Glycosyl Phosphatidylinositol – anchored T-Cadherin Mediates Calcium-dependent, Homophilic Cell Adhesion

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Abstract. Cadherins are a family of cell adhesion molecules that exhibit calcium-dependent, homophilic binding. Their function depends on both an HisAlaVal sequence in the first extracellular domain, EC1, and the interaction of a conserved cytoplasmic region with intracellular proteins. T-cadherin is an unusual member of the cadherin family that lacks the HisAlaVal motif and is anchored to the membrane through a glycosyl phosphatidylinositol moiety (Ranscht, B., and M. T. Dours-Zimmermann. 1991. *Neuron.* 7:391–402). To assay the function of T-cadherin in cell adhesion, we have transfected T-cadherin cDNA into CHO cells. Two proteins, mature T-cadherin and the uncleaved T-cadherin precursor, were produced from T-cadherin cDNA. The T-cadherin proteins differed from classical cadherins in several aspects. First, the uncleaved T-cadherin precursor was expressed, together with mature T-cadherin, on the surface of the transfected cells. Second, in the absence of calcium, T-cadherin was more resistant to proteolytic cleavage than other cadherins. Lastly, in contrast to classical cadherins, T-cadherin was not concentrated into cell–cell contacts between transfected cells in monolayer cultures. In cellular aggregation assays, T-cadherin induced calcium-dependent, homophilic adhesion which was abolished by treatment of T-cadherin-transfected cells with phosphatidylinositol-specific phospholipase C. These results demonstrate that T-cadherin is a functional cadherin that differs in several properties from classical cadherins. The function of T-cadherin in homophilic cell recognition implies that the mechanism of T-cadherin–induced adhesion is distinct from that of classical cadherins.

C ELL recognition plays a major role in the regulation of tissue morphogenesis during which the various tissue structures are formed through the selective association and segregation of embryonic cells. In the nervous system, cell recognition is important in orchestrating events ranging from the initial segregation of neural tissue from surrounding ectoderm to the establishment of neuronal circuitry. The molecular basis for cellular recognition is attributed, in part, to cell adhesion molecules that selectively interact with ligands in their extracellular milieu. One class of cell adhesion molecules, the cadherins, mediates calcium-dependent adhesion between adjacent cells and is strongly implicated in the control of tissue morphogenesis (Takeichi, 1988; Takeichi, 1990; Edelman and Crossin, 1991; Ranscht, 1991 for reviews). A large number of cadherin molecules have already been molecularly characterized (Nagafuchi et al., 1987; Ringwald et al., 1987; Gallin et al., 1987; Nose et al., 1987; Heimark et al., 1990; Donalies et al., 1991; Hatta et al., 1988; Inuzuka et al., 1991; Napolitano et al., 1991; Choi et al., 1990; Angres et al., 1991; Ginsberg et al., 1991; Suzuki et al., 1991; Ranscht and Dours-Zimmermann, 1991).

Typically, cadherins are transmembrane proteins that share extensive sequence similarity. The extracellular domains of cadherins are responsible for the specificity of the calcium-dependent, homophilic recognition required to link together cells of the same cadherin type. The region that determines the binding specificity of E- and P-cadherin has been localized to the amino-terminal 113 amino acids of the mature proteins (Nose et al., 1990). Expression of molecules containing amino acid substitutions within this region indicates that the residues flanking the conserved HisAlaVal sequence contribute to, but are not sufficient for, homophilic recognition (Nose et al., 1990). The HisAlaVal motif itself is implicated to be important in cell binding because of its conservation between cadherins and the functional regions of hemagglutinins (Blaschuk et al., 1990*a*) and the ability of synthetic peptides containing HisAlaVal to inhibit cell adhesion (Blaschuk et al., 1990*b*; Doherty et al., 1991).

At the ultrastructural level, cadherins are concentrated at cell–cell junctions of the intermediate or adherens type, which are characterized by a well-developed undercoat of actin filaments (Volk and Geiger, 1984; Boller et al., 1985). Work from several laboratories has established that cadherins are functionally associated through their cytoplasmic domain with a group of submembranous proteins, the catenins (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989). One of these molecules, α-catenin, is related to vinculin (Nagafuchi et al., 1991; Herrenknecht et al., 1991), a component of the undercoat of cell–cell and cell–substrate ad-

herens junctions (Bandori et al., 1989). Another catenin, β-catenin, shares structural similarity with both plakoglobin, a component of adherens-type junctions and desmosomes, and with the product of the *Drosophila* segmentation gene *armadillo* (McCrea et al., 1991). The similarity of these catenins to components of the cytoskeleton supports their proposed role in providing a linkage between transmembrane cadherins and components of the cytoskeleton. The importance of this linkage is illustrated by the behavior of fibroblasts expressing mutant E-cadherin lacking the cytoplasmic region. In contrast to intact E-cadherin that concentrates at sites of cell–cell contact, the mutant E-cadherin is distributed over the entire cell surface and is not functional in cell adhesion (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990).

T-cadherin is an unusual member of the cadherin family that shares the exodomain organization with other cadherins but does not contain the HisAlaVal motif in the extracellular domain EC1 (Ranscht and Dours-Zimmermann, 1991). The major distinguishing structural feature of T-cadherin is that it lacks the conserved cytoplasmic region present in classical cadherins and is attached to the neuronal plasma membrane through a glycosyl phosphatidylinositol moiety (GPI)[1] (Ranscht and Dours-Zimmermann, 1991).

T-cadherin is a membrane component of both neural and nonneural tissues. In developing chicken embryos, T-cadherin is expressed in a temporally and spatially restricted pattern. For example, during the migration of neural crest cells and motor axons through the rostral regions of each somitic sclerotome, T-cadherin is expressed in a regular segmental pattern in the corresponding caudal sclerotome regions (Ranscht and Bronner-Fraser, 1991). The initial expression of T-cadherin in the caudal sclerotome correlates with the time neural crest cells immigrate into the rostral sclerotome. This expression pattern is consistent with the possibility that T-cadherin affects the metameric migration pattern of neural crest cells and motor axons, and thus controls specific events during tissue morphogenesis.

To understand the role of T-cadherin in tissue morphogenesis, we decided to first determine whether T-cadherin functions in cell adhesion. This is an even more pressing question in light of the requirement of the cytoplasmic tail for the function of other cadherins. We have expressed T-cadherin in CHO cells and report here that T-cadherin induces calcium-dependent, homophilic adhesion in cellular aggregation assays. This result demonstrates that T-cadherin is a functional member of the cadherin family and indicates that T-cadherin induces cell adhesion by a mechanism that is distinct from that of classical cadherins.

Materials and Methods

Expression Vectors and Transfection of CHO Cells

To generate full-length T-cadherin plasmid DNA, T-cadherin cDNA-266 (Ranscht and Dours-Zimmermann, 1991) was released from λgt.11 by partial digestion with EcoR1 (0.0625 U/μg DNA for 30 min) and subcloned into Bluescript KS⁺ (Stratagene, La Jolla, CA). For expression in eukaryotic cells, a plasmid, pcD-Tcad, containing the coding region of T-cadherin

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1. *Abbreviations used in this paper*: GPI, glycosyl phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

was generated. A T-cadherin DNA fragment was excised from Bluescript by digestion with NotI and StuI and ligated into partially digested XmaIII/EcoRV polylinker sites of the eukaryotic expression vector pcDNA1 (Invitrogen, San Diego, CA).

CHO-DG44 cells were transfected by calcium phosphate coprecipitation with pcD-Tcad and pSV2neo, a plasmid carrying neomycin resistance (American Type Tissue Culture Collection, Rockville, MD). Cells were grown in alpha formulated MEM (Gibco Laboratories, Gaithersburg, MD or Sigma Chemical Co., St. Louis, MO) containing 10% FCS (Tissue Culture Biologicals, Tulare, CA) plus 1× HT supplement (Sigma Chemical Co.), 2 mM L-glutamine, 1 mM sodium pyruvate, and nonessential amino acids (Gibco Laboratories) and plated at a density of 4.5×10^5 cells/6-cm dish. After 16 h in culture, 5 μg calcium phosphate–precipitated pcD-Tcad or pcDNA1 plus 1 μg pSV2neo were added in fresh culture medium. After a further 24 h in culture, the cells were split 1:3 into 10-cm dishes and G418 (Geneticin; Gibco Laboratories) was added to a final concentration of 1 mg/ml. After 12–15 d G418-resistant colonies were isolated using cloning chambers and examined for cell surface expression of T-cadherin by indirect immunofluorescence with anti-T-cadherin antiserum. Several colonies were picked, and one, C4C6, was enriched by fluorescence-activated cell sorting (FACS) for the cells expressing the highest levels of T-cadherin. These cells were used for all of the experiments described. For controls, CHO cells were transfected with both pcDNA1 and pSV2neo and the cells from the G418-resistant colonies were examined by Southern blot analysis for the incorporation of pcDNA1 into genomic DNA. One pcDNA1 positive colony was chosen as control. Control CHO cells do not express T-cadherin, as determined by indirect immunofluorescence and Western blot analysis.

Antibodies

The rabbit polyclonal anti-T-cadherin antiserum generated against 95-kD mature T-cadherin (Ranscht and Dours-Zimmermann, 1991) was used for most of the experiments.

An additional antiserum (anti-pre) was generated against the prepeptide region of recombinant T-cadherin. A BsmI/SspI fragment corresponding to the prepeptide region of T-cadherin was prepared for subcloning by blunt ending with T4 DNA polymerase and the addition of phosphorylated EcoRI linkers (12 mer; Boehringer Mannheim Biochemicals, Indianapolis, IN). The linkers were digested with EcoRI and the fragment was ligated into EcoRI cut pGEX-2T (Smith and Johnson, 1988). The resulting plasmid, pGEX-2 preT, was grown in XL1-Blue cells (Stratagene). Proper orientation and frame were verified by double-stranded dideoxy sequencing of the plasmid (Sanger et al., 1977). The resulting fusion protein was isolated essentially as described (Smith and Johnson, 1988) with the following modifications. An 80-ml overnight culture of pGEX-2 preT in XL1-Blue was added to ∼720 ml of fresh media and grown at 37°C for 1 h followed by induction with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG; Sigma Chemical Co.) for 5 h. Cells were pelleted at 5K for 10 min at 4°C in a GSA rotor and lysed in 20 ml MTPBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, pH 7.3) containing 20 mM EDTA and 5 mg/ml lysozyme at room temperature for 1 h. The concentrations of NaCl and Triton X-100 were adjusted to 0.5 M and 1%, respectively, and the lysate was incubated at room temperature for 30 min. Insoluble material was removed by centrifugation in a Sorvall SS34 rotor at 10K for 10 min at 4°C. The cleared lysate was incubated with 2 ml MTPBS-washed 50% solution of glutathione agarose (Sigma Chemical Co.) by rotating at 4°C overnight. Unbound protein was removed by three washes with 50 ml MTPBS and bound protein was eluted twice with 3 ml of 5 mM reduced glutathione (Sigma Chemical Co.) in 50 mM Tris, pH 8.0. About 600 μg of fusion protein was recovered from an 800 ml culture. The purity of the protein was examined by SDS-PAGE on a 12% acrylamide gel and staining with Coomassie blue R250. A New Zealand white rabbit was immunized by both subcutaneous and intramuscular injections using 100 μg of fusion protein in Freund's complete adjuvant, followed by a boost with 100 μg in Freund's incomplete adjuvant 21 d after the first injection. The animal was bled 10 days after the first boost. The resulting anti-prepeptide antiserum was adsorbed on bovine liver acetone powder.

Immunofluorescence

Cells grown on uncoated glass coverslips were incubated for 20 min at room temperature with DME containing 10% heat-inactivated goat serum, 25 mM Hepes, 2 mM CaCl₂, and 0.03% sodium azide. The cells were then incubated for 45 min at room temperature with anti-T-cadherin antiserum diluted 1:80 or antiprepeptide antiserum diluted 1:1 in the above DME solution. The coverslips were washed with PBS (140 mM NaCl, 3 mM KCl,

1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, pH 7.4) and incubated for 1 h with fluorescein-conjugated (FITC) goat F(ab')$_2$ anti-rabbit IgG (Tago Immunologicals, Burlingame, CA) diluted 1:50. The coverslips were again washed with PBS and the cells were fixed by incubating with cold acetic ethanol (ethanol/acetic acid, 95:5) at −20°C for 10 min. After washing, the coverslips were mounted with 90% glycerol in PBS containing 1 mg/ml p-phenylenediamine (Adams and Pringle, 1984).

Immunoblot Analysis of the T-Cadherin Protein Expression by the Transfected CHO Cells

Both the T-cadherin-transfected and control cells and their conditioned media were examined for T-cadherin expression. Cells in 10-cm dishes were washed with PBS and lysed by the addition of 3 ml of lysis buffer (50 mM Tris, pH 7.6, 2 mM CaCl$_2$, 1% NP-40, 1 mM PMSF, 50 μM leupeptin, 5 μM pepstatin, and 4 μg/ml aprotinin) (all protease inhibitors were from Boehringer Mannheim Biochemicals). The cell lysates were spun at 11,000 rpm in a Sorvall SS34 rotor for 30 min at 4°C to remove cellular debris. For serum-free conditioned media, cells were washed once with serum-free alpha MEM and incubated for 24 h with 5 ml of serum-free alpha MEM. The media were collected and cellular debris was removed by centrifugation as described for cell lysates. Media and cell lysate samples (100 μl of each) were mixed with 50 μl PAGE loading buffer (0.25 M Tris HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, 50% glycerol, 0.01% bromophenol blue), boiled for 3 min, cooled, run into a reducing 8% SDS-polyacrylamide gel, and electrophoretically transferred to Immobilon PVDF membrane (Millipore Corporation, Bedford, MA) (Towbin et al., 1979). Following transfer, the membrane was blocked for 1 h in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 2% Carnation nonfat dry milk. The blocked membrane was then incubated with anti-T-cadherin antiserum (1:500 in TBST) or anti-prepeptide antiserum (1:50) for 1 h at room temperature. The blot was washed four times with TBST and incubated with peroxidase-conjugated donkey anti-rabbit Ig (1:400; Amersham Corp., Arlington Heights, IL) for 1 h. The blots were then washed twice with TBST and processed for chemiluminescence with the ECL detection system (Amersham Corp.).

Phosphatidylinositol-specific Phospholipase C Release of T-Cadherin from CHO Cells

Equal numbers of cells were grown in 3-cm dishes. The cells were washed once with PBS, once with 0.5 ml 0.1 N NaOH, pH 11.0, to remove peripheral membrane proteins, and again with PBS. This was followed by the addition of 0.5 ml incubation buffer (50 mM Tris, pH 7.6, 0.15 mM CaCl$_2$, protease inhibitors) with or without 40 μl of 9.42 U/ml phosphatidylinositol-specific phospholipase C (PI-PLC) (ICN Biomedicals, Inc., Costa Mesa, CA) and incubation at 37°C for 60 min. The solution was removed and the cells were washed with an additional 0.5 ml of buffer. The two were combined. The cells were lysed in 0.5 ml of lysis buffer and the dishes rinsed with 500 μl of the same buffer. The two were combined and spun in an Eppendorff microfuge at 12K and 4°C for 30 min. 100-μl samples were analyzed by immunoblotting with anti-T-cadherin antiserum as described above.

^{3}H-ethanolamine Label and PI-PLC Release of T-Cadherin from CHO Cells

Equal numbers of cells, grown in two 3-cm dishes per clone, were washed twice with 1 ml of serum-free alpha MEM. 1 ml of labeling media (alpha MEM with 10% dialyzed FCS and 100 μCi [^{3}H]-ethanolamine (ICN, 1.0 mCi/ml) was added to each dish and the cells were incubated for 14-16 h under normal tissue culture conditions. The PI-PLC treatment and subsequent cell lysis were performed as described above. After removal of cell debris, 5 μl of anti-T-cadherin antiserum were added per sample and the samples were incubated rotating at 4°C for 2 h. At this time, 100 μl of a 10% solution of Protein G agarose (Calbiochem-Behring Corp., La Jolla, CA) were added and the samples were rotated at 4°C overnight. The resulting complexes were washed twice with 1 ml RIPA (0.05 M Tris-HCl, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, pH 8.5) and once with 1 ml NET (0.01 M Tris HCl, 0.15 M NaCl, 5 mM EDTA, pH 7.4). The complexes were dissociated by the addition of 50 μl PAGE buffer and separated by SDS-PAGE into 8% reducing gels. The gels were stained with Coomassie blue R250, destained, and incubated with Amplify (Amersham Corp.) for 30 min before being dried and processed for fluorography using Kodak XAR-5 film. The fluorographs were exposed for 21 wk.

Trypsin Sensitivity

Cells in 24-well dishes were washed twice with 0.5 ml Ca^{2+}/Mg^{2+}-free HBSS (Gibco Laboratories) supplemented with either 1 mM CaCl$_2$ or 1 mM EDTA. Trypsin (Type IX, crystallized; Sigma Chemical Co.) was added at dilutions of 0, 0.1, 0.01, 0.001, or 0.0001% (wt/vol) in 25 mM Hepes-buffered HBSS (HHBSS) containing either 1 mM CaCl$_2$ or 1 mM EDTA and the cells were incubated at 37°C for 15 min. The reactions were stopped by the addition of 0.1% soybean trypsin inhibitor (Sigma Chemical Co.) in HHBSS. The cells were washed with HHBSS plus trypsin inhibitor and lysed in 100 μl 50 mM Tris, pH 7.6, 2 mM CaCl$_2$, 1.5% NP-40 plus protease inhibitors. Cellular debris was removed by centrifugation in a microfuge at 12,000 rpm for 30 min at 4°C. PAGE loading buffer (30 μl) was added and the samples were processed and analyzed by immunoblotting as described above.

Aggregation Assays

For aggregation assays, cells were washed twice with 5 ml HBSS containing 1 mM CaCl$_2$ and suspended by incubation at 37°C for 20 min in HHBSS containing 0.014% trypsin and 0.9 mM CaCl$_2$. The reaction was stopped by the addition of an equal volume of 0.02% soybean trypsin inhibitor in HHBSS. The cells were washed, resuspended at 4°C in HHBSS containing 1 mg/ml BSA, and incubated with 50 μg/ml DNase I and 1 mM MgCl$_2$ for 30 min at 37°C to remove any residual DNA that might increase the background aggregation. Cells (1 × 10^5) in HHBSS plus BSA were incubated in a volume of 0.5 ml in Linbro uncoated 24-well dishes at 37°C and rotated at 90 rpm for 30 min. Aggregation was started by the addition of 1 mM CaCl$_2$ and was stopped by the addition of 500 μl of 5% glutaraldehyde in HHBSS (final concentration of 2.5%). The aggregates were mixed gently and the particle numbers were determined on a Coulter Model Z$_f$ with a 100-μ aperture. The percent aggregation was determined by the formula N_0-N_t/N_0 × 100%, where N_t was the particle number at time t = 30 minutes and N_0 was the starting particle number. The cell viability was determined by trypan blue exclusion before the cells were aliquoted into the 24-well dishes.

When the cells were to be treated with PI-PLC before aggregation, 40 μl of 9.42 U/ml PI-PLC were added before the 30-min incubation with DNase I. For those experiments where cytoskeleton disrupting agents were used, either cytochalasin D (Calbiochem-Behring Corp.) or nocodazole (Calbiochem-Behring Corp.) were added along with the DNase I and the cells were incubated at 37°C for 30 min. The aggregation assays were then performed as described. Because both cytochalasin D and nocodazole were dissolved in DMSO, controls were performed in the presence of DMSO alone. DMSO had no effect on the aggregation of either control or T-cadherin-transfected CHO cells.

For mixing experiments, control cells were labeled after trypsin treatment by incubation for 30 min at 37°C with 10 pM carboxyfluorescein diacetate succinimyl ester (CFSE; Molecular Probes, Inc., Junction City, OR) in HHBSS containing 1 mg/ml BSA. Control cells and transfected cells were mixed in a 1:1 ratio at 1-2 × 10^5 cells per cell type in a total of 500 μl of HHBSS, 1 mg/ml BSA, 1 mM CaCl$_2$. Photographs were taken of live cells using a heated (37°C) microscope stage on a Zeiss Axiovert 405M.

To determine the surface distribution of T-cadherin subsequent to cell aggregation, cells were processed and allowed to aggregate as described above. After 10 min, formaldehyde was added to 3% and the cells were rotated for 15-20 min. The cells were carefully washed with PBS, resuspended in PBS containing anti-T-cadherin antiserum (1:100), and incubated at room temperature with gentle rocking for 40 min. The cells were washed with PBS twice and incubated with FITC-conjugated goat F(ab')$_2$ anti-rabbit IgG diluted 1:50 for 40 min. After washing, the cells were resuspended in PBS containing 2% 1,4-Diazabicyclo [2.2.2]-octane (Aldrich Chemical Co., Milwaukee, WI) for examination.

Results

Characterization of Proteins Encoded by T-Cadherin cDNA

To study the biochemical and functional properties of T-cadherin, T-cadherin cDNA was cloned into the eukaryotic expression vector pcDNA1 and cotransfected with pSV2neo into CHO cells. Cell lines expressing T-cadherin were selected from neomycin resistant colonies by fluorescence-
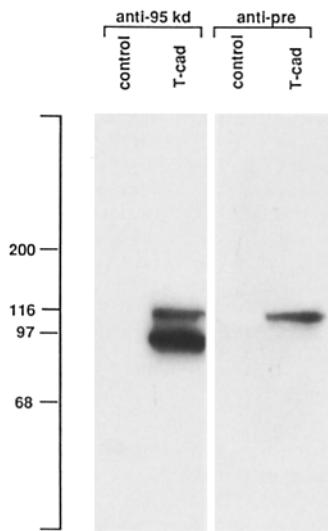
Figure 1. T-cadherin proteins of 95 and 100 kD are expressed by transfected CHO cells. Proteins of 95 and 110 kD react with the anti-T-cadherin antiserum (*anti-95-kD*). The 95-kD protein is mature T-cadherin, while the 100-kD polypeptide is recognized specifically by the anti-prepeptide antiserum (*anti-pre*) and, therefore, is the T-cadherin precursor. Molecular mass markers are indicated on the left.

activated cell sorting and one cell line, C4C6, was used in this study. In Western blots of the T-cadherin–transfected cells, proteins of 95 and 110 kD reacted with polyclonal anti-T-cadherin antiserum, generated against mature 95-kD T-cadherin from embryonic chick brains (Ranscht and Dours-Zimmermann, 1991) (Fig. 1). No immunoreactive polypeptides were observed in either parental or control (mock transfected) CHO cells. As the sizes of the endogenous T-cadherin proteins in neural and nonneural tissues are 95 and 110 kD (Ranscht and Dours-Zimmermann, 1991), the recombinant polypeptides appear to represent faithfully processed forms of T-cadherin.

Cadherins are initially synthesized as precursors containing a prepeptide that is proteolytically cleaved to produce the mature molecules. Amino-terminal sequence analysis of the 95-kD T-cadherin protein from brain revealed that it is the mature T-cadherin (Ranscht and Dours-Zimmermann, 1991). To investigate the possibility that the 110-kD polypeptide is the T-cadherin precursor, an antiserum (anti-pre) was generated against a bacterial fusion protein containing the T-cadherin prepeptide. The anti-prepeptide antiserum reacted specifically with the 110-kD, but not the 95-kD, polypeptide on immunoblots of T-cadherin–transfected CHO cells (Fig. 1) and tissues expressing endogenous T-cadherin (not shown). These results indicate that the 95-kD polypeptide is the mature protein and the 110-kD polypeptide, containing the prepeptide region, is the precursor.

Both mature T-cadherin and the precursor protein were expressed on the surface of CHO cells (Fig. 2, *A* and *C*). Despite the occasional observation of more intense T-cadherin-specific staining at the regions of contact between adjacent cells, there was no convincing relocalization of T-cadherin to these regions even when the cells were grown to confluency. This was true whether the cells were prefixed (not shown) or stained live in the presence of azide (Fig. 2 *A*). The fact that T-cadherin does not cluster at adjacent cell surfaces appears to be a property of T-cadherin itself as N-cadherin, expressed in the CHO-DG44 cells used for this study, concentrates into cell contact sites (Vestal, D. J., and B. Ranscht, unpublished observations).

T-Cadherin Expressed in CHO Cells Is Attached to the Cell Membrane through a GPI Moiety

Most, if not all, T-cadherin expressed by cultured sympathetic neurons and primary fibroblasts and myoblasts is anchored to the plasma membrane via a GPI moiety (Ranscht and Dours-Zimmermann, 1991; Vestal, D. J., and B. Ranscht. 1990. *Soc. Cell Biol.* 11:158a). To determine if T-cadherin cDNA encodes the predicted GPI-anchored T-cadherin protein(s), transfected cells were treated with PI-PLC, an enzyme that specifically cleaves GPI anchors (Low, 1989). PI-PLC treatment released both the 95-kD mature protein and the 110-kD precursor from the cell surface (Fig. 3 *A*). The molecular masses of the PI-PLC released molecules on SDS-PAGE gels were indistinguishable from those of the cell-associated forms, indicating that the proteins were cleaved at or close to the membrane anchor. On longer exposures of the autoradiographs, a small amount of T-cadherin was detected in the absence of PI-PLC (not shown), indicating that it was spontaneously released. Additional evidence for the membrane attachment of T-cadherin through a GPI-moiety was obtained by metabolic labeling of the GPI-anchored proteins with ^{3}H-ethanolamine. The cells were subsequently treated with PI-PLC and T-cadherin was immunoprecipitated from cell lysates and incubation buffer. As shown by SDS-PAGE and fluorography, both the mature 95-kD polypeptide and the 110-kD precursor incorporated ^{3}H-ethanolamine and were released by PI-PLC (Fig. 3 *B*). This demonstrates the presence of the GPI anchor in both the precursor and the mature protein. Some proportion of T-cadherin remained cell-associated after PI-PLC treatment and may represent either incompletely digested cell surface or intracellular T-cadherin. Release of the 110-kD precursor from the cell surface by PI-PLC supports the finding that the unprocessed precursor is present on the cell surface (see Fig. 2 *C*).

Significant amounts of both the mature and precursor T-cadherin proteins were released by transfected cells into the culture media over a 24 hour period (Fig. 3 *C*). This release parallels observations with primary myoblast and fibroblast cultures (Vestal, D. J., and B. Ranscht. 1990. *Soc. Cell Biol.* 11:158a), and cultured sympathetic and retinal neurons (B. Ranscht, unpublished observations). To investigate the possibility that the released T-cadherin is cleaved from the cell surface by endogenous phospholipases, T-cadherin from conditioned media was examined for the presence of the GPI anchor after metabolic labeling with ^{3}H-ethanolamine. T-cadherin was immunoprecipitated from conditioned media with the anti-T-cadherin antiserum and the presence of the GPI anchor was examined by fluorography. No ^{3}H-ethanolamine-labeled T-cadherin was detected in the culture medium (data not shown), implying that the spontaneously released T-cadherin may be either a secreted form or generated by protease cleavage. However, another possible explanation is that the labeling time was insufficient and the level of released T-cadherin containing a labeled GPI anchor was below detection limits.

Trypsin Sensitivity of T-Cadherin Is Modulated by Calcium

One of the hallmark features of cadherins is their resistance to proteolytic degradation in the presence of calcium (Grunwald et al., 1982; Yoshida-Noro et al., 1984; Nose and
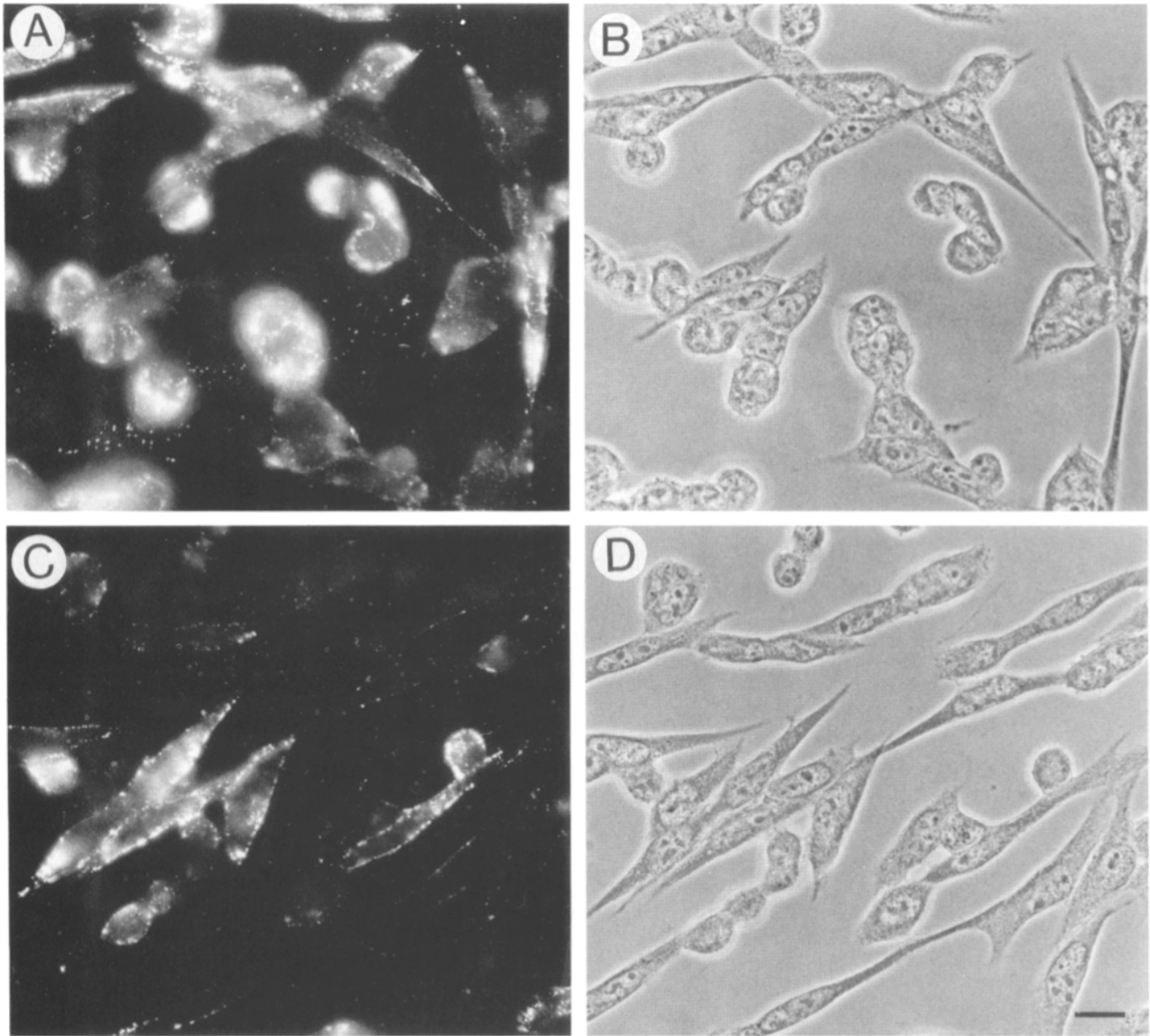
Figure 2. T-cadherin is expressed on the surface of transfected CHO cells. T-cadherin–transfected CHO cells were examined for surface expression of T-cadherin by indirect immunofluorescence with anti-T-cadherin (*A*) or anti-prepeptide (*C*) antisera. The phase-contrast photomicrographs of the corresponding fields are shown in *B* and *D*, respectively. The distribution of T-cadherin is punctate on both cells stained alive in the presence of azide (*A* and *C*) and on prefixed cells (not shown). The anti-prepeptide antiserum detects the T-cadherin precursor on the surface of the transfected cells (*C*). There is significant variability in the level of precursor expression, which is consistent with the finding that the precursor is less stable than mature T-cadherin (see Fig. 4). At low cell densities, T-cadherin staining is observed in a punctate pattern on the tissue culture substrate. This extracellular, substrate-attached T-cadherin may be deposited by cells during cell retraction, or alternatively, T-cadherin released into the conditioned media of transfected cells (see Fig. 3 *C*) may bind to the substrate. Bar, 10 μm.

Takeichi, 1986). To determine if T-cadherin shares this characteristic, intact transfected cells were treated with trypsin in the presence or absence of calcium. In the absence of calcium, detectable amounts of both the precursor and mature T-cadherin proteins were removed from the cell surface by as little as 0.0001% trypsin (Fig. 4). However, while a significant amount of mature T-cadherin was preserved at 0.0001% trypsin, the 110-kD precursor was almost completely digested at this trypsin concentration. At trypsin concentrations of 0.001% and greater, the levels of the cell-associated 110-kD T-cadherin precursor were comparable to those obtained at 0.0001% trypsin, indicating that the remaining precursor is inaccessible to trypsin and probably intracellular. Progressively more mature T-cadherin was digested as the trypsin concentration was increased from 0.001% through 0.1%. Surprisingly, a significant amount of mature T-cadherin was resistant to digestion with 0.1% trypsin, even in the absence of calcium. It is not clear, however, if this represents surface-attached or intracellular T-cadherin.

In the presence of calcium, T-cadherin was protected from proteolytic cleavage up to a trypsin concentration of 0.01%. At 0.01% trypsin most of the precursor protein was removed
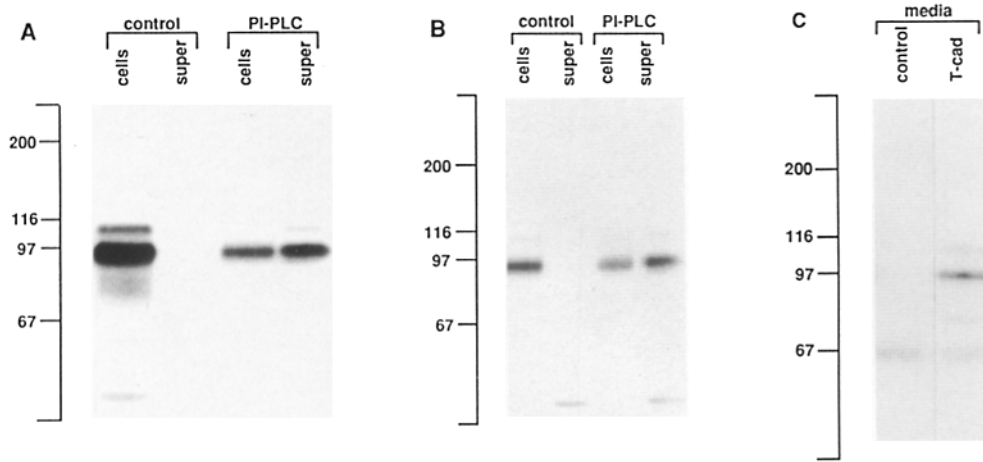
Figure 3. T-cadherin is attached to the cell membrane through a GPI anchor. (*A*) Both the 95-kD mature protein and the 110-kD precursor of T-cadherin are released from the surface of intact cells by PI-PLC. Approximately equal numbers of T-cadherin-transfected cells were incubated in the absence (*control*) or presence of PI-PLC and equal amounts of cell-associated (*cells*) and released (*super*) material were analyzed. (*B*) Both the 95- and the 110-kD T-cadherin proteins incorporate ^{3}H-ethanolamine and are released by PI-PLC. T-cadherin-transfected cells were metabolically labeled with ^{3}H-ethanolamine and the labeled cells were treated with PI-PLC. Cell-associated (*cells*) and released (*super*) material were immunoprecipitated and analyzed by reducing SDS-PAGE and fluorography. (*C*) Both the precursor and the mature T-cadherin proteins are released into the culture media. Serum-free culture medium was conditioned over a 24-h period and 100 μl out of 5 ml were analyzed. Molecular weight markers are indicated on the left.

from the cell surface, but very little of the mature protein was affected. At higher trypsin concentrations, additional proteolytic fragments of T-cadherin were generated. A prominent proteolytic fragment of ~80 kD was detected at 0.01% trypsin and became more prevalent at 0.1% trypsin. Because this fragment was cell-associated, it probably represents a portion of mature T-cadherin from which an amino-terminal fragment has been proteolytically removed.

These experiments show that, like other cadherins, T-cadherin is protected from proteolytic degradation by Ca^{2+}, and that the T-cadherin precursor, present on the surface of the transfected cells, is more sensitive to trypsin digestion than mature T-cadherin. In comparison to classical cadherins, T-cadherin appears to be more resistant to proteolysis, even in the absence of Ca^{2+}.
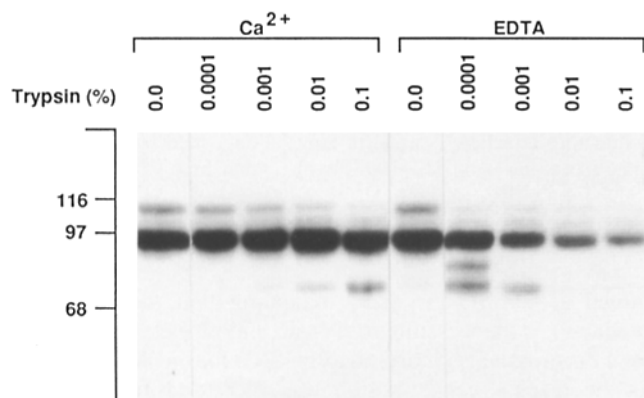

Figure 4. Cell surface T-cadherin is protected from trypsin digestion by calcium. Equal numbers of T-cadherin-transfected cells were treated with increasing percentages (wt/vol) of trypsin in the presence of either 1 mM calcium or EDTA. Cell-associated T-cadherin was analyzed by indirect immunoblotting with anti-T-cadherin antiserum. T-cadherin is protected from proteolysis in the presence of calcium. Molecular weight markers are indicated on the left.

T-Cadherin Induces Calcium-dependent, Homophilic Cell Adhesion

The structural features of T-cadherin, in particular its GPI anchor and lack of the HisAlaVal recognition sequence, raise the question of its ability to function in cell adhesion. To determine if T-cadherin is competent to mediate adhesive interactions, T-cadherin-transfected CHO cells were examined for their ability to aggregate. Under conditions that have been used to suspend cells from monolayers in the study of other cadherins (0.01% trypsin in the presence of 1-2 mM $CaCl_2$), control CHO cells display a low level of endogenous calcium–dependent aggregation. To lower this background aggregation, the relative stability of T-cadherin to proteolytic degradation (Fig. 4) was exploited by treating cells with 0.014% trypsin in the presence of 0.9 mM calcium. Under these conditions most, if not all, of the T-cadherin precursor was removed from the surface and a small proportion of the mature T-cadherin was degraded into an 80-kD proteolytic fragment. The majority of mature T-cadherin remained intact (data not shown).

Aggregation of T-cadherin-transfected cells was initiated by the addition of 1 mM calcium. The cells aggregated within 10 min and the size of the aggregates increased over time. Aggregates formed 30 min after induction with calcium are shown in Fig. 5. Although T-cadherin-transfected cells formed large aggregates compared to controls, many cells expressing T-cadherin on their cell surface as determined by immunofluorescence, remained single. The degree of aggregation of other cadherins is closely correlated with their level of cell surface expression (Nose et al., 1988), therefore, we attribute this to variations in T-cadherin cell surface expression between individual cells. Within the selected clone, the level of T-cadherin expression follows a Gaussian distribution as determined by fluorescence-activated cell sorting (data not shown). To quantitate the extent of aggregation, aliquots of cells were fixed before and after the aggregation assay and counted with a Coulter Counter. Aggregation was calculated as percent reduction in particle number over the
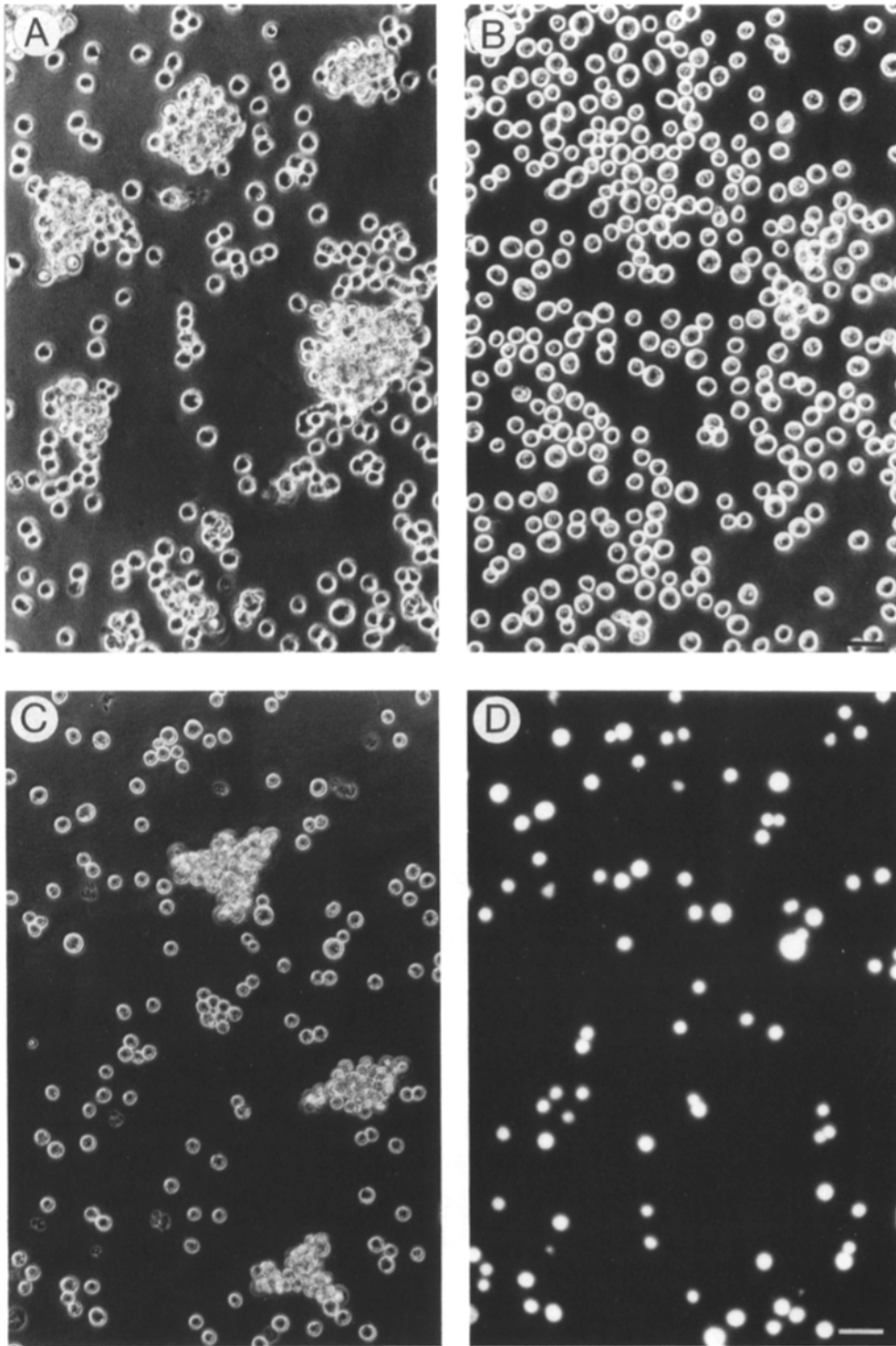
Figure 5. T-cadherin induces aggregation between T-cadherin transfected cells. (*A* and *B*) T-cadherin-transfected (*A*) and control (*B*) CHO cells were induced to aggregate in the presence of 1 mM CaCl₂. T-cadherin–transfected cells form large aggregates while most of the control cells remain single. (*C* and *D*) Equal numbers of unlabeled T-cadherin–transfected and carboxyfluorescein diacetate succinyl ester–labeled control cells were mixed and induced to aggregate. *C* is the phase-contrast photomicrograph of the resulting aggregates. *D* is the fluorescence photomicrograph of the same field. The aggregates contained only T-cadherin–transfected cells, therefore, T-cadherin binding is homophilic. Bar, 50 μm.

30-min incubation time. The number of individual cells was reduced 43.6 ± 9.2 percent for T-cadherin–expressing cells compared to 12.7 ± 6.9 percent for control CHO cells (Fig. 6). The aggregation of T-cadherin–transfected cells was calcium-dependent, as in the absence of calcium no aggregates formed. Adhesion between T-cadherin–transfected cells could be induced both at physiological temperature (37°C) (Fig. 6) and at room temperature (33.0 ± 8.1 percent, $n = 3$). To test for the specificity of T-cadherin-mediated adhesion, T-cadherin–transfected cells were treated with PI-PLC before aggregation was initiated. The removal of a large proportion of T-cadherin molecules from the cell surface (see Fig. 3 *A* for comparison) resulted in the reduction of aggregate formation to background levels ($9.8 \pm 0.3\%$; Fig. 6). No significant reduction of T-cadherin–induced aggregation was observed in the presence of the available anti-T-cadherin antiserum, which we attribute to the fact that the antiserum was raised against the denatured protein (Ranscht and Dours-
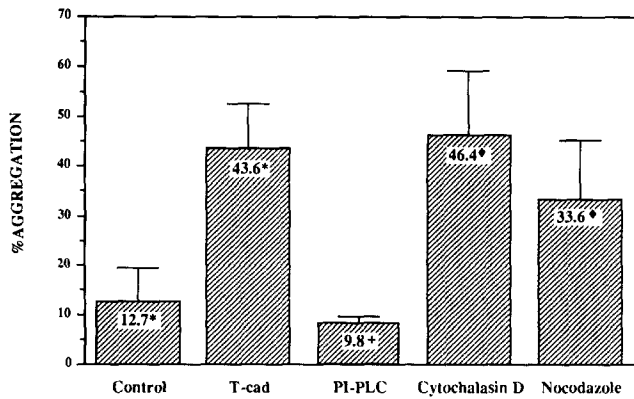
Figure 6. Percent aggregation of T-cadherin–transfected cells. Aggregation assays were performed as described and percent aggregation was determined as a reduction in particle number. Cells were treated with PI-PLC, 1 μg/ml cytochalasin D or 1 μg/ml nocodazole where indicated. The results are averages ± standard deviation. Each assay was performed n number of times, each in triplicate. (*) $n = 12$, (+) $n = 2$, and (◆) $n = 3$.

Zimmermann, 1991) and therefore may not recognize the binding site of T-cadherin in its native configuration.

Generally, cadherin-mediated cell adhesion is the result of homophilic binding between identical cadherin molecules on the surfaces of apposing cells (Takeichi, 1988). To determine whether T-cadherin binding is homophilic or heterophilic, aggregation assays were performed after mixing control and

T-cadherin-transfected cells. To distinguish between the two cell types, the control cells were labeled with the fluorescent dye carboxyfluorescein diacetate succinimyl ester. Analysis of the resulting aggregates revealed that only T-cadherin-expressing cells were present in the aggregates, while the control cells remained single (Fig. 5, C and D). Only on rare occasions were individual control cells mixed within the T-cadherin aggregates. These experiments document that T-cadherin–mediated adhesion is the result of homophilic binding between T-cadherin molecules.

Although in substrate-attached transfected CHO cells T-cadherin appears not to be relocalized to the boundaries between adjacent cells, its distribution could be different when cells aggregate in three dimensions. To determine if this is the case, the distribution of T-cadherin was examined immunohistochemically in formaldehyde-fixed aggregates. T-cadherin began to accumulate at the boundaries between many apposing cells within 10 min of aggregate formation (Fig. 7). Analysis of the aggregates by confocal microscopy revealed that the level of T-cadherin immunoreactivity between many of the apposing cell surfaces was indeed higher than at most cell surface regions facing the outside of the aggregates (data not shown). This observation was extended to one additional T-cadherin–transfected CHO cell line, and was even more striking than that shown in the figure (data not shown). The concentration of T-cadherin at sites of cell–cell contact was accompanied by an apparent increase in the area of cell surface contact between adjacent cells and the assumption of a less spherical cell morphology. In comparison with other cadherins, however, the localization of T-cad-
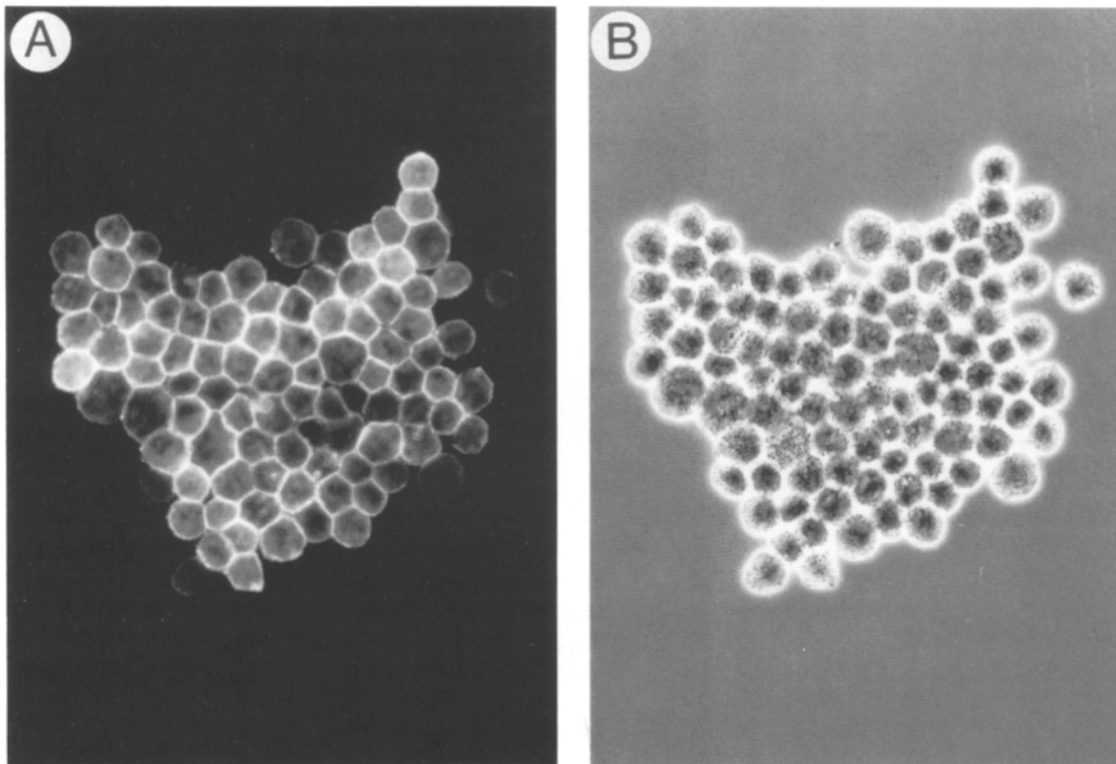
Figure 7. T-cadherin concentrates at adjacent cell boundaries within aggregates. (*A*) The distribution of T-cadherin within the aggregates was determined by indirect immunofluorescence with anti-T-cadherin antiserum 10 min after calcium induction. T-cadherin is concentrated at many areas of cell-cell contact although this distribution is not exclusive. (*B*) Phase contrast of the aggregates.

herin was not exclusive to cell–cell contacts, and the T-cadherin aggregates seemed not as tightly packed as those composed of cells heterologously expressing classical cadherins (Nose et al., 1988; Inuzuka et al., 1991).

T-Cadherin–induced Adhesion Is Not Affected by Cytoskeleton-disrupting Agents

Adhesion mediated by classical cadherins depends on the interaction of the cadherin cytoplasmic region with an intact cellular cytoskeleton (Hirano et al., 1987; Nagafuchi and Takeichi, 1988; Ozawa et al., 1990), and disruption of the actin-based microfilament system blocks N-cadherin– and L-CAM–induced cell aggregation (Matsuzaki et al., 1990). T-cadherin was originally isolated from chick embryo brains as part of an actin-containing polypeptide complex resistant to extraction in nonionic detergents (Ranscht et al., 1984; Ranscht and Dours-Zimmermann, 1991). This property raises the possibility that T-cadherin may be indirectly linked, through associated proteins, with components of the cytoskeleton. To determine whether T-cadherin–mediated adhesion depends on an intact cytoskeleton, aggregation assays were performed after treatment with, and in the presence of, cytochalasin D or nocodazole, drugs that disrupt actin filaments and microtubules, respectively. The drug concentrations were chosen to be comparable or exceed those shown to be effective in the disruption of N-cadherin– or L-CAM–mediated cell aggregation (Matsuzaki et al., 1990). Treatment with 1 μg/ml of either cytochalasin D or nocodazole did not reduce the level of T-cadherin–induced cell aggregation (Fig. 6). In one experiment, the assays were performed after treatment of cells with 5 μg/ml, instead of 1 μg/ml, of cytochalasin D or nocodazole with similar results (percent aggregations for this experiment were: untreated T-cadherin transfected cells, 31.8%; 5 μg/ml cytochalasin D, 43.7%; 5 μg/ml nocodazole, 33.1%). To confirm an adequate level of drug activity, the substrate-attached T-cadherin–expressing cells were examined subsequent to treatment. Nocodazole treatment (1 μg/ml) resulted in the disruption of microtubules, as determined by indirect immunofluorescence with anti-α-tubulin mAbs (not shown; antibodies were the gift of Dr. Bessie Wong, Research Institute of Scripps Clinic, La Jolla, CA). With cytochalasin D (1 μg/ml) the cells rounded up and changed morphology (not shown). These changes indicated that the drug concentrations were sufficient to disrupt the integrity of the cytoskeleton. Together, these observations suggest that T-cadherin–mediated cell adhesion is independent of the drug-sensitive cytoskeleton.

Discussion

T-cadherin is an unusual member of the cadherin family of cell adhesion molecules that is anchored to the plasma membrane through a GPI moiety (Ranscht and Dours-Zimmermann, 1991). When transfected into CHO cells, T-cadherin shares with classical cadherins the ability to induce calcium-dependent, homophilic cell-cell adhesion. Heterologously expressed T-cadherin differs from other cadherins in the following characteristics: First, the T-cadherin precursor is expressed on the cell surface; second, the structural integrity of T-cadherin is preserved at lower calcium concentra-

tions than that of other cadherins; and lastly, T-cadherin does not accumulate at cell–cell contacts between transfected cells in monolayer cultures. These results demonstrate that T-cadherin is a functional cadherin and suggest that it operates by a mechanism distinct from that of classical cadherins.

T-cadherin cDNA transfected into CHO cells encoded GPI-anchored surface glycoproteins of 95 and 110 kD (Figs. 2 and 3). Their sizes are indistinguishable from the T-cadherin polypeptides detected in a number of neural and nonneural tissues (Ranscht and Dours-Zimmermann, 1991). The 95-kD protein from chick brain is known to be mature T-cadherin (Ranscht and Dours-Zimmermann, 1991), while the 110-kD protein, both in CHO cells and in chick brain, contains the prepeptide region and therefore is the T-cadherin precursor. The cell surface expression of the T-cadherin precursor is surprising, as the precursor for E-cadherin is cleaved intracellularly and not transported to the cell surface (Ozawa and Kemler, 1990). Importantly, when mutant E-cadherin containing the prepeptide is expressed on the surface of L-cells, it is not functional (Ozawa and Kemler, 1990). The cleavage site for endogenous proteases, ArgLysGlnArg, is conserved in T-cadherin, but the residues carboxy terminal of the cleavage site diverge substantially from the amino-terminal residues conserved in other cadherins (Ranscht and Dours-Zimmermann, 1991; Ozawa and Kemler, 1990). It could be speculated that the different flanking sequences render endoprotease cleavage of T-cadherin less efficient and thus some proportion of the T-cadherin precursors reach the cell surface intact. Whether the T-cadherin precursor functions in cell adhesion remains to be determined.

In the presence of calcium, T-cadherin induced adhesion between T-cadherin–expressing cells, indicating that one ligand for T-cadherin is T-cadherin itself (Fig. 5). Homophilic binding is the predominant mechanism of cadherin-mediated cell adhesion (Nose et al., 1988). The region for homophilic recognition and binding specificity of E- and P-cadherin has been localized to the amino-terminal 113 amino acids of the mature proteins (Nose et al., 1990). Amino acid substitutions within this region indicate that the amino acids flanking the conserved HisAlaVal tripeptide contribute to homophilic recognition (Nose et al., 1990). The HisAlaVal motif itself is implicated in homophilic cadherin binding as HisAlaVal-containing peptides block cadherin function (Blaschuk et al., 1990b; Doherty et al., 1991). T-cadherin does not contain the HisAlaVal sequence, therefore, its binding function is independent of this tripeptide. This observation may extend to other cadherins lacking this tripeptide sequence (Suzuki et al., 1991; Donalies et al., 1991). When the sequences in the positions corresponding to HisAlaVal were compared between T-cadherin and these latter cadherins, no common motif was evident. This may indicate that cell binding is regulated by other regions of these molecules.

Although several features of T-cadherin, such as the dependence of its stability and homophilic binding on calcium, are common to all known cadherins, T-cadherin differs from classical cadherins in one major aspect. T-cadherin is not concentrated to sites of cell–cell contact in monolayer cultures of transfected CHO cells. This appears to represent a genuine difference between GPI-linked T-cadherin and N-cadherin, as N-cadherin is concentrated at cell contact sites in transfected CHO cells (Geiger et al., 1990; Vestal,

D. J., and B. Ranscht, unpublished observations). Several explanations are possible for the lack of T-cadherin concentration between adjacent substrate-attached cells. First, as relatively large quantities of T-cadherin are released into the culture medium, the concentration of membrane-bound T-cadherin molecules to sites of cell–cell contact may be prevented by competition with released T-cadherin. Soluble forms of cadherin cell adhesion molecules, such as the 80-kD extracellular fragment of E-cadherin/cell CAM 120/80, can neutralize the homophilic binding between membrane-bound molecules (Wheelock et al., 1986). Second, CHO-DG44 cells may contain a negative regulator of T-cadherin homophilic binding, which is not present when cells aggregate in three dimensions as the cells are treated with trypsin before the aggregation assays. Third, homophilic T-cadherin-binding may represent a relatively weak interaction, which is observed only under favorable assay conditions. Future investigations will clarify this issue.

Adhesive interactions induced by T-cadherin do not require the cytoplasmic region required for the function of classical cadherins. The cadherin cytoplasmic segment is thought to associate with the cytoskeleton to control cell adhesion by clustering cadherin molecules at sites of cell–cell contact, which, in turn, generates the force for adhesive interactions (Kemler and Ozawa, 1989; Takeichi, 1991). Indeed, cell adhesion induced by either heterologously expressed N-cadherin or L-CAM is disrupted to a significant degree when actin polymerization is prevented with cytochalasin (Matsuzuki et al., 1990), although the drug is ineffective at disintegrating established cell contacts (Hirano et al., 1987). Because of its membrane attachment through a GPI moiety, the mechanism of T-cadherin–induced cell adhesion must differ from that of classical cadherins. One possibility is that T-cadherin is indirectly linked, through associated proteins, with components of the cytoskeleton. In line with this possibility, T-cadherin was originally isolated as part of a detergent-insoluble polypeptide complex that includes actin (Ranscht and Dours-Zimmermann, 1991). Insolubility in nonionic detergents has also been observed for a proportion of GPI anchored Thy-1 (Ishara et al., 1987), for which a linkage with a submembranous cytoskeletal component has been reported (Bourguignon et al., 1986). However, disruption of actin filaments or microtubules did not affect the aggregation of T-cadherin-transfected cells (Fig. 6). This result strongly indicates that the drug-sensitive cytoskeleton is not required for the function of T-cadherin, although an interaction with other cytoskeletal components can not be excluded.

How can T-cadherin mediate cell adhesion while classical cadherins without their cytoplasmic domain are functionally inactive? Several explanations are possible. One model is that T-cadherin has a higher affinity for itself than other cadherins. This is unlikely, as in the aggregation assays a large number of cells do not participate in the formation of T-cadherin-induced aggregates even though they express T-cadherin on their cell surface. A more likely explanation is that T-cadherin is laterally associated with auxillary molecules that cause the clustering of T-cadherin, as observed in many regions of the T-cadherin aggregates (Fig. 7), and thereby induce the local critical receptor density needed for functional activity.

Although numerous GPI-anchored molecules have been demonstrated to function in cell adhesion (Elkins et al., 1990; Furley et al., 1990; Gennarini et al., 1991), the mechanism of this function is largely unexplored. GPI-anchored proteins are also involved in leukocyte activation where they have been suggested to activate a signal transduction pathway (Robinson, 1991) and, in line with this suggestion, GPI-anchored proteins in leukocytes are complexed to an Src-related protein tyrosine kinase (Stefanova et al., 1991). Future work will demonstrate if GPI-anchored cell adhesion molecules activate similar pathways. Although the mechanism for T-cadherin function remains obscure, the current study has established that T-cadherin, in contrast to classical cadherins, can function without a cytoplasmic domain. In light of its selective distribution in developing embryos, T-cadherin is therefore a genuine candidate to control important aspects of tissue morphogenesis.

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