast *Schizosaccharomyces pombe*: Myo52 is concerned with growth polarity and cytokinesis, Myo 51 is a component of the cytokinetic actin ring. J. Cell Sci. *114*, 69–79.

Yang, P., Qyang, Y., Bartholomeusz, G., Zhou, Z., and Marcus, S. (2003). The novel Rho1 GTPase-activating protein family, Rga8, provides a potential link between cdc42/p21-activated kinase and Rho signaling pathways in the fission yeast, *Schizosaccharomyces pombe*. J. Biol. Chem. *278*, 48821–48830.

Yeh, B. J., Rutigliano, R. J., Deb, A., Bar-Sagi, D., and Lim, W. A. (2007). Rewiring cellular morphology pathways with synthetic guanine nucleotide exchange factors. Nature *447*, 596–600.

Zaitsevskaya-Carter, T., and Cooper, J. A. (1997). Spm1, a stress-activated MAP kinase that regulates morphogenesis in *Schizosaccharomyces pombe*. EMBO J. *16*, 1318–1331.

CAPÍTULO IV

En Schizosaccharomyces pombe, la proteína Rgf2p es un GEF de Rho1p, necesaria para la maduración de la pared de la espora y el mantenimiento de la integridad celular en ausencia de Rgf1p

Antecedentes

Las células de *S. pombe* en condiciones de escasez de nutrientes, especialmente nitrógeno, detienen su crecimiento en G1. Posteriormente conjugan y llevan a cabo dos divisiones meióticas para dar lugar a cuatro esporas haploides. En cada uno de estos pasos es necesaria la síntesis de nueva membrana y pared celular, para preservar la forma e integridad de las células.

Muchas de las proteínas que intervienen en la formación de la pared de la espora, son necesarias para que la esporogénesis se produzca de forma correcta. Una de ellas es la proteína Bgs2p, que es la única β-glucán sintasa esencial para la síntesis y maduración de la pared de la espora. En su ausencia las esporas presentan un aspecto inmaduro y carecen de la refringencia que se aprecia en las esporas silvestres. Además, las ascas *bgs2Δ/bgs2Δ* no liberan las esporas al medio espontáneamente y éstas son incapaces de germinar en medio rico (Liu et al., 2000; Martín et al., 2000).

Cuando realizamos una comparación de las secuencias de los genes *rgf3*$^+$ y *rgf1*$^+$ con la base de datos (http://www.genedb.org/genedb/pombe/index.jsp), encontramos otra ORF (SPAC1006.06) con un porcentaje de identidad significativo, a la que denominamos *rgf2*$^+$. La expresión de este gen aumenta en esporulación (Mata et al., 2002) y la proteína Rgf2p posee un dominio RhoGEF, por lo que podría ser un regulador de alguna de las GTPasas de la familia Rho. Al igual que *rgf3*$^+$ y *rgf1*$^+$, la caracterización del gen *rgf2*$^+$, nos pareció interesante para estudiar el modo de regulación de las GTPasas de la familia Rho en *S. pombe*.

Trabajo Experimental

1. Rgf2p es esencial para el desarrollo de la pared de la espora

Rgf2p pertenece a la familia de factores GEFs de *S. pombe* y presenta cuatro posibles dominios funcionales (DEP, DH, PH y CNH), con una distribución casi idéntica a la de Rgf1p. Además el porcentaje de identidad al comparar los dominios RhoGEF de Rgf1p y Rgf2p es mucho mayor, que el obtenido al comparar los mismos dominios de Rgf1p y Rgf3p, que son GEFs con la misma especificidad de sustrato.

Para determinar la función de Rgf2p examinamos los fenotipos de las células *rgf2Δ*. No observamos ninguna diferencia entre la cepa silvestre y el mutante *rgf2Δ* durante el crecimiento vegetativo, pero sí durante el proceso de esporulación. En ascas homozigóticas *rgf2Δ* la meiosis tiene lugar de manera normal, así como el desarrollo de la membrana de la proespora. Sin embargo, estas ascas presentan aspecto inmaduro y dan lugar a cuatro ascosporas que carecen de la refringencia característica de las esporas silvestres. La morfología de las ascas mutantes es muy parecida a la observada en las ascas *bgs2Δ*, (mutante nulo para la β-glucán sintasa esencial en esporulación) (Liu et al., 2000; Martín et al., 2000). Además, la gran mayoría de las ascas mutantes (*rgf2Δ*) no liberan espontáneamente las esporas al medio y cuando éstas se liberan mediante tratamiento enzimático, son incapaces de germinar.

Todos estos datos indican que Rgf2p está participando en el proceso de síntesis de la pared de las ascosporas y que en su ausencia éstas presentan una pared inmadura que les impide sobrevivir, incluso en condiciones ambientales favorables.

2. La actividad GS está disminuida en diploides *rgf2Δ*

Nos propusimos entonces, comprobar si Rgf2p podría ser un regulador de la actividad βGS durante la esporulación. Para conseguir una meiosis sincrónica del cultivo utilizamos un mutante *pat1-114*. En estas células, después de 6 horas de incubación a 34ºC, se inactiva la quinasa Pat1p que es un inhibidor de la meiosis, por lo que los diploides empiezan a esporular de manea sincrónica. En la cepa silvestre se produce un gran aumento de la actividad βGS, coincidiendo con la formación de la pared de las esporas, mientras que en las células *rgf2Δ* este pico de actividad es mucho menor. Estos datos demuestran que Rgf2p contribuye en gran medida a la actividad βGS durante la esporulación y por consiguiente, también a la síntesis de la pared de la ascospora.

Además, Rgf2p-GFP se observa solamente en la periferia de las ascosporas una vez que éstas están claramente delineadas y coincidiendo con la síntesis de la pared.

3. La sobreexpresión de *rgf2*$^+$ produce un aumento de Rho1p activo durante el crecimiento vegetativo

Dada la similitud que presentan Rgf2p y Rgf1p, nos preguntamos si Rgf2p podría actuar también como activador de Rho1p durante el crecimiento vegetativo.

La sobreexpresión de *rgf2*$^+$ es letal para las células, dando lugar a células más largas de lo normal, multiseptadas y con septos más engrosados. Además, pudimos comprobar que el nivel de Rho1p activo en estas células era muy superior al de la cepa silvestre y que también presentaban una actividad βGS incrementada.

4. Rgf2p es necesaria para el mantenimiento de la integridad en ausencia de Rgf1p

Con el propósito de investigar si Rgf2p tenía alguna función redundante con otros GEFs de Rho1p, decidimos construir todas las combinaciones posibles de mutantes dobles entre estas tres proteínas: Rgf1p, Rgf2p y Rgf3p. Sólo los genes *rgf1*$^+$ y *rgf2*$^+$ resultaron ser sintéticos letales. Además, al igual que les ocurre a las células con niveles de Rho1p muy bajos (Arellano et al., 1997), la letalidad de la cepa *rgf1*Δ*rgf2*Δ no pudo ser suprimida añadiendo un estabilizador osmótico al medio.

Estos resultados sugieren que Rgf1p y Rgf2p comparten un papel esencial como activadores de Rho1p durante el crecimiento vegetativo.

A continuación, nos preguntamos si Rgf1p y Rgf2p poseían funciones intercambiables. Para ello, aumentamos los niveles de *rgf2*$^+$ en una cepa *rgf1*Δ y observamos que todos los defectos propios de estas células fueron suprimidos en mayor o menor medida.

Por otro lado, quisimos comprobar si lo contrario también era cierto, es decir, si Rgf1p podía realizar las funciones de Rgf2p en esporulación. Para lo cual, expresamos *rgf1*$^+$ bajo el control del promotor de *rgf2*$^+$, de forma que hubiera una expresión suficiente de este gen durante la esporulación. En estas condiciones Rgf1p pudo también suprimir, aunque sólo parcialmente, los defectos de germinación de las ascosporas *rgf2*Δ.

Podemos concluir por tanto, que Rgf1p y Rgf2p son parcialmente intercambiables, aunque sólo cuando se expresan a unos niveles adecuados en la célula.

Fission Yeast Rgf2p Is a Rho1p Guanine Nucleotide Exchange Factor Required for Spore Wall Maturation and for the Maintenance of Cell Integrity in the Absence of Rgf1p

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ABSTRACT

Schizosaccharomyces pombe Rho1p is essential, directly activates β-(1,3)-glucan synthase, and participates in the regulation of morphogenesis. In *S. pombe,* Rho1p is activated by at least three GEFs (guanine nucleotide exchange factors): Rgf1p, Rgf2p and Rgf3p. In this study we show that Rgf2p is a Rho1p GEF required for sporulation. The *rgf2*$^+$ deletion did not affect forespore membrane formation and the nuclei were encapsulated properly. However, the mutant ascospores appeared dark and immature. The *rgf2*Δ zygotes were not able to release the ascospores spontaneously, and the germination efficiency was greatly reduced compared to wild-type spores. This phenotype resembles that of the mutants in *bgs2*$^+$, which encodes a sporulation-specific glucan synthase subunit. In fact, glucan synthase activity was diminished in sporulating *rgf2*Δ diploids. Rgf2p also plays a role in β-glucan biosynthesis during vegetative growth. Overexpression of *rgf2*$^+$ specifically increased GTP-bound Rho1p, caused changes in cell morphology, and elicited an increase in β-1,3-glucan synthase activity. Moreover, the simultaneous disruption of *rgf1*$^+$ and *rgf2*$^+$ was lethal and both proteins (Rgf1p and Rgf2p) were able to partially substitute for each other. Our results suggest that Rgf1p and Rgf2p are alternative GEFs with an essential overlapping function in Rho1p activation during vegetative growth.

SCHIZOSACCHAROMYCES pombe cells are rod shaped, grow mainly by elongation of their ends, and divide by binary fission after forming a centrally placed division septum (HAYLES and NURSE 2001). Upon nutrient starvation, especially that of nitrogen, *S. pombe* cells exit the mitotic cycle at G1 and proceed through mating and two meiotic divisions to generate four haploid spores (EGEL 2004; SHIMODA and NAKAMURA 2004; YAMAMOTO 2004). In each of these polarization states, a new membrane and cell wall are necessary to preserve cellular shape and integrity.

The cell wall is a rigid structure that protects yeast cells, controlling all communication with the extracellular world. However, the cell wall must be loosened to allow expansion during periods of polarized growth, while it needs to be constrained when cells are growing in poor substrate conditions (LATGÉ 2007; LEVIN 2005). The *S. pombe* cell wall mainly consists of an outer layer rich in galactomannoproteins and an inner layer of β-1,3-, β-1,6- and α-1,3-glucans (DURÁN and PÉREZ 2004; MANNERS and MEYER 1977). Among the polysaccharides, we focused on the β-1,3-glucan fibrillar network. β-1,3-glucan is the major structural component and is also the first polymer to be synthesized in regenerating protoplasts (OSUMI *et al.* 1989) and in the *S. pombe* spore wall (GARCIA *et al.* 2006; MARTIN *et al.* 2000). β-1,3-glucan biosynthesis is carried out by the β-1,3-glucan synthase complex (GS), a multimeric enzyme composed of both catalytic and regulatory subunits. The catalytic component is encoded by the *bgs* family of genes (*bgs1*$^+$, *bgs2*$^+$, *bgs3*$^+$ and *bgs4*$^+$) (CORTES *et al.* 2005; CORTÉS *et al.* 2007; LE GOFF *et al.* 1999; LIU *et al.* 2002; LIU *et al.* 2000b; MARTIN *et al.* 2003; MARTIN *et al.* 2000), while the regulatory component is the GTPase Rho1p (ARELLANO *et al.* 1996). The biochemistry of the GS complex has been characterized, but the assembly process and the activation mechanisms that polarize cell wall extension only to certain areas of the cell wall remain largely unknown. There is accumulating evidence that Bgs (β-GS) might be controlled by local/temporal activation of Rho1p. Rho1p acts as a binary switch, cycling between an inactive GDP-bound and an active GTP-bound conformational state, stimulating GS in its GTP-bound prenylated form (ARELLANO *et al.* 1997; ARELLANO *et al.* 1996; NAKANO *et al.* 1997). During vegetative growth, Rho1p travels to the growth sites, poles, and septum to meet Bgs1p, Bgs3p and Bgs4p. The three GS catalytic subunits localize to the poles during tip elongation and to the septum during cytokinesis; all of them are large integral membrane proteins whose levels do not fluctuate along the cell cycle (LIU *et al.* 2002; MARTIN *et al.* 2003). Bgs1p is required for primary septum formation (CORTÉS *et al.*

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2007), while Bgs3p and Bgs4p are good candidates for the synthesis of the β-1,3-glucan of the surrounding cell wall and the secondary septum, both with a similar composition (CORTES *et al.* 2005; HUMBEL *et al.* 2001). β-1,3-glucan also accounts for 38% of the polysaccharides present in the spore wall (GARCIA *et al.* 2006). In spores, β-1,3-glucan synthesis is carried out by Bgs2p, the GS catalytic subunit specific for sporulation (LIU *et al.* 2000; MARTIN *et al.* 2000). Bgs2p is required for proper spore wall maturation and in its absence the spores are not released from the ascal sac and are unable to germinate (MARTIN *et al.* 2000).

For GS activation, as well as for other functions, Rho1p must be precisely regulated in response to temporally preceding upstream signals. This regulation mainly involves two types of proteins: GEFs (guanine nucleotide exchange factors) and GAPs (GTPase activating proteins) (BOS *et al.* 2007; ROSSMAN *et al.* 2005a). GEFs turn on signalling by catalyzing the exchange from G-protein-bound GDP to GTP, whereas GAPs terminate signalling by inducing GTP hydrolysis. All these proteins are multidomain proteins and play important roles in the specificity of Rho functions. In *S. pombe* there are at least three GEFs (Rgf1p, Rgf2p and Rgf3p) (GARCÍA *et al.* 2006b), and several putative GAPs (Rga1p, Rga5p and Rga8p) (CALONGE *et al.* 2003; NAKANO *et al.* 2001; YANG *et al.* 2003), all of them specific for Rho1p.

Among the activators, *rgf3*$^+$ was first cloned in our laboratory by complementation of a mutant (*ehs2-1*) hypersensitive to drugs that interfere with cell wall biosynthesis (TAJADURA *et al.* 2004). *rgf3*$^+$ is essential for cell viability and the protein localizes exclusively to the middle region of the cell (MORRELL-FALVEY *et al.* 2005; MUTOH *et al.* 2005; TAJADURA *et al.* 2004). Rgf3p activates GS and increases the amount of cell wall β-1,3-glucan. Thus, it is probable that Rgf3p stimulates Rho1p-mediated activation of a type of GS activity that is crucial for proper septum function (TAJADURA *et al.* 2004). Rgf1p is not essential for viability, but it does play an important role in regulating the growth pattern of fission yeast cells and signals upstream from the Pmk1 mitogen-activated protein kinase pathway (GARCÍA *et al.* 2006a; GARCÍA *et al.* 2009). Rgf1p localizes to the cell tips in interphase cells and to the division septum in mitotic cells, and it activates the β-GS complex containing the catalytic subunit Bgs4p (GARCÍA *et al.* 2006a; MORRELL-FALVEY *et al.* 2005; MUTOH *et al.* 2005). Moreover, Rgf1p is required for actin reorganization necessary for cells to change from monopolar to bipolar growth during NETO (New End Take Off), thus coupling a cell polarity transition to cell wall biogenesis (GARCÍA *et al.* 2006a). Based on two-hybrid analysis and its protein sequence (MUTOH *et al.* 2005), Rgf2p has been proposed to be a Rho1p GEF. Rgf1p and Rgf2p are the closest relatives -63.4% identity within the DH domains- but while *rgf1*Δ cells are hypersensitive to cell wall-damaging agents and other types of stress, *rgf2*Δ cells are similar to wild-type cells and Rgf2p is not required for cell elongation or assembly of the division septum.

Here we report that simultaneous depletion of Rgf1p and Rgf2p is lethal in vegetative cells and that mild overexpression of *rgf2*$^+$ fully rescues the lysis and hypersensitivity to caspofungin, which interferes with cell wall integrity, of *rgf1*Δ cells. Our data strongly suggest that Rgf1p and Rgf2p share an essential function as Rho1p activators during vegetative growth. In addition, Rgf2p appears to play an essential function as a GS activator during the sporulation process. Following meiosis, *rgf2*-null mutants failed to properly assemble the spore wall, resulting in the formation of immature spores. The subcellular localization of Rgf2p supports its role in spore wall assembly.

MATERIALS AND METHODS

Media, reagents and genetics: The genotypes of the *S. pombe* strains used in this study are listed in Table 1. The complete yeast growth medium (YES), selective medium (MM) supplemented with the appropriate requirements and sporulation medium (MEA) have been described elsewhere (MORENO *et al.* 1991). Caspofungin (Csp) was stored at -20° in a stock solution (2.5 mg/ml) in H_2O and was added to the media after autoclaving at the corresponding final concentration. Crosses were performed by mixing appropriate strains directly on MEA plates and diploids allowed to sporulate at 28º. Recombinant strains were obtained by tetrad analysis. For synchronous meiosis, diploid strains homozygous for *pat1-114*^{ts} were cultured in MM-N at 24º for 18h, after which the temperature was shifted to 34º to induce meiosis (IINO *et al.* 1995). For overexpression experiments using the *nmt1* promoter, cells were grown to logarithmic phase in EMM containing 15μM thiamine. Cells were harvested, washed three times with water, and inoculated in fresh medium (without thiamine) at an OD_{600}=0.01.

Disruption of the *rgf2*$^+$ gene: The *rgf2::ura4*$^+$ disruption construct was obtained in a two-step process. The 5´ non-coding region of the *rgf2*$^+$ ORF [nucleotides (nt) -1475 to -23] was amplified by PCR, inserting the *Apa*I and *Sal*I sites (one at each end), and was ligated into the same sites of the SK-*ura4*$^+$ vector. The 3´ flanking region of the *rgf2*$^+$ ORF (nt +3604-5241) was amplified by PCR, inserting the *Bam*HI and *Not*I sites as above, and was cloned into the same sites of pSK-*ura*$^+$ with the 5´ end, to yield pGR2. *rgf2*$^+$ gene disruption was accomplished using the 4.7 Kb fragment from pGR2 cut with *Apa*I and *Not*I and transforming the YS165 diploid strain. Transformants were replica-plated five times consecutively on YES medium to eliminate cells that had not integrated the construct. Integration was analysed by PCR using the following oligonucleotides: IPCR-b (5´-CACCATGCCAAAAATTACACAAGATAG AAT-3´) in the *ura4*$^+$ gene; Rom3-ext-3´ (5´-GAGACGGTAAAATCACG-3´) downstream from nucleotide +5311, and therefore external to the disruption cassette; Rom3-int-3´ (5´-TCCAGCAAATGCAGCAG-3´) in the *rgf2*$^+$ gene. A diploid strain heterozygous for the *rgf2*::*ura4*$^+$ allele was subjected to tetrad analysis. 2*ura4*$^+$:2*ura4*$^-$ segregation indicating that *rgf2*$^+$ is not essential for vegetative growth. To make the *rgf2::his* disruption construct (pRZ38), the upstream *Apa*I-*Sal*I fragment and the downstream 1.6 Kb *Bam*HI-*Not*I fragment were ligated into the same sites of pSK-*his3*$^+$. Plasmid pRZ38 was digested with *Apa*I and *Not*I and the linear DNA containing the cassette was used transform a haploid strain (YS64). *rgf2*::*his3* disruptants (GRG55) were tested for stability and analysed by PCR.

Construction of plasmids and strains: *rgf2*$^+$ was obtained from cosmid SPA1006. First, upstream (1.4 Kb) and downstream (1.6 Kb) flanking sequences from *rgf2*$^+$ obtained by PCR amplification were subcloned into *Apa*I-*Sal*I and *Bam*HI-*Not*I from pAL-KS (*S. pombe ars1*$^+$ and *S. cerevisiae LEU2* selection) to make plasmid pGR3. Then, we cloned a 3.7 Kb *Xho*I-*Sph*I fragment from cosmid

TABLE 1

***S. pombe* strains used in this work**

Strains	Genotype	Source
YSM180	h$^-$ 972	Our collection
PG242	h$^+$ *rgf2::ura4*$^+$	This work
YS64	h$^-$ *leu1-32 ade6M210 ura4D-18 his3D1*	Our collection
YS71	h$^+$ *leu1-32 ade6M210 ura4D-18 his3D1*	Our collection
PG1	h$^-$ *leu1-32 ade6M210 his3D1 ura4D-18 rgf2::ura4*$^+$	This work
PG5	h$^+$ *leu1-32 ade6M210 his3D1 ura4D-18 rgf2::ura4*$^+$	This work
PG9	h^{90} *leu1-32 ade6M210 his3D1 ura4D-18 rgf2::ura4*$^+$	This work
YS260	h^{90} *leu1-32 ade6M210 his3D1 ura4D-18*	Our collection
YS527	h$^-$ *leu1-32 ade6M210 his3D1*	H. Valdivieso lab
YS165	h$^+$/h$^-$ *leu1-32/ leu1-32 ade6M210/ade6M216 ura4D-18/ura4D-18 his3D1/his3D1*	Our collection
PG3	h$^+$/h$^-$ *leu1-32/ leu1-32 ade6M210/ade6M216 rgf2::ura4*$^+$*/rgf2*$^+$ *ura4D-18/ura4D-18 his3D1/his3D1*	This work
PG107	h$^-$/h$^-$ *pat1-114/pat1-114 leu1-32/leu1-32 ade6M-210/ade6M-216*	S. Moreno lab
PG110	h$^-$/h$^-$ *rgf2::ura4*$^+$*/rgf2::ura4*$^+$ *pat1-114/pat1-114 leu1-32/leu1-32 ade6M-210/ade6M-216*	This work
PG115	h- *leu1-32 his3D1 ura4D-18 ade6M210 P81Xnmt-rgf1*$^+$	This work
VT88	h$^-$ *leu1-32 ade6M210 ura4D-18 his3DI 81 nmt-rgf3*$^+$*-ura4*$^+$	Our collection
PG94	h$^-$ *leu1-32 ade6M210 ura4D-18 his3DI rgf1::his3*$^+$ *P81Xnmt-rgf3*$^+$*-ura4*$^+$	This work
PG395	h$^-$ *leu1-32 ade6M210 ura4D-18 his3DI rgf2::his3*$^+$ *P81Xnmt-rgf3*$^+$*-ura4*$^+$	This work
GRG33	h$^-$ *leu1-32 ade6M210 his3D1 ura4D-18 rgf2::ura4*$^+$*P81Xnmt-rgf1*$^+$	This work
GRG55	h$^-$ *leu1-32 ade6M210 his3D1 ura4D-18 rgf2::his3*$^+$	This work
GI1	h$^-$ *ehs2-1 leu1-32*	Our collection
PG105	h$^-$ *ehs2-1 leu1-32 ura4D-18 rgf2::ura4*$^+$	This work
MS228	h^{90} *leu1-32 his3D1 bgs2::his3*$^+$	Our collection
NG188	h^{90} *leu1-32 ade6M210 his3D1 ura4D-18 rgf2::ura4*$^+$ *bgs2::his3*$^+$	This work
PG280	h^{90} *leu1-32 ade6M210 his3D1 ura4D-18 rgf2::ura4*$^+$*chs1::his3*$^+$	This work
PG222	h^{90} *leu1-32 ade6M210 his3D1 ura4D-18 mok12::ura4*$^+$ *rgf2::his3*$^+$	This work
PPG1.60	h$^-$ *HA:rho1 leu1-32*	P. Pérez lab
PPG2.17	h$^+$ *leu1-32 ura4D-18 rho1::ura4*$^+$ p41X-*rho1*$^+$	P. Pérez lab
PG260	h^{90} *leu1-32 ura4D18 rgf2-GFP::kan*^{R}	This work
VT14	h$^-$ *leu1-32 ade6M210 ura4D-18 his3D1 rgf1::his3*$^+$	Our collection
PG88	h$^+$ *leu1-32 ura4D-18 cdc10-129 rgf1::his3*$^+$	Our collection
MS168	h$^-$ *leu1-32 ura4D-18 cdc10-129*	Our collection

SPA1006 (containing the *rgf2*$^+$ ORF) into pGR3 (cut with *Xho*I-*Sph*I) to obtain pGR13, bearing the entire *rgf2*$^+$ ORF and flanking sequences. To tag Rgf2p at the C-terminus with enhanced green fluorescent protein (engineered with 8 alanines at the N-terminus) and with the triple repeat of the influenza virus haemagglutinin epitope (HA) (CRAVEN *et al.* 1998), pAL-*rgf2*$^+$ (pGR13) was modified by site-directed mutagenesis. We destroyed the *Not*I site at the multiple cloning site and created a *Not*I site by site-directed mutagenesis three nt before the TAG stop codon of *rgf2*$^+$ (pGR39). The 8ala-GFP and HA epitopes were inserted in-frame at the *Not*I site of pGR39. pGR76 (pAL-*rgf2*$^+$-8ala-*GFP*) and pGR51 (pAL-*rgf2*$^+$*-HA*) fully complemented the *rgf2*Δ phenotypes. Strain (PG260), containing a chromosomal copy of *rgf2*$^+$ tagged with 8ala-GFP, was obtained using a PCR-based approach, as described by (BÄHLER *et al.* 1998). The primers 5´-CAATTCCATTTCATCTGGAGAAAATCCTATAGTTCATTCTTTAATACTTCCTCCAGCAAATGCAGCAGGTCCTGCACTTGGTGGTGGTGGTGGGATCCCCGGGTTAATTAA3´ and 5´-GGTCATAGATTCTTGAAGCTTAGATTGTAATAGCATATATATAATCAATTGATGTGGCATGCAAAAGATGTCCGGAGTAGGAATTCGAGCTCGTTTAAAC-3´ were used to amplify GFP-kan from pFA6a-GFP(S65T)-kanMX6. The same approach was used to construct the strain (PG115) with the endogenous *rgf1*$^+$ gene driven by the 81X*nmt* promoter. The primers 5´-GTAACCAATGCGAACGCAATTAAAATAAAATAAGTCAATAGCATAGTCATAGAAATCGATCAATTGTTATCCGGAGAATTCGAGCTCGTTTAAA-3´ and 5´-CTTGGAGCACCAAAAATTTCCTCATAAGCACGCGAGCTCTCATTCACACCGAGTGCATTTGGATGCCGTAATCCATTGCCATGATTTAACAAAGCGACTATA-3´ were used to amplify *kan-P81xnm*t from pFA6a-kanMX6-P81nmt. To make strain GRG33, *P81nmt-rgf1 rgf2*Δ, the *rgf2*::*ura4*$^+$ disruption cassette from pGR2 was used to transform PG115 cells carrying the endogenous *rgf1*$^+$ promoter replaced by the *P81nmt* promoter. To make strain PG94, *P81nmt-rgf3 rgf1*Δ, the *rgf1*::*his3*$^+$ disruption cassette (GARCÍA *et al.* 2006a) was used to transform VT88 cells carrying the *rgf3*$^+$ gene driven by the *P81nmt* promoter (TAJADURA *et al.* 2004). Strain PG395, *P81nmt-rgf3 rgf1*Δ, was obtained from VT88 cells carrying the *rgf3*$^+$ gene driven by the *P81nmt* promoter, transformed with the *rgf2*::*his3*$^+$ disruption cassette and analyzed by PCR.

For *rgf2*$^+$ overexpression, pGR13 (pAL-*rgf2*$^+$) was modified by site-directed mutagenesis, introducing *Xho*I and *Sma*I sites flanking the *rgf2*$^+$ ORF and thus creating pGR69. The *rgf2*$^+$ ORF from pGR69 was cloned into the same sites of pREP3X, pREP41X and pREP81X, thus making pGR70, pGR71 and pGR72 respectively. For pREP3X-*rgf2-PTTR*Δ (pGR93), the *rgf2*$^+$ ORF from pGR69 was mutagenized by site-directed mutagenesis to eliminate the sequence "cctacgactcgc". The mutated ORF was subcloned as a *Xho*I-*Sma*I fragment into the same sites of pREP3X. To overexpress *rgf2*$^+$

tagged with HA or GFP, the *rgf2*$^+$ ORF was obtained from pGR51 (pAL-*rgf2*$^+$-*HA*) or pGR76 (pAL-*rgf2*$^+$-8ala-*GFP*) respectively. pGR83 is pREP41X-*rgf2*$^+$-HA; pGR84 is pREP41X-*rgf2*$^+$-8ala-GFP; pGR85 is pREP3X-*rgf2*$^+$-HA, and pGR86 is pREP3X-*rgf2*$^+$-8ala-GFP. Plasmid pNG45, bearing the *rgf1*$^+$ ORF under the control of the *rgf2*$^+$ promoter, was obtained by eliminating the *nmt*1 promoter from pGR33 (pREP3X-*rgf1*$^+$) by digestion with *Pst*I-*Nco*I and replacing it with the *rgf2*$^+$ promoter obtained by PCR with *Pst*I-*Nco*I tails. pREP3X-*rho1*$^+$ was kindly provided by P. Pérez and is described in (ARELLANO *et al.* 1996).

Cell wall analyses: Enzyme preparations and GS assays were essentially performed as described previously (MARTIN *et al.* 2000). 1×10^9 cells were harvested, washed twice with buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol) and resuspended in 100 µl of the same buffer. Lysis was achieved in a Fast-Prep device, using 0.4 g of glass beads and spinning three times for 15 s at a speed setting of 5. The resulting homogenates were collected by adding 3 ml of buffer A. The cell walls were removed by low-speed centrifugation (5000 *g* for 5 min at 4°C). The supernatant was centrifuged for 30 min at 48 000 *g*, and the membrane pellet was resuspended in 0.3 ml of buffer A containing 33% glycerol and stored at −70°C. Standard GS assays contained 15-25 µg protein of enzyme extract (3-5 mg protein/ml) in a total volume of 40 µl, and the reaction mixture was incubated at 30º for 60-90 min. All reactions were carried out in duplicate and values were calculated from two independent cell cultures. One unit of activity was measured as the amount that catalyses the uptake of 1 µ mol of substrate (UDP-D-glucose) min^{-1} at 30º.

Pull-down assay for GTP-bound Rho proteins: The expression vector pGEX-C21RBD (rhotekin-binding domain)(REID et al. 1996) was used to transform *Escherichia coli* cells. The fusion protein was produced according to the manufacturer´s instructions and immobilized on glutathione-Sepharose 4B beads (Amersham). After incubation, the beads were washed several times and the bound proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. The amount of GTP-bound Rho proteins was analysed using the Rho-GTP pull-down assay modified after (REN et al. 1999). Briefly, extracts from 50-ml cultures of wild-type cells transformed with pREP3X, pREP3X-rgf2$^+$ (pGR70) or pREP3X-rgf2-PTTRΔ (pGR93), containing HA-rho1$^+$ expressed from its own promoter, were obtained using 200 µl of lysis buffer (50 mM Tris, pH 7.5, 20 mM NaCl, 0.5% NP-40, 10% glycerol, 0.1 mM dithiothreitol, 1 mM NaCl, 2 mM $MgCl_2$, containing 100 µM p-aminophenyl methanesulfonyl fluoride, leupeptin, and aprotinin). 100 µg of GST-RBD fusion protein coupled to glutathione-agarose beads was used to immunoprecipitate 1.5 mg of cell lysates. The extracts were incubated with GST-RBD beads for 2 h. The beads were then washed with lysis buffer four times, and bound proteins were blotted against 1:5000-diluted 12CA5 mAb as primary antibody to detect HA-Rho1p. The total amount of HA-Rho1p was monitored in whole-cell extracts (25 µg of total protein), which were used directly for Western blot and were developed with 12CA5 mAb. Immunodetection was accomplished using the ECL detection kit (Amersham Biosciences).

Microscopy techniques: The localization of Rgf2p-GFP was visualized in living cells. For Calcofluor staining, exponentially growing S. pombe cells were harvested, washed once, and resuspended in water with Calcofluor white (Cfw) at a final concentration of 20µg/ml for 5 min at room temperature. After washing with water, the cells were observed under a DMRXA microscope (Leica, Wetzlar, Germany).

RESULTS

Rgf2p is required for ascospore development: Rgf2p belongs to a family of guanine nucleotide exchange factors (GEFs) in *S. pombe* (GARCÍA *et al.* 2006b) (http://www.genedb.org/genedb/pombe/index.jsp). Rgf2p contains a putative Dbl homology domain (DH) (amino acid residues -aa- 461-633) found in proteins responsible for the activation of Rho-family GTPases). Adjacent and C-terminal to the DH domain is a pleckstrin homology domain (PH) (aa 670-805), which has been proposed to localize GEFs proteins to the plasma membrane and to regulate their GEF activity through allosteric mechanisms (ROSSMAN and SONDEK 2005b). Apart from the DH-PH module, Rgf2p contains at least two additional predicted functional domains: a DEP (*Dishevelled*, *Egl-10*, and *Pleckstrin*) domain (aa 212-287) and a CNH (*Citron and NIK1-like kinase homology domain*) (aa, 827-1120) (Figure 1A). Within the GEF family, the closest relative to Rgf2p is Rgf1p (GARCÍA *et al.* 2006b). The percent identity between the deduced amino acid sequence of the DH domain of Rgf2p and Rgf1p is 63.4%, while the identity is less than 20% between the DH domain of Rgf2p and Rgf3p, Scd1p and Gef1p. Moreover, the distribution of the DEP, DH-PH and CNH domains is similar in both proteins, suggesting that the N-terminus is shorter in Rgf2p, although this has yet to be proved.

To investigate the function of *rgf2*$^+$ in greater depth, we constructed and examined the phenotypes of *S. pombe* strains in which *rgf2*$^+$ had been deleted (see MATERIALS AND METHODS) (Figure 1). Haploid *rgf2*Δ transformants were obtained as readily as diploid transformants, indicating that *rgf2*$^+$ was not required for cell growth. *rgf2*Δ cells did not exhibit any evident morphological changes, as judged by light microscopy, and the *rgf2*Δ strain grew well under standard growth conditions at either 28º or 37º and entered stationary phase at the same time as the wild-type cultures. We noticed that *rgf2*Δ cells even grew slightly faster than wild-type cells (not shown). We also examined cell viability of stationary phase *rgf2*Δ and *rgf2*$^+$ cultures incubated for 4 days at 28º; both strains were found to be >90% viable during this period.

Next, we looked at the effect of the mutation on mating and sporulation. The mating rate was not affected in *rgf2*Δh$^+$ x *rgf2*Δh$^-$ crosses (data not shown), but we did observe a sporulation defect. In wild-type, 70% of the culture showed refringent ascospores after 24 hours. By contrast, no examples of mature asci were observed in the *rgf2*Δ mutant. The *rgf2*Δ zygotes appeared dark, although it was possible to differentiate the outline of the four ascospores inside the asci (Figure 1B). The sporulation defect seen in *rgf2*Δ homozygous zygotes was rescued by the plasmid carrying the *rgf2*$^+$ gene (pGR13), but not by the vector alone (pAL). Moreover, heterozygous zygotes (*rgf2*Δ/*rgf2*$^+$) generated phenotypically wild-type asci, with four viable and refringent ascospores. This result indicates that the *rgf2*Δ allele is recessive and suggests that *rgf2*$^+$ expression takes place before the time of prospore enclosure. To pinpoint the exact time at which *rgf2*$^+$ begins to play its role in sporulation, we examined the meiotic time-course of *rgf2*Δ gene expression by Northern analysis. Synchronous meiosis was induced using the *pat1-114* mutation (see MATERIALS AND METHODS). As shown in Figure 1C, *rgf2*$^+$ mRNA was detectable in vegetative cells (at 0 h) and *rgf2*$^+$ mRNA peaked at 5 h when cells were in

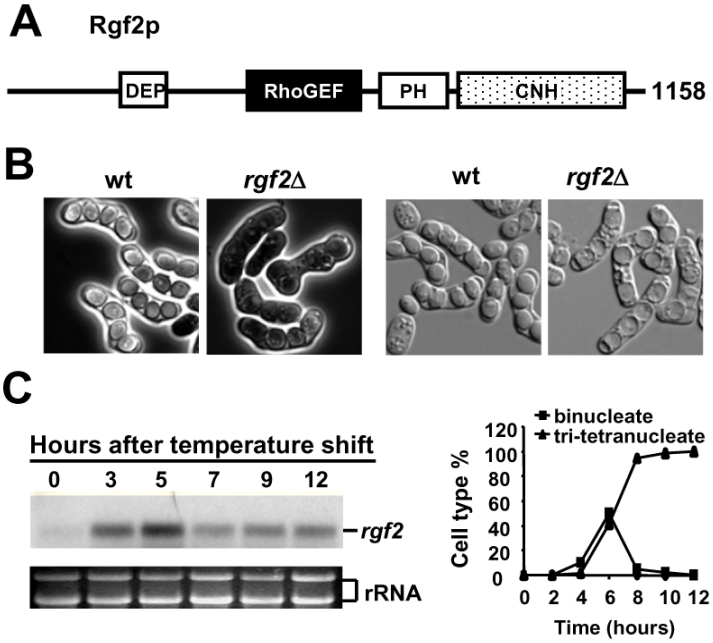

FIGURE 1.—Rgf2p is required for ascospore development. (A) Schematic representation of the structural features of the Rgf2 protein analysed by the SMART program (http://smart.embl-heidelberg.de/). Domains are indicated: DEP, is present in signalling molecules and is responsible for mediating intracellular protein targeting (CHEN and HAMM 2006); RhoGEF/DH domain (Dbl homology domain), conserved among GEFs for Rho/Rac/Cdc42-like GTPases; PH, pleckstrin homology domain (ROSSMAN and SONDEK 2005b); CNH, citron homology domain, required for protein-protein interaction (TAIRA *et al.* 2004). (B) Sporulation phenotype of wild-type (YS64 x YS71) and *rgf2*Δ (PG1 x PG5) cells incubated for 24h on MEA. Phase contrast micrographs are shown on the left and Nomarsky micrographs are shown on the right. (C) *rgf2*$^+$ mRNA is highly induced during meiosis. Synchronous meiosis was induced in diploid cells carrying the *pat1-114*^{ts} temperature-sensitive allele by a temperature upshift (34º) after overnight incubation in nitrogen-free medium at the permissive temperature (25º). The kinetics of meiosis in the *pat1-114*^{ts} mutant was followed by staining a portion of the culture every 2 h with Hoechst stain and is shown in the graphic. Total RNA samples were prepared at the indicated times and subjected to Northern blot analysis. Ethidium bromide staining of rRNA is presented as a loading control.

meiosis II, although a certain level of *rgf2*$^+$ mRNA was maintained during spore wall maturation. The *rgf2*$^+$ induction profile was similar to that described by (MATA *et al.* 2002), available on the *S. pombe* gene database (http://www.genedb.org/genedb/pombe/index.jsp). Taken together, these observations indicate that *rgf2*$^+$ is required for spore development and that its induction profile is that of a late gene whose expression is induced just before the bulk of refringent ascospores appear.

Rgf2p is required for the assembly of functional spore walls: The induction profile of *rgf2*$^+$ and the fact that RhoGTPases play important roles in the regulation of protein transport and membrane recycling, prompted us to test the possibility that the *rgf2*Δ mutation might be affecting forespore membrane (FSM) expansion during the early stages of sporulation. To this end, the envelopes of the developing spores were monitored using a GFP-tagged marker protein (Mde10 fused to GFP in plasmid A799) and nuclei stained with the Hoechst DNA-specific fluorophore. We constructed an *rgf2*Δ h^{90} (PG9) and the isogenic h^{90} wild-type parental strain (YS260). Both homothallic strains were transformed with plasmid A799 (kindly provided by Dr Hiraoka, Kobe, Japan, (DING *et al.* 2000; NAKAMURA *et al.* 2004)), cultured in MM (-N) for 10h, and stained with Hoechst reagent. Both the first and the second meiotic divisions in the mutants proceeded with similar kinetics to those of wild-type cells (not shown). Moreover, 86% of the *rgf2*Δ asci showed four spore-like structures outlined by GFP signals (n=40 asci labeled with GFP) (Figure 2A). Our results clearly indicate that the *rgf2*Δ mutation does not impair meiosis or the normal development of the FSM.

In wild-type cells, refringent spore walls were first seen after 12 h in sporulation medium. In contrast, microscopic examination of *rgf2*Δ cells revealed dark cells, suggesting that the mutants were blocked during the assembly of the spore wall: the structure that confers the spore refringence and a high degree of resistance to stressful conditions. This phenotype suggests a delay in ascospore maturation. Even after prolonged incubation in sporulation medium no increase in the number of refringent spores was observed.

We then examined whether the *rgf2*Δ mutant diploid was able to give rise to viable ascospores capable of germinating in rich medium. We noted that the *rgf2*Δ asci were not dehiscent, which meant that we could not release single ascospores by simple micromanipulation. To ascertain spore survival, we isolated and individually micromanipulated 200 zygotes of each strain onto rich solid medium (YES or YES plus sorbitol). Most of the wild-type zygotes were viable and formed colonies (82%), whereas the *rgf2*Δ zygotes largely failed to germinate (18% viability) (Figure 2B). The percent germination was similar, regardless of the presence or absence of sorbitol. To test the possibility that the *rgf2*Δ spores were impaired in germination because they failed to break the ascal sac, we treated the sporulating cultures with helicase, an enzyme that destroys vegetative cells and releases single ascospores from the ascus. The *rgf2*Δ ascospores released after treatment (0.2% helicase, o/n at room temperature) were grey and showed an aberrant morphology, different from that of the wild-type ascospores, which were round and refringent. The plating efficiency of *rgf2*Δ spores was less than 1% as compared to 81% for the wild-type spores (Figure 2B), and it was independent of the presence of sorbitol in the medium. We then examined spore integrity by

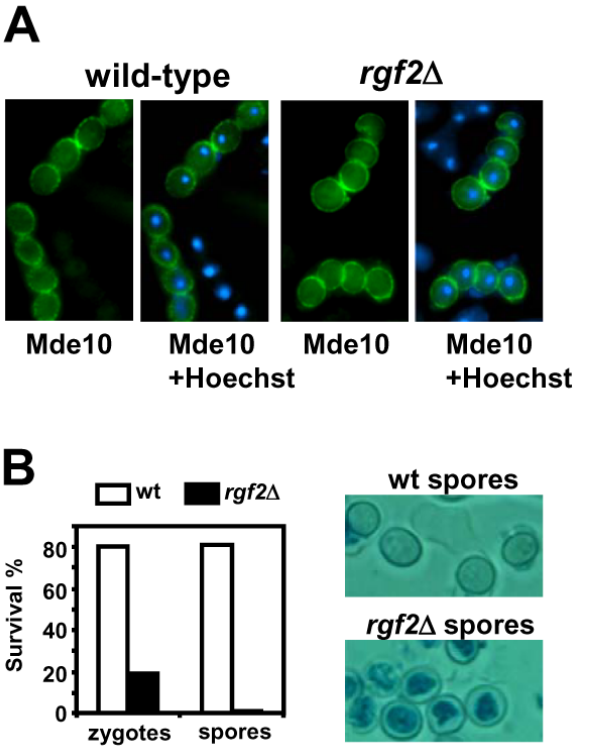

FIGURE 2.—Characterization of the phenotype of the *rgf2* disruptants in sporulation. (A) Encapsulation of nuclei in *rgf2*Δ spores. The homothallic haploid strains YS260 (h^{90} *rgf2*$^+$) and PG9 (h^{90} *rgf2*Δ) harbouring the A799 plasmid were incubated in MM-N and samples were taken after 12 h. FSM (fore-spore membranes) were visualized by fluorescence (Mde10-GFP) and nuclei by Hoechst staining. In *rgf2*Δ cells, >80% of the spore envelope encapsulated a single nucleus (n=40 sporulated cells). (B) Efficiency of zygotes and ascospore germination in *rgf2*Δ homothallic strains. In the left panel, strains YS260 (h^{90} *rgf2*$^+$) and PG9 (h^{90} *rgf2*Δ) were incubated on MEA plates and the zygotes from 2-day sporulation plates were micromanipulated individually (n= 200 of each strain), spotted onto YES plates, and incubated at 28º. For spore survival, cells from 3-day sporulation cultures were resuspended in 1 ml of water to an OD_{600} 20, treated with helicase (0.2%) for 12 h, and diluted with water. The same dilutions of each strain were spread onto YES plates and incubated for 3 days. In the right panel, the spores treated with helicase as above were stained with Methylene Blue (0.01% in 0.2M acetate buffer, pH 5). Lysed cells appear dark blue due to staining with the vital dye.

staining spores with the vital dye Methylene Blue (0.01%, w/v), a dye that only enters dead cells. Figure 2B shows that while most of the wild-type spores were impermeable to the Methylene Blue stain, more than 95% of the *rgf2*Δ spores were stained. These results suggest that ascospore maturation does not proceed properly in the *rgf2*Δ/*rgf2*Δ homozygous zygotes and that these spores probably lacked one or more of the wall layers responsible for spore resistance and refringency.

Glucan synthase activity is diminished in sporulating *rgf2*Δ diploids: We next analyzed whether Rgf2p might be acting as a glucan synthase (GS) regulator during sporulation. First, we examined the effects of *rgf2*Δ disruption on GS activity during ascospore development. To synchronize the cells, we obtained diploid *rgf2*$^+$ and *rgf2*Δ strains harboring a *pat1-114* mutation (PG107 and PG110 respectively). Cells grown in MM without nitrogen were incubated at the restrictive temperature (34º) and portions of the culture were sampled every 2 h. Between 4 and 8 h after the induction of sporulation, and coinciding with the appearance of the *rgf2*$^+$ transcript, GS activity increased; this reached a maximum at 6h. After 10 h, GS activity declined, with the widespread appearance of mature asci. This activity peak was strongly diminished in the *rgf2*Δ diploid strain (Figure 3A, left). This result indicates that Rgf2p is involved in β–glucan biosynthesis during sporulation.

We also observed that the *rgf2*Δ phenotype, characterized by dark, immature spores, resembled that previously seen in the *bgs2*Δ mutants, which are defective for the glucan synthase catalytic subunit (GS) (LIU *et al.* 2000; MARTIN *et al.* 2000). We therefore reasoned that if Rgf2p was acting as a Rho1p-GEF, and was thus activating β–glucan synthesis, the double mutant *rgf2*Δ*bgs2*Δ would be blocked at the same stage as each individual mutant. This can be seen in Figure 3A (right): the *rgf2*Δ*bgs2*Δ homozygous asci are apparently very similar to the individual *rgf2*Δ and *bgs2*Δ homozygous asci. We have previously shown that the *bgs2*Δ phenotype is epistatic to the *chs1*Δ phenotype (chitin synthase mutant) (ARELLANO *et al.* 2000), and to the *mok12*Δ phenotype (α-glucan synthase mutant) in homozygous diploids, suggesting that β-glucan synthesis occurs within the prospore membrane before the deposition of other layers on the spore surface (GARCIA *et al.* 2006; MARTIN *et al.* 2000). In a similar approach, we observed that the morphology of the spores from homothallic strains *rgf2*Δ*chs1*Δ (PG280) and *rgf2*Δ*mok12*Δ (PG222) was dark and uniform, very similar to that of the *rgf2*Δ spores (data not shown).

To determine the intracellular localization of Rgf2p, the coding sequence of GFP was fused in-frame before the stop codon of *rgf2*$^+$ using a PCR-based approach (BÄHLER *et al.* 1998). The resulting strain (PG260) contained the fusion under the control of the native *rgf2*$^+$ promoter and the fusion protein was fully functional. As shown in Figure 3B, the fluorescence signal appeared to be localized uniformly at the periphery of the spore, probably associated with the forespore inner membrane. Consistent with the timing of expression of its mRNA, Rgf2-GFP fluorescence appeared in the fraction of cells that had already undergone meiosis I and II, where the spore outline was perfectly defined (see Nomarsky photos). The fluorescence signal was completely absent in vegetative cells grown in rich medium. To ascertain whether the lack of signal might be a consequence of the low expression of Rgf2p-GFP during vegetative growth, we expressed the Rgf2p-GFP on a multicopy plasmid (pAL-*rgf2*$^+$-8ala-*GFP*). Under these conditions, Rgf2p fluorescence localized to the

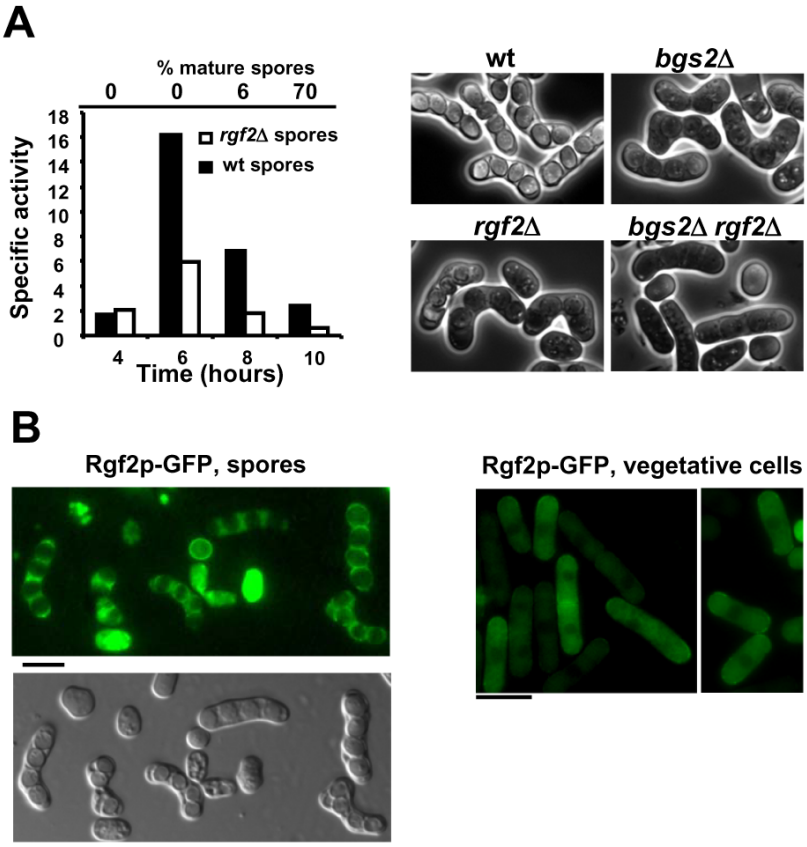

FIGURE 3.—Glucan synthase activity is diminished in sporulating *rgf2*Δ diploids. (A) In vitro β-GS activity in wt and *rgf2*Δ sporulating strains (left). The diploid strains *rgf2*$^+$ and *rgf2*Δ harboring a *pat1-114* mutation (PG107 and PG110 respectively) were grown in MM-N and incubated at the restrictive temperature (34º). Then, 100 ml portions of the culture were sampled every 2 h and assayed for GS activity, starting 4 hours after the temperature shift (see MATERIALS AND METHODS). The values shown are from one experiment, but similar results were obtained in 2 additional independent experiments. Specific activity is expressed as mU/mg protein. The final GTP concentration in the assay was 150 μM. Sporulation phenotype of wild-type (YS260), *bgs2*Δ (MS228), *rgf2*Δ (PG9) and *rgf2*Δ*bgs2*Δ (NG188) homothallic haploid strains (right). (B) Localization of Rgf2p during sporulation. Left panel: Rgf2p was detected in cells of strain PG260 carrying the chromosomal copy of Rgf2p tagged with GFP and incubated in sporulation medium for 14 h. The Rgf2p-GFP signal was observed when the spore outline was defined. Right panel: Localization of Rgf2p in vegetative cells. Early log-phase cells containing the *rgf2*$^+$*-GFP* fusion allele on a plasmid (pGR76) were visualized for GFP fluorescence. Rgf2p localizes to the growing regions: one or both poles and the septum. The scale bar represents 10 μm.

growing ends, the septum, and also slightly spread out along the cell surface (Figure 3B, right panel).

***rgf2*$^+$ overexpression elicits an aberrant morphology, promotes the GDP-GTP exchange, and increases β(1,3)-glucan synthase during vegetative growth:** The above results prompted us to investigate whether Rgf2p might play a role as a Rho1p activator during vegetative growth. For these experiments, the *rgf2*$^+$ gene was cloned under the thiamine-repressible *nmt1* promoter in the pREP3X vector. Plasmid pREP3X (empty) or pREP3X-*rgf2*$^+$ (overexpressor) were transformed into wild-type cells expressing HA-*rho1*$^+$ from its own promoter. It has been reported that overexpression of *rho1*$^+$ is not lethal but produces swollen and multiseptated cells, with thick walls and thick septa (ARELLANO *et al.* 1996; NAKANO *et al.* 1997). Surprisingly, when thiamine was eliminated to enhance *rgf2*$^+$ expression, the cells were unable to produce visible colonies on plates. After 18 hours of induction in liquid culture, the cells were larger than wild-type cells and displayed multiple abnormal septa. These cells also showed a general increase in Calcofluor white (Cfw) fluorescence, most of them containing aberrant depositions of Cfw-stainable material (see cells marked with an arrow in Figure 4A).

Next, we analysed the in vivo amount of GTP-bound Rho1p in cells overexpressing Rgf2p. After induction of the *nmt1* promoter for 20 h, the amount of Rho1p bound to GTP was precipitated with GST-C21RBD, the rhotekin-binding domain (which had previously been obtained and purified from bacteria), and blotted with anti-HA antibody (Figure 4B). Western blots of whole extracts (25 μg protein) showed that the total amount of Rho1p was similar in all strains. However, the amount of active Rho1p increased considerably in the strain overexpressing Rgf2p as compared with the wild-type strain (Figure 4B). As a control we examined whether the GEF domain was required for the Rgf2p-related overexpression phenotypes. We created a deletion mutant in the RhoGEF domain of Rgf2p (*rgf2-PTTR*Δ). The four amino acids deleted in the *rgf2-PTTR*Δ mutant (proline-threonine-threonine-arginine, PTTR) have been predicted to be located on helix H8 (CR3), which is the most highly conserved region of the DH domain and is where many mutations that decrease nucleotide exchange activity map (LIU *et al.* 1998; SOISSON *et al.* 1998). We have previously shown that a similar mutation in Rgf1p (*rgf1-PTTR*Δ mutant) produces a loss-of-function phenotype (GARCÍA *et al.* 2006a; GARCÍA *et al.* 2009). As expected, overexpression of the *rgf2-PTTR*Δ mutant in a pREP3X vector produced viable cells and no multiseptated phenotype was seen, even at very long times of derepresion in the absence of thiamine (Figure 4A). Moreover, overexpression of the mutated version (*rgf2-PTTR*Δ) did not increase the amount of GTP-Rho1p (active-Rho1p) in a "pull down" binding assay (Figure 4B).

Finally, we analysed glucan synthase (GS) activity

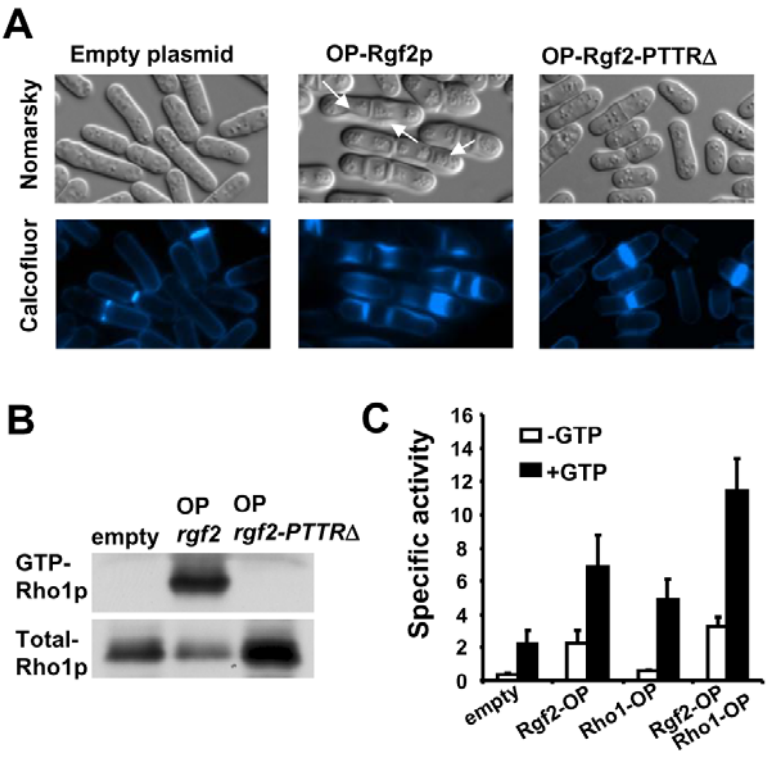

FIGURE 4.—*rgf2*$^+$ overproduction (OP) produces aberrant cells and increases β–GS activity. (A) Phenotype of Rgf2p OP. Overexpression of *rgf2*$^+$ causes cell growth arrest and an abnormal accumulation of cell wall material. Nomarsky and Calcofluor-stained UV micrographs of wild-type cells transformed with pREP3X (empty plasmid), pREP3X-*rgf2*$^+$ (Rgf2-OP) or pREP3X-*rgf2RTTP*Δ (Rgf2RTTPΔ-OP) grown without thiamine for 20 h. Overexpression of the Rgf2-PTTR protein (mutated in the RhoGEF domain) gave rise to wild-type morphology. (B) The level of Rgf2p modulates the amount of GTP-bound Rho1p *in vivo*. Wild-type cells expressing HA-*rho1*$^+$ from its own promoter (PPG1.60) were transformed with plasmid pREP3X, pREP3X-*rgf2*$^+$ or pREP3X-*rgf2RTTP*Δ as above. GTP-Rho1p was pulled down from the cell extracts with GST-C21RBD and blotted against 12CA5, an anti-HA mAb. (C) *In vitro* glucan synthase (GS) activity assayed with the membrane fraction of wild-type cells (YS64) transformed with pREP3X (empty plasmid), pREP3X-*rgf2* (Rgf2-OP), pREP4X-*rho1* (Rho1-OP), or both pREP3X-*rgf2* and pREP4X-*rho1* (Rgf2-OP and Rho1-OP). Extracts were prepared from cells grown in MM without thiamine at 32º for 18 hours. Specific activity is expressed as milliunits/mg protein. Values are means of at least three independent experiments with duplicate samples, and error bars represent standard deviations (SDs).

during *rgf2*$^+$ overexpression. GS activity was threefold higher than that observed in the wild-type strain (Figure 4C). To corroborate these results, we also studied the activity in cells overexpressing *rho1*$^+$ and *rgf2*$^+$ at the same time (transformed with pREP4X-*rho1* and pREP3X-*rgf2* plasmid). As described previously, cells overexpressing *rho1*$^+$ showed an increase in GS activity (Figure 4C) (ARELLANO *et al.* 1996). This increase was considerably higher (9-fold) in cells that overexpressed *rgf2*$^+$ at the same time (Figure 4C). These results clearly indicate that Rgf2p is involved in the regulation of β(1,3)-glucan biosynthesis.

Synthetic lethality of *rgf2*::*ura4*$^+$ and *rgf1*::*his3*$^+$: We used genetic approaches to ascertain whether Rgf2p showed any functions overlapping with the other Rho1p GEFs, Rgf1p and Rgf3p. First, we investigated whether *rgf1*$^+$ and *rgf2*$^+$ interacted genetically. After analysing tetrads of a *rgf1*::*his3*$^+$ h$^-$ x *rgf2*::*ura*$^+$ h$^+$ cross, we failed to find any double mutant spore (*rgf1*Δ*rgf2*Δ). Of 18 asci dissected: 11 yielded one *his*$^-$ *ura*$^-$ spore, one *his*$^+$ *ura*$^-$ spore, one *his*$^-$ *ura*$^+$, and one unviable spore predicted to be *his*$^+$ *ura*$^+$. Three asci yielded 4 viable parental-type spores, and 2 asci yielded 3 viable parental-type spores. None of the 11 spores predicted to be *rgf1*::*his3*$^+$ *rgf2*::*ura*$^+$ was viable, strongly supporting the idea that simultaneous disruption of *rgf1*$^+$ and *rgf2*$^+$ is lethal. To eliminate the possibility that these mutations might be affecting only sporulation or germination, we also tested for synthetic lethality during vegetative growth. We created a strain, *P81nmt-rgf1 rgf2*Δ (GRG33), deleted for the *rgf2*$^+$ gene and with the endogenous *rgf1*$^+$ promoter replaced by the *P81nmt* promoter (*P81nmt* is a thiamine-regulatable and reduced expression-rate promoter derived from the *nmt1* promoter, (FORSBURG 1993). As shown in Figure 5A, the cells displayed normal morphology when *rgf1*$^+$ was expressed in the absence of thiamine (promoter on). Six hours after the addition of thiamine to repress *rgf1*$^+$ expression, 68% of the cells had shrunk, and after 9 hours the whole culture had lysed (Figure 5A). The lysis phenotype seen in the *P81nmt-rgf1rgf2*Δ double mutant was similar to that observed in the *rgf1*Δ mutant, and in cells depleted for Rgf3p or Rho1p. However, while Rho1p depletion causes cell death mainly after cytokinesis, in this case most of the cells lysed as single and long cells, a phenotype characteristic of the *rgf1*$^+$ null mutants and probably related to their NETO defect (ARELLANO *et al.* 1997; GARCÍA *et al.* 2006a). The cell death due to Rho1p depletion cannot be prevented by an osmotic stabilizer (ARELLANO *et al.* 1997) and (Figure 5B), while the same phenotype produced by Rgf3p depletion is prevented by 1.2 M sorbitol (TAJADURA *et al.* 2004). We therefore examined whether the double mutant *P81nmt-rgf1rgf2*Δ shut-off phenotype could be rescued by osmotic support. As shown in Figure 5B, both Rgf1p Rgf2p-depleted, and Rho1p-depleted cells were unable to grow in YES medium (promoter off) regardless of the presence or absence of 1.2M sorbitol.

We also tested whether any other combination between mutations of Rho1p activators might cause cell death. Rgf3p is essential, so we used a strain with a TS mutation in *rgf3*$^+$, the *ehs2-1* mutation that stands for "echinocandin-hypersensitive". At 37º in liquid

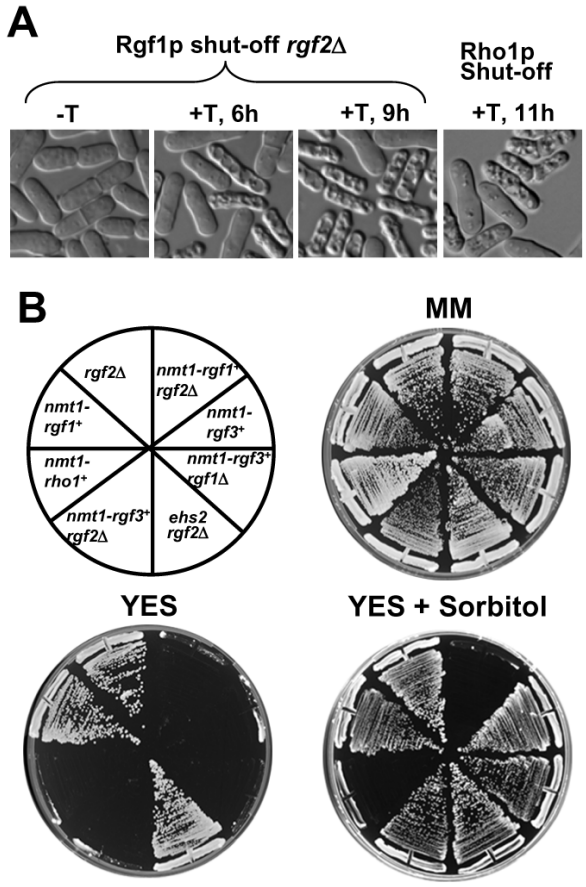

FIGURE 5.—Double disruption of Rgf1p and Rgf2p is essential for cell viability. (A) Depletion of Rgf1p in an *rgf2*Δ background leads to a lysis phenotype similar to depletion of Rho1p. Lethal phenotype of the *P81 nmt-rgf1rgf2*Δ (GRG33) and *P41 nmt-rho1* (PPG2.17) shut-off mutants. Cells grown at 28º in MM were supplemented with thiamine to repress the *nmt* promoter. For Rgf1p shut-off, Nomarsky micrographs were taken before and after the addition of thiamine (6 h and 9 h). For Rho1p shut-off, the micrographs were taken after 11 h in MM with thiamine. (B) Strains *nmt1-rgf1*$^+$ *rgf2*Δ (GRG33), *nmt1-rgf3*$^+$ (VT88), *nmt1-rgf3*$^+$*rgf1*Δ (PG94), *ehs2-1 rgf2*Δ (PG105), *nmt1-rgf3*$^+$*rgf2*Δ (PG395), *nmt1-rho1*$^+$ (PPG2.17), *nmt1-rgf1*$^+$ (PG115), *rgf2*Δ (GRG55) were streaked onto MM (w/o thiamine), YES and YES plus Sorbitol 1.2M

medium the *ehs2-1* cells showed a lytic thermosensitive phenotype (TAJADURA *et al.* 2004). We first created a strain lacking *rgf2*$^+$ with a TS mutation in *rgf3*$^+$ (*rgf2*Δ, *ehs2-1*). This strain, PG105, was viable and phenotypically indistinguishable from the *ehs2-1* mutant (Figure 5B). In addition, we constructed a strain deleted for *rgf2*$^+$ with the *rgf3*$^+$ gene under the control of the *P81nmt* promoter *P81nmt-rgf3rgf2*Δ (PG395) (MATERIALS AND METHODS). As expected for the *rgf3*$^+$ shut-off, the cells died in the presence of thiamine (promoter off). However, their growth was rescued in the presence of sorbitol (Figure 5B). Finally, we searched for an interaction between Rgf1p and Rgf3p. To this end, we constructed a strain, *P81nmt-rgf3rgf1*Δ (PG94), in which the *rgf3*$^+$ gene was under the control of the *P81nmt* promoter and the *rgf1*$^+$ gene was deleted (see MATERIALS AND METHODS). As above, growth of the *P81nmt-rgf3rgf1*Δ strain in rich medium (promoter off) was dependent on the presence of 1.2 M sorbitol (Figure 5B).

Our results indicate that Rgf1p and Rgf2p share an essential role as Rho1p activators, and they suggest that in the absence of Rgf1p, Rgf2p takes over the essential functions for Rho1p during vegetative growth.

Rgf2p behaves as a functional homologue of Rgf1p when expressed during vegetative growth: We next examined whether the functions of Rgf1p and Rgf2p were interchangeable. Previous studies had shown that the *rgf1*$^+$ deletion causes cell lysis, hypersensitivity to the antifungal drug Caspofungin (Csp), and defects in the establishment of bipolar growth (GARCÍA *et al.* 2006a). We overexpressed *rgf2*$^+$ in an *rgf1*Δ background. Figure 6A (upper panels) shows that *rgf2*$^+$ expressed from plasmids, containing the *rgf2*$^+$ genomic promoter (pGR13) or the strongest *nmt1* promoter (pGR70), fully rescued the lysis and the Csp hypersensitivity of *rgf1*Δ cells in medium containing thiamine. We had previously observed that the overexpression of *rgf2*$^+$ (under the high-strength *nmt1* promoter) was lethal by itself in a wild-type background; this is shown in Figure 6A (MM upper panel). To avoid this problem, we made use of the *rgf2*$^+$ driven by the *P81nmt* promoter (low level, pGR72) and the *P41Xnmt* promoter (medium level, pGR71). Both constructs produced viable cells (in the absence of thiamine: promoter on), and complementation of the hypersensitivity to Csp was found even in the presence of thiamine (promoter off) (Figure 6A, lower panels). We therefore wondered whether *rgf2*$^+$ would be able to rescue the monopolar-to-bipolar switch defect in the *rgf1*Δ mutants. To address this, we transformed *rgf2*$^+$ expressed from its own promoter (pGR13) or the empty plasmid (pAL) in the double mutant *cdc10-129 rgf1*Δ and synchronized cells in G1 by arrest at 37º. Strain *cdc10-129 rgf1*$^+$ was used as a wild-type control of proper bipolar growth and it was also transformed with plasmid pGR13. The areas in which new cell wall deposition, and hence growth, was occurring were visualized using the fluorescent dye Calcofluor white (Cfw). Ninety minutes after being shifted to the permissive temperature of 25º, 47% of *cdc10-129rgf1*Δ cells overexpressing *rgf2*$^+$ were bipolar, whereas only 10% was observed in the *cdc10-129 rgf1*Δ cells bearing the empty plasmid (Figure 6B). Thus, mild overexpression of *rgf2*$^+$ was able to suppress the bipolar growth defect of *rgf1*Δ mutants.

Consistent with the hypothesis that Rgf1p and Rgf2p are able to substitute for each other during vegetative growth, we found that the expression of *rgf1*$^+$ driven by the *rgf2*$^+$ promoter on a multicopy plasmid partially complemented the sporulation defect of an *rgf2*Δ h^{90} strain (PG9) (22% ascopore survival compared with the 1% of survival with the empty plasmid) (Figure

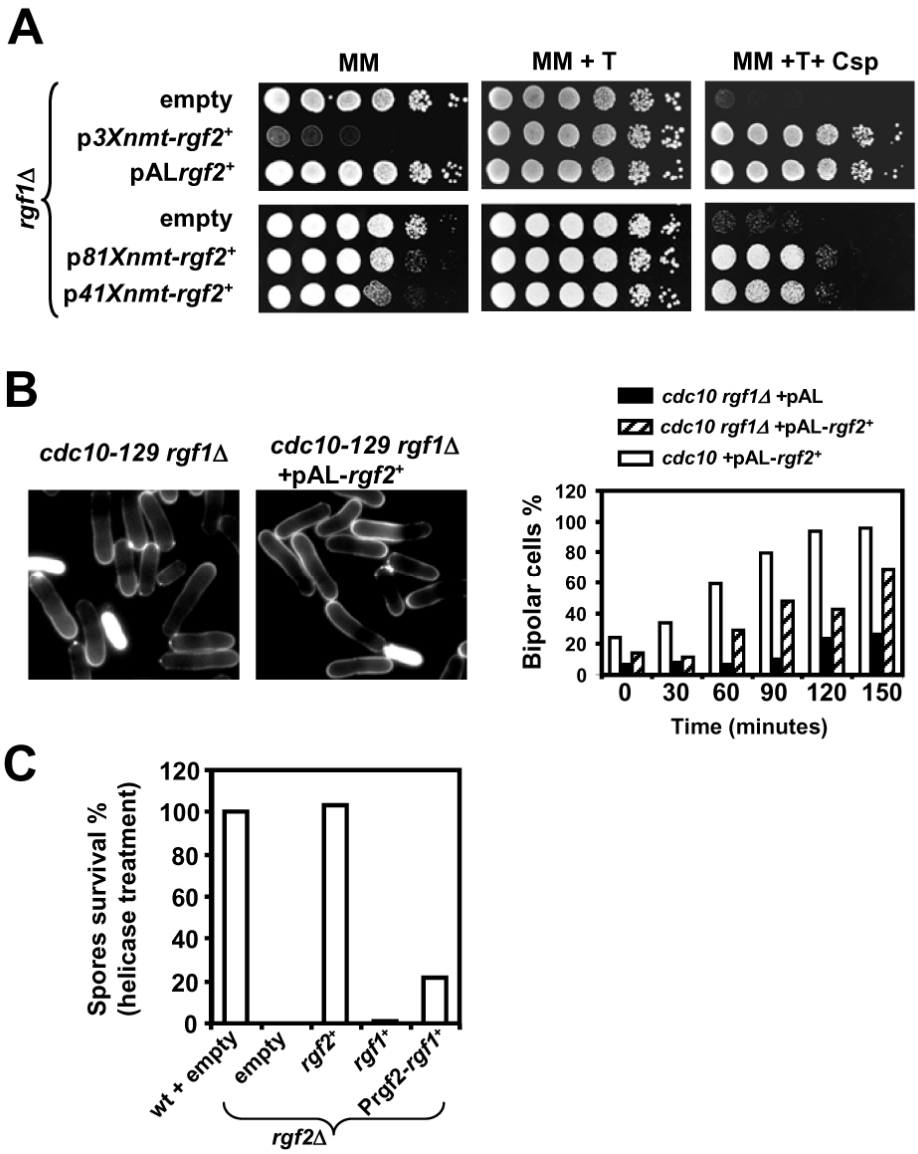

FIGURE 6.—The Caspofungin–hypersensitive growth phenotype of *rgf1*Δ mutants is suppressed by overexpresion of *rgf2*$^+$. (A) *rgf1*Δ (VT14) was transformed with pREP3X (empty vector), pREP3X-*rgf2*$^+$ (pGR70), pAL-*rgf2*$^+$ (pGR13), pREP81X-*rgf2*$^+$ (pGR72), and pREP41X-*rgf2*$^+$ (pGR71). Transformants were spotted onto MM, MM plus thiamine and MM plus thiamine and 1.5 μg/ml of Caspofungin plates as serial dilutions (8 x10^4 cells in the left row, and then 4x 10^4, 2 x 10^4, 2 x 10^3, 2 x 10^2 and 2x10^1 in each subsequent spot) and incubated at 28º for 3 days. (B) *cdc10-129 rgf1*Δ (PG88) transformed with pAL (control) and pAL-*rgf2*$^+$, and *cdc10-129* (MS168) transformed with plasmid pAL-*rgf2*$^+$ were grown at 25º to OD_{600} 0.15, shifted to 37º for 4 h, and then grown at 25º for 150 min. Aliquots of cells were harvested before and every 30 min after the shift to 25º. The graphic (on the right) represents the percentages of bipolar cells at each time-point. Micrographs show Cfw-stained *cdc10-129 rgf1*Δ plus empty plasmid (pAL) and pAL-*rgf2*$^+$ at 90 min after the shift to 25º. (C) Rgf1p partially complemented the sporulation defect seen in the *rgf2*Δ mutants. The homothallic haploid strain PG9 (h^{90} *rgf2*Δ) transformed with different plasmids, empty (pAL), pAL-*rgf2*$^+$, pAL*rgf1*$^+$ and pAL-P*rgf2-rgf1*$^+$, and the YS260 (h^{90}) strain carrying the empty plasmid pAL were incubated on MEA plates. After 4 days, spores were resuspended in 1 ml of water to the same OD_{600} 20, treated with helicase (0.2%) for 12 h, and diluted in water. The same dilutions of each strain were spread onto YES plates and incubated for 3 days. In the experiment, the spore survival of each strain was the average of three platings. The experiment was repeated at least three times.

6C). This complementation was most likely dependent on the expression of *rgf1*$^+$ during sporulation, since we failed to observe complementation when *rgf1*$^+$ was driven by its own promoter (Figure 6C).

DISCUSSION

Guanine nucleotide exchange factors (GEFs) are directly responsible for the activation of Rho-family GTPases in response to diverse stimuli, and ultimately they regulate many cellular responses, such as proliferation, differentiation and movement (ROSSMAN *et al.* 2005a). In fission yeast there are seven proteins with a Rho-GEF domain: *scd1*$^+$, *gef1*$^+$, *gef2*$^+$, *gef3*$^+$, *rgf1*$^+$, *rgf2*$^+$ and *rgf3*$^+$ (http://www.genedb.org/genedb/pombe/ index.jsp) (IWAKI *et al.* 2003). Of these, *scd1*$^+$ and *gef1*$^+$ are Cdc42p-specific GEFs, and Rgf3p and Rgf1p have been described as GEFs for Rho1p, while *gef2*$^+$ and *gef3*$^+$ have not yet been assigned to any

known GTPase (GARCÍA *et al.* 2006b). Considering that there are four additional Rho GTPases and at least two biochemically uncharacterized GEFs, it will take considerable effort in the future to sort out the biochemical specificities, cellular roles, and regulation of each Rho-GEF. Our data provide new evidence to show that Rgf2p acts as an exchange factor for Rho1p and that this activity is necessary for the development of the ascospore wall.

Deletion of *rgf2*$^+$ blocks spore-wall development: Sexual reproduction proceeds through mating and meiosis, and it culminates with the formation of ascospores, which in itself is a process of differentiation into a specialized cell form. Spores are metabolically inert and tolerant to severe environmental stresses. One of the most intriguing aspects of this process is the *de novo* synthesis of a plasma membrane and cell wall. During mitotic cell division, the plasma membrane of the mother cell is inherited by the two daughter cells and cell wall synthesis therefore extends from an old cell-wall precursor. By contrast, sporulation requires the *de novo* establishment of wall-producing plasma membranes within the mother cell cytoplasm, and the cell wall materials accumulate -without precursor- in the lumen of these membranes (EGEL 2004; SHIMODA and NAKAMURA 2004).

Here we show that Rgf2p is required for proper spore wall formation and that it is involved in spore maturation. First, no mature or refractile spores were formed in *rgf2*Δ homozygous asci after incubation in sporulation medium for several days. The appearance of the mutant spores was always very similar, suggesting they arrest at a particular stage of development. This phenotype was almost identical to that seen in spores lacking *bgs2*$^+$, the sporulation-specific GS catalytic subunit, and this observation suggests that both proteins function in the same process. As described previously for *bgs2*Δ asci, *rgf2*Δ asci were able to complete meiosis I and meiosis II, and each of the spores was bounded by a forespore membrane, thus indicating that early events in spore morphogenesis were normal. Moreover, the localization of the α–glucan synthases Mok12-GFPp and Mok13-GFPp to the four nuclear envelopes was similar in *rgf2*Δ and wild-type cells (not shown).

Second, *rgf2*Δ zygotes were not able to release ascospores spontaneously, and only 18% of the zygotes gave rise to colonies as compared to the 83% survival of the wild-type zygotes. When the ascospores were released from the ascal sac by mild treatment with helicase (0.2%), only 1% of the *rgf2*Δ spores survived, indicating that they were unable to assemble a functional spore wall. These results suggest that the spore wall is not only the cellular structure responsible for the extreme resistance to stress conditions but that proper assembly of the spore wall is itself important for spore survival. Third, *rgf2*$^+$ expression was induced 15-fold (MATA *et al.* 2002) after the second meiotic division (at about 5h) and the transcript was maintained until mature spores appeared between 10-12 h. Rgf2p tagged with GFP localized to structures around each of the four nuclear lobes and appeared when the spore outline was visible under phase contrast microscopy.

The coincidence of the expression of *rgf2*$^+$ with the synthesis of the spore wall, the fact that Rgf2p has been proposed as a GEF for Rho1p based on two-hybrid analysis (MUTOH *et al.* 2005), together with the phenotypes described above all suggest that the *rgf2*$^+$ gene is directly involved in β-1,3-glucan synthesis. We observed that mutation of the *rgf2*$^+$ gene resulted in a 2.5 reduction in β–GS activity that peaked at 6 hours in synchronous sporulation cultures. Moreover, Rgf2p overproduction in vegetative cells raised the amount of Rho1p bound to GTP and elicited a phenotype similar to that of the constitutively active allele *Rho1G15V* in wild-type *S. pombe* cells, providing confirmatory results for the hypothesis that Rgf2p indeed activates β-glucan during sporulation. It is likely that β-glucan synthesis would occur within the prospore membrane before the deposition of other layers on the spore surface, such as α-glucans or chitin-like material. In agreement with this, we observed that the *rgf2*Δ phenotype was epistatic to the *mok12*Δ (the α-GS sporulation specific) or the *chs1*Δ (the chitin-synthase sporulation-specific) phenotype in *rgf2*Δ*mok12*Δ or *rgf2*Δ*chs1*Δ double mutants, respectively. Taken together, all these results indicate that Rgf2p functions in sporulation, when the assembly and maturation of the spore wall occurs.

Role of *rgf2*$^+$ in vegetative growth: Rgf1p and Rgf2p are the closest related members among the GEF family in *S. pombe*. Both proteins share the same domain structure and 63% identity within the DH domain (Dbl Homology domain). This is very unusual, since DH domains, also called "Rho GEF domains", generally share little sequence identity with each other. In *S. pombe*, the percent identity between the deduced amino acid sequence of the DH domain that belongs to GEFs with the same substrate specificity was less than 20% between Rgf1p and Rgf3p, and Scd1p and Gef1p, respectively. Despite this, crystallographic and NMR analyses of several DH domains have revealed a highly related three-dimensional structure (SCHMIDT and HALL 2002).

Previous studies have shown that Rho1p depletion causes cell death concomitant with a decrease in β-1,3-GS activity, and that the lysis in these conditions cannot be prevented by an osmotic stabilizer (ARELLANO *et al.* 1997). Among the Rho1p GEFs described to date, only Rgf3p is essential for cell survival. However, the Rgf3p shut-off was rescued by the presence of sorbitol, suggesting that in the presence of an osmotic support Rho1p may be activated by other GEFs (TAJADURA *et al.* 2004).

Mutoh *et al.* reported that double deletion of *rgf1*$^+$ and *rgf2*$^+$ is synthetically lethal and our work has shown that the lysis-and-death-phenotype seen in Rgf1p- and Rgf2p-depleted vegetative cells is not prevented by sorbitol. These results strongly suggest that both proteins have an essentially overlapping function, probably as Rho1p activators. This genetic interaction is specific to Rgf1p and Rgf2p, since we observed that other mutant combinations were not essential for survival. Cells depleted for Rgf1p and Rgf3p, or Rgf3p and Rgf2p at the same time were able to survive in the presence of sorbitol. Moreover, Rgf1p and Rgf3p were not functionally interchangeable. Moderate expression of *rgf1*$^+$ did not suppress lysis at 37º of the *rgf3* mutant (*ehs2-1*), and the *rgf3*$^+$ gene driven either by its own promoter or by the *nmt1* promoter was not able to suppress the hypersensitivity to the presence of Caspofungin or the lysis of *rgf1*Δ cells (TAJADURA *et al.* 2004).

We also found that Rgf2p appears to be fully competent to substitute for Rgf1p, when expressed at a high enough level. Rgf2p fully suppressed the Caspofungin-hypersensitive phenotype and, to a lesser extent, bipolar growth activation in *rgf1*Δ mutants. On the other hand, the sporulation defect of *rgf2*Δ was not complemented by extra copies of *rgf1*$^+$. Rgf1p is probably poorly expressed during sporulation, but the sporulation defect seen in the *rgf2*Δ mutants was partially suppressed when *rgf1*$^+$ was driven by the *rgf2*$^+$ promoter. These results imply that, if expressed, Rgf1p can functionally replace Rgf2p in the synthesis of spore wall glucan. Interestingly, while *bgs2*Δ mutants (affected in the β–GS catalytic subunit) were completely defective in spore germination (MARTIN *et al.* 2000), 5% of *rgf2*Δ spores germinated (w/o helicase treatment), suggesting that the redundancy among Rgf1p and Rgf2p could account for Rho1p activation during sporulation. Together, these results further suggest that Rgf1p and Rgf2p may be alternative GEFs with overlapping functions.

Biochemical data also support this view. It has been shown that Rgf1p acts as a Rho1p GEF; both proteins coimmunoprecipitate and overexpression of *rgf1*$^+$ increases the GTP-bound Rho1p and causes a large increase in β-1,3-GS activity (GARCÍA *et al.* 2006a). We observed that the overexpression of Rgf2p in vegetative cells behaved in the same way, increasing the level of GTP-Rho1p *in vivo* and β-1,3-GS activity, which was 3-fold higher than that of the wild-type cells. Moreover, the overexpression of *rgf2*$^+$ was lethal and produced long, multiseptated and highly refringent cells, with a phenotype similar to that of the constitutively active allele *Rho1G15V* or the overexpression of *rgf1*$^+$.

Differential expression of *rgf1*$^+$ and *rgf2*$^+$: Since Rgf1p and Rgf2p seem to activate the synthesis of β–glucan at different points of the yeast cycle, it is interesting to note that their regulation often appears to follow opposite trends: when the level of one is high, that of the other is low. Data from our lab and others suggest that both genes are differentially expressed (GARCÍA *et al.* 2006a; MATA *et al.* 2002; MUTOH *et al.* 2005). For Rgf1p, the mRNA level is constant along the cell cycle (our unpublished results), but protein localization has been shown to be regulated in a cell-cycle-dependent manner, moving from the old end to the new end and then back to the septum during cytokinesis (GARCÍA *et al.* 2006a). For Rgf2p, the *rgf2*$^+$ mRNA was highly induced in sporulation (after meiosis II), while it showed a low profile in vegetative cells. Accordingly, the Rgf2 protein tagged with GFP was hardly seen in vegetative wild-type cells. Only the mild overexpression of Rgf2p in a multicopy plasmid with its own promoter revealed weak fluorescence in the areas of growth and across the whole cell surface.

The differential expression observed may also account for the fact that the redundancy of Rgf1p and Rgf2p appears to be partial in both directions. Disruption of *rgf1*$^+$ results in a slow growth pattern at 28º, and the viability of the *rgf1*Δ cells was 55% as compared to that of that of the wild-type isogenic strain (GARCÍA *et al.* 2006a). Thus, *rgf2*$^+$ only partially compensates for the lack of *rgf1*$^+$ during vegetative growth. In the absence of *rgf2*$^+$, only 5% of the spores were able to germinate under laboratory conditions. In this sense, *rgf1*$^+$ only partially compensated for the absence of *rgf2*$^+$ in sporulation.

Many questions, however, still remain unanswered. Are Rgf1p or Rgf2p involved in the synthesis of the same type of β–glucan or in that of a slightly different type of β–glucan? Are GEFs only important for the temporal or spatial activation of Rho1p, acting independently of the catalytic subunits? Rgf2p is specifically involved in β–glucan biosynthesis during sporulation, probably doing its job in the local activation of Rho1p/Bgs2p. If Rgf2p function during vegetative growth is exclusively related to Rho1p/Bgs2p activation, then the double mutation in *rgf1*$^+$ and *bgs2*$^+$ should be synthetically lethal. We observed that *rgf1*Δ*bgs2*Δ mutant cells behaved like the *rgf1*Δ mutants as regards sensitivity to Caspofungin and lysis. However, they were thermosensitive for growth while none of the individual mutants were. The results thus suggest that Rgf2p might be activating Rho1p, but that it does not function exclusively through Bgs2p to cope with stress situations during polarized growth.

In conclusion, the differences in the phenotypes caused by the deletion of *rgf1*$^+$ and *rgf2*$^+$ and the differences in the regulation of these genes suggest that these two GEFs have different functions within the cell.

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LITERATURE CITED

ARELLANO, M., H. CARTAGENA-LIROLA, M. A. NASSER HAJIBAGHERI, A. DURÁN and M. H. VALDIVIESO, 2000 Proper ascospore maturation requires the *chs1*$^+$ chitin synthase gene in *Schizosaccharomyces pombe*. Mol. Microbiol **35:** 79-89.

ARELLANO, M., A. DURÁN and P. PÉREZ, 1997 Localization of the *Schizosaccharomyces pombe* Rho1 GTPase and its involvement in the organization of the actin cytoskeleton. J. Cell. Sci. **110:** 2547-2555.

ARELLANO, M., A. DURÁN and P. PÉREZ, 1996 Rho1 GTPase activates the (1-3)β-D-glucan synthase and is involved in *Schizosaccharomyces pombe* morphogenesis. EMBO J. **15:** 4584-4591.

BÄHLER, J., J.-Q. WU, M. S. LONGTINE, N. G. SHAH, A. MCKENZIE III *et al.*, 1998 Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast **14:** 943-951.

BOS, J. L., H. REHMANN and A. WITTINGHOFER, 2007 GEFs and GAPs: critical elements in the control of small G proteins. Cell **129:** 865-877.

CALONGE, T. M., M. ARELLANO, P. M. COLL and P. PÉREZ, 2003 Rga5p is a specific Rho1p GTPase-activating protein that regulates cell integrity in *Schizosaccharomyces pombe*. Mol. Microbiol. **47:** 507-518.

CORTÉS, J. C., E. CARNERO, J. ISHIGURO, Y. SÁNCHEZ, A. DURÁN *et al.*, 2005 The novel (1,3)β-D-glucan synthase catalytic subunit Bgs4p from fission yeast is essential during both cytokinesis and polarized growth. J. Cell Sci. **118:** 157-174.

CORTÉS, J. C., M. KONOMI, I. M. MARTINS, J. MUÑOZ, M. B. MORENO *et al.*, 2007 The (1,3)beta-D-glucan synthase subunit Bgs1p is responsible for the fission yeast primary septum formation. Mol. Microbiol. **65:** 201-217.

CRAVEN, R. A., D. J. GRIFFITHS, K. S. SHELDRICK, R. E. RANDALL, I. M. HAGAN *et al.*, 1998 Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. Gene **221:** 59-68.

CHEN, S., and H. E. HAMM, 2006 DEP Domains: More Than Just Membrane Anchors. Dev Cell **11:** 436-438.

DING, D. Q., Y. TOMITA, A. YAMAMOTO, Y. CHIKASHIGE, T. HARAGUCHI *et al.*, 2000 Large-scale screening of intracellular protein localization in living fission yeast cells by the use of a GFP-fusion genomic DNA library. Genes Cells **5:** 169-190.

DURÁN, A., and P. PÉREZ, 2004 Cell wall synthesis, pp. 269-276 in *The molecular biology of Schizosaccharomyces pombe. Genetics, genomics and beyond.*, edited by R. EGEL. Springer.

EGEL, R., 2004 Fission yeast in general genetics, pp. 1-10 in *The molecular biology of Schizosaccharomyces pombe. Genetics, genomics and beyond*., edited by R. EGEL. Springer, Berlin.

FORSBURG, S. L., 1993 Comparison of *Schizosaccharomyces pombe* expression systems. Nucleic Acids Res **21:** 2955-2956.

GARCÍA, I., V. MARTÍN, V. TAJADURA, T. TODA and Y. SÁNCHEZ, 2006 Synthesis of α-glucans in fission yeast spores is carried out by three alpha-glucan synthase paralogs, Mok12p, Mok13p and Mok14p. Mol. Microbiol **59:** 836-853.

GARCÍA, P., V. TAJADURA, I. GARCÍA and Y. SÁNCHEZ, 2006a Rgf1p is a specific Rho1-GEF that coordinates cell polarization with cell wall biogenesis in fission yeast. Mol Biol Cell **17:** 1620-1631.

GARCÍA, P., V. TAJADURA, I. GARCÍA and Y. SÁNCHEZ, 2006b Role of Rho GTPses and Rho-GEFs in the regulation of cell shape and integrity in fission yeast. Yeast **23:** 1031-1043.

GARCÍA, P., V. TAJADURA and Y. SÁNCHEZ, 2009 The Rho1p exchange factor Rgf1p signals upstream from the Pmk1 mitogen-activated protein kinase pathway in fission yeast. Mol. Biol. Cell **20:** 721-731.

HAYLES, J. A., and P. NURSE, 2001 A journey into space. Nat. Rev. Mol. Cell Biol. **2:** 647-656.

HUMBEL, B. M., M. KONOMI, T. TAKAGI, N. KAMASAWA, S. A. ISHIJIMA *et al.*, 2001 In situ localization of β-glucans in the cell wall o*f Schizosaccharomyces pomb*e. Yeast. **18:** 433-444.

IINO, Y., Y. HIRAMINE and M. YAMAMOTO, 1995 The role of cdc2 and other genes in meiosis in *Schizosaccharomyces pombe*. Genetics **4:** 1235-1245.

IWAKI, N., K. KARATSU and M. MIYAMOTO, 2003 Role of guanine nucleotide exchange factors for Rho family GTPases in the regulation of cell morphology and actin cytoskeleton in fission yeast. Biochem. Biophys. Res. Commun. **312:** 414-420.

LATGÉ, J. P., 2007 The cell wall: a carbohydrate armour for the fungal cell. Mol. Microbiol. **66:** 279-290.

LE GOFF, X., A. WOOLLARD and V. SIMANIS, 1999 Analysis of the *cps1* gene provides evidence for a septation checkpoint in *Schizosaccharomyces pombe.* Mol. Gen. Genet. **262:** 163-172.

LEVIN, D., 2005 Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **69:** 262-291.

LIU, J., X. TANG, H. WANG and M. K. BALASUBRAMANIAN, 2000 Bgs2p, a 1,3-β-glucan synthase subunit is essential for maturation of ascospore wall in *Schizosaccharomyces pombe*. FEBS Lett. **478:** 105-108.

LIU, J., X. TANG, H. WANG, S. OLIFERENKO and M. K. BALASUBRAMANIAN, 2002 The localization of the integral membrane protein Cps1p to the cell division site is dependent on the actomyosin ring and the septation-inducing network in *Schizosaccharomyces pombe*. Mol. Biol. Cell **13:** 989-1000.

LIU, J., H. WANG and M. K. BALASUBRAMANIAN, 2000b A checkpoint that monitors cytokinesis in *Schizosaccharomyces pombe*. J Cell Sci. **113:** 1223-1230.

LIU, X., H. WANG, M. EBERSTADT, A. SCHNUCHEL, E. T. OLEJNICZAK *et al.*, 1998 NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor. Trio. Cell **95:** 269-277.

MANNERS, D. J., and M. T. MEYER, 1977 The molecular structures of some glucans from the cell wall of *S. pombe*. Carbohyd. Res. **57:** 189-203.

MARTÍN, V., B. GARCÍA, E. CARNERO, A. DURÁN and Y. SÁNCHEZ, 2003 Bgs3p, a putative 1,3-β-glucan synthase subunit, is required for cell wall assembly in *Schizosaccharomyces pombe.* Eukaryotic Cell **2:** 159-169.

MARTÍN, V., J. C. RIBAS, E. CARNERO, A. DURÁN and Y. SÁNCHEZ, 2000 Bgs2$^+$, a sporulation-specific glucan synthase homologue is required for proper ascospore wall maturation in fissión yeast. Mol. Microbiol. **38:** 308-321.

MATA, J., R. LYNE, G. BURNS and J. BAHLER, 2002 The transcriptional program of meiosis and sporulation in fission yeast. Nat. Genet. **32 (1):** 143-147.

MORENO, S., A. KLAR and P. NURSE, 1991 Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Meth. Enzymol. **194:** 795-823.

MORRELL-FALVEY, J. L., L. REN, A. FEOKTISTOVA, G. D. HAESE and K. L. GOULD, 2005 Cell wall remodeling at the fission yeast cell division site requires the Rho-GEF Rgf3p. J. Cell Sci. **118:** 5563-5573.

MUTOH, T., K. NAKANO and I. MABUCHI, 2005 Rho1-GEFs Rgf1 and Rgf2 re involved in formation of cell wall and septum, while Rgf3 is involved in cytokinesis in fission yeast. Genes Cells **10:** 1189-1202.

NAKAMURA, T., H. ABE, A. HIRATA and C. SHIMODA, 2004 ADAM family protein Mde10 is essential for development of spore envelopes in the fission yeast *Schizosaccharomyces pombe*. Eukaryotic Cell **3:** 27-39.

NAKANO, K., R. ARAI and I. MABUCHI, 1997 The small GTP binding protein Rho1 is a multifunctional protein that regulates actin localization, cell polarity, and septum formation in the fission yeast *Schizosaccharomyces pombe*. Genes Cells **2:** 679-694.

NAKANO, K., T. MUTOH and I. MABUCHI, 2001 Characterization of GTPase-activating proteins for the function of the Rho-family small GTPases in the fission yeast *Schizosaccharomyces pombe*. Genes Cells **6:** 1031-1042.

OSUMI, M., N. YAMADA, H. KOBORI, A. TAKI, N. NAITO *et al.*, 1989 Cell wall formation in regenerating protoplast of *Schizosaccharomyces pombe*: study by high resolution, low voltage scanning electron microscopy. J. Electron Microsc. **38:** 457-468.

REID, T., T. FURIYASHIKI, T. ISHIZAKI, G. WATANABE, N. WATANABE *et al.*, 1996 Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and

rhophilin in the rho-binding domain. J. Biol. Chem. **271:** 13556-13560.

REN, X. D., W. B. KIOSSES and M. A. SCHWARTZ, 1999 Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J. **18:** 578-585.

ROSSMAN, K. L., J. D. CHANNING and J. SONDEK, 2005a GEF means go: turning on Rho GTPases with guanine nucleotide-exchange factors. Nat. Rev. Mol. Cell Biol. **6:** 167-180.

ROSSMAN, K. L., and J. SONDEK, 2005b Larger than Dbl: new structural insights into RhoA activation. Trends Biochem. Sci. **30:** 163-165.

SCHMIDT, A., and A. HALL, 2002 Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev. **16:** 1587-1609.

SHIMODA, C., and T. NAKAMURA, 2004 Control of late meiosis and ascospore formation, pp. 311-325 in *The molecular biology of Schizosaccharomyces pombe. Genetics, genomics and beyond.*, edited by R. EGEL. Springer.

SOISSON, S. M., A. S. NIMNUAL, M. UY, D. BAR-SAGI and J. KURIYAN, 1998 Crystal structure of the Dbl and pleckstrin homology domains from the human son of sevenless protein. Cell **95:** 259-268.

TAIRA, K., M. UMIKAWA, K. TAKEI, B.-E. MYAGMAR, M. SHINZATO *et al.*, 2004 The Traf2- and Nck-interacting Kinase as a Putative Effector of Rap2 to Regulate Actin Cytoskeleton J. Biol. Chem. **279:** 49488-49496.

TAJADURA, V., B. GARCÍA, I. GARCÍA, P. GARCÍA and Y. SÁNCHEZ, 2004 *Schizosaccharomyces pombe* Rgf3p is a specific Rho1 GEF that regulates cell wall β-glucan biosynthesis through the GTPase Rho1p. J. Cell Sci. **117:** 6163-6174.

YAMAMOTO, M., 2004 Initiation of meiosis, pp. 297-309 in *The molecular biology of Schizosaccharomyces pombe. Genetics, genomics and beyond.*, edited by R. EGEL.

YANG, P., Y. QYANG, G. BARTHOLOMEUSZ, Z. ZHOU and S. MARCUS, 2003 The novel Rho1 GTPase-activating protein family, Rga8, provides a potential link between cdc42/p21-activated kinase and Rho signaling pathways in the fission yeast, *Schizosaccharomyces pombe*. J. Biol. Chem. **278:** 48821-48830.

DISCUSIÓN GENERAL

1. Rgf1p

1.1. Papel de Rgf1p como activador de Rho1p

Quizás la conclusión más importante de este trabajo ha sido atribuir a Rgf1p el papel de regulador positivo o GEF de Rho1p. Esta GTPasa interviene en numerosos procesos esenciales para la célula, como la regulación de la síntesis de la pared celular, la polimerización del citoesqueleto de actina y el mantenimiento de la integridad celular. Sin embargo, con la excepción de su papel como activador de la β-glucan sintasa, su contribución específica en procesos tales como la polaridad o la integridad celular es prácticamente desconocida. Este desconocimiento se debe en parte a que Rho1p es esencial para la viabilidad celular y a que carecemos de mutantes condicionales para estudiar los fenotipos derivados de su falta de función. El estudio de la proteína Rgf1p y sobre todo la caracterización de los fenotipos de los mutantes *rgf1*Δ, nos ha permitido aventurar posibles funciones de Rho1p, desconocidas hasta ahora, como la participación en la transición del crecimiento monopolar a bipolar o la regulación de la cascada de integridad.

La mayoría de los defectos observados durante la represión o sobreexpresión del gen *rgf1*$^+$ pueden ser explicados, de manera más o menos evidente, por su acción como regulador de Rho1p.

Uno de los fenotipos más característicos de la cepa *rgf1*Δ es la presencia de numerosas células lisadas en cultivos líquidos. Nuestros datos indican que esta lisis se produce de manera mayoritaria por la debilidad en la pared de uno de los polos de la célula. En primer lugar, las células lisadas son largas y no forman parejas, como sucede en los mutantes en los que la lisis se produce por el septo. Otro dato, quizás más concluyente, es que cuando teñimos estas células con un colorante vital (azúl de metileno) vemos que éste penetra en las células lisadas sólo por uno de sus polos (datos no mostrados). Pensamos que esta debilidad en la pared podría ser consecuencia de la ausencia de Rho1p activo en esas zonas. Hasta hace poco el único GEF de Rho1p conocido era Rgf3p, que se localiza exclusivamente en el septo y es esencial para el proceso de citoquinesis (Morrell-Falvey et al., 2005; Tajadura et al., 2004). Sin embargo, Rgf1p se localiza también en los polos y es el mejor candidato para actuar como GEF de Rho1p en esos lugares.

La lisis celular producida por la ausencia de Rgf1p se ve agravada en el momento en el que debería producirse la transición del crecimiento monopolar a bipolar. Posiblemente esto es debido a que exista una cierta tensión en esa zona de expansión que aumenta al aumentar la longitud de la célula. La lisis se producirá por el lugar más débil, que es el polo en el que no se está ensamblando correctamente ni la actina, ni la pared celular, en ausencia de Rgf1p. Otra explicación podría ser que aunque en el segundo polo no haya apenas síntesis de pared en células *rgf1*Δ, como se puede observar mediante tinción con Calcoflúor, sí podría estar dándose una cierta elongación de la membrana en esa zona, que a su vez ejercería una cierta tensión, sin la suficiente protección de la pared celular.

Por otro lado, las células *rgf1*Δ son extremadamente sensibles a Caspofungina (incapaces de crecer a una concentración de 0,03 μg/ml, mientras que la estirpe silvestre puede tolerar hasta 7,5 μg/ml en medio sólido). Esto podría ser consecuencia de la suma de dos defectos. Por un lado, su incapacidad de activar la cascada de integridad en respuesta a daños en la pared y por otro, la disminución de la cantidad de Rho1p activo y el efecto que esta disminución podría tener sobre la activación del componente catalítico de la βGS. En primer lugar, hay que tener en cuenta que la Caspofungina es un agente capaz de estimular la ruta de señalización de Pmk1p, y que lo hace en gran medida a través de Rgf1p. Por lo tanto, si las células *rgf1*Δ no son capaces de activar la ruta de integridad en respuesta a este daño (con el fin de contrarrestarlo), esto las haría más sensibles a la droga que las células de la cepa silvestre. Esta explicación está respaldada por el hecho de que los demás mutantes en genes de esta ruta son también sensibles a esta droga. Pero entonces ¿por qué la cepa *rgf1*Δ es más sensible que las otras? La respuesta posiblemente es que la Caspofungina es un antifúngico que inhibe específicamente la actividad β(1,3)-glucán sintasa. Aunque no se ha demostrado experimentalmente en *S. pombe*, es lógico pensar que las células *rgf1*Δ tengan una menor cantidad de β-glucano debido a la disminución de la cantidad de Rho1p activo. En este contexto, la adición de Caspofungina al medio empeoraría la situación, al inhibir la parte de βGS que todavía podría ser activada. Esto explicaría por qué las células *rgf1*Δ son mucho más vulnerables a esta droga que otros mutantes de la ruta de integridad, en los que la síntesis del β-glucano no está afectada.

En conclusión, *rgf1*$^+$ es necesario para mantener la integridad de la célula especialmente durante la fase de elongación (crecimiento por los polos) que es un momento crítico del ciclo de vida de *S. pombe*.

1.2. Rgf1p en el establecimiento de NETO

En *S. pombe* la GTPasa Rho1p se ha relacionado con la regulación del citoesqueleto de actina, ya que una disminución de Rho1p provoca el desensamblaje de los parches de actina. Sin embargo, no se conocen los efectores a través de los cuales

realiza esta acción (Arellano et al., 1999a; Arellano et al., 1997).

Nos preguntamos si Rgf1p, como activador de Rho1p, también podría ejercer alguna función en este proceso. Curiosamente, observamos que las células *rgf1*Δ, además de lisarse por uno de los polos, mostraban defectos en la reorganización de la actina necesaria para la transición del crecimiento monopolar a bipolar. Por lo tanto otra de las conclusiones principales de este trabajo es que Rgf1p es fundamental para que se produzca la transición conocida como NETO (*New End Take Off*), pero ¿Cuál es su función en este proceso?. Por desgracia nuestros resultados no nos proporcionan una respuesta concluyente de momento, pero sí ciertos datos interesantes.

En primer lugar, podemos decir que Rgf1p no funciona como un marcador de polaridad necesario para definir los polos de crecimiento. Cuando se tratan células del mutante *cdc10-129* bloqueadas en G1 durante unos minutos con Latrunculina A, se liberan monómeros de actina. Una vez eliminada la droga se produce una repolimerización de la actina en el segundo polo y las células son capaces de crecer de forma bipolar, saltándose así el bloqueo en G1 (Rupes et al., 1999). Cuando realizamos este experimento en un mutante *rgf1*Δ y en una cepa silvestre, observamos que la activación del segundo polo se produce independientemente de la presencia o ausencia de Rgf1p. Esto nos está indicando que Rgf1p no es estrictamente necesaria para el reconocimiento del polo nuevo de crecimiento, ni para la polimerización de la actina en ese polo. Por tanto, parece que Rgf1p es necesaria fundamentalmente en el desencadenamiento de la señal que da lugar al crecimiento bipolar. Una pregunta obvia que surge en este momento es ¿en qué consiste esa señal?. Por el momento no se conoce cuál es su naturaleza, aunque en el proceso podría intervenir la despolimerización de la actina, ya que ésa es la forma por la que conseguimos de manera "artificial" evitar el bloqueo en G1 y que tenga lugar NETO en células *cdc10-129* (Rupes et al., 1999).

En experimentos de localización hemos observado que los marcadores de polaridad Tea1p y Pom1p se localizan de forma normal en la cepa *rgf1*Δ, es decir en ambos polos. Sin embargo, otras proteínas importantes para NETO, como la formina For3p, algunas de las proteínas del complejo Arp2/3 (Wsp1p y ArcC5p), así como la subunidad catalítica de la βGS, Bgs4p, sólo aparecen en un polo cuando no hay Rgf1p en la célula. Las proteínas del complejo Arp2/3 son necesarias para la polimerización de los parches de actina (revisado en (Moseley and Goode, 2006)), y la β GS es esencial en la construcción de la pared celular. La ausencia de ambos tipos de proteínas en el polo nuevo podría explicar el defecto en la polimerización de la actina en ese polo, así como la fragilidad de la pared celular. En cuanto a los cables de actina, si bien no podemos decir que en el mutante *rgf1*Δ hayan desaparecido, sí que parecen más finos y escasos en las zonas en las que los parches están ausentes, probablemente como consecuencia de la ausencia de For3p, la formina encargada de la polimerización de estos cables (Feierbach and Chang, 2001).

El hecho de que sistemáticamente todas las proteínas (For3p, Myo52p, Wsp1p, ArpC5p y Bgs4p) cuya localización hemos analizado en el mutante *rgf1*Δ presenten una distribución monopolar, con la excepción de los marcadores de polaridad Tea1p y Pom1p, sugiere que Rgf1p podría estar actuando en los primeros estadios de esta transición del crecimiento.

También hemos observado que la función de Rgf1p durante el establecimiento del crecimiento bipolar depende de su actividad GEF.

Hemos obtenido un mutante puntual en el dominio DH (dominio catalítico), que es incapaz de activar a Rho1p y que se comporta como el mutante nulo (*rgf1*Δ) en cuanto a la activación del crecimiento bipolar. Es muy probable pues que Rgf1p ejerza su función en NETO activando/localizando a alguna de las GTPasas de la familia Rho. La mejor candidata es desde luego Rho1p, ya que es la diana conocida de Rgf1p y se sabe que interviene en la organización del citoesqueleto de actina (Arellano et al., 1999a). Sin embargo, no podemos descartar completamente la posibilidad de que Rgf1p esté actuando sobre alguna de las otras GTPasas de la familia Rho, como Rho3p o Cdc42p. Esta última está estrechamente relacionada con la polimerización del citoesqueleto de actina a través de sus reguladores y efectores (Gef1p y Pak2p/ Orb2p) y es necesaria para activar y localizar a For3p, encargada de la formación de los cables de actina y fundamental para el establecimiento del crecimiento bipolar (Coll et al., 2003; Feierbach and Chang, 2001; Kim et al., 2003). Un mutante puntual en esta GTPasa, *cdc42-1625*, presenta unos cables de actina muy cortos y finos (Martin et al., 2007). Nosotros hemos observado que el aumento en la expresión de *rgf1*$^{+}$ provoca una mayor cantidad de Cdc42p activo en la célula (datos no mostrados), aunque aún no podemos decir si esta relación es directa o una consecuencia de la activación de Rho1p, por ejemplo. También hemos visto que el doble mutante *rgf1*Δ*gef1*Δ es sintético letal a 37ºC (datos no mostrados).

Sorprendentemente, ni el aumento en la cantidad de Rho1p ni el de Cdc42p, produce un incremento del número de células con crecimiento bipolar en la cepa *rgf1*Δ (datos no mostrados). Esto podría deberse a que se necesita que Rho1p o Cdc42p estén activos en un momento o lugar determinados, y el simple aumento en la cantidad de estas proteínas no es suficiente para suprimir el defecto.

Otra GTPasa candidata podría ser Rho3p, ya que interacciona con la formina For3p y modula las

funciones del exocisto (Nakano et al., 2002). Sin embargo, no creemos que sea el caso, ya que un aumento de la cantidad de Rgf1p en la célula, no parece influir en la activación de esta GTPasa (datos no mostrados).

Por otro lado, nuestros datos sugieren que Rgf1p podría realizar su acción en el establecimiento de NETO, antes de localizarse en el segundo polo. La proteína Rgf1p siempre se localiza en los polos que están creciendo activamente, de forma que en un mutante *cdc10-129* bloqueado en G1 sólo aparece en el polo que esta creciendo y en un mutante *cdc25-22* bloqueado en G2, se localiza en los dos. La proteína Rgf1PTTRΔp (mutada en el dominio catalítico y marcada con GFP) no es capaz de promover el crecimiento bipolar en un mutante *rgf1*Δ y además se localiza de manera monopolar (en el polo de crecimiento). Curiosamente, en la cepa silvestre la proteína mutada Rgf1PTTRΔp se localiza en ambos polos. Esto sugiere que su ausencia en el segundo polo no se debe a un defecto en el anclaje de la proteína mutada, sino que es una consecuencia del defecto de activación de NETO. Solamente cuando está la copia silvestre de Rgf1p, ésta sí es capaz de activar el crecimiento y de esta manera arrastrar a la proteína mutada al polo nuevo. Finalmente estos datos podrían indicar que Rgf1p activaría NETO desde el polo antiguo, esta actividad promovería el crecimiento en el polo nuevo y como consecuencia de esto, ella misma se localizaría en este segundo polo, activando la biosíntesis de la pared.

La hipótesis con la que trabajamos, que aún necesita ser probada, es que Rgf1p podría activar a una cierta GTPasa, cuya acción daría lugar a una señal que a su vez desencadenaría la concentración de las proteínas necesarias para NETO en el segundo polo y la polimerización de la actina en éste. A continuación Rgf1p también aparecería en ese polo, hacia donde se desplaza toda la maquinaria biosintética, de una forma dependiente de la actina. Una vez allí, podría realizar su función como GEF de Rho1p y activar la síntesis de la pared celular, necesaria en último término para el crecimiento del polo y también podría actuar activando la cascada de integridad en respuesta a ciertos tipos de estrés.

1.3. Rgf1p en la ruta de integridad celular

Otra de las conclusiones de esta memoria es el papel de Rgf1p como nuevo miembro de la cascada de MAPKs de Pmk1p. En *S. cerevisiae* se sabe desde hace años que Rom2p es un GEF de Rho1p que señaliza hacia la cascada de integridad liderada por la MAPK Slt2p (Bickle et al., 1998). En *S. pombe* los primeros trabajos en los que se describieron los tres componentes de la cascada Mkh1p, Pek1p/Shk1p y Pmk1p/Spm1p (Loewith et al., 2000; Sengar et al., 1997; Sugiura et al., 1999; Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997) no hallaron una relación directa entre Pck2p (homólogo en *S. pombe* a Pkc1p de *S. cerevisiae*) y la ruta de MAPKs. Incluso en algunos trabajos se alude a un posible papel antagónico entre Pck2p y la cascada de integridad (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). Sólo recientemente, a partir del trabajo del grupo de Sugiura y Kuno en Japón (Ma et al., 2006), se identificó el gen *cpp1*$^{+}$ que codifica una farnesiltransferasa cuya función es favorecer la localización en la membrana de la GTPasa Rho2p. El mutante *cpp1* fue identificado por mostrar el fenotipo *vic* (viable en presencia de FK506 y $MgCl_2$) que presentan todos los mutantes nulos de los componentes de la cascada de integridad, incluidos los mutantes *pck2*Δ y *rho2*Δ.

La relación entre Pck2p y la cascada y el hecho de que Pck2p sea un efector de Rho1p y un supresor de la hipersensibilidad a Caspofungina del mutante *rgf1*Δ, nos animó a seguir por esa vía y descubrir la relación de Rgf1p y la cascada de integridad. Varias líneas de evidencias indican que Rgf1p participa en la regulación de la cascada de integridad en *S. pombe*, pero sólo comentaremos algunas de ellas. La cepa *rgf1* Δ presenta una serie de fenotipos comunes a los mutantes nulos de las quinasas de esta ruta. Todos ellos son sensibles al estrés osmótico y al estrés producido por daños en la pared celular, pero el fenotipo más característico y distintivo de los miembros de la cascada de integridad es el fenotipo *vic* (*viable in the presence of immunosuppressant and choride ion*) que también presentan las células *rgf1*Δ. Además, la fosforilación y por tanto activación de la MAPK de esta cascada (Pmk1p), en respuesta a estrés osmótico o daño en la pared celular, depende de Rgf1p y concretamente de su actividad GEF.

Otro dato importante es la relación entre la cascada de integridad y la GTPasa Rho1p. En *S. cerevisiae*, Rho1p es el principal activador de la ruta de integridad, mientras que en *S. pombe* no se sabe nada al respecto. Nuestros datos indican que Rgf1p podría estar activando la ruta a través de su GTPasa diana, Rho1p. Hemos observado que tanto niveles bajos, como niveles altos de Rho1p, producen su efecto en la señalización de la cascada, por ejemplo, un mutante de Rgf1p en el dominio Rho-GEF, que presenta niveles muy bajos de Rho1p activo, es incapaz de fosforilar a la MAPK Pmk1p en respuesta a estrés osmótico. Por otro lado, un aumento de los niveles de Rho1p producido, tanto por la sobreexpresión de *rho1*$^{+}$, como por la sobreexpresión de cualquiera de sus activadores (Rgf1p, Rgf2p o Rgf3p) provoca la activación de Pmk1p de una forma dependiente de Pck2p y Mkh1p.

Rgf1p es el único GEF de Rho1p conocido que participa en la activación de la cascada de integridad

en *S. pombe*; ni las células *rgf2*Δ, ni las del mutante *ehs2-1* (afectado en el gen *rgf3*$^{+}$), muestran los fenotipos característicos de los mutantes en genes de esta ruta, ni presentan defectos a la hora de activar a Pmk1p en respuesta a una gran variedad de estreses. Este resultado demuestra la especificidad de Rgf1p, y sugiere que un GEF determinado y no otro, es capaz de activar específicamente alguna de las funciones de Rho1p y no otras. De esta forma, los GEFs podrían estar regulando en el espacio o en el tiempo, o en ambos a la vez, a las GTPasas y determinando de esta manera que actúen sobre unos efectores u otros.

Además, Rgf1p actúa de forma específica sobre la ruta de MAPKs de Pmk1p y no parece tener ninguna influencia en la activación de otra ruta de MAPKs activada por estrés conocida como SAPK (*Stress Activated Protein Kinase*), cuya MAPK es Spc1p y que también responde a estrés osmótico (Shiozaki and Russell, 1995).

2. Rgf2p

Un capítulo de esta memoria se ha destinado al estudio del gen *rgf2*$^{+}$ en *S. pombe*. Hemos demostrado que la proteína codificada por este gen, es esencial durante el proceso de formación de la pared de la ascospora. Rgf2p participa en la síntesis del β-glucano durante la esporulación y probablemente lo hace a través de su capacidad para activar a Rho1p. En este sentido sabemos que la actividad βGS medida a lo largo del proceso de esporulación es mucho menor en las ascas del mutante *rgf2*Δ que en la cepa silvestre, y que los defectos observados en las ascosporas *rgf2*Δ son muy similares a los de las ascosporas del mutante *bgs2*Δ, que carecen de la βGS de esporulación.

En ausencia de *rgf2*$^{+}$, las esporas no son capaces de sintetizar una pared adecuada y pierden la integridad, incluso en condiciones favorables para la célula. Estos resultados sugieren que la pared de la espora no sólo es la estructura responsable de la resistencia extrema a las condiciones de estrés de las esporas, sino que también es esencial para su supervivencia.

Además, la mayoría de las ascas *rgf2*Δ no son capaces de romper su envuelta para liberar las esporas al medio. La función de Rgf2p en este proceso no está clara, es posible que Rgf2p esté actuando en la degradación de esta envuelta. Sin embargo, es mucho más probable que la célula tenga algún tipo de mecanismo de control, por el cual bloquee o retrase la rotura de la envuelta del asca, hasta que no detecte que la maduración de las esporas se ha completado correctamente (Liu et al., 2000; Martín et al., 2000).

Hemos visto la relación de Rgf2p con la biosíntesis de β-glucano en esporulación, pero además existen otros datos que sugieren que Rgf2p es un GEF de Rho1p. Por un lado, un ensayo de dos híbridos realizado por Mutoh y colaboradores en 2005 (Mutoh et al., 2005) mostró una interacción entre el dominio DH de Rgf2p y el alelo *rho1T20N*, que da lugar a una proteína Rho1p constitutivamente unida a GDP; como correspondería para un factor GEF. En segundo lugar, hemos demostrado que el aumento en los niveles de Rgf2p tiene como consecuencia una subida de los niveles de Rho1-GTP, al menos durante el crecimiento vegetativo, y que esto depende de la actividad de su dominio DH y no de alguno de sus otros dominios. Durante el proceso de esporulación es probable que Rgf2p también esté activando a Rho1p para llevar a cabo su función sobre la síntesis de la pared de la espora, pero por desgracia esto no se ha podido demostrar experimentalmente debido a problemas técnicos.

3. GEFs de Rho1p

Además de Rgf1p y Rgf2p, existe otra proteína identificada anteriormente como GEF de Rho1p, denominada Rgf3p (Morrell-Falvey et al., 2005; Mutoh et al., 2005; Tajadura et al., 2004). La existencia de tres factores GEFs diferentes para una misma GTPasa puede parecer sorprendente, pero es un fenómeno frecuente. En humanos se han identificado 22 GTPasas pertenecientes a la familia Rho y 69 proteínas con un dominio DH (Rossman et al., 2005a). Una posible explicación para esto es que los distintos RhoGEFs podrían estar determinando la especificidad de la GTPasa para activar unos efectores u otros. Esto lo realizarían gracias a su capacidad de unión a diferentes proteínas y de reclutamiento de la GTPasa en distintos lugares de la célula, o en diferentes momentos de su ciclo de vida (Schmidt and Hall, 2002). En *S. pombe* ya existen ejemplos de este mecanismo de acción, como en el caso de los GEFs Ste6p y Efc25p, que actúan regulando diferencialmente dos rutas de señalización, modulando a Ras1p para que se una a determinados efectores (Papadaki et al., 2002).

A lo largo de esta memoria, hemos descrito en varias ocasiones una forma de actuación similar a esta para los activadores de Rho1p. Hemos demostrado que Rgf1p y Rgf2p son capaces de activar a Rho1p, al igual que se había visto anteriormente con Rgf3p (Tajadura et al., 2004). Sin embargo, las funciones de estas tres proteínas son muy distintas. Sólo Rgf1p y Rgf2p son parcialmente intercambiables, pero su patrón de expresión es muy diferente. Rgf1p se expresa mayoritariamente durante el crecimiento vegetativo, de forma constante, mientras que Rgf2p, se induce durante el proceso de esporulación y en vegetativo

tiene una expresión basal. Esto en la práctica, hace que la presencia de una de ellas en ausencia de la otra, sea capaz de evitar la muerte de las células, pero no evita que presenten graves defectos.

Aunque estas tres proteínas, Rgf1p, Rgf2p y Rgf3p actúen como GEFs de Rho1p, cada una de ellas es importante para un proceso distinto. Rgf3p es esencial para la viabilidad celular porque participa en un momento crítico, la división. Cuando se reprime la expresión de *rgf3*$^{+}$ las células se lisan en parejas, probablemente a nivel del septo (Morrell-Falvey et al., 2005; Tajadura et al., 2004). Rgf3p se expresa justo antes de la separación celular y se localiza exclusivamente en el septo, mientras que Rho1p y Rgf1p también se pueden observar en los polos de crecimiento (Arellano et al., 1997; Tajadura et al., 2004). Las diferencias en la localización y expresión de Rgf3p y Rgf1p podrían ser las responsables de que cada una de ellas active a Rho1p en momentos y procesos diferentes. Rgf1p parece ser más importante en los polos celulares, especialmente en el mantenimiento de la integridad y en la transición del crecimiento monopolar a bipolar. *rgf3*$^{+}$ es un gen esencial mientras que *rgf1*$^{+}$ no lo es, sin embargo es muy posible que la proteína Rgf1p sea la encargada de la activación de la mayor parte del Rho1p presente en la célula. En un mutante de *rgf3*$^{+}$ (*ehs2-1*) apenas hay disminución en la cantidad de Rho1p activo con respecto a una cepa silvestre, por el contrario en un mutante *rgf1*Δ los niveles de Rho1p activo son mínimos; (Tajadura et al., 2004) y esta memoria.

La proteína Rgf2p por su parte, parece estar activando a Rho1p durante la formación de la pared de la espora. Es lógico que sea ésta la proteína encargada de activar a Rho1p durante este momento del ciclo de vida de *S. pombe*, ya que es la única de las tres cuya expresión aumenta de forma muy significativa durante la esporulación. Rgf2p además podría estar activando a Rho1p de forma basal suplantando en parte las funciones de Rgf1p, de esta forma se explicaría por qué las células presentan una viabilidad del 50% cuando carecen de *rgf1*$^{+}$ y son inviables en ausencia de ambos genes. Además, la letalidad de este doble mutante no se corrige en presencia de un estabilizador osmótico, al igual que le ocurre a la cepa con una expresión disminuida de *rho1*$^{+}$, lo cual podría indicar que en ausencia de Rgf1p y Rgf2p los niveles de Rho1p activo son tan bajos que no permiten a la célula sobrevivir (Arellano et al., 1997).

En resumen, existen tres GEFs que activan a Rho1p en *S. pombe*, los tres presentan una estructura y distribución de dominios similar, pero cada uno de ellos participa en un proceso diferente. Estas diferencias podrían explicarse como consecuencia de su distinto patrón de expresión y/o localización en la célula, que condicionaría a su vez la activación espacio-temporal de Rho1p.

CONCLUSIONES

CONCLUSIONES

- La proteína Rgf1p es un activador positivo de la GTPasa Rho1p.

- Esta proteína es necesaria para el mantenimiento de la integridad celular y para el establecimiento del crecimiento bipolar.

- La localización de varios factores de polaridad en el polo nuevo, depende de la presencia de Rgf1p en las células.

- Rgf1p es una proteína perteneciente a la cascada de señalización de MAPK de Pmk1p y es necesaria para trasmitir la señal en respuesta a cambios en la osmolaridad y a daño en la pared celular.

- La proteína Rgf2p es un GEF de Rho1p esencial para la maduración de la pared de la espora en *S. pombe.*

- Durante el crecimiento vegetativo, Rgf1p y Rgf2p comparten una función esencial en el mantenimiento de la integridad celular.

BIBLIOGRAFÍA

Abruzzo, G. K., Gill, C. J., Flattery, A. M., Kong, L., Leighton, C., Smith, J. G., Pikounis, V. B., Bartizal, K. and Rosen, H. (2000). Efficacy of the echinocandin caspofungin against disseminated aspergillosis and candidiasis in cyclophosphamide-induced immunosuppressed mice. *Antimicrob. Agents Chemother.* **44**, 2310-8.

Adamo, J. E., Rossi, G. and Brennwald, P. (1999). The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. *Mol. Biol. Cell* **10**, 4121-4133.

Alfa, C., Fantes, P., Hyams, J., McLeod, M. and Warbrick, E. (1993). Experiments with fission yeast: a laboratory course manual: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Arai, R., Nakano, K. and Mabuchi, I. (1998). Subcellular localization and possible function of actin, tropomyosin and actin-related protein 3 (Arp3) in the fission yeast *Schizosaccharomyces pombe*. *Eur. J. Cell Biol.* **76**, 288-295.

Arellano, M., Cartagena-Lirola, H., Nasser Hajibagheri, M. A., Durán A. and Valdivieso, M. H. (2000). Proper ascospore maturation requires the *chs1*$^{+}$ chitin synthase gene in *Schizosaccharomyces pombe*. *Mol. Microbiol* **35**, 79-89.

Arellano, M., Coll, P. M. and Pérez, P. (1999a). Rho GTPases in the control of cell morphology, cell polarity, and actin localization in fission yeast. *Microsc. Res. Tech.* **47**, 51-60.

Arellano, M., Durán, A. and Pérez, P. (1997). Localization of the *Schizosaccharomyces pombe* Rho1 GTPase and its involvement in the organization of the actin cytoskeleton. *J. Cell. Sci.* **110**, 2547-2555.

Arellano, M., Durán, A. and Pérez, P. (1996). Rho1 GTPase activates the (1-3)β-D-glucan synthase and is involved in *Schizosaccharomyces pombe* morphogenesis. *EMBO J.* **15**, 4584-4591.

Arellano, M., Valdivieso, M. H., Calonge, T. M., Coll, P. M., Durán, A. and Pérez, P. (1999b). *Schizosaccharomyces pombe* protein kinase C homologues, pck1p and pck2p, are targets of rho1p and rho2p and differentially regulate cell integrity. *J. Cell Sci.* **112**, 3569-3578.

Bähler, J. and Pringle, J. R. (1998). Pom1p, a fission yeast protein kinase that provides positional information for both polarized growth and cytokinesis. *Genes Dev.* **12**, 1356-1370.

Balasubramanian, M. K., Helfman, D. M. and Hemmingsen, S. M. (1992). A new tropomyosin essential for cytokinesis in the fission yeast *S. pombe*. *Nature* **360**, 84-87.

Barba, G., Soto, T., Madrid, M., Núñez, A., Vicente, J., Gacto, M. and Cansado, J. (2008). Activation of the integrity pathway is channelled through diverse signalling elements in fission yeast. *Cell signal* **20**, 748-757.

Bi, E., Chiavetta, J. B., Chen, H., Chen, G. C., Chan, C. S. and Pringle, J.R. (2000). Identification of novel, evolutionarily conserved Cdc42p-interacting proteins and of redundant pathways linking Cdc24p and Cdc42p to actin polarization in yeast. *Mol Biol Cell* **11**, 773-793.

Bickle, M., Delley, P. A., Schmidt, A. and Hall, M. N. (1998). Cell wall integrity modulates Rho1 activity via the exchange factor ROM2. *EMBO J.* **17**, 2235-2245.

Bobbit, T. F., Nordin, J. H., Roux, M., Revol, J. F. and Marchessault, R.H. (1977). Distribution and conformation of crystalline nigeran in hyphal walls of *Aspergillus niger* and *A. awamori*. *J. Bacteriol.* **132**, 691-703.

Bos, J. L., Rehmann, H. and Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. *Cell* **129**, 865-877.

Bourne, H. R., Sanders, D. A. and F., M. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature* **349**, 117-127.

Bourne, H. R., Sanders, D. A. and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* **348**, 125-132.

Browning, H., Hayles, J., Mata, J., Aveline, L., Nurse, P. and McIntosh, J. R. (2000). Tea2p is a kinesin-like protein required to generate polarized growth in fission yeast. *J. Cell Biol.* **151**, 15-27.

Brunner, D. and Nurse, P. (2000). CLIP170-like tip1p spatially organizes microtubular dynamics in fission yeast. *Cell* **102**, 695-704.

Bush, D. A., Horisberger, M., Horman, I. and Wursch, P. (1974). The wall structure of *S. pombe*. *J. Gen. Microbiol.* **81**, 199-206.

Bustelo, X. R. (2000). Regulatory and signaling porperties of the Vav family. *Mol. Cell. Biol.* **20**, 1461-1477.

Bustelo, X. R., Sauzeau, V. and Berenjeno, I. M. (2007). GTP binding proteins of the Rho/Rac family: regulation, effectors and function in vivo. *Bioessays* **29**, 356-370.

Cabib, E., Drgonova, J. and Drgon, T. (1998). Role of small G proteins in yeast cell polarization and wall biosynthesis. *Annu. Rev. Biochem.* **67**, 307-333.

Calonge, T. M., Arellano, M., Coll, P. M. and Pérez, P. (2003). Rga5p is a specific Rho1p GTPase-activating protein that regulates cell integrity in *Schizosaccharomyces pombe*. *Mol. Microbiol.* **47**, 507-518.

Calonge, T. M., Nakano, K., Arellano, M., Arai, R., Katayama, S., Toda, T., Mabuchi, I. and Pérez, P. (2000). *Schizosaccharomyces pombe* Rho2 GTPase regulates the cell wall α-glucan biosynthesis, through the protein kinase Pck*2*. *Mol. Biol. Cell* **11**, 4393-4401.

Carnero, E., Ribas, J. C., García, B., Durán, A. and Sánchez, Y. (2000). *Schizosaccharomyces pombe* Ehs1p is involved in maintaining cell wall integrity and in calcium uptake. *Mol. Gen. Genet.* **264**, 173-183.

Castagnetti, S., Behrens, R. and Nurse, P. (2005). End4/Sla2 is involved in establishment of a new growth zone in *Schizosaccharomyces pombe*. *J. Cell Sci.* **118**, 1843-1850.

Coll, P. M., Trillo, Y., Ametzazurra, A. and Pérez, P. (2003). Gef1p, a new guanine nucleotide exchange factor for Cdc42p, regulates polarity in *Schizosaccharomyces pombe*. *Mol. Biol. Cell* **14**, 313-323.

Cortes, J. C., Carnero, E., Ishiguro, J., Sánchez, Y., Durán, A. and Ribas, J. C. (2005). The novel (1,3)β-D-glucan synthase catalytic subunit Bgs4p from fission yeast is essential during both cytokinesis and polarized growth. *J. Cell Sci.* **118**, 157-174.

Cortes, J. C., Ishiguro, J., Durán, A. and Ribas, J. C. (2002). Localization of the (1,3)beta-D-glucan synthase catalytic subunit homologue Bgs1p/ Cps1p from fission yeast suggests that it is involved in septation, polarized growth, mating, spore wall formation and spore germination. *J. Cell Sci.* **115**, 4081-4096.

Cortés, J. C., Konomi, M., Martins, I. M., Muñoz, J., Moreno, M. B., Osumi, M., Durán, A. and Ribas, J. C. (2007). The (1,3)beta-D-glucan synthase subunit Bgs1p is responsible for the fission yeast primary septum formation. *Mol. Microbiol.* **65**, 201-217.

Chang, E. C., Barr, M., Wang, Y., Jung, V., Xu, H. P. and Wigler, M. H. (1994). Cooperative interaction of *S. pombe* proteins required for mating and morphogenesis. *Cell* **79**, 131-141.

Chang, F. (2001). Establishment of a cellular axis in fission yeast. *Trends Genet.* **15**, 273-278.

Chang, F. and Verde, F. (2004). Control of cell polarity and morphogenesis in fission yeast. In *The molecular biology of Schizosaccharomyces pombe. Genetics, genomics and beyond.*, (ed. R. Egel), pp. 255-268: Springer.

Chang, F., Woollard, A. and Nurse, P. (1996). Isolation and characterization of fission yeast mutants defective in the assembly and placement of the contractile actin ring. *J. Cell. Sci.* **109**, 131-142.

Dong, Y., Pruyne, D., and Bretscher, A. (2003). Formin-dependent actin assembly is regulated by distinct modes of Rho signaling in yeast. *J Cell Biol.* **161**, 1081-1092.

Douglas, C. M. (2001). Fungal β(1,3)-D-glucan synthesis. *Medical Micology* **39 (Suplement)**, 55-66.

Douglas, C. M., Foor, F., Marrinan, J. A., Morin, N., Nielsen, J. B., Dahl, A. M., Mazur, P., Baginsky, W., Li, W., El-Sherbeini, M. et al. (1994). The *Saccharomyces cerevisiae FKS1 (ETG1)* gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. *Proc. Natl. Acad. Sci. USA* **91**, 12907-12911.

Drewes, G. and Nurse, P. (2003). The protein kinase kin1, the fission yeast orthologue of mammalian MARK/PAR-1, localises to new cell ends after mitosis and is important for bipolar growth. *FEBS Lett.* **554**, 45-49.

Drummond, D. R. and Cross, R. A. (2000). Dynamics of interphase microtubules in *Schizosaccharomyces pombe*. *Curr. Biol.* **10**, 766-775.

Durán, A. and Pérez, P. (2004). Cell wall synthesis. In *The molecular biology of Schizosaccharomyces pombe. Genetics, genomics and beyond.*, (ed. R. Egel), pp. 269-276: Springer.

Egel, R. (1994). Regulation of meiosis and sporulation in *Schizosaccharomyces pombe*. In *The Mycota I*, (ed. Wessels-Meinhardt), pp. 251-265. Berlin. Heidelberg: Springer-Verlag.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629-635.

Eva, A. and Aaronson, S. A. (1985). Isolation of a new human oncogene from a diffuse B-cell lymphoma. *Nature* **316**, 273-275.

Feierbach, B. and Chang, F. (2001). Roles of the fission yeast formin For3 in cell polarity, actin cable formation and symmetric cell division. *Curr. Biol.* **11**, 1656-1665.

Feierbach, B., Verde, F. and Chang, F. (2004). Regulation of a formin complex by the microtubule plus end protein tea1p. *J. Cell Biol.* **165**. 697-707

Fischer, R., Zekert, N. and Takeshita, N. (2008). Polarized growth in fungi-interplay between the cytoskeleton, positional markers and membrane domains. *Mol Microbiol* **68**, 813-826.

Fujita, A. (2008). ADP-ribosylation factor arf6p may function as a molecular switch of new end take off in fission yeast. *Biochem. Biophys. Res. Commun.* **366**, 193-198.

Fukui, Y. and Yamamoto, M. (1988). Isolation and characterization of *Schizosaccharomyces pombe* mutants phenotypically similar to ras1. *Mol. Gen. Genet.* **215**, 26-31.

García, I., Martín, V., Tajadura, V., Toda, T. and Sánchez, Y. (2006). Synthesis of α-glucans in fission yeast spores is carried out by three alpha-glucan synthase paralogs, Mok12p, Mok13p and Mok14p. *Mol. Microbiol* **59**, 836-853.

García, P., Tajadura, V., García, I. and Sánchez, Y. (2006a). Rgf1p is a specific Rho1-GEF that coordinates cell polarization with cell wall biogenesis in fission yeast. *Mol. Biol. Cell* **17**, 1620-1631.

García, P., Tajadura, V., García, I. and Sánchez, Y. (2006b). Role of Rho GTPses and Rho-GEFs in the regulation of cell shape and integrity in fission yeast. *Yeast* **23**, 1031-1043.

Glynn, J. M., Lustig, R. J., Berlin, A. and Chang, F. (2001). Role of bud6p and tea1p in the interaction between actin and microtubules for the establishment of cell polarity. *Curr. Biol.* **11**, 836-845.

Gulli, M. P. and Peter, M. (2001). Temporal and spatial regulation of Rho-type guanine-nucleotide exchange factors: the yeast perspective. *Genes Dev.* **4**, 365-379.

Guo, W., Tamanoi, F. and Novick, P. (2001). Spatial regulation of the exocyst complex by Rho1 GTPase. *Nat. Cell Biol.* **3**, 353-360.

Hagan, I. M. (1998). The fission yeast microtubule cytoesqueleton. *J Cell Sci* **111 (Pt 12)**, 1603-12.

Hagan, I. M. and Hyams, J. S. (1988). The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **89**, 343-357.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.

Heasman, S. J. and Ridly, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat. Rev. Mol. Cell Biol.* **9**, 690-701.

Hirota, K., Tanaka, K., Ohta, K. and Yamamoto, M. (2003). Gef1p and Scd1p, the two GDP-GTP exchange factors for Cdc42p, form a ring structure that shrinks during cytokinesis in *Schizosaccharomyces pombe*. *Mol. Biol. Cell* **14**, 3617-3627.

Hochstenbach, F., Klis, F. M., Van den Ende, H., Van Donselaar, E., Peters, P. J. and Klausner, R. D. (1998). Identification of a putative alpha-glucan synthase essential for cell wall construction and morphogenesis in fission yeast. *Proc. Natl. Acad. Sci. USA* **95**, 9161-9166.

Hogan, L. H. and Klein, B. S. (1994). Altered expression of surface α(1,3)-glucan in genetically related strains of *Blastomyces dermatitidis* that differ in virulence. *Infect. Immun.* **62**, 3543-3546.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* **66**, 300-372.

Horisberger, M., Vonlanthen, M. and Rosset, J. (1978). Localization of α-galactomanan and of wheat germ agglutinin receptors in *Schizosaccharomyces pombe. Arch. Microbiol.* **119**, 107-111.

Humbel, B. M., Konomi, M., Takagi, T., Kamasawa, N., Ishijima, S. A. and Osumi, M. (2001). In situ localization of β-glucans in the cell wall of *Schizosaccharomyces pombe. Yeast*. **18**, 433-444.

Iden, S. and Collard, J. G. (2008). Crosstalk between small GTPases and polarity proteins in cell polarization. *Nat. Rev. Mol. Cell Biol.* **9**, 846-859.

Imamura, H., Tanaka, K., Hihara, T., Umikawa, M., Kamei, T., Takahashi, K., Sasaki, T. and Takai, Y. (1997). Bni1p and Bnr1p: downstream targets of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in *Saccharomyces cerevisiae*. *EMBO J.* **16**, 2745-2755.

Ishiguro, J., Saitou, A., Durán, A. and Ribas, J. C. (1997). *cps1*$^{+}$, a *Schizosaccharomyces pombe* gene homolog of *Saccharomyces cerevisiae FKS* genes whose mutation confers hypersensitivity to cyclosporin A and papulacandin B. *J. Bacteriol.* **179**, 7653-7662.

Iwaki, N., Karatsu, K. and Miyamoto, M. (2003). Role of guanine nucleotide exchange factors for Rho family GTPases in the regulation of cell morphology and actin cytoskeleton in fission yeast. *Biochem. Biophys. Res. Commun.* **312**, 414-420.

Jaffe, A. B. and Hall, A. (2005). Rho GTPases: Biochemistry and Biology. *Ann. Rev. Cell Dev. Biol.* **21**, 247-269.

Johnson, B. F., Calleja, G. B., Zuker, M. and MacDonal, T. J. (1982). Cell division: key to cellular morphogenesis in the fission yeast *Schizosaccharomyces pombe*. *Int. Rev. Cytol.* **75**, 167-208.

Kamada, Y., Jung, U. S., Piotrowski, J. and Levin , D. E. (1995). The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. *Genes Dev.* **9**, 1559-1571.

Katayama, S., Hirata, D., Arellano, M., Pérez, P. and Toda, T. (1999). Fission yeast α-glucan synthase Mok1 requires the actin cytoskeleton to localize the sites of growth and plays an essential role in cell morphogenesis downstream of protein kinase C function. *J. Cell Biol.* **144**, 1173-1186.

Kim, H., Yang, P., Catanuto, P., Verde, F., Lai, H., Du, H., Chang, F. and Marcus, S. (2003). The kelch repeat protein, Tea1, is a potential substrate target of the p21-activated kinase, Shk1, in the fission yeast, *Schizosaccharomyces pombe*. *J. Biol. Chem.* **278**, 30074-30082.

Klimpel, K. R. and Goldman, W. E. (1988). Cell wall from avirulent variants of *Histoplasma capsulatum* lack α(1,3)-glucan. *Infect. Immun.* **56**, 2997-3000.

Kohno, H., Tanaka, K., Mino, A., Umikawa, M., Mamura, H., Fujiwara, T., Fujita, Y., Hotta, K., Qadota, H., Watanabe, T. et al. (1996). Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. *EMBO J.* **15**, 6060-6068.

Kopecka, M., Fleet, G. H. and Phaff, H. J. (1995). Ultrastructure of the cell wall of *Schizosaccharomyces pombe* following treatment with various glucanases. *J. Struct. Biol* **114**, 140-152.

Latgé, J. P. (2007). The cell wall: a carbohydrate armour for the fungal cell. *Mol Microbiol.* **66**, 279-90.

Le Goff, X., Utzig, S. and Simanis, V. (1999). Controlling septation in fission yeast: finding the middle, and timing it right. *Curr. Genet.* **35**, 571-584.

Lessage, G. and Bussey, H. (2006). Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev* **70**, 317-343.

Leupold, U. (1950). Die Vererbung von Homothallie und Heterrothallie bei *Schizosaccharomyces pombe*. *C. R. Lab. Carlsberg, Sér. Physiol.* **24**, 381-480.

Levin, D. (2005). Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **69**, 262-291.

Levin, D. E. and Errede, B. (1995). The proliferation of MAP kinase signaling pathways in yeast. *Curr. Opin. Cell Biol.* **7**, 197-202.

Liu, J., Tang, X., Wang, H. and Balasubramanian, M. K. (2000). Bgs2p, a 1,3-β-glucan synthase subunit is essential for maturation of ascospore wall in *Schizosaccharomyces pombe*. *FEBS Lett.* **478**, 105-108.

Liu, J., Tang, X., Wang, H., Oliferenko, S. and Balasubramanian, M. K. (2002). The localization of the integral membrane protein Cps1p to the cell division site is dependent on the actomyosin ring and the septation-inducing network in *Schizosaccharomyces pombe*. *Mol. Biol. Cell* **13**, 989-1000.

Liu, J., Wang, H. and Balasubramanian, M. K. (2000b). A checkpoint that monitors cytokinesis in *Schizosaccharomyces pombe*. *J Cell Sci.* **113**, 1223-1230.

Liu, J., Wang, H., MacCollum, D. and Balasubramanian, M. K. (1999). Drc1p/Cps1p, a 1,3-beta-glucan synthase subunit, is essential for division septum assembly in *Schizosaccharomyces pombe*. *Genetics* **153**, 1193-1203.

Liu, X., Wang, H., Eberstadt, M., Schnuchel, A., Olejniczak, E. T., Meadows, R. and Fesik, S. W. (1998). NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor Trio. *Cell* **95**, 269-277.

Loewith, R., Hubberstey, A. and Young, D. (2000). Skh1, the MEK component of the Mkh1 signaling pathway in *Schizosaccharomyces pombe*. *J. Cell Sci.* **113**, 153-160.

Ma, Y., Kuno, T., Kita, A., Asayama, Y. and Sugiura, R. (2006). Rho2 is a target of the farnesiltransferase Cpp1 and acts upstream of Pmk1 mitogen-activated protein kinase signaling in fission yeast. *Mol. Biol. Cell* **17**, 5028-5037.

Madrid, M., Soto, T., Khong, H. K., Franco, A., Vicente, J., Pérez, P., Gacto, M. and Cansado, J. (2006). Stress-induced response, localization, and regulation of the Pmk1 cell integrity pathway in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **281**, 2033-2043.

Manners, D. J. and Meyer, M. T. (1977). The molecular structures of some glucans from the cell wall of *S. pombe*. *Carbohyd. Res.* **57**, 189-203.

Marcus, S., Polverino, A., Chang, E., Robbins, D., Cobb, M. H. and Wigler, M. (1995). Shk1, a homolog of the Saccharomyces cerevisiae Ste20 and mammalian p65PAK protein kinases, is a component of a Ras/Cdc42 signaling module in the fission yeast *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. USA* **92**, 6180-6184.

Marks, J. and Hyams, J. S. (1985). Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. *Eur. J. Cell Biol.* **39**, 27-32.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179-185.

Martin, S. G. and Chang, F. (2005). New End Take Off. Regulating cell polarity during the fission yeast cell cycle. *Cell Cycle* **4**, 1046-1049.

Martin, S. G. and Chang, F. (2006). Dynamics of the formin for3p in actin cable assembly. *Curr. Biol.* **16**, 1161-1170.

Martin, S. G., McDonald, W. H., Yates III, J. and Chang, F. (2005). Tea4p links microtubule plus ends with the formin For3p in the establishment of cell polarity. *Develop. Cell* **8**, 479-491.

Martin, S. G., Rincón, S. A., Basu, R., Pérez, P. and Chang, F. (2007). Regulation of the Formin for3p by cdc42p and bud6p. *Mol Biol Cell* **18**, 4155-4167.

Martín, V., García, B., Carnero, E., Durán, A. and Sánchez, Y. (2003). Bgs3p, a putative 1,3-β-glucan synthase subunit, is required for cell wall assembly in *Schizosaccharomyces pombe*. *Eukaryotic Cell* **2**, 159-169.

Martín, V., Ribas, J. C., Carnero, E., Durán, A. and Sánchez, Y. (2000). $Bgs2^{+}$, a sporulation-specific glucan synthase homologue is required for proper ascospore wall maturation in fissión yeast. *Mol. Microbiol.* **38**, 308-321.

Mata, J., Lyne, R., Burns, G. and Bähler, J. (2002). The transcriptional program of meiosis and sporulation in fission yeast. *Nat Genet.* **32 (1)**, 143-147.

Mata, J. and Nurse, P. (1997). tea1 and the microtubular cytoskeleton are important for generating global spatial order within the fission yeast. *Cell* **89**, 939-950.

McCollum, D., Feoktiskova, A., Morphew, M., Balasubramanian, M. K. and Gould, K. L. (1996). The *Schizosaccharomyces pombe* actin-related protein, Arp3, is a component of the cortical actin cytoskeleton and interacts with profilin. *EMBO J.* **15**, 6438-6446.

Medina-Redondo, M., Arnaiz-Pita, Y., Fontaine, T., Latgé, J. P. and Vazquez de Aldana, C. R. (2008). The β-1,3-glucanosyltransferase gas4p is essential for ascospore wall maturation and spore viability in *Schizosaccharomyces pombe*. *Mol Microbiol.* **68**, 1283-1299.

Merla, A. and Johnson, D. I. (2001). The *Schizosaccharomyces pombe* Cdc42p GTPase signals through Pak2p and the Mkh1p-Pek1p-Spm1p MAP kinase pathway. *Curr. Genet.* **39**, 205-209.

Miller, P. J. and Johnson, D. I. (1994). Cdc42p GTPase is involved in controlling polarized growth in *Schizosaccharomyces pombe*. *Mol. Cell Biol.* **14**, 1075-1083.

Mitchison, J. M. and Nurse, P. (1985). Growth in cell length in the fission yeast *Schizosaccharomyces pombe*. *J. Cell. Sci* **75**, 357-376.

Moon, S. Y. and Zheng, Y. (2003). Rho GTPases-activating proteins in cell regulation. *Trends Cell Biol.* **13**, 13-22.

Moreno, S., Ruiz, T., Sánchez, Y., Villanueva, J. R. and Rodriguez, L. (1985). Subcellular localization and glycoprotein nature of the invertase from the fission yeast *Schizosaccharomyces pombe*. *Arch. Microbiol.* **142 (4)**, 370-374.

Morrell-Falvey, J. L., Ren, L., Feoktistova, A., Haese, G. D. and Gould, K. L. (2005). Cell wall remodeling at the fission yeast cell division site requires the Rho-GEF Rgf3p. *J. Cell Sci.* **118**, 5563-5573.

Moseley, J. B. and Goode, B. L. (2006). The

yeast actin cytoskeleton: from cellular function to biochemical mechanism. *Microbiol. Mol. Biol. Rev.* **70**, 605-645.

Motegi, F., Arai, R. and Mabuchi, I. (2001). Identification of two type V Myosins in fission yeast, one of which functions in polarized cell growth and moves rapidly in the cell. *Mol. Biol. Cell* **12**, 1367-1380.

Mulvihill, D. P., Edward, S. R. and Hyams, J. S. (2006). A critical role for the type V myosin, Myo52, in septum deposition and cell fission during cytokinesis in *Schizosaccharomyces pombe*. *Cell Motil Cytoskeleton* **63**, 149-161.

Murray, J. M. and Johnson, D. I. (2001). The Cdc42p GTPase and Its Regulators Nrf1p and Scd1p Are Involved in Endocytic Trafficking in the Fission Yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **276**, 3004-3009.

Mutoh, T., Nakano, K. and Mabuchi, I. (2005). Rho1-GEFs Rgf1 and Rgf2 are involved in formation of cell wall and septum, while Rgf3 is involved in cytokinesis in fission yeast. *Genes Cells* **10**, 1189-1202.

Nakamura, T., Abe, H., Hirata, A. and Shimoda, C. (2004). ADAM family protein Mde10 is essential for development of spore envelopes in the fission yeast *Schizosaccharomyces pombe*. *Eukaryotic Cell* **3**, 27-39.

Nakano, K., Arai, R. and Mabuchi, I. (1997). The small GTP binding protein Rho1 is a multifunctional protein that regulates actin localization, cell polarity, and septum formation in the fission yeast *Schizosaccharomyces pombe*. *Genes Cells* **2**, 679-694.

Nakano, K., Arai, R. and Mabuchi, I. (2005). Small GTPase Rho5 is a functional homologue of Rho1, which controls cell shape and septation in fission yeast. *FEBS Lett.* **579**, 5181-5186.

Nakano, K., Mai, J., Arai, R., Toh-E, A., Matsui, Y. and Mabuchi, I. (2002). The small GTPase Rho3 and the diaphanous/formin For3 function in polarized cell growth in fission yesat. *J. Cell Sci.* **115**, 4629-4639.

Nakano, K., Mutoh, T., Arai, R. and Mabuchi, I. (2003). The small GTPase Rho4 is involved in controlling cell morphology and septation in fission yeast. *Genes to Cells* **8**, 357-370.

Nakano, K., Mutoh, T. and Mabuchi, I. (2001). Characterization of GTPase-activating proteins for the function of the Rho-family small GTPases in the fission yeast *Schizosaccharomyces pombe*. *Genes Cells* **6**, 1031-1042.

Nicholas, R. O., Williams, D. W. and Hunter, P. A. (1994). Investigation of the value of β-glucan-specific fluorochromes for predicting the β-glucans content of the cell wall of zoopathogenic fungi. *Mycol. Res.* **98**, 694-698.

Nolen, B. J. and Pollard, T. D. (2008). Structure and biochemical properties of fission yeast Arp2/3 complex lacking the Arp2 subunit. *J. Biol. Chem* **283**, 26490-8.

Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A. and Takai, Y. (1995). A downstream target of *RHO1* small GTP-binding protein is *PKC1*, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. *EMBO J.* **14**, 5931-5938.

Osumi, M. (1998). The ultrastructure of yeast: cell wall structure and formation. *Micron.* **29**, 207-233.

Osumi, M., Sato, M., Ishijima, S. A., Konomi, M., Tanagi, T. and Yaguchi, H. (1998). Dynamics of cell wall formation in fission yeast, *Schizosaccharomyces pombe*. *Fungal Genetics and Biology* **24**, 178-206.

Osumi, M., Yamada, N., Kobori, H., Taki, A., Naito, N., Baba, M. and Nagatani, T. (1989). Cell wall formation in regenerating protoplast of *Schizosaccharomyces pombe*: study by high resolution, low voltage scanning electron microscopy. *J. Electron Microsc* **38**, 457-468.

Ottilie, S., Miller, P. J., Johnson, D. I., Creasy, C. L., Sells, M. A., Bagrodia, S., Forsburg, S. and Chernoff, J. (1995). Fission yeast *pak1*$^{+}$ encodes a protein kinase that interacts with Cdc42p and is involved in the control of cell polarity and mating. *EMBO J.* **14**, 5908-5919.

Papadaki, P., Pizon, V., Onken, B. and Chang, E. (2002). Two ras pathways in fission yeast are differentially regulated by two ras guanine nucleotide exchange factors. *Mol. Cell Biol.* **22**, 4598-4606.

Park, H. O. and Bi, E. (2007). Central roles of small GTPases in the development of cell polarity in yeast and beyond. *Microbiol. Mol. Biol. Rev.* **71**, 48-96.

Pelham, R. J. J. and Chang, F. (2001). Role of actin polymerization and actin cables in actin-patch movement in *Schizosaccharomyces pombe*. *Nat. Cell*

Biol. **3**, 235-244.

Pollard, T. D. (2008). Progress towards understanding the mechanism of cytokinesis in fission yeast. *Biochem Soc Trans* **36**, 425-430.

Pruyne, D. and Bretscher, A. (2000). Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. *J. Cell Sci* **113**, 365-375.

Reese, A. J. and Doering, T. L. (2003). Cell wall α-1,3-glucan is required to anchor the *Cryptococcus neoformans* capsule. *Mol. Microbiol.* **50**, 1401-1409.

Rincón, S., Santos B. and Pérez, P. (2005). Fission yeast Rho5p GTPase is a functional paralogue of Rho1p that plays a role in survival of spores and stationary-phase cells. *Eukaryotic Cell* **5**, 435-446.

Rossman, K. L., Channing, J. D. and Sondek, J. (2005a). GEF means go: turning on Rho GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* **6**, 167-180.

Rupes, I., Jia, Z. and Young, P. (1999). Ssp1 promotes actin depolymerization and is involved in stress response and new end take-off control in fission yeast. *Mol. Biol. Cell* **10(5)**, 1495-510.

San-Blas, G., San-Blas, F. and Serrano, L. E. (1977). Host parasite relationships in the yeastlike form of *Paracoccidioides brasiliensis* strain IVIC Pb9. *Infect. Immun.* **15**, 343-346.

Santos, B., Gutierrez, J., Calonge, T. M. and Pérez, P. (2003). Novel Rho GTPase involved in cytokinesis and cell wall integrity in the fission yeast *Schizosaccharomyces pombe*. *Eukaryotic Cell* **2**, 521-533.

Santos, B., Martín-Cuadrado, A. B., Vazquez de Aldana, C. R., del Rey, F. and Pérez, P. (2005). Rho4 GTPase is involved in secretion of glucanases during fission yeast cytokinesis. *Eukaryotic Cell* **4**, 1639-1645.

Sawin, K. (1999). Miss-specification of cortical identity in a fission yeast PAK mutant. *Curr. Biol.* **9**, 1335-1338.

Sawin, K. E. and Nurse, P. (1998). Regulation of cell polarity by microtubules in fission yeast. *J Cell Biol.* **142**, 457-471.

Sawin, K. E. and Tran, P. T. (2006). Cytoplasmic microtubule organization in fission yeast. *Yeast* **23**, 1001-1014.

Sayers, L. G., Katayama, S., Nakano, K., Mellor, H., Mabuchi, I., Toda, T. and Parker, P. J. (2000). Rho-dependence of *Schizosaccharomyces pombe* Pck2. *Genes Cells* **5**, 17-27.

Schmidt, A. and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev.* **16**, 1587-1609.

Sells, M. A., Barratt, J. T., Caviston, J., Ottilie, S., Leberer, E. and Chernoff, J. (1998). Characterization of Pak2p, a pleckstrin homology domain-containing, p21-activated protein kinase from fission yeast. *J. Biol. Chem.* **273**, 18490-18498.

Sengar, A. S., Markley, N. A., Marini, N. J. and Young, D. (1997). Mkh1, a MEK kinase required for cell wall integrity and proper response to osmotic and temperature stress in *Schizosaccharomyces pombe*. *Mol. Cell Biol.* **17**, 3508-3519.

Shimoda, C. (2004). Forespore membrane assembly in yeast: coordinating SPBs and membrane trafficking. *J. Cell Sci.* **117**, 389-396.

Shiozaki, K. and Russell, P. (1995b). Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. *Nature* **378**, 739-743.

Sirotkin, V., Beltzner, C. C., Marchand, J. B. and Pollard, T. D. (2005). Interactions of WASp, myosin-I, and verprolin with Arp2/3 complex during actin patch assembly in fission yeast. *J. Cell Biol.* **170**, 637-648.

Skoumpla, K., Coulton, A. T., Lehman, W., Geeves, M. A. and Mulvihill, D. P. (2007). Acetylation regulates tropomyosin function in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci* **120**, 1635-45.

Snaith, H. and Sawin, K. (2003). Fission yaest mod5p regulates polarized growth through anchoring of tea1 at the cell tips. *Nature* **423**, 647-651.

Soisson, S. M., Nimnual, A. S., Uy, M., Bar-Sagi, D. and Kuriyan, J. (1998). Crystal structure of the Dbl and pleckstrin homology domains from the human son of sevenless protein. *Cell* **95**, 259-268.

Streiblova, E. and Girbardt, M. (1980). Microfilaments and cytoplasmic microtubules in cell division cycle mutants of *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **26**, 250-254.

Sugawara, T., Sato, M., Takagi, T., Kamasaki, T., Ohno, N., and Osumi. M. (2003). In situ localization of cell wall α-1,3-glucan in the fission yeast *Schizosaccharomyces pombe*. *J. Electron Micros.*

52, 237-242.

Sugiura, R., Toda, T., Dhut, S., Shuntoh, H. and Kuno, T. (1999). The MAPK kinase Pek1 acts as a phosphorylation-dependent molecular switch. *Nature* **399**, 479-483.

Sugiura, R., Toda, T., Shuntoh, H., Yanagida, M. and Kuno, T. (1998). *pmp1*$^{+}$, a suppressor of calcineurin deficiency, encodes a protein similar to the *cdc10*$^{+}$ and *SWI4* gene products. *EMBO J.* **17**, 140-148.

Tajadura, V., García, B., García, I., García, P. and Sánchez, Y. (2004). *Schizosaccharomyces pombe* Rgf3p is a specific Rho1 GEF that regulates cell wall β-glucan biosynthesis through the GTPase Rho1p. *J. Cell Sci.* **117**, 6163-6174.

Tanaka, K. and Hirata, A. (1982). Ascospore development in the fission yeast *Schizosaccharomyces pombe* and *S. japonicus*. *J. Cell. Sci.* **56**, 263-279.

Toda, T., Dhut, S., Superti-Furga, G., Gotoh, Y., Nishida, E., Sugiura, R. and Kuno, T. (1996). The fission yeast *pmk1*$^{+}$ gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the Protein Kinase C pathway. *Mol. Cell. Biol.* **16**, 6752-6764.

Toda, T., Shimanuki, M. and Yanagida, M. (1991). Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/ MAP2 and budding yeast FUS3 and KSS1 kinases. *Genes Dev.* **5**, 60-73.

Toda, T., Shimanuki, M. and Yanagida, M. (1993). Two novel protein kinase C-related genes of fission yeast are essential for cell viability and implicated in cell shape control. *EMBO J.* **12**, 1987-1995.

Tran, P. T., Marsh, L., Doyle, V., Inoue, S. and Chang, F. (2001). A mechanism for nuclear positioning in fission yeast based upon microtubule pushing. *J. Cell Biol.* **153**, 397-411.

Verde, F., Mata, J. and Nurse, P. (1995). Fission yeast cell morphogenesis: identification of new genes and analysis of their role during the cell cycle. *J. Cell Biol.* **131**, 1529-1538.

Verde, F., Wiley, D. J., Nurse, P. (1998). Fission yeast Orb6, a ser/thr protein kinase related to mammalian Rho kinase and myotonic dystrophy kinase, is required for maintainance of cell polarity and coordinates cell morphogenesis with the cell cycle. *Proc. Natl. Acad. Sci. USA* **95**, 7526-7531.

Wang, H., Tang, X. and Balasubramanian, M. K. (2003). Rho3p regulates cell separation by modulating exocyst function in *Schizosaccharomyces pombe*. *Genetics* **164**, 1323-1331.

Waskiewicz, A. J. and Cooper, J. (1995). Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Op. Cell Biol.* **7**, 798-805.

Win, T. Z., Gachet, Y., Mulvihill, D. P., May, K. M. and Hyams, J. S. (2001). Two type V myosins with non-overlapping functions in the fission yeast *Schizosaccharomyces pombe*: Myo52 is concerned with growth polarity and cytokinesis, Myo51 is a component of the cytokinetic actin ring. *J. Cell Sci.* **114**, 69-79.

Wood, V. Gwilliam, R. Rajandream, M. A. Lyne, M. Lyne, R. Stewart, A. Sgouros, J. Peat, N. Hayles, J. Baker, S. et al. (2002). The genome sequence of Schizosaccharomyces pombe. *Nature* **415**, 871-80.

Worthylake, D. K., Rossman, K. L. and Sondek, J. (2000). Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. *Nature* **408**, 682-688.

Wu, J. Q., Kuhn, J. R., Kovar, D. R. and Pollard, T. D. (2003). Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. *Dev Cell* **5**, 723-734.

Yanagida, M. (2002). The model unicellular eukaryote, *Schizosaccharomyces pombe*. *Genome Biol* **3**.

Yang, P., Kansra, S., Pimental, R. A., Gilbreth, M. and Marcus, S. (1998). Cloning and characterization of *shk2*, a gene encoding a novel p21-activated protein kinase from fission yeast. *J. Biol. Chem.* **273**, 18481-18489.

Yang, P., Qyang, Y., Bartholomeusz, G., Zhou, Z., and Marcus, S. (2003). The novel Rho1 GTPase-activating protein family, Rga8, provides a potential link between cdc42/p21-activated kinase and Rho signaling pathways in the fission yeast, *Schizosaccharomyces pombe*. *J. Biol. Chem.* **278**, 48821-48830.

Yeh, B. J., Rutigliano, R. J., Deb, A., Bar-Sagi, D. and Lim, W. A. (2007). Rewiring cellular morphology pathways with synthetic guanine nucleotide exchange factors. *Nature* **447**, 596-600.

Yoo, B. Y., Calleja, G. B. and Johnson, B. F.

(1973). Ultrastructural changes of the fission yeast (*Schizosaccharomyces pombe*) during ascospore formation. *Arch. Microbiol.* **91**, 1-10.

Zaitsevskaya-Carter, T. and Cooper, J. A. (1997). Spm1, a stress-activated MAP kinase that regulates morphogenesis in S. pombe. *EMBO Journal* **16**, 1318-1331.