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Rapid Communication

T-Cadherin 2: Molecular Characterization, Function in Cell Adhesion, and Coexpression With T-Cadherin and N-Cadherin

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Cadherins are integral membrane glycoproteins that mediate calcium-dependent, homophilic cell-cell adhesion and are implicated in controlling tissue morphogenesis. T-cadherin is anchored to the membrane through a glycosyl phosphatidylinositol (Ranscht B, Dours-Zimmermann MT: Neuron 7:391–402, 1991) and expressed in a restricted pattern in developing embryos (Ranscht B, Bronner-Fraser M: Development 111:15–22, 1991). We report here the molecular and functional characterization of the T-cadherin isoform, T-cadherin 2 (Tcad-2) and the expression of the corresponding mRNA. Tcad-2 cDNA differs in its 3′ nucleotide sequence from T-cadherin cDNA and encodes a protein in which the carboxy terminal Leu of T-cadherin is substituted by Lys and extended by the amino acids SerPheProTyrVal. By RNase protection, mRNAs encoding the T-cadherin isoforms are coexpressed in heart, muscle, liver, skin, somites, and in neural tissue. Many tissues contain both T-cadherin and Tcad-2 mRNAs in conjunction with N-cadherin transcripts, and T-cadherins and N-cadherin proteins are coexpressed on the surface of individual neurons in vitro. Expression in Chinese hamster ovary cells (CHO) revealed that Tcad-2 is a glycosyl phosphatidylinositol-anchored membrane protein that functions in calcium-dependent, homophilic cell adhesion. The identification of a functional T-cadherin isoform and the coexpression of T-cadherins and N-cadherin by individual cells suggest that specific adhesive interactions of embryonic cells may involve a complex interplay between multiple cadherins.
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Key words: cell adhesion molecule, calcium dependence, GPI anchor, homophilic binding, T-cadherin isoforms

INTRODUCTION

Development of the vertebrate nervous system depends on complex and selective embryonic cell-cell interactions in which cell adhesion molecules have been implicated to play a key role (Jessell, 1988; Edelman and Crossin, 1991; Hynes and Lander, 1992). Cadherins are a family of calcium-dependent cell adhesion molecules both within and outside the nervous system that mediate homophilic cell-cell adhesion (Takeichi, 1988, 1990, 1991). Originally, three cadherin subclasses have been characterized: E-cadherin or uvomorulin (epithelial cadherin) (Nagafuchi et al., 1987; Ringwald et al., 1987; Mansouri et al., 1988) and its chick homologue L-CAM (Gallin et al., 1987), P-cadherin (placental cadherin) (Nose et al., 1987), and N-cadherin (neural cadherin) (Hatta et al., 1988). The function and binding specificity of cadherins in cell adhesion depend on regions in both their extracellular and cytoplasmic domains (Takeichi, 1991; Ranscht, 1991). In particular, the cytoplasmic domain has been implicated to control cell adhesion by providing a linkage of the transmembrane cadherin proteins to cortical actin filaments (Ozawa et al., 1989, 1990; Nagafuchi and Takeichi, 1988, 1989). The association with the cytoskeleton is suggested to cluster cadherins at sites of cell contact and thus provide the force for adhesive interactions (Kemler and Ozawa, 1989).

Each molecular cadherin species shows a distinct pattern of expression in developing embryos that frequently correlates with the aggregation and segregation of cells as they form specific tissue structures (Takeichi, 1988). For example, E-cadherin, contained in the ectoderm of the early developing embryo, is gradually lost

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from the dorsal portion of ectoderm that begins to form the neural tube. The loss of E-cadherin from neuralized tissue is accompanied by the complementary expression of N-cadherin in the neural tube to turn into a mutually exclusive expression of E-cadherin in ectoderm and N-cadherin in neural tissue by the time of neural tube closure (Hatta and Takeichi, 1986; Duband et al., 1988; Hatta et al., 1987). Similarly, N-cadherin is expressed in epithelial somites, but is lost as the cells migrate and rearrange to form the sclerotome and dermomyotome (Hatta et al., 1987; Duband et al., 1987, 1988). Based on their complementary expression and their selective binding, cadherins are suggested to control the aggregation and segregation of embryonic cells and thereby regulate tissue morphogenesis (Takeichi, 1988, 1990, 1991). Experimental support for this suggestion comes from two lines of functional studies. First, when cells expressing different cadherin types are mixed, they efficiently sort out into distinct cell populations (Nagafuchi et al., 1987; Edelman et al., 1987; Nose et al., 1988; Hatta et al., 1988; Jaffe et al., 1990). Second, the ectopic expression of N-cadherin in developing *Xenopus* embryos leads to severe defects in developing ectodermal and neural tissue (Detrick et al., 1990; Fujimori et al., 1990).

N-cadherin is widely expressed in the nervous system and has been implicated in multiple functions the specification of neural tissue after induction, neural retina development (Matsunaga et al., 1988) and the control of neurite growth and fasciculation (Bixby et al., 1987; Matsunaga et al., 1988; Bixby and Zhang, 1990; Drazba and Lemmon, 1990). One model explaining the multiple and different functions of one molecular cadherin species is that the adhesive specificities of diverse cell types are generated by the cooperation of different cadherin molecules (Takeichi, 1991). A wide range of cadherin cell adhesion molecules have been identified recently in the nervous system including the cadherins 4–11 (Suzuki et al., 1991), B-cadherin (Napolitano et al., 1991), R-cadherin (Inuzuka et al., 1991a,b), and T-cadherin (Ranscht and Dours-Zimmermann, 1991). Although the cellular distribution of these new cadherins has not been studied extensively, these molecules are good candidates to cooperate with N-cadherin in the establishment and maintenance of neural architecture.

T-cadherin is a functional cadherin that differs from classical cadherins in its membrane attachment through a phosphatidylinositol glycan (GPI) (Ranscht and Dours-Zimmermann, 1991; Vestal and Ranscht, 1992). In the few instances where the expression of T-cadherin has been studied in developing embryos, its temporal and spatial expression correlates with the formation of specific tissue structures. For example, T-cadherin is observed in the caudal somite regions as they dissociate from an epithelial organization to form the dermomyotome and sclerotome (Ranscht and Bronner-Fraser, 1991). The onset of T-cadherin expression in the caudal sclerotome correlates inversely with the loss of N-cadherin from epithelial somites (Hatta et al., 1987; Duband et al., 1987, 1988) and coincides with the immigration of neural crest cells and motor axons into the corresponding rostral regions (Ranscht and Bronner-Fraser, 1991). This pattern is consistent with the model that the aggregation and segregation of embryonic cells is controlled by different cadherins.

We have now identified a T-cadherin isoform, T-cadherin 2 (Tcad-2), and report its molecular and functional characterization and the expression of the corresponding mRNA in relation to T-cadherin and N-cadherin. The discovery of functional cadherin isoforms indicates a complex mechanism for the regulation of cell adhesion in developing embryos.

MATERIALS AND METHODS

Cloning and Sequence Analysis

The cDNA for Tcad-2 was isolated from a λgt.11 cDNA expression library representing transcripts from 13-day-old (E13) chick embryo brain as described previously (Ranscht and Dours-Zimmermann, 1991). Restriction fragments of Tcad-2 cDNA were subcloned into compatible sites of pBluescript KS^+ (Stratagene, La Jolla, CA) for sequence analysis by the dideoxy chain termination method (Sanger et al., 1977) using a Sequenase Kit (United States Biochemical Corp., Cleveland, OH). T3 and T7 primers and Tcad-2-specific oligonucleotide primers (DNA facility, La Jolla Cancer Research Foundation) were used for sequencing.

RNA Extraction

Total RNA was extracted from chick tissues using the guanidinium isothiocyanate procedure of Chirgwin et al. (1979). From tissues of early developing chicken embryos and cultures of sympathetic neurons, RNA was isolated by lithium chloride precipitation (Cathala et al., 1983). $Poly(A)^+$ RNA was selected using standard oligo-(dT) cellulose chromatography (Sambrook et al., 1989).

Polymerase Chain Reaction (PCR) Amplification

Five micrograms total or 1 μg enriched $poly(A)^+$ RNA was used as template for first strand cDNA synthesis using an 18-mer poly (dT) oligonucleotide in the presence of reverse transcriptase (Invitrogen, Red Module Kit 090915) and ^{32}P-α-dCTP. One fifth of the first strand cDNA was used as template for PCR amplification. Figure 2 shows the position of the primers in relation to the cDNAs. The 5′ primer 1 corresponds to nucleotides (nt) 2083–2108 of Tcad-2 cDNA and to nt

2088–2113 of T-cadherin cDNA (Ranscht and Dours-Zimmermann, 1991), respectively, and encodes carboxy-terminal amino acids of the extracellular domain EC5. The sequence is 5′-GGAATTCTAGAGCGGCCGCTG-CAAGAAATCCAGAATGGACTGCAG-3′. This primer was used with each one of the 3′ primers 3, 4, 5, and 6. Primer 3 (5′-GGGAATTCTCGAGCGGCCGC-GAATTCAAGTGGGGAGAGAT-3′) and primer 4 (5′-GGGAATTCTCGAGCGGCCGCCGTACCCTCCCGG-CACTGAG-3′) are complementary to nt 2461–2480 and 2959–2978 of Tcad-2 and T-cadherin, respectively. Primers 5 (5′-GGGAATTCTCGAGCGGCCGCRT-CRTCCTCVCCDCCRCCRTA-3′) and 6 (5′-GGGAAT-TCTCGAGCGGCCGCRTCYTCYTCNCCNCCNCCY-TCYTC-3′) are degenerate oligonucleotides corresponding to the two most conserved cytoplasmic domains of classical cadherins, YGGGEDD and EEG-GGEED, respectively (Y = C + T, R = G + A, V = A + C + G, D = A + G + T, N = A + C + G + T). Primer 2 (5′-CGGAATTCTAGAGCGGCCGCAG-GATCAGGTTCCTGGAGGCT-3′) represents nt 2051–2071 of N-cadherin cDNA (Hatta et al., 1988) and encodes a region of the N-cadherin extracellular domain EC5. The underlined sequences correspond to the restriction sites Eco RI, Xba I, and Not I in primers 1 and 2, and Eco RI, Xho I, and Not I in primers 3–6, which were added to facilitate subcloning of the amplified DNA fragments. PCR amplifications were carried out in a 100 μl reaction volume with 50 mM KCl, 10 mM Tris, pH 8.0, 0.01% bovine serum albumin (BSA), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 μM of each primer, and 2.5 U Taq polymerase (Perkin Elmer, Cetus, Norwalk, CT). Thirty-five cycles of hybridization (1 min at 45–62°C, depending on the primer-specific hybridization temperature), extension (72°C for 2–3 min), and denaturation (94°C for 1 min) were carried out unless otherwise indicated. Reaction products were separated on agarose gels and cloned into the pBluescript KS^+ vector. Sequence analysis was carried out as described by Sanger et al. (1977) using a T7 sequencing Kit (Pharmacia, Gaithersburg, MD).

Northern and Southern Blot Analyses

Ten micrograms of enriched poly$(A)^+$ RNA from E13 chick embryo brain and 10 μg of the corresponding poly$(A)^-$ RNA were resolved on 1% agarose/formaldehyde gels, transferred by capillary blotting to a nylon membrane (GeneScreen Plus, New England Nuclear, Cambridge, MA), and hybridized with three different probes. The probes were labeled with ^{32}P-α-dCTP by random priming (Kit from Amersham Corporation, Arlington Heights, IL). The T-cadherin/Tcad-2 probe was an Eco RI restriction fragment corresponding to the propeptide region common to both Tcad-2 and T-cadherin (nt 167–434 in Fig. 1, and nt 172–440 in Ranscht and Dours-Zimmermann, 1991); the Tcad-2 probe corresponded to the 3′ Hpa I/Eco RI restriction fragment of Tcad-2 cDNA (nt 2256–2774), and the third probe, Tcad, corresponded to a 3′ Pst I restriction fragment of T-cadherin cDNA (nt 2263–2831, Ranscht and Dours-Zimmermann, 1991). Hybridization was performed in 1% BSA, 1 mM EDTA, 0.5 mM $NaHPO_4$, pH 7.2, and 7% sodium dodecyl sulfate (SDS) at 65°C for 16 hr. The blots were washed twice in 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, and 5% SDS for 15 min each at 65°C and followed by 8 changes of 10 min each in 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, and 1% SDS at 65°C (Church and Gilbert, 1984). Filters were exposed to Kodak XAR-5 films for 24 hr at −70°C.

For Southern blot analysis, chicken genomic DNA was isolated from several newly hatched chicken brains (Blin and Stafford, 1976) and digested (10 μg) with Eco RI, Eco RV, Hind III, Sac I, and Xba I. Corresponding digestions of λDNA into the predicted fragments served as controls. Digested DNA was electrophoresed on 0.7% agarose gels, blotted to nitrocellulose membranes (Nitro-Pure, Micron Separations, Inc., Westboro, MA), and hybridized with a ^{32}P-labeled Taq I/Nsi I fragment (nt 237–399, Fig. 1; nt 242–404, Ranscht and Dours-Zimmermann, 1991) which is comprised in a single exon of a T-cadherin genomic DNA clone (Sacristán and Ranscht, unpublished data). This fragment was excised from Tcad-2 cDNA. Hybridization conditions were as described above.

pcDNA1 Cloning and Transfection of Tcad-2 Into CHO Cells

The coding region of Tcad-2 cDNA was obtained by partial digestion of the λ Tcad-2 cDNA with Eco R1 (0.0625 U/μg DNA for 30 min) and cloned into Eco RI-digested pBluescript KS^+. A clone with the 3′ end toward the T3 promoter was selected. The 2.7 kb cDNA insert was excised with Not I/Xho I and directionally cloned into Not I/Xho I-digested pcDNA1 (Invitrogen, La Jolla, CA). For the isolation of cell lines expressing Tcad-2, the pcDNA/Tcad-2 plasmid was cotransfected with pSV2 neo into CHO-DG44 cells as described previously (Vestal and Ranscht, 1992). G418 resistant colonies were isolated and characterized by indirect immunofluorescence with rabbit polyclonal anti-T-cadherin antiserum (Ranscht and Dours-Zimmermann, 1991). Control CHO cells contained the pcDNA vector without an insert and were selected as described (Vestal and Ranscht, 1992). Control CHO cells do not express T-cadherin, as determined by indirect immunofluorescence and Western blot analysis.

Phospholipase Digestion of Tcad-2-Transfected CHO Cells

Phospholipase digestions and [^{3}H]-ethanolamine labeling of transfected and control CHO cells were carried out as described in detail by Vestal and Ranscht (1992).

Aggregation Assays

Cells were pretreated as described (Vestal and Ranscht, 1992) and incubated at a density of 1×10^5 cells/0.5 ml in Hepes-buffered HBSS (HHBSS) containing 1 mM $CaCl_2$ and 1 mg/ml BSA in Linbro uncoated 24 well dishes at 37°C with rotation at 90 rpm for 30 min. For mixing experiments, control cells were labeled by incubation for 30 min at 37°C with 10 pM carboxyfluorescein diacetate succinimyl ester (CFSE; Molecular Probes, Inc., Junction City, OR) and incubated at a density of $1–2 \times 10^5$ cells per cell type in a total of 0.5 ml HHBSS, 1 mg/ml BSA, 1 mM $CaCl_2$, as above. Photographs of living cells were taken on a Zeiss Axiovert 405 M microscope with a heated (37°C) stage.

RNase Protection Assays

T-cadherin and Tcad-2 RNA transcripts were detected by RNase protection (Melton et al., 1984). Three RNA probes corresponding either to a part of the common T-cadherin/Tcad-2 prepeptide region or either of the two specific 3′ untranslated regions of T-cadherin and Tcad-2 were generated in vitro by transcribing the appropriate cDNA template in presence of T7 RNA polymerase and ^{32}P-α-rUTP. The prepeptide probe was generated from a 267 bp Eco RI restriction fragment cloned into pBluescript KS$^+$ and linearized by digestion with Hind III in the polylinker region. The template for the 3′ T-cadherin probe was obtained by removing 1.5 kb of untranslated sequence from the extreme 3′ end of T-cadherin cDNA with Stu I/Sma I restriction digestions and religation of the blunt ends. A template of 168 bp was generated after linearizing the shortened T-cadherin cDNA with Sfa NI. The 3′ Tcad-2 probe was synthesized from the 3′ 298 bp EcoRI restriction fragment of Tcad-2 cDNA cloned into pBluescript KS$^+$. The DNA was linearized by digestion with Acc I in the polylinker region. Chicken β-actin cDNA (Cleveland et al., 1980; Kost et al., 1983) was used as control. A Kpn I/Hind III β-actin cDNA restriction fragment was cloned into the SP72 vector (Melton et al., 1984) and linearized by digestion with Pvu II. For RNase protection assays with RNA from developing brain, a shorter β-actin probe (124 bp) was used. This probe was generated by linearizing the Kpn I/Hind III β-actin restriction fragment with Hae III restriction endonuclease. N-cadherin RNA transcripts were detected with RNA probes generated from regions of the N-cadherin cDNA that either encode the extracellular domain EC1 *and* part of the prepeptide, or part of the prepeptide alone (Hatta et al., 1988). The cDNA for chicken N-cadherin was obtained from Dr. C. Kintner, Salk Institute, La Jolla, CA (personal communication). The former template for transcription was constructed by cloning a 1.3 kb Eco RI N-cadherin cDNA restriction fragment into pBluescript KS$^+$. A 370 bp template DNA was linearized by digestion with Nco I and then transcribed with T3 RNA polymerase in the presence of ^{32}P-α-rUTP. The latter template was generated from a ~500 bp Eco RI/Hind III restriction fragment cloned into the SP72 vector. The template was linearized by digestion with Bam HI and transcribed using SP6 RNA polymerase in the presence of ^{32}P-α-rUTP. The antisense RNA probes were purified on polyacrylamide gels. Ten micrograms of total RNA was hybridized for 16–18 hr with a 2% aliquot of the total probe in hybridization buffer (80% formamide, 400 mM NaCl, 4 mM PIPES, and 1 mM EDTA) at 45°C. Single-stranded sequences were digested by RNases A and T1. Protected RNA fragments were resolved on polyacrylamide gels and then exposed to Kodak XAR-5 film with intensifying screens at −70°C. For RNase protection analysis during neural development, hybridization with the Tcad-2 and T-cadherin-specific probes was carried out simultaneously. An LKB Ultrascan XL laser densitometer (Alameda, CA) was used for scanning the films.

Cell Culture and Immunofluorescence

For cultures of sympathetic neurons, sympathetic ganglia were collected from 10-day-old chicken embryos, dissociated into single cells after trypsin treatment, and cultured on laminin-coated tissue culture dishes or glass coverslips covered with the extracellular matrix from bovine corneal endothelial cells essentially as described (Ranscht et al., 1984). Leibovitz's L-15 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin (100 U/ml), 2 mM glutamine, 0.6 g/l glucose, and nerve growth factor (NGF) was used as culture medium.

For double staining with antibodies against T- and N-cadherin, living cells were incubated for 30 min with L-15 medium containing 10% heat-inactivated goat serum, 2 mM $CaCl_2$, and 0.02% sodium azide. The cells were then incubated for 45 min with either FA-5 ascites (obtained from Sigma, St. Louis, MO; Volk and Geiger, 1984) diluted 1:80 in the above L-15 medium or NCD-2 ascites (1:80) (cell line kindly provided by Dr. M. Takeichi; Hatta et al., 1985), washed with phosphate-buffered saline (PBS) containing 0.5 mM $MgCl_2$ and 0.6 mM $CaCl_2$ and incubated for 45 min with either rhodamine-conjugated goat F(ab′)$_2$ anti-mouse IgG (1:50) (Cappel, West Chester, PA) adsorbed against rabbit immunoglob-

ulin or rhodamine-conjugated goat $F(ab')_2$ anti-rat IgG (1:80) (Cappel), respectively. The coverslips were again washed in PBS and incubated with anti-T-cadherin antiserum (1:80) (Ranscht and Dours-Zimmermann, 1991) for 45 min. T-cadherin labeling was detected after washes by incubation with fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-rabbit IgG (1:50) (Tago Immunologicals, Burlingame, CA) adsorbed against mouse immunoglobulin. Cells were then fixed by incubation with cold acetic ethanol (ethanol:acetic acid, 95:5) at $-20°C$ for 15 min and mounted, after washing, with 90% glycerol in PBS containing 1 mg/ml p-phenylenediamine (Adams and Pringel, 1984). For controls, preimmune rabbit serum or ascites fluid from myeloma cells was used instead of the specific primary antibodies, and the inappropriate fluorochrome-conjugated secondary antibodies were applied after binding of the specific primary antibodies. In none of the controls were immunofluorescence signals detected.

RESULTS

Molecular Cloning and Sequence Analysis of Tcad-2

Tcad-2 represented 1 of 15 cross-hybridizing T-cadherin cDNA clones isolated from a λgt.11 cDNA expression library representing transcripts of 13-day-old chick embryo brain (Ranscht and Dours-Zimmermann, 1991). In Figure 1, the nucleotide and protein sequence for Tcad-2 is shown in comparison with T-cadherin. The cDNA for Tcad-2 consists of 2779 nucleotides and contains an uninterrupted 2151 bp open reading frame (ORF) for a protein of 717 amino acids. The ORF starts with a methionine codon at nucleotide 40 and terminates at position 2190. At the 3′ end, the cDNA contains a 589 bp untranslated sequence that terminates with a poly(A) tail of which the three first residues are shown in Figure 1 preceding the Eco RI linker. T-cadherin and Tcad-2 cDNA sequences are identical over the first 2165 residues with the exception of four nucleotides at positions 730, 978, 1671, and 1848 of Tcad-2, which, however, does not alter the corresponding amino acid (Fig. 1). The cDNAs for Tcad-2 and T-cadherin diverge at nucleotides 2173 and 2178, respectively, and differ in their 3′ end regions (Fig. 1).

To eliminate the possibility that Tcad-2 was generated as an artifact during cDNA cloning, we determined by reverse transcriptase-polymerase chain reaction (RT-PCR) if Tcad-2 is represented in the mRNA pool. A 5′ primer common for both T-cadherin and Tcad-2 (primer 1, see Fig. 2) and specific 3′ primers for Tcad-2 (primer 3) and T-cadherin (primer 4) were used in separate reactions. With primer 1 and the Tcad-2-specific primer 3, a DNA fragment with the nucleotide sequence shown in Figure 1 was amplified from reverse transcribed mRNA templates from E13 chick brain, newly hatched muscle and spinal cord, and E8 dorsal root ganglia (data not shown). Similarly, the 3′ end region of T-cadherin cDNA (Ranscht and Dours-Zimmermann, 1991) was amplified from mRNA of these tissues with primers 1 and 4 (not shown). PCR amplification of DNA representing Tcad-2 and T-cadherin cDNAs demonstrates that mRNAs for both forms are contained in various tissues.

Tcad-2 Lacks the Conserved Cadherin Cytoplasmic Region

The ORF of the Tcad-2 cDNA predicts a protein consisting of a signal peptide, a precursor peptide, and the five extracellular domains characteristic of cadherin cell adhesion molecules. The amino acid sequence of these regions is identical to that of T-cadherin (Ranscht and Dours-Zimmermann, 1991). At the carboxy terminus, the ORF for Tcad-2 differs from that of T-cadherin in that the carboxy terminal Leu of T-cadherin is changed into Lys and extended by the amino acids SerPheProTyrVal (Fig. 1). Thus, like T-cadherin, Tcad-2 does not encode the cytoplasmic region conserved in classical cadherins.

To possibly identify a T-cadherin form containing the typical cadherin cytoplasmic sequences, RT-PCR was performed with the T-cadherin/Tcad-2 5′ primer 1 and two degenerate primers representing either one of the most conserved amino acid clusters in the cytoplasmic region of classical cadherins, YGGGEDD and EEGGGEED (primers 5 and 6, respectively, see Fig. 2 and Materials and Methods). At the primer-specific annealing temperatures, no DNA fragments were amplified from E13 chick brain or hatched muscle mRNAs. Decreasing the renaturing temperature to 45°C, no specific DNA products were generated, as judged by cloning and sequence analysis. Variation of either the cDNA template amount, the time for renaturing and extension

Fig. 1. The complete nucleotide sequence of Tcad-2 DNA is shown in comparison with that of T-cadherin (Ranscht and Dours-Zimmermann, 1991) up to nucleotide 2172. The nucleotide sequence of Tcad-2 diverges from that of T-cadherin at nucleotide 2173 to encode a protein in which the carboxy terminal T-cadherin Leu is changed into a Lys and the protein sequence extended by the amino acids SerPheProTyrVal (boxed). Identical nucleotide residues are indicated with a dot; nucleotide differences are boxed, the position of the stop codon is marked with an asterisk. Hydrophobic domains at both the amino and carboxy termini are underlined; double-underlined residues correspond to the amino terminal amino acid sequence determined by microsequencing of the mature T-cadherin polypeptide. Triangles indicate potential glycosylation sites.

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    1 GAATTCCAAAAAGCCTCTGGTACGTTCTAGTCTGGCAAAATGCAGCACAAAACTCAACTTACTCTGTCCTTTCTGCTGTCCCAGGTTCTG
  -22                                          M  Q  H  K  T  Q  L  T  L  S  F  L  L  S  Q  V  L

   91 TTGCTTGCGTGTGCAGAAGATTTAGAATGCACCCCTGGATTCCAGCAAAAGGTTTTTATATTGAACAGCCATTTGAATTCACAGAGGAC
   -5 L  L  A  C  A  E  D  L  E  C  T  P  G  F  Q  Q  K  V  F  Y  I  E  Q  P  F  E  F  T  E  D

  181 CAGCCAATTCTGAACCTGGTGTTTGATGACTGCAAGGGGAATAACAAATTGAACTTCGAAGTTTCTAACCCAGACTTTAAGGTGGAACAC
   26 Q  P  I  L  N  L  V  F  D  D  C  K  G  N  N  K  L  N  F  E  V  S  N  P  D  F  K  V  E  H

  271 GATGGATCTTTAGTTGCACTGAAGAATGTATCAGAAGCTGGCAGAGCTTTGTTTGTCCATGCACGGTCTGAGCATGCTGAGGATATGGCA
   56 D  G  S  L  V  A  L  K  N  V  S  E  A  G  R  A  L  F  V  H  A  R  S  E  H  A  E  D  M  A

  361 GAAATTTTGATTGTTGGAGCTGATGAGAAGCACGATGCATTAAAGGAAATCTTTAAGATAGAAGGCAACCTTGGAATTCCAAGACAAAAA
   86 E  I  L  I  V  G  A  D  E  K  H  D  A  L  K  E  I  F  K  I  E  G  N  L  G  I  P  R  Q  K

  451 AGGGCTATTCTGGCGACTCCAATATTAATTCCAGAAAATCAAAGACCACCATTTCCCAGATCAGTTGGCAAGGTCATCAGGAGTGAAGGG
  116 R  A  I  L  A  T  P  I  L  I  P  E  N  Q  R  P  P  F  P  R  S  V  G  K  V  I  R  S  E  G

  541 ACAGAGGGAGCAAAGTTCCGACTCTCTGGTAAGGGAGTAGATCAAGACCCGAAAGGAATTTTTAGAATCAATGAGATCAGTGGGGATGTC
  146 T  E  G  A  K  F  R  L  S  G  K  G  V  D  Q  D  P  K  G  I  F  R  I  N  E  I  S  G  D  V

  631 TCTGTGACCCGACCCCTGGATAGAGAAGCAATAGCCAATTATGAGCTGGAAGTTGAAGTAACGGATTTAAGTGGGAAAATCATTGATGGC
  176 S  V  T  R  P  L  D  R  E  A  I  A  N  Y  E  L  E  V  E  V  T  D  L  S  G  K  I  I  D  G
               T
  721 CCAGTCCGCCTAGATATTTCTGTTATTGATCAAAATGATAACAGGCCGATGTTCAAAGAAGGACCCTATGTTGGTCACGTCATGGAAGGA
  206 P  V  R  L  D  I  S  V  I  D  Q  N  D  N  R  P  M  F  K  E  G  P  Y  V  G  H  V  M  E  G

  811 TCCCCTACAGGAACAACTGTGATGCGGATGACAGCATTTGATGCTGATGATCCTAGCACAGACAACGCTCTTCTGCGGTATAACATCCTC
  236 S  P  T  G  T  T  V  M  R  M  T  A  F  D  A  D  D  P  S  T  D  N  A  L  L  R  Y  N  I  L
                                                                                   A
  901 AAGCAGACACCTACCAAACCTTCCCCAAATATGTTCTACATTGACCCAGAAAAGGGAGATATTGTCACAGTGGTGTCGCCTGTACTGCTG
  266 K  Q  T  P  T  K  P  S  P  N  M  F  Y  I  D  P  E  K  G  D  I  V  T  V  V  S  P  V  L  L

  991 GATCGTGAGACAATGGAAACGCCGAAGTACGAGCTGGTTATTGAAGCCAAGGATATGGGCGGCCATGATGTGGGACTTACTGGAACTGCA
  296 D  R  E  T  M  E  T  P  K  Y  E  L  V  I  E  A  K  D  M  G  G  H  D  V  G  L  T  G  T  A

 1081 ACTGCCACTATTCTTATTGATGACAAAAACGACCACCCACCAGAATTTACCAAGAAGGAGTTTCAGGCCACAGTAAAGGAAGGAGTCACA
  326 T  A  T  I  L  I  D  D  K  N  D  H  P  P  E  F  T  K  K  E  F  Q  A  T  V  K  E  G  V  T

 1171 GGAGTAATAGTAAACTTAACTGTTGGTGACCGAGATGACCCAGCAACTGGAGCATGGAGAGCTGTCTACACTATTATTAACGGAAATCCA
  356 G  V  I  V  N  L  T  V  G  D  R  D  D  P  A  T  G  A  W  R  A  V  Y  T  I  I  N  G  N  P

 1261 GGGCAGAGTTTTGAAATCCATACCAATCCCCAGACTAATGAGGGAATGCTCTCTGTTGTCAAACCTTTAGACTATGAGATTTCAGCATTT
  386 G  Q  S  F  E  I  H  T  N  P  Q  T  N  E  G  M  L  S  V  V  K  P  L  D  Y  E  I  S  A  F

 1351 CACACATTGCTGATAAAAGTAGAAAATGAAGACCCGTTGATTCCAGACATAGCCTACGGTCCCAGTTCCACAGCAACAGTTCAGATCACC
  416 H  T  L  L  I  K  V  E  N  E  D  P  L  I  P  D  I  A  Y  G  P  S  S  T  A  T  V  Q  I  T

 1441 GTTGAGGATGTGAATGAAGGCCCTGTTTTCCACCCAAACCCAATGACAGTGACAAAACAAGAGAACATCCCTATTGGCAGCATTGTGTTA
  446 V  E  D  V  N  E  G  P  V  F  H  P  N  P  M  T  V  T  K  Q  E  N  I  P  I  G  S  I  V  L

 1531 ACAGTAAATGCCACTGATCCAGATACTTTGCAACATCAGACTATCAGGTATTCAGTTTACAAGGATCCAGCAAGCTGGCTAGAGATTAAT
  476 T  V  N  A  T  D  P  D  T  L  Q  H  Q  T  I  R  Y  S  V  Y  K  D  P  A  S  W  L  E  I  N
                                                        T
 1621 CCCACCAATGGTACCGTTGCCACCACTGCTGTCCTGGATCGGGAATCTCCGCATGTTCAGGATAACAAATACACTGCTCTCTTCCTGGCA
  506 P  T  N  G  T  V  A  T  T  A  V  L  D  R  E  S  P  H  V  Q  D  N  K  Y  T  A  L  F  L  A

 1711 ATAGACAGTGGTAACCCTCCTGCTACAGGTACAGGAACTTTACACATCACCTTGGAGGACGTCAATGACAATGTCCCCTCCCTTTACCCA
  536 I  D  S  G  N  P  P  A  T  G  T  G  T  L  H  I  T  L  E  D  V  N  D  N  V  P  S  L  Y  P
                                                     A
 1801 ACACTGGCAAAAGTCTGTGATGATGCTAAAGATCTCAGAGTAGTGGTTCTAGGAGCATCAGACAAAGACCTCCATCCCAACACAGATCCA
  566 T  L  A  K  V  C  D  D  A  K  D  L  R  V  V  V  L  G  A  S  D  K  D  L  H  P  N  T  D  P

 1891 TTTAAATTTGAACTGAGTAAGCAATCTGGTCCAGAAAAGTTATGGAGAATCAACAAGCTTAACAATACTCATGCCCAGGTTGTCCTGCTT
  596 F  K  F  E  L  S  K  Q  S  G  P  E  K  L  W  R  I  N  K  L  N  N  T  H  A  Q  V  V  L  L

 1981 CAAAACCTGAAAAAGGCCAATTACAACATCCCAATCTCAGTGACAGATTCTGGAAAACCACCTCTGACTAACAACACAGAACTGAAATTA
  626 Q  N  L  K  K  A  N  Y  N  I  P  I  S  V  T  D  S  G  K  P  P  L  T  N  N  T  E  L  K  L

 2071 CAAGTGTGTTCCTGCAAGAAATCCAGAATGGACTGCAGTGCAAGTGATGCCCTTCATATCAGCATGACTCTTATCCTTCTTTCACTCTTC
  656 Q  V  C  S  C  K  K  S  R  M  D  C  S  A  S  D  A  L  H  I  S  M  T  L  I  L  L  S  L  F

 2161 AGTTTATTTTGTAAGTCTTTTCCTTATGTGTAAGCATTGAACGTTATTTATCTGCTTGCTTTTGCACTATAAGAAACCTTACCAAGAGAG
  686 S  L  F  C  K  S  F  P  Y  V  *
                  L  *
 2251 AAGTTAACTTTATTTTTTCCCTGCGGTAGATGCTATACAGAAGTAGGAGGGGAGGGATTTTTCACAGTCAAAAAATAGCAACAAATGCCG
 2341 GGTTGTCAAATTAAGAAATAGAAGCAATAATTCTAGGAAGAATCAAAGAGAATTAAAGCTAGCATATGATAAACTAAGAAGTACCAGCTG
 2431 TAGTAACAGATTTCTGAGATGCTTTCTTTCATCTCTCCCCACTTGAATTCAATTCAAAAGCAGAAACTGAAGATTAAAAGGTGTTCTTGT
 2521 AACAATAACTGTTCTGGGTCACCATGAAAATGAGTACTGTCTGCTTCAATCTATTTGTCCGTAAAGTGCGCGAGCAATTGGAACATAAGG
 2611 AACTTACTGAAGATTCTGGGTTTAGAGACACTCAAACTGATAACCAGAATAGCAGGTCTGTGTTGAGGGAGAGAGAACTGATGCATAAAG
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Fig. 1.

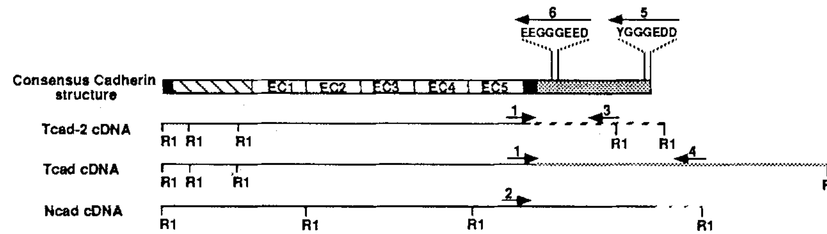

Fig. 2. Oligonucleotide primers used for PCR analysis. Tcad-2, T-cadherin (Tcad), and N-cadherin (Ncad) cDNAs are aligned to the cadherin consensus structure. Untranslated regions of the cDNAs are indicated by different dotted lines. The primers (numbered arrows) are indicated above the corresponding cDNAs and the cadherin consensus structure and extend into the direction of the arrow.

steps, or the number of cycles produced no specific DNA fragments from either brain or muscle mRNAs. In contrast, the corresponding DNA fragments for N-cadherin were amplified in control reactions using a 5′ oligonucleotide specific for N-cadherin (nt 2051–2071, Hatta et al., 1988, primer 2) and either 3′ primers 5 or 6. Moreover, no DNA fragments other than those containing the nucleotide sequences of the previously identified T-cadherin cDNAs were amplified using the T-cadherin/Tcad-2 5′ primer 1 and poly(dT) under different reaction conditions. Thus, using these techniques, no additional forms of T-cadherin were detected.

T-Cadherin Isoforms Are Derived From Different mRNAs

The size and multiplicity of mRNAs for T-cadherin and Tcad-2 were analyzed on Northern blots of enriched poly(A)$^+$ RNA from E13 chick brain with three different probes labeled by random priming: Tcad and Tcad-2 probes corresponded to parts of the specific 3′ untranslated regions of T-cadherin and Tcad-2 cDNAs, respectively, and the Tcad/Tcad-2 probe represented part of the prepeptide region common to both Tcad-2 and T-cadherin. With all probes, several mRNA species were detected (Fig. 3A). In agreement with previous results (Ranscht and Dours-Zimmermann, 1991), the T-cadherin-specific probe hybridized to a major band of ~7.5 kb and three less prominent ones of ~10, ~4.6, and ~3.5 kb. With the Tcad-2-specific probe, a different set of mRNAs was detected consisting of a broad mRNA band of ~4.9 kb and three smaller ones of ~3.3, ~2.8, and ~2.3 kb. When the common Tcad/Tcad-2 probe was used, the sum of all the bands detected with the Tcad and Tcad-2 probes was hybridized. No signal was detected in the corresponding poly(A)$^-$ RNA with any of the probes. The relationship between the hybridized mRNAs and the isolated Tcad-2 and T-cadherin cDNAs is not evident. The existence of multiple mRNA species for T-cadherin and Tcad-2 may indicate the presence of additional T-cadherin variants generated by alternative splicing of one or several mRNA precursors or the existence of multiple, T-cadherin-related genes.

To distinguish between these possibilities, chick genomic DNA was probed in Southern blots with a T-cadherin/Tcad-2 probe. Genomic DNA was digested with the restriction enzymes Eco RI, Eco RV, Hind III, Sac I, and Xba I and hybridized with a 162 bp Taq I/Nsi I fragment common for the two T-cadherin cDNAs (see Materials and Methods). This fragment was chosen for hybridization because its nucleotide sequence is contained within a single exon of a T-cadherin genomic DNA clone and it does not include the restriction sites selected for genomic DNA digestions (Sacristán and Ranscht, unpublished data). Single DNA fragments were hybridized with Hind III, Sac I, and Xba I-digested genomic DNA, while in Eco RI and Eco RV digestions double bands were observed (Fig. 3B). Because only single bands were hybridized in several restriction digests of the genomic DNA, T-cadherin and Tcad-2 are likely to be derived from a single gene. The hybridization of two bands in the Eco RI and Eco RV digests can most likely be attributed to polymorphism, although the existence of more than one T-cadherin gene cannot be excluded.

Membrane Attachment and Function of Tcad-2

The cDNA for Tcad-2 encodes a protein that contains at its carboxy terminus a hydrophobic domain followed by Lys and the amino acid sequence SerPheProTyrVal. This motif raises the possibility that Tcad-2 is a transmembrane protein with a short cytoplasmic tail (Sabatini et al., 1982). Alternatively, it is conceivable that Tcad-2, like T-cadherin, is also a GPI-anchored membrane protein. To distinguish between these possibilities, Tcad-2 was expressed in CHO cells and characterized biochemically. Three proteins of 95, 105, and 110 kD were generated from Tcad-2 cDNA. Cadherins are initially synthesized as precursors from which the prepeptide is proteolytically cleaved to produce the mature protein (Takeichi, 1988, 1991). Amino terminal sequencing of the 95 kD protein from brain had revealed that it is

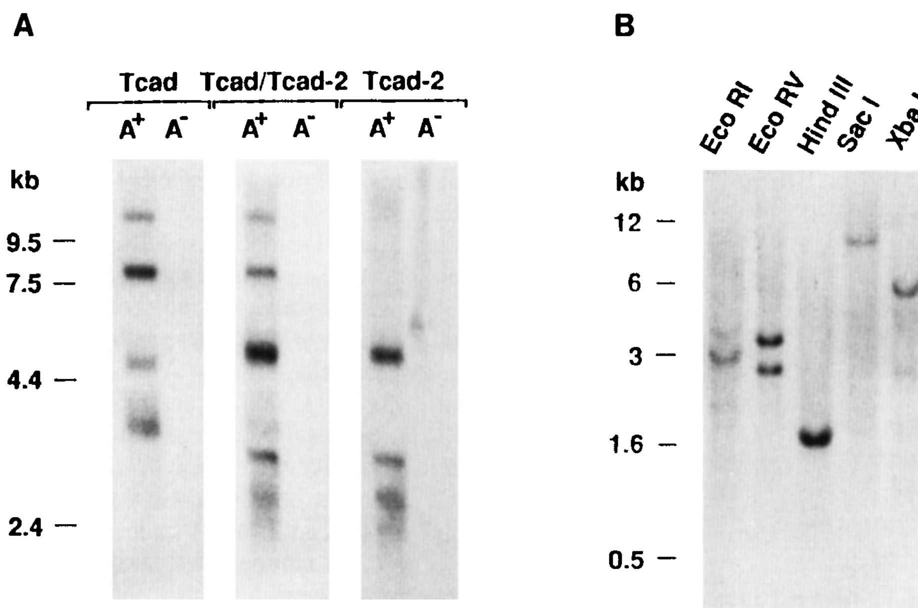

Fig. 3. Northern and Southern blot analyses. **A:** T-cadherin (Tcad) and Tcad-2-specific probes hybridize to different mRNA species. Enriched poly(A)$^+$ and poly(A)$^-$ RNAs (10 μg) extracted from E13 chicken embryo brain were consecutively probed with three different ^{32}P-labeled DNA fragments: Tcad, a Pst I fragment covering nt 2263–2831 which are unique to T-cadherin (Ranscht and Dours-Zimmermann, 1991); Tcad-2, an Hpa I/Eco RI fragment covering nt 2256–2774 specific of Tcad-2 cDNA; and Tcad/Tcad-2, an Eco RI fragment representing a region of the prepeptide (nt 167–434) common to both Tcad-2 and T-cadherin cDNAs. Following hybridization with one of the cDNA fragments, the blot was washed in 2 mM Tris/EDTA, pH 8.2, 0.1% SDS for 20 min at 65°C and exposed to XAR-5 film for 36 hr to check the background before reprobing with the next probe. **B:** Genomic DNA was isolated from newly hatched chicken brains, digested with the indicated restriction enzymes, and resolved on a 0.7% agarose gel. The gel was transferred to nitrocellulose and probed with a 162 bp DNA fragment corresponding to nt 237–399 in Figure 1, which are contained within a single exon of a T-cadherin genomic DNA clone. Single bands are hybridized in Hind III, Sac I, and Xba I digests, while in Eco RI and Eco RV digests two DNA fragments are detected.

mature T-cadherin (Ranscht and Dours-Zimmermann, 1991). To test if either or both the 105 kD and 110 kD proteins are forms of the Tcad-2 precursor, the transfected cells were probed with an anti-prepeptide antiserum (Vestal and Ranscht, 1992). Both the 105 and 110 kD proteins, but not mature 95 kD Tcad-2, reacted with this antiserum (data not shown). The differences between the two Tcad-2 precursor forms are unclear. Precursor and mature Tcad-2 proteins were detected on the surface of transfected cells by immunofluorescence with anti-T-cadherin-specific antisera (data not shown; for comparison see Vestal and Ranscht, 1992). Tcad-2 was uniformly distributed on the cell surface, and, in contrast to classical cadherins, no convincing relocalization of the heterologously expressed protein was observed at the sites of cell-cell contact in monolayer cultures.

To examine the membrane attachment of Tcad-2, transfected CHO cells were treated with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves GPI anchors. This treatment released a large portion of 95 kD mature Tcad-2 and the 110 kD precursor from the cell surface (Fig. 4). The 105 kD precursor was insusceptible to PI-PLC treatment, either because it is intracellular or the cleavage site is inaccessible. Further evidence for the GPI anchor of Tcad-2 was obtained by metabolic labeling of the GPI-anchored proteins in transfected cells with [^{3}H]-ethanolamine, subsequent PI-PLC treatment, and immunoprecipitation of labeled Tcad-2 with the anti-95 kD T-cadherin antiserum (data not shown). These results demonstrate the membrane attachment of Tcad-2 through a GPI moiety.

One important issue is the function of Tcad-2 as a cell adhesion molecule. To test this function, Tcad-2-transfected cells were assayed for their ability to aggregate in three dimensions. After the induction with 1 mM calcium, Tcad-2-expressing cells formed large aggregates over controls. Aggregate formation was not observed in the absence of calcium. The anti-T-cadherin antiserum (Ranscht and Dours-Zimmermann, 1991) was ineffective in blocking aggregation, possibly because it does not recognize the binding site of Tcad-2 in its native configuration.

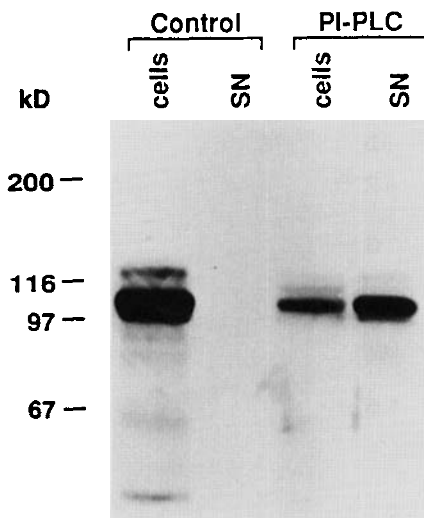

Fig. 4. Release of Tcad-2 from the surface of transfected CHO cells with PI-PLC. Tcad-2-transfected cells were treated with PI-PLC, and cell lysates (cells) and culture medium (SN) were analyzed by Western blotting. Three proteins of 95, 105, and 110 kD were generated from Tcad-2 cDNA. In the cell lysates of untreated cells shown in the figure, the 95 and 105 kD proteins are not resolved into single bands (left lane, cells). Upon PI-PLC treatment, both the 95 kD Tcad-2 mature protein and the 110 kD precursor are released into the culture medium (right lane, SN), while the 105 kD precursor form remains cell-associated. The Tcad-2 precursor is highly susceptible to proteolytic degradation (data not shown), and thus some of the precursor may be lost during PI-PLC treatment. In controls (no enzyme), mature and precursor Tcad-2 are detected only in cell lysates (left lane, cells).

To examine if Tcad-2-expressing cells confer homophilic recognition, the transfected cells were mixed with CFSE-labeled control CHO cells and assayed for aggregation. Analysis of the resulting aggregates revealed that they are composed exclusively of Tcad-2-transfected cells, while the labeled control cells remain single (Fig. 5A,B). Thus, Tcad-2 induces calcium-dependent, homophilic cell adhesion. To determine the localization of Tcad-2 within the aggregates, cells were fixed with 3% formaldehyde after 10 min of aggregation and stained in suspension with anti-T-cadherin antiserum followed by FITC-labeled goat anti-rabbit immunoglobulin. In contrast to the uniform distribution of Tcad-2 in substrate-attached transfected cells (not shown), Tcad-2 was clustered at sites of cell-cell contact in three-dimensional aggregates (Fig. 5C,D). This concentration at contact sites, however, was not exclusive and a small portion of Tcad-2 remained present on the free surfaces of the aggregates.

Expression of T-Cadherin Isoforms in Neural and Non-Neural Tissues

The identification of a T-cadherin isoform raises the possibility that Tcad-2 and T-cadherin are differentially expressed in specific tissues or during development and thus function in a distinct cellular context. To address this issue, we have analyzed the distribution of Tcad-2 and T-cadherin-specific mRNAs by RNase protection. Antisense RNA was transcribed from parts of the respective T-cadherin and Tcad-2-specific 3′ untranslated regions of the corresponding cDNAs (probes 2 and 3, respectively, Fig. 6). A probe representing the common prepeptide region for Tcad-2 and T-cadherin (probe 1, Fig. 6) was used as a control. The amounts of mRNA in each sample were normalized against chicken β-actin (Cleveland et al., 1980; Kost et al., 1983). The distribution of Tcad-2 and T-cadherin mRNAs is shown in Figure 6. In non-neural tissues, Tcad-2 and T-cadherin transcripts were protected in E15 heart and muscle, newly hatched liver (Fig. 6), skin and dorsal aorta (data not shown). Possibly the same is true for kidney from hatched animals, although mRNA protected with the Tcad-2 probe cannot be clearly distinguished (Fig. 6). In all tissues, the mRNA for T-cadherin was significantly more abundant than Tcad-2 mRNA.

In neural tissues, Tcad-2 and T-cadherin mRNAs were protected in samples prepared from E13 brain, newly hatched and stage 24 spinal cord, newly hatched retina (Fig. 6), forebrain, optic lobe, cerebellum, and sciatic nerve (data not shown). Moreover, RNA for both isoforms was protected in samples prepared from pure cultures of chick sympathetic neurons (Fig. 6).

The expression of T-cadherin in the caudal halves of each somitic sclerotome correlates with the immigration of neural crest cells and motor axons into the corresponding rostral regions (Ranscht and Bronner-Fraser, 1991). To determine if one or both T-cadherin isoforms are expressed in somites, RNA from the somites of stage 24 chick embryos was examined and found to be protected by both the T-cadherin and the Tcad-2-specific probes (Fig. 6). Similarly, in the spinal cord of stage 24 chick embryos, when T-cadherin is strongly expressed in the floor plate (Ranscht and Dours, 1989; Kanekar and Ranscht, in preparation), RNA was protected with the specific probes for both T-cadherin isoforms. Thus, in all samples examined, T-cadherin and Tcad-2 mRNAs were codetected.

T-Cadherin Isoforms Are Coexpressed With N-Cadherin

The overall tissue distribution of the T-cadherin and Tcad-2 isoforms overlaps to a large extent with that reported for N-cadherin (Hatta and Takeichi, 1986). In

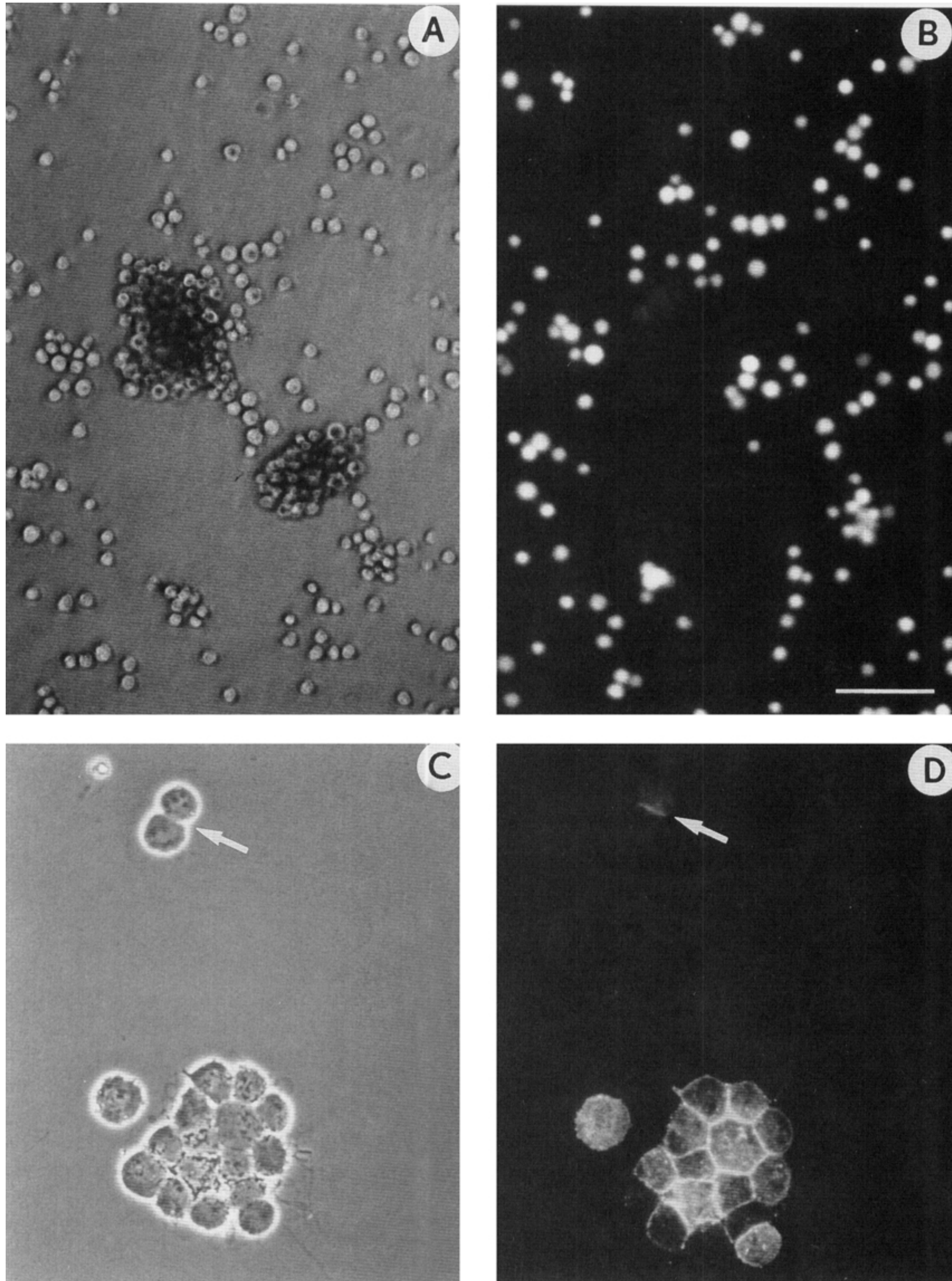

Fig. 5. **A,B:** Homophilic adhesion between Tcad-2-transfected cells. Equal numbers of non-labeled Tcad-2-transfected and CFSE-labeled control cells were mixed and induced to aggregate with 1 mM calcium. Living cells were viewed in suspension, thus the position of individual cells in the microscopic field may have changed during the exposure time. **A:** Phase contrast photomicrograph of the resulting aggregates. **B:** Fluorescence photomicrograph of the same field. The aggregates contain almost exclusively non-labeled Tcad-2-expressing cells. Bar = 50 μm. **C,D:** Tcad-2 is concentrated at sites of cell-cell contact in aggregates. Tcad-2-transfected cells were induced to aggregate and stained by indirect immunofluorescence with anti-T-cadherin antiserum for the localization of Tcad-2. Tcad-2 is concentrated in areas of cell-cell contact between aggregated cells, although this distribution is not exclusive. **C:** Phase contrast photomicrograph. **D:** Fluorescence photomicrograph. Note the cluster of Tcad-2 between two aggregated cells (arrow).

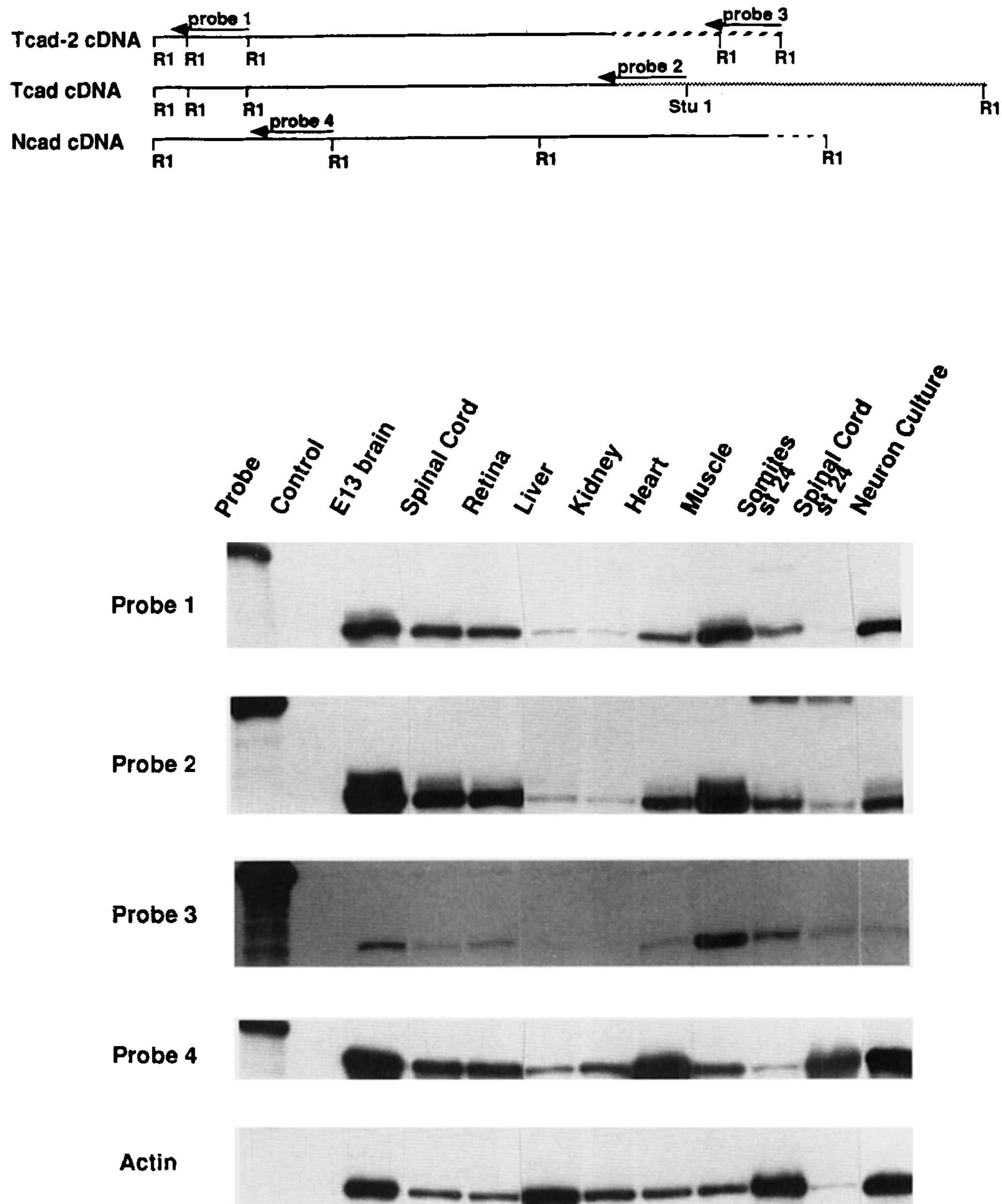

Fig. 6. RNase protection of T-cadherin, Tcad-2, and N-cadherin mRNAs. RNA from various tissues was assayed for the presence of T-cadherin, Tcad-2, and N-cadherin transcripts. Samples were hybridized separately with the five RNA probes indicated in the scale drawing. The micrograms of total RNA was used, except with the actin probe where only 5 μg was hybridized. Control samples lack the RNA. The position of the undigested probes is shown in the left lanes. Film exposure times for probes 2 and 3 were identical. Longer exposure times were used for probes 2 and 3 than for probes 1 and 4 to reveal the faint band protected in liver with the Tcad-2-specific probe.

order to investigate the possibility that T-cadherin isoforms and N-cadherin are coexpressed within the same tissues, the samples above were assayed, in parallel, for the expression of N-cadherin transcripts. For this, N-cadherin probes corresponding to either the extracellular domain EC1 and part of the prepeptide (Fig. 6, probe 4) or a region of the prepeptide (see Materials and Methods; data not shown) were used. With both probes identical results were obtained. In non-neural tissues, mRNAs for both T-cadherin isoforms and N-cadherin were detected in heart, muscle, and liver (Fig. 6). The detection of N-cadherin by RNase protection in liver can possibly be

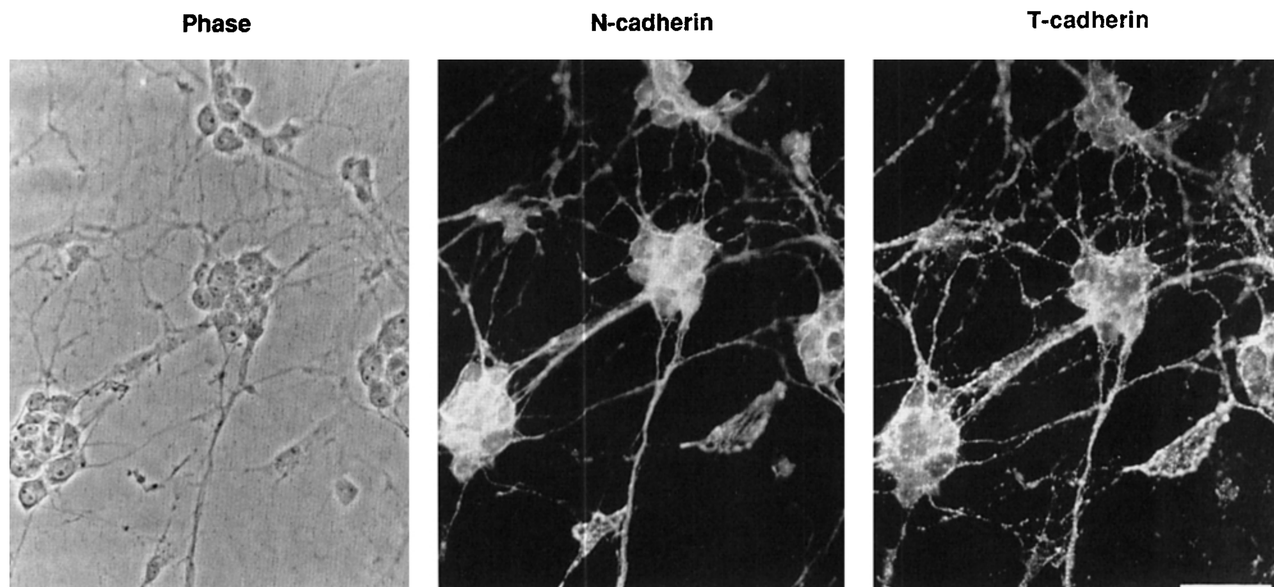

Fig. 7. Coexpression of T- and N-cadherin proteins on the surface of sympathetic neurons. Sympathetic neurons were analyzed for expression of T- and N-cadherins by indirect immunofluorescence with anti-T-cadherin antiserum and NCD-2 monoclonal antibody, respectively. The phase contrast photomicrograph of the same field is shown. T-cadherins and N-cadherin are coexpressed by individual neurons in culture. Bar = 50 μm.

explained by the high sensitivity of this assay in comparison to Northern analysis, which previously did not reveal N-cadherin transcripts in this tissue (Hatta et al., 1988; Dalseg et al., 1990). In neural tissues, N-cadherin mRNA was coexpressed with both T-cadherin isoforms in samples of the spinal cord of hatched chicks and stage 24 embryos, hatched retina, E13 chick embryo brain, stage 24 somites, and cultures of sympathetic neurons (Fig. 6).

To address the question if T-cadherins and N-cadherin are coexpressed by the same cells, pure cultures of chick sympathetic neurons were examined by indirect double immunofluorescence with the anti-N-cadherin NCD-2 monoclonal antibody (Hatta et al., 1985) and anti-T-cadherin antiserum (Ranscht and Dours-Zimmermann, 1991). T-cadherins and N-cadherin were codistributed on the cell bodies and neurites of individual cells (Fig. 7). The same staining pattern for N-cadherin was obtained with the FA-5 monoclonal antibody (Volk and Geiger, 1984; data not shown). Together with the RNase protection assays, these results show that sympathetic neurons in culture coexpress the two T-cadherin isoforms and N-cadherin.

mRNAs for T-Cadherin and Tcad-2 Increase During Brain Development

The coexpression of the T-cadherin isoforms led us to examine the possibility that Tcad-2 and T-cadherin mRNAs are differentially expressed during neural development. Total RNA was extracted from the brains of chick embryos between developmental stages 10 (33–38 hr of development; Hamburger and Hamilton, 1951) and 46 (hatching). The RNA samples were hybridized with antisense probes corresponding to part of the respective 3′ untranslated regions of T-cadherin and Tcad-2 (probes 2 and 3, respectively, Fig. 6). The amount of RNA in each sample was normalized against the corresponding hybridization signal for chicken β-actin. Three independent experiments were conducted with essentially identical results. In Figure 8, one typical experiment is shown. The transcripts for Tcad-2 and T-cadherin are codetected at all stages examined, and, except for stage 24 when the levels for both forms are about equal, the level of T-cadherin mRNA exceeds that of Tcad-2. Laser densitometry of the autoradiographs and normalization to β-actin mRNA confirm the difference in the developmental profile for Tcad-2 and T-cadherin mRNA expression at early stages. The level of T-cadherin mRNA decreases at stage 24 and then rises about 40-fold between stages 28 and 44. In contrast, the levels of Tcad-2 mRNA decline only at stage 28 and then progressively increase about 14-fold up to hatching. The highest levels of both Tcad-2 and T-cadherin are observed in hatched brain where T-cadherin and Tcad-2 are expressed in a ratio of $\geq$15:1. Therefore, Tcad-2 and T-cadherin mRNAs are coexpressed in different ratios throughout neural development.

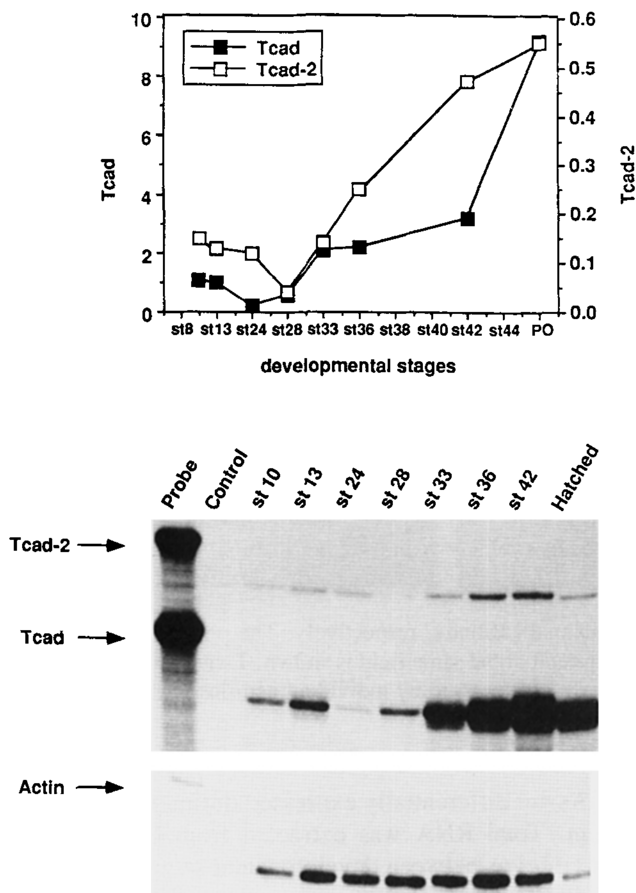

Fig. 8. Expression of T-cadherin and Tcad-2 mRNAs in the developing chick brain. Total RNA isolated from embryonic chick brain at various developmental stages was assayed for the presence of both T-cadherin and Tcad-2 transcripts. Samples were assayed simultaneously with probes 2 and 3 (Fig. 6) and in parallel with the β-actin probe. The position of the undigested probes is indicated by arrows. The top part of the figure shows the quantitation of both T-cadherin and Tcad-2 mRNA levels normalized to β-actin mRNA. The lower part of the figure shows the corresponding RNase protection autoradiographs. mRNAs for both T-cadherin and Tcad-2 isoforms are coexpressed throughout development and levels for both isoforms increase.

DISCUSSION

We report here the molecular characterization and function in cell adhesion of Tcad-2 and its mRNA distribution in comparison with T-cadherin and N-cadherin.

T-cadherin and Tcad-2 are represented by multiple and non-overlapping mRNAs in E13 chick brain. Several explanations are possible for the multiplicity of T-cadherin and Tcad-2 transcripts: the two isoforms are generated by alternative splicing of one or several mRNA precursors, use alternative polyadenylation sites, or are derived from different genes. We cannot distinguish between these possibilities. Experimental support for the hypothesis that the highly conserved T-cadherin and Tcad-2 isoforms are derived from one gene is the hybridization of a single DNA fragment in Southern blots of chick genomic DNA using a probe contained within a single exon (Fig. 3B). However, in addition, double bands were hybridized in two of the genomic DNA restriction digests. Two explanations are possible for this. First, T-cadherin and Tcad-2 could be derived from different, but closely related genes that evolved by gene duplication. Second, and more likely, the hybridization of two restriction DNA fragments could be attributed to polymorphism. The analysis of genomic T-cadherin DNA clones will clarify this issue. It should be noted that multiple mRNAs were also detected by some authors for chick, mouse, human, and bovine N-cadherin (Hatta et al., 1988; Miyatani et al., 1989; Liaw et al., 1990; Walsh et al., 1990; Dalseg et al., 1990) although, thus far, only one N-cadherin cDNA has been isolated.

Using RT-PCR, we have obtained no evidence for additional T-cadherin variants in brain and muscle. In particular, we could rule out the presence of a T-cadherin isoform that contains the cytoplasmic region of classical cadherins. The cadherin family includes the desmosomal glycoproteins desmoglein and desmocollin that share the cadherin structure in their extracellular domain but differ from classical cadherins in their cytoplasmic regions (Koch et al., 1990; Collins et al., 1991). Although we did not use specific primers for the desmoglein or desmocollin cytoplasmic region, the existence of a T-cadherin isoform with a cytoplasmic region resembling these proteins is unlikely as no DNA fragments other than the already identified cDNAs were detected by amplification of T-cadherin 3′ end sequences with a 3′ poly(dT) primer.

In the cDNA for Tcad-2 the codon for the carboxy terminal Leu of T-cadherin is changed into Lys and the ORF extended by the amino acids SerPheProTyrVal. As the presence of a Lys in this position could indicate a putative membrane spanning domain (Sabatini et al., 1982), we have expressed Tcad-2 cDNA in CHO cells to characterize the membrane linkage of the encoded protein. Three proteins—95 kD mature Tcad-2 and two precursor forms of 105 and 110 kD—were expressed by CHO cells. The structural differences between the two precursor proteins are unclear; we suspect that the generation of two precursor forms is the result of aberrant protein processing by CHO cells. Both the 95 kD mature Tcad-2 and the 110 kD precursor were susceptible to PI-PLC treatment, indicating their membrane attachment through a GPI moiety. In contrast, the 105 kD precursor form remained cell-associated after PI-PLC treatment, either because it is intracellular or inaccessible to the

enzyme. The consensus amino acid for GPI anchor attachment is either a Ser, Asp, Asn, Cys, Gly, or Ala located 15–23 amino acid residues amino terminal of a hydrophobic domain (Low, 1989; Moran and Caras, 1991). In T-cadherin and Tcad-2, this site is located amino terminal of the divergence point between the two cDNAs, and thus appears to be used by both T-cadherin and Tcad-2. Therefore, apparently two identical proteins are generated from T-cadherin and Tcad-2 mRNAs and expressed on the cell surface, although differences in their post-translational processing and GPI-anchor composition cannot be excluded.

Classical cadherins are clustered at cell-cell junctions both in vivo and in vitro (Takeichi, 1988, 1991). In contrast, Tcad-2 (like T-cadherin) was uniformly distributed on the cell surface of transfected CHO cells in monolayer cultures. This localization represents a genuine difference between classical cadherins containing the cytoplasmic region and GPI-anchored Tcad-2, as N-cadherin is concentrated at cell-cell contacts in CHO cells transfected with the corresponding cDNA (Vestal and Ranscht, unpublished observation). In cellular aggregation assays, Tcad-2 induces calcium-dependent aggregation between transfected cells. Tcad-2 shares this principal function with T-cadherin, although subtle differences, possibly due to variations between individual cell lines, were noted in the characteristics of cell adhesion mediated by Tcad-2 and T-cadherin. Interestingly, Tcad-2 was clustered at sites of cell-cell contact when the transfected cells formed three-dimensional aggregates. Homophilic cell adhesion exerted by classical cadherins critically depends on their cytoplasmic region as mutated cadherins lacking this region do not promote cell adhesion (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989). The cadherin cytoplasmic region interacts with submembranous components that are thought to provide a linkage with the cytoskeleton (Nagafuchi et al., 1991; Herrenknecht et al., 1991; McCrea et al., 1991). This interaction is proposed to be responsible for the clustering of cadherin receptors on the cell surface, which, in turn, provides the strength for adhesive interactions. The mechanism by which T-cadherin and Tcad-2 function in cell adhesion without a cytoplasmic tail is not clear, although several explanations are conceivable. One possibility is that T-cadherin-mediated adhesion occurs because the binding affinity between T-cadherin molecules is higher than that between classical cadherins. This is unlikely as a number of T-cadherin-expressing cells do not participate in the formation of aggregates in our assays. An alternative—and more likely—hypothesis is that T-cadherins are associated with auxiliary proteins that either provide an indirect linkage with components of the cytoskeleton or connect the T-cadherin cell adhesion molecules with proteins that transmit signals to the inside of cells. The linkage to a signal transduction pathway has been suggested as the mechanism for the function of GPI-anchored proteins in T-cell activation (Robinson, 1991) and may constitute a general mechanism for the function of GPI-anchored proteins.

The fact that two different cDNAs encode essentially identical proteins with apparently similar functions raises the possibility of the tissue-specific use of the corresponding mRNAs. However, mRNAs for T-cadherin and Tcad-2 were codetected by RNase protection, in a ratio of $\geq$15:1, in all tissues examined. The developmental profile of T-cadherin and Tcad-2 mRNA expression in chick brain (reported here) and spinal cord (Sacristán and Ranscht, unpublished observations) also revealed no stage-specific expression of either mRNA. However, differences were observed during neural development in the ratio of T-cadherin:Tcad-2 mRNA expression. Like in non-neural tissues, levels of T-cadherin mRNAs exceeded those of Tcad-2 after stage 28, while the two isoforms were expressed at comparable levels at stage 24. This observation may indicate different roles of T-cadherin isoforms during early and late phases of neural development. An exciting possibility is that T-cadherin and Tcad-2 are expressed by different cell populations within the nervous system. This possibility is currently under investigation.

Because of the differential expression of classical cadherins in embryonic tissues, cadherins are thought to regulate the aggregation and segregation of cells during development and thereby control tissue morphogenesis (Takeichi, 1988, 1990). We codetected mRNAs for both T-cadherin isoforms in many tissues together with N-cadherin (Fig. 6). This finding can be explained in two ways. First, the isolated tissues contain specialized subregions expressing either of the T-cadherins or N-cadherin, or second, the same cells simultaneously express multiple cadherins. We have obtained evidence for both suggestions. Tissue sections of stage 24 chick embryos showed that the expression pattern of T-cadherin and N-cadherin proteins differs significantly. For example, although N-cadherin mRNA is detected at stage 24 in the somite tissue of developing chicken embryos, its distribution by indirect immunofluorescence only overlaps with T-cadherins in the myotome, while T-cadherin is expressed in both the caudal somitic sclerotome and, at lower levels, in the myotome (Ranscht, 1991, and unpublished observation; for comparison see Ranscht and Bronner-Fraser, 1991). Similarly, the expression pattern of T-cadherin and N-cadherin proteins differs particularly during later stages of spinal cord development (Ranscht, 1991, and unpublished observation). Therefore, the expression of T-cadherin and N-cadherin proteins in different subregions of somite and spinal cord

tissue could serve to define and segregate particular cell subpopulations and tissue structures.

On the other hand, we detected similar staining patterns by indirect immunofluorescence of both T-cadherin and N-cadherin proteins in tissue sections of heart and muscle (Ranscht, unpublished observation). T-cadherin and N-cadherin proteins were even codetected on the surface of individual cells. Cultured sympathetic neurons (Fig. 7) and myoblasts (Vestal and Ranscht, 1990) express both N- and T-cadherin proteins over their entire cell surface. Provided both cadherins are functional, the coexpression of multiple cadherins is likely to affect the overall adhesiveness of these cells. Although both T-cadherin isoforms are competent to induce homophilic cell adhesion in vitro, their biological function may extend beyond this interaction. Because of their GPI-membrane attachment, it is conceivable that T-cadherin proteins either modulate the function of classical cadherins or regulate cell adhesion by interacting with heterotypic ligands.

The characterization of a T-cadherin isoform that functions in homophilic recognition and is coexpressed with T-cadherin and N-cadherin indicates that the generation and maintenance of specific tissue structures may be far more complex than previously appreciated. Understanding the biological role, mechanism of function, and regulation of the GPI-anchored T-cadherin adhesion molecules will therefore be an important contribution in elucidating the complex molecular interplay in the generation of highly ordered tissue structures.

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