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Brief Report

Human Cdc14A Reverses CDK1 Phosphorylation of Cdc25A on Serines 115 and 320

Verónica Esteban[1]
María D. Vázquez-Novelle[1]
Enrique Calvo[3]
Avelino Bueno[1,2]
María P. Sacristán[1,*]

[1]Centro de Investigación del Cáncer; [2]Departamento de Microbiología y Genética; Universidad de Salamanca/CSIC, Salamanca, Spain

[3]Unidad de Proteómica; Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III; Madrid, Spain

*Correspondence to: María P. Sacristán; Centro de Investigación del Cáncer; Campus Miguel de Unamuno; 37007 Salamanca, Spain; Tel.:34.92.329.4805; Fax: 34.92.329.4743; Email: msacristan@usal.es

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KEY WORDS

Cdc14A, Cdc25A, Cdk1/Cyclin-B1, dephosphorylation, cell cycle

ABBREVIATIONS

APC/C anaphase-promoting complex/cyclosome
Cdk cyclin-dependent kinase
SCF Skp1/Cul1/F-box

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ABSTRACT

Human Cdc14A is an evolutionary conserved dual-specificity protein phosphatase that reverses the modifications effected by cyclin-dependent kinases and plays an important role in centrosome duplication and mitotic regulation. Few substrates of Cdc14A have been identified, some of them with homologues in yeast that, in turn, are substrates of the *Saccharomyces cerevisiae* Cdc14 homologue, a protein phosphatase essential for yeast cell viability owing its role in mitotic exit regulation. Identification of the physiological substrates of human Cdc14A is an immediate goal in order to elucidate which cellular processes it regulates. Here, we show that human Cdc14A can dephosphorylate Cdc25A in vitro. Specifically, the Cdk1/Cyclin-B1-dependent phosphate groups on Ser115 and Ser320 of Cdc25A were found to be removed by Cdc14A. Cdc25A is an important cell cycle-regulatory protein involved in several cell cycle transitions and checkpoint responses and whose function and own regulation depend on complex phosphorylation/dephosphorylation-mediated processes. Importantly, we also show that the upregulation of Cdc14A phosphatase affects Cdc25A protein levels in human cells. Our results suggest that Cdc14A may be involved in the cell cycle regulation of Cdc25A stability.

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INTRODUCTION

The dual-specificity phosphatase Cdc14 is conserved from yeast to mammals and is involved in a variety of functions controlling the cell division cycle. Cdc14 is essential for the survival of *S. cerevisiae* since it is required for the cell to exit from mitosis.[1,2] ScCdc14 dephosphorylates and activates several Cdk1/Cdc2 substrates such Hct1/Cdh1 protein, a component of the anaphase promoting complex/cyclosome (APC/C), or the Cdk1 inhibitor Sic1p, in both cases to downregulate Cdk1 activity at the end of mitosis.[2-4] In *Schizosaccharomyces pombe*, the Cdc14 homologue Flp1/Clp1[5,6] also regulates mitotic events, although through a distinct mechanism. Flp1/Clp1 antagonizes mitotic CDK activity by dephosphorylation and subsequent degradation of SpCdc25, the inducer of mitotic kinase, and also substrate of Cdk1/Cdc2.[7,8] Homologues of Cdc14 with roles in cell division processes and cytokinesis have also been identified in *Caenorhabditis elegans* and *Xenopus*.[9-11] In human cells, two Cdc14 isoforms -Cdc14A and Cdc14B- have been identified.[12] The function of these phosphatases remains poorly understood, although it is known that Cdc14A plays an important role in the centrosome cycle and mitotic regulation.[13,14] An important goal to elucidate the functions of human Cdc14 phosphatases is the identification of their substrates. A few substrates for Cdc14A have been described, and these include Cdh1/Hct1 and INCENP proteins[15,16] and the tumor suppressor p53.[17,18] Moreover, human Cdc14A can rescue *flp1/clp1* deficiency and dephosphorylate SpCdc25 protein,[19] suggesting that some conserved mechanisms must exist with the fission yeast Cdc14 homologue. Mammalian cells have three Cdc25 isoforms; Cdc25A, B and C.[20-22] All three are dual-specificity phosphatases that activate their physiological substrates, the CDKs,[23] although each Cdc25 family member has unique characteristics and specific roles in cell cycle regulation.

Here, we show that human Cdc14A can dephosphorylate Cdc25A on Ser115 and Ser320, two Cdk1/Cyclin-B1 phosphorylation sites, and that these protein modifications are involved in the stability of Cdc25A.

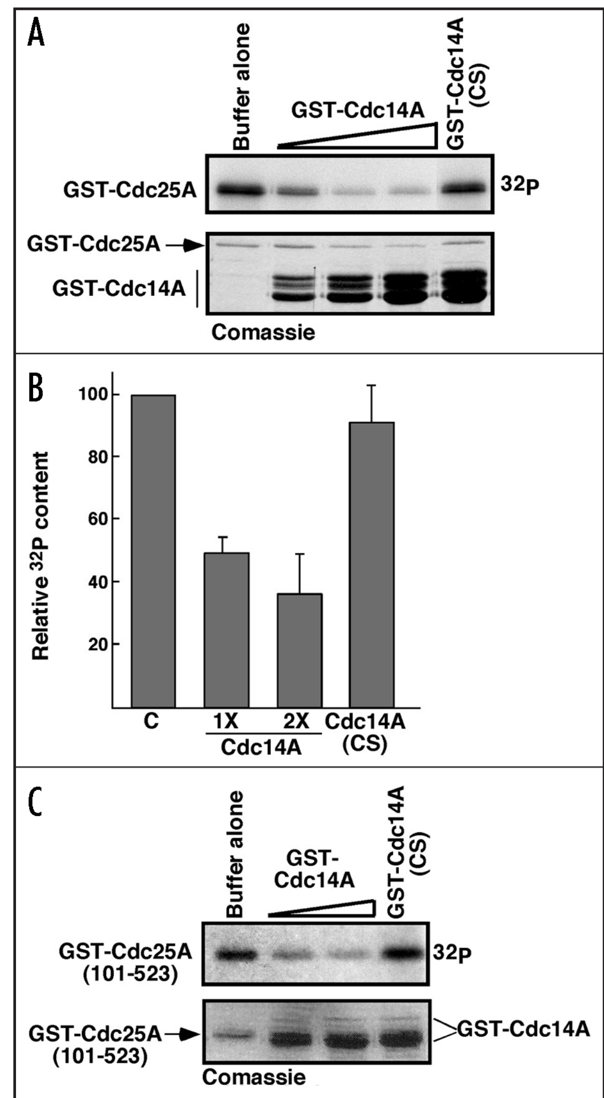

Figure 1. Human Cdc14A dephosphorylates in vitro Cdc25A previously phosphorylated by Cdk1/Cyclin-B1. (A) GST-Cdc25A purified from *E. coli* was phosphorylated in vitro by the Cdk1/Cyclin-B1 complex and subsequently used in a phosphatase assay with three increasing amounts of GST-Cdc14A, the catalytically inactive GST-Cdc14A(CS) form, or buffer alone as control. Reactions were separated by SDS-PAGE and analyzed by autoradiography (upper panel) and Coomassie blue staining (lower panel). (B) the relative amount of radioactive GST-Cdc25A protein after the in vitro kinase and phosphatase assays was quantified. Bars show the means from three independent experiments of normalized ratio between ^{32}P-GST-Cdc25A radiactivity and the total amount of protein. 1X corresponds to the lowest amount of GST-Cdc14A shown in (A), 2X corresponds to double the amount. (C) GST-Cdc25A(101-523), lacking the first 100 N- terminal amino acids, was used as the Cdc14A phosphatase substrate as in A. The GST-C-terminal fragment of Cdc25A was phosphorylated by Cdk1/Cyclin-B1 complex and subsequently incubated with two amounts of GST-Cdc14A, GST-Cdc14A-(CS), or buffer alone. The levels of phosphorylated and total GST-fusion protein were detected by autoradiography and Coomassie blue staining respectively.

MATERIALS AND METHODS

Kinase and phosphatase assay. Wild-type or Cdc25A protein mutants were expressed and purified from *E. coli* as GST-Cdc25A fusion proteins (GST-Cdc25A, GST-Cdc25A$^{(1-100)}$, GST-Cdc25A$^{(101-523)}$, GST-Cdc25A^{S115A}, GST-Cdc25A^{S320A} and GST-Cdc25A$^{S115-320A)}$) using a pGEX-4T vector (the full-length pGEX-4T-Cdc25A was a gift from P. Lazo´s laboratory). The constructs for GST-hCdc14A and its phosphatase-inactivating mutant, GST-Cdc14A-CS, with the cysteine 278 mutated to serine, have been described previously.[19] GST-purification was carried out as previously described.[24] The CDK substrates, GST-Cdc25A constructs, were incubated with 2U of Cdk1/cyclin-B1 human complex (New England Biolabs) in kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 50 mM ATP) in the presence of 0.15 μCi of $\gamma(^{32}P)$ATP for 30´ at 30°C. When kinases assays were performed for mass spectrometry, only cold ATP was used. Samples were then washed three times with 1 ml of phosphatase buffer (50 mM imidazole, pH 6.9, 1 mM EDTA, and 1 mM DTT) and GST-hCdc14 was added and incubated for 40´ at 30°C. Reactions were stopped by the addition of loading buffer and boiling for 5´at 95°C. Proteins were resolved by SDS-PAGE and visualized by autoradiography and Comassie staining.

Characterization of phosphorylation by mass spectrometry. Protein bands were incubated for several minutes in ultra-pure water and digested according with protocol of Schevchenko et al[25] with some minor variations. Gel pieces were equilibrated in 50 mM ammonium bicarbonate (ABC) prior to reduction with 10 mM DTT and alkylation with 100 mM iodoacetamide, both in 50 mM ABC. Modified porcine trypsin at a final concentration of 0.4 μg per reaction in 50 mM ABC was added to dried bands and the digestion proceeding at 37°C overnight. Finally, tryptic peptides were extracted with 0.5% trifluoroacetic acid. The resulting tryptic peptides were on line injected onto a C-18 reversed-phase nano-column (Discovery® BIO Wide pore, Supelco, Bellafonte, PA) and analyzed in a continous acetonitrile gradient. A flow rate of ca. 300 nl/min was used to elute peptides from the reversed-phase nano-column to a PicoTip™ emitter nano-spray needle (New Objective, Woburn, MA) for real-time ionization and precursor ion scanning analysis on a 4000 Q-Trap LC-MS/MS hybrid system (Applied Biosystems, MDS, Sciex) mass spectrometer.

Plasmids, mutagenesis and transfections. The Cdc25A construct was generated by PCR from pGEX-4T-Cdc25A and cloned into the EcoRI restriction site of pCEFL-HA vector. pcDNA3-His6-Myc-hCdc14A was provided by J. Lukas. The Cdc14A phosphatase-dead version (C278S) and all the S/E Cdc25A mutants (S115E, S320E, S115/320E, S17/115E, and S17,115,320E) were generated using QuikChange Site-directed mutagenesis (Stratagene), according to manufacturer´s protocols. The pCMV-GFP construct was from F. Pimentel. 293-T cells were transfected using Lipofectamine 2000 (Invitrogen) and harvested 40h post-transfection. To monitor transfection efficiency and protein loading, 0.8 μg of pCMV-GFP was included in each transfection. The following antibodies were used: anti-Cdc25A (F6; Santa Cruz); anti-Cdc14A (NeoMarkers-Lab Vision) anti-GFP (JL-8; BD Biosciences).

RESULTS AND DISCUSSION

While many aspects have been described concerning the function of Cdc14 and the molecular mechanisms by which this phosphatase acts regulating cell division in the budding yeast *S. cerevisiae*, little is known about the cellular processes carried out by Cdc14 homologues in human cells. We have previously described that the two human Cdc14 isoforms are able to complement the lack of the *flp1/clp1*$^+$ gene in *S. pombe* and that human Cdc14A dephosphorylates SpCdc25

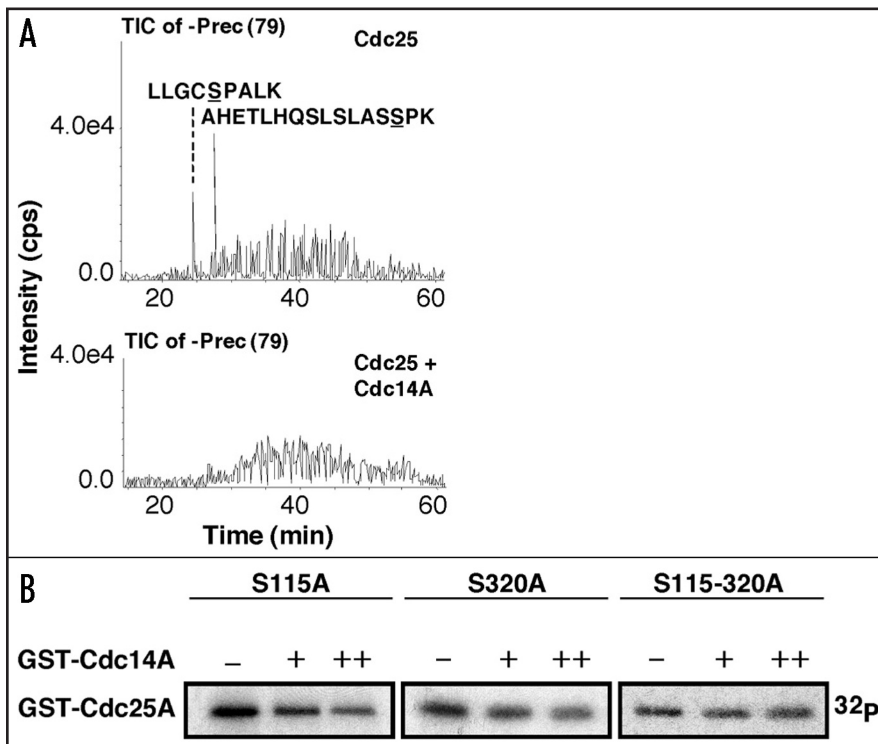

Figure 2. Cdc14A reverses the Cdk1/Cyclin-B1 phosphorylation of serines 115 and 320 of Cdc25A protein. (A) Total ion chromatogram of the precursor ion scanning of the phosphate group in negative mode at 79 Da. GST-Cdc25A protein was phosphorylated by Cdk1/Cyclin-B1 complex (two peaks in upper panel), subsequently dephosphorylated by Cdc14A phosphatase (lower panel) and analyzed by triple-quadrupole hybrid mass spectrometry for phosphorylation state. The ions corresponding to each chromatographic peak were fragmented and the phosphorylation sites were characterized (underlined residues). (B) Two individual mutants (S115A and S320A) and a double (S115/320A) mutant of GST-Cdc25A containing one or both serine residues substituted by alanine were used as substrates of Cdc14A phosphatase. Two amounts of GST-Cdc14A were used (+ and ++).

protein to deprotect it from degradation[19] in a similar manner to Flp1/Clp1 does in fission yeast.[7,8] In the present work, we wondered whether Cdc14A was able to dephosphorylate human Cdc25A, a critical regulator of the cell cycle progression, involved in both S-phase and mitotic cellular events and whose contribution to G_2/M transition requires its stability at this point of the cell cycle by mitotic CDK phosphorylation.[26] To this end, we used purified GST-Cdc14A and Cdk1/Cyclin-B1-phosphorylated GST-Cdc25A in some in vitro phosphatase assays. As shown in Figure 1A and B, Cdc14A, but not its catalytically inactive form, Cdc14A-CS, was able to dephosphorylate Cdc25A. It has been described that in mitosis Cdc25A is phosphorylated by Cdk1/Cyclin-B1 on two specific residues: serine 17 and serine 115.[26] In order to study whether these two known Cdc2/CyclinB1-dependent phosphate groups could be removed by Cdc14A, we dissected the Cdc25A protein into two fragments, one of them corresponding to the first 100 N-amino terminal residues, where Ser17 is included, and a second construct comprising the rest of the protein, the GST-Cdc25A$^{(101-523)}$ mutant, which contains the Ser115 residue. We then assayed the ability of Cdc14A to dephosphorylate these two GST-Cdc25A constructs and found that the C-terminal GST-Cdc25A$^{(101-523)}$ mutant was efficiently dephosphorylated by GST-Cdc14A (Fig. 1C). However, Cdc14A did not dephosphorylate the N-terminal GST-Cdc25A$^{(1-100)}$ fragment (data not shown). These data indicated that Cdk1/Cyclin-B1-phosphorylated serine 115 might be a target of Cdc14A, but not the phosphate group of serine 17. The negative result concerning the dephosphorylation of Ser17 by Cdc14A was also checked by immunoblotting with a specific antibody to Ser17-phosphorylated Cdc25A (data not shown).

To identify the specific Cdk1/Cyclin-B1-dependent phosphorylated residues of Cdc25A targeted by Cdc14A, we set up a kinase/phosphatase assay as described in Figure 1, followed by a mass spectrometric analysis to detect phospho-amino acids. We found that serine 115 and serine 320 were dephosphorylated by Cdc14A (Fig. 2A). Unexpectedly, Ser320 is a new identified in vitro residue targeted by mitotic Cdk1/Cyclin-B1 complexes, that can also be removed by Cdc14A phosphatase. Next, we tested these two specific Cdc14A dephosphorylation sites by additional in vitro kinase/phosphatase assays, using three full-length GST-Cdc25A mutants in which the serines identified (115 and 320) were substituted by alanines. While the two individual mutants showed some level of dephosphorylation by Cdc14A, the mutation of both Ser115 and Ser320 appeared to abolish the Cdc14A dephosphorylation effect on Cdc25A (Fig. 2B). Thus, human Cdc14A can dephosphorylate the Cdk1/Cyclin-B1 phosphorylation sites of Cdc25A, Ser115 and Ser320 in vitro.

Cdc25A is an unstable protein that is subject to constant turnover in cycling cells by periodic synthesis and ubiquitin-mediated proteolysis. It first appears in late G_1 as a result of E2F-1- and c-Myc-mediated transcriptional activation[27-29] after which it accumulates until mitosis. Counterbalancing the de novo synthesis, the ubiquitin ligase complexes APC/C^{Cdh1}, upon exit from mitosis, and $SCF^{\beta TrCP}$, during interphase and upon DNA damage, mediate its ubiquitin-proteasome degradation.[30,31] Moreover, multiple phosphorylation sites of Cdc25A by several kinases seem to tightly regulate its degradation mechanisms and the final protein levels, which could afford the cell different thresholds of activity, depending on the cell cycle phase or stress-induced situations (reviewed in ref. 32).

As mentioned above, the phosphorylation of serines 17 and 115 by Cdk1/Cyclin-B1 appears to be crucial for the stability of Cdc25A at the G_2/M transition.[26] According to these data and our own results, the dephosphorylation of Cdc25A by Cdc14A, particularly on Ser115 and Ser320 residues, could be involved in the regulation of Cdc25A stability. In order to check this possibility, we cotransfected 293-T cells with wild-type Cdc25A or different Cdc25A mutant forms (S115E, S320E and S115/320E), in which serines 115 and 320 had been substituted by a phosphomimicking amino acid -glutamic acid- together with either Cdc14A or an empty plasmid, and we then tested Cdc25A protein accumulation. We observed that under these experimental conditions the overexpression of Cdc14A, but not of the phosphatase-dead form Cdc14A-CS, resulted in a significant decrease in the level of wild-type Cdc25A (Fig. 3A). This effect was also observed in both individual mutants, S115E or S320E, but was very reduced in the double mutant S115/320E, whose protein level was much less affected by the overexpression of

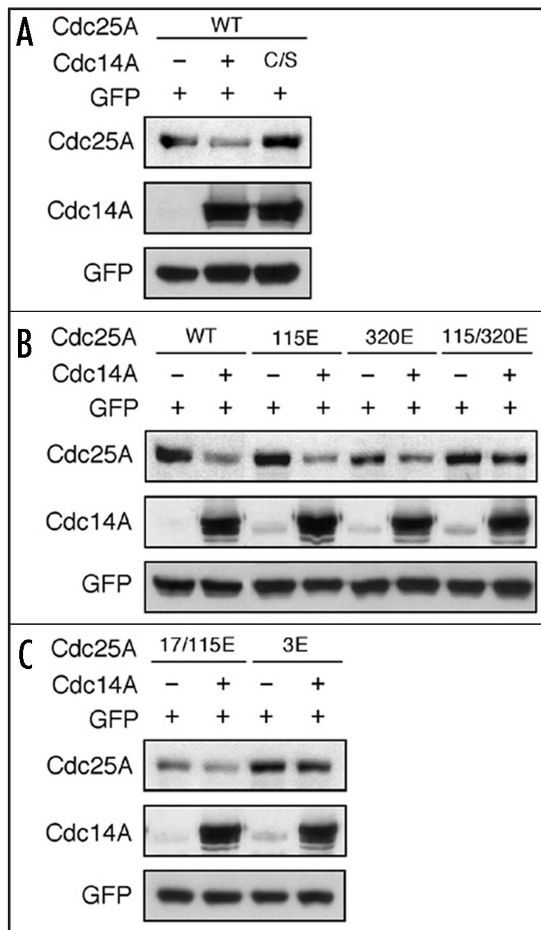

Figure 3. The overexpresion of Cdc14A reduces the protein levels of Cdc25A in a dephosphorylation-dependent manner. Extracts were prepared from 293-T cells transfected with plasmids encoding wild-type Cdc25A (A and B) or any of the different Cdc25A mutants, where specific serine residues were substituted by glutamic acid (Ser115E, Ser320E, Ser115/320E Ser17/115E and Ser17/115/320E (3E) (B and C) and either Myc-Cdc14A, Myc-Cdc14A-CS variant (A) or the empty plasmid together with GFP as a control of transfection efficiency and sample loading. Cdc25A, Cdc14A and GFP proteins were analyzed by Western blotting.

Cdc14A (Fig. 3B). These results suggest that Cdc14A activity would be involved in some Cdc25A regulatory mechanism, acting at least in part through the dephosphorylation of Ser115/Ser320 residues. We then tested whether overexpression of Cdc14A affected the protein levels of a Cdc25A mutant in which serines 17 and 115, whose phosphorylation is crucial for mitotic Cdc25A stability, had been mutated to glutamic acid (S17/115E). Figure 3C shows that although the stability of this mutant was higher than that of the wild-type under Cdc14A overexpressing conditions, cells overexpressing Cdc14A accumulated lower levels of this Cdc25A mutant than controls. Finally, when S320 was also mutated to obtain a triple S17/115/320E mutant (3E), the overexpression of Cdc14A had a smaller effect and the Cdc25A mutant protein level remained more stable. These results are consistent with the interpretation that Cdc14A would be able to dephosphorylate Cdc25A in vivo on at least S115 and S320 in order to regulate its cellular levels during the cell division cycle, counteracting Cdk1/Cyclin-B1 activity on the mitotic inducer.

Both the lability and stability of Cdc25A protein rely on phosphorylation of certain specific amino-acid residues. It has been shown that the phosphorylation-dependent degradation of Cdc25A occurs in both normal interphase cells and S-phase cells subjected to different stress conditions.[33-38] Thus, a fixed threshold of Cdc25A activity is maintained in a normal cell cycle, and, in response to cell damage, the accelerated phosphorylation-dependent degradation of Cdc25A induces the inactivation of CDK complexes and the consequent arrest of cell division. Mitotic stabilization of Cdc25A also requires some phosphorylation events. Thus, at around the G_2/M transition Cdc25A switches from a labile to a stable form through Cdk1/Cyclin-B1 phosphorylation.[26] As a result of this, CDK activity peaks, leading to entry into mitosis. Later on, at the mitotic exit and early G_1 Cdc25A is degraded in an APC/C^{Cdh1}-dependent manner[30] at the time when Cdk1/Cyclin-B1 activity drops. Here we report that in vitro Cdc14A is able to dephosphorylate Cdk1/CyclinB1-dependent Cdc25A phosphorylated Serine 115 and 320 residues and that overexpression of Cdc14A induces Cdc25A degradation in a dephosphorylation-dependent manner. Our work suggests that Cdc14A may be involved in the control of the cell cycle- regulated Cdc25A stability.

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