Insights into the Low Adhesive Capacity of Human T-cadherin from the NMR Structure of Its N-terminal Extracellular Domain*§

Sonja A. Dames 1,2, Eunjung Bang 1,2, Daniel Häussinger 1, Thomas Ahrens 3, Jürgen Engel 1, and Stephan Grzesiek 4,4

From the Departments of 1 Structural Biology and 2 Biophysical Chemistry, Biozentrum, University of Basel, Klingelbergstr. 70, 4056 Basel, Switzerland

T-cadherin is unique among the family of type I cadherins, because it lacks transmembrane and cytosolic domains, and attaches to the membrane via a glycosphingolipid anchor. The N-terminal cadherin repeat of T-cadherin (Tcad1) is ≈30% identical to E-, N-, and other classical cadherins. However, it lacks many amino acids crucial for their adhesive function of classical cadherins. Among others, Trp-2, which is the key residue forming the canonical strand-exchange dimer, is replaced by an isoleucine. Here, we report the NMR structure of the first cadherin repeat of T-cadherin (Tcad1). Tcad1, as other cadherin domains, adopts a β-barrel structure with a Greek key folding topology. However, Tcad1 is monomeric in the absence and presence of calcium. Accordingly, Ile-2 binds into a hydrophobic pocket on the same protomer and participates in an N-terminal β-sheet. Specific amino acid replacements compared to classical cadherins reduce the size of the binding pocket for residue 2 and alter the backbone conformation and flexibility around residues 5 and 15 as well as many electrostatic interactions. These modifications apparently stabilize the monomeric form and make it less susceptible to a conformational switch upon calcium binding. The absence of a tendency for homoassociation observed by NMR is consistent with electron microscopy and solid-phase binding data of the full T-cadherin ectodomain (Tcad1–5). The apparent low adhesiveness of T-cadherin suggests that it is likely to be involved in reversible and dynamic cellular adhesion-deadhesion processes, which are consistent with its role in cell growth and migration.

T-cadherin, also known as truncated, H-, or heart cadherin is an unusual member of the family of type I cadherins that function in calcium-dependent homophilic cell-cell adhesion and thereby govern processes important for tissue morphogenesis such as cell recognition, sorting, coordinated cell movements, and polarity (1). T-cadherin is widely expressed in the brain and the cardiovascular system, but absent or strongly depleted in many cell cancer lines (2–5). The expression of T-cadherin is regulated in response to growth factor signals, aryl hydrocarbon receptor ligands, and oxidative stress (6–8). T-cadherin shares the extracellular five cadherin repeats with other type I cadherins but lacks the transmembrane and cytosolic domains and instead attaches to the membrane via a glycosphingolipid (GPI) anchor (Fig. 1A) (9). Classical cadherins accumulate at adherens junctions, where they interact via their cytoplasmic domain with integrins, catenins, and specific kinases. In contrast, T-cadherin has been localized within lipid rafts of the plasma membrane, is targeted to the apical surface in polarized epithelial cells, and redistributed to the leading edge of migrating cells (10–12). Based on these observations, it has been suggested that T-cadherin functions as a signaling molecule involved e.g. in angiogenesis, cell growth, proliferation, migration, and survival (8, 13, 14). T-cadherin is a receptor for the hexameric and higher oligomeric forms of adiponectin/Acrp30 (15) and for low-density lipoproteins (LDL) (13, 16). Moreover, it has been shown to influence signaling via the RhoA/ROCK, Rac, Erk1/2, and PI3K/Akt/mTOR pathways (8, 13, 17).

Cell-cell adhesion mediated by classical cadherins is induced by the presence of calcium. Current models (18–20) of this process postulate an initial oligomerization at the cell surface (cis) followed by the formation of the adhesive contact via intercellular cadherin dimers (trans). In these strand-exchange dimers, which can be observed by x-ray crystallography (Fig. 3A) (19, 21, 22), Trp-2 from one cad1 domain binds into a hydrophobic pocket presented by the cad1 domain from a

* This work was supported in part by the Canton of Basel and Swiss National Science Foundation Grants 31-109712 (to S. G.) and 31-49281.96 (to J. E.).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental data, references, and Figs. S1–S5.

The atomic coordinates and structure factors (code 2V37) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 Both authors contributed equally to this work.

2 To whom correspondence may be addressed: Dept. of Structural Biology, Biozentrum, University of Basel, Klingelbergstr. 70, 4056 Basel, Switzerland. Tel.: 41-61-2672106; Fax: 41-61-2672109; E-mail: sonja.dames@unibas.ch.

3 Supported by the Korea Research Foundation (Grant KRF-2003-214-C00236). Present address: Korea Basic Science Institute, Seoul Center, Seoul 136-701, Korea.

4 To whom correspondence may be addressed: Dept. of Structural Biology, Biozentrum, University of Basel, Klingelbergstr. 70, 4056 Basel, Switzerland. Tel.: 41-61-2672100; Fax: 41-61-2672109; E-mail: stephan.grzesiek@unibas.ch.

5 The abbreviations used are: GPI, glycosphingolipid; ABTS, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid; cad, cadherin; cmp, coiled-coil trimerization domain of cartilage matrix protein; comp, coiled-coil pentamerization domain of cartilage oligomeric matrix protein; EM, electron microscopy; HRP, horseradish peroxidase; LDL, low-density lipoprotein; PDB, Protein Data Bank.
homologous cadherin on an opposing cell. The calcium-dependent adhesion mechanism was first identified in N- and E-cadherin (21, 23) and recently confirmed for other cadherins (19, 24, 25). The alignment of amino acid sequences of the N-terminal extracellular domain of human T- and classical cadherins reveals ≈30% sequence identity (Fig. 1B). Residues important for calcium binding such as Glu-11 and the calcium binding motifs LDRE and DXND are well conserved. In contrast, amino acids important for the adhesive function of classical cadherins are not shared by T-cadherin (Fig. 1B). These include Trp-2 and residues of the hydrophobic binding pocket of cad1 (Ile-24, Ser-26, Tyr-36, Ala-80, Asp-90, and Met-92), which accommodates Trp-2 in the intercellular strand-exchange dimer (19–21, 26, 27). Similarly, residues in cad1 and cad2 that make contacts in the crystal structure of C-cadherin (19) and which have been proposed to represent the cis-interaction interface are mostly replaced by other amino acids (Fig. 1B and supplemental Fig. S1). Based on the unusual amino acid composition, domain organization, and different cellular localization, it has been suggested that the adhesive capacity of T-cadherin is lower and that the mechanism of T-cadherin-induced calcium-dependent cell-cell adhesion should be different from those of classical cadherins (28).

Here we report the NMR characterization of the structure, dynamics, and calcium binding properties of the N-terminal cadherin domain of human T-cadherin (Tcad1, residues 1–105 of the mature form, residues 139–243 of the unprocessed precursor protein). Tcad1, as the other characterized cadherin domains, adopts a β-sandwich structure with a Greek key folding topology. However, Tcad1 is monomeric, both in the absence and presence of calcium. This is supported by additional EM and solid-phase binding data of the full T-cadherin ectodomain (Tcad1–5). The structure of Tcad1 shows that isoleucine 2, which is conserved in T-cadherins from different organisms (Fig. 1C), occupies a hydrophobic pocket on the same monomer. The latter is formed by equivalent residues to those lining the binding pocket for Trp-2 in the strand-exchange dimer of classical cadherins. Based on the presented NMR data of Tcad1, the higher stability of the monomeric form and its lower susceptibility for calcium-induced conformational changes are rationalized.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs for NMR Studies**—The DNA fragment encoding for the first two extracellular domains of T-cadherin (1–223, Tcad12) preceded by a His tag/Factor Xa cleavage site (MGH10SSGHID2KHMEGGR) was obtained by PCR using a cDNA of human T-cadherin. The fragment was cloned into Ndel/BamHI restriction sites of the *Escherichia coli* expression vector pET-19b (Novagen). A plasmid encoding residues 1–105 in the Tcad1–5. The structure of Tcad1 was prepared with XPLOR-NIH (33) using torsion angle molecular dynamics. Distance restraints were generated in NMRView and classified according to NOE-cross-peak intensities. Upper bounds were 2.8, 3.5, 4.5, and 5.5 Å. The lower bound was always 1.8 Å. For all NOE-restraints r<sup>−6</sup> sum averaging was used. Backbone dihedral angle restraints for φ and ψ were derived based on 3<sup>1</sup>H<sub>NMe</sub>NOE, the determined 13<sup>C</sup> and 1<sup>H</sup> chemical shifts, and on initial structure calculations. Stereospecific assignments were obtained for 13 β-methylene and 8 valine γ-methyl proton pairs. Based on 3<sup>1</sup>H<sub>N</sub>δ<sub>2/3</sub> and 3<sup>1</sup>HNδ<sub>2/3</sub> coupling constants and NOE data, side chain χ<sub>1</sub> angles were restrained to one of the staggered conformations (60°, 180°, −60°) ± 30°. For regions with β-sheet conformation, hydrogen bonds (H-bonds) were defined by H-N distance bounds of 1.8–2.3 Å, and N-O distance bounds of 2.6–3.1 Å. The 20 lowest energy structures out of 200 calculated ones were finally refined in a water shell (34, 35).

**Protein Expression and Purification for EM and Solid-phase Binding Studies**—Several (fusion) protein constructs were generated to carry out *in vitro* EM and solid-phase binding studies (Fig. 1A). These comprise His-tagged (-H) and Strep-tagged (-S) versions of T-cadherin domains 1–3 (Tcad1–3–S), fusions...
containing the first five domains of T-cadherin and the coiled-coil trimerization domain of cartilage matrix protein (Tcad1–5cmp-H/S), a fusion containing the first T-cadherin domain, domains 2–5 of E-cadherin, and the trimerization domain of cartilage matrix protein (Tcad1Ecad2–5cmp-H), as well as a fusion containing the first five domains of E-cadherin and the trimerization domain of cartilage matrix protein (Ecad1–5cmp-H) or the pentamerization domain of cartilage oligomeric matrix protein (Ecad1–5cmp-S). Vectors for expression of these constructs in human embryonal kidney cells were obtained as described in the supplemental data.

Human embryonal kidney (HEK) 293-EBNA cells (Invitrogen) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) F12 supplemented with 10% fetal bovine serum, 1% glutamine and 10 mg/ml penicillin/streptomycin (all obtained from Invitrogen). Transfectants were generated as described before (36), and bulk cultures were used for protein expression. Confluent cells were maintained in 23 ml of DMEM F12, 1% glutamine, 10 mg/ml penicillin/streptomycin for 48 h. Then the medium was collected, and new medium was added. This procedure was repeated 8–10 times. Supernatants were centrifuged at 2500 × g for 10 min, buffered in 20 mM Hepes, pH 7.1, and stored at −20 °C.

Supernatants containing recombinant proteins were sterile-filtered, dialyzed against 50 mM Tris, pH 7.9, and concentrated and stored at 20 °C.

Solid-phase Binding Assays—200 μl of trimeric Ecad1–5cmp-H (50 nM), Tcad1–5cmp-H, or Tcad1Ecad2–5cmp-H (50 nM) in 20 mM Tris, pH 7.4 were coated at 4 °C overnight on 96-well flat bottom plates (Becton Dickinson). All buffers contained 2 mM Ca2+. Plates were then blocked with 20 mM Tris, pH 8.1 and 3% (w/v) bovine serum albumin for 2 h at room temperature. After three washing steps, different concentrations of the Strep-Tactin®-Sepharose columns (IBA, Göttingen). Purified proteins were stored at −20 °C in 20 mM Tris, pH 7.4 and 0.5 mM CaCl2. The expression and folding of T-cadherin constructs in eukaryotic HEK 293 cells was analyzed on Western blots, silver-stained SDS gels, and circular dichroism data (supplemental Fig. S3).

RESULTS

Description of the Structure of Tcad1—Initial NMR studies on a T-cadherin construct containing the first two cadherin repeats (Tcad12) indicated that the second repeat was unfolded (supplemental Fig. S2). The reason for this behavior is currently unclear. Under similar conditions, the second domain of Ecad12 with equivalent domain boundaries (1–219) is folded (22). Therefore the domain boundaries should have been defined correctly. It has however been observed that in an E-cadherin construct encompassing the 2nd and the 3rd cadherin repeat, the latter is unfolded (37). Because the second domain in Tcad12 is unfolded, we restricted the structural characterization to Tcad1. According to NMR relaxation, EM, and solid-phase binding data, this Tcad1 domain is monomeric in solution in the presence and absence of calcium (see below).

The NMR structure of the calcium-free form of the Tcad1 monomer together with a topology diagram is shown in Fig. 2. Corresponding structural statistics are given in Table 1. Tcad1 adopts a β-barrel structure with a Greek key folding topology (Fig. 2B). The β-sheets formed by strands F–G–C and B–I–H–E, colored in green and blue in Fig. 2A, are also found in the known structures of classical cadherins (19, 21, 22, 27) and CNR/protocalderin–α (38). Conserved proline and glycine residues in the cad1 domains of cadherins (Figs. 1B and 2C) strongly determine the overall fold by marking the ends of β-sheet strands and restricting the conformation of loop regions. In Tcad1, prolines 16, 18, 46, and 90 show a cis-peptide bond conformation. This is evident from strong NOE-correlations between the α-protons of the respective proline and the preceding residue and the observation of characteristic proline 13Cβ and 13Cγ chemical shifts (39). In addition, glycines 39, 41, 48, 57, 84, 89 as well as asparagine 12 have positive Φ-angles. Such cis-peptide bonds in conserved prolines and positive Φ-angles in conserved glycines and asparagines are also found in the crystal structures of N-cadherin (PDB code 1NCH; Pro-16, Pro-18, Pro-47, Gly-40, Gly-42, Gly-49, Gly-58, Gly-85, Asn-12), C-cadherin (PDB code 1L3W; Pro-18, Pro-47, Gly-40, Gly-42, Gly-49, Gly-58, Gly-85, Asn-12), and E-cadherin (PDB code 1FFS; Pro-18, Pro-

### Table 1

| Experimental restraints | Distance restraints | All (assigned + ambiguous) |
|-------------------------|--------------------|--------------------------|
| Total                   | 3843 (3437 + 406) |                           |
| 1N-NOESY                | 1069 (925 + 144)  |
| Aliphatic 1H-NOESY      | 2665 (2431 + 234) |
| Aromatic 1H-NOESY       | 109 (81 + 28)     |
| Hydrogen bond           | 40                 |
| ϕ angle restraints      | 57                 |
| ψ angle restraints      | 46                 |
| χ1 angle restraints     | 32                 |

| Structural statisticsa | Total Energy (kcal mol−1) | −3932 ± 91 |
|------------------------|---------------------------|------------|
| R.m.s. deviations from experimental restraints | Distance (Å) 0.0142 ± 0.0005 | Dihedral angle (°) 0.224 ± 0.047 |
| Bonds | A angles 1.174 ± 0.021 |
| Angles 1.740 ± 0.080 |
| Improper 1.370 ± 0.080 |

| Average r.m.s.d. to mean structure (Å) | Residues 6–33 (backbone/heavy) 0.53/1.09 |

| None of the structures had distance restraints violations > 0.3 Å or dihedral angle violations > 5°. |
| R.m.s.(d.), root mean square (deviation). |

| Corresponding structural statistics are given in Table 1. Tcad1 adopts a β-barrel structure with a Greek key folding topology (Fig. 2B). The β-sheets formed by strands F–G–C and B–I–H–E, colored in green and blue in Fig. 2A, are also found in the known structures of classical cadherins (19, 21, 22, 27) and CNR/protocalderin–α (38). Conserved proline and glycine residues in the cad1 domains of cadherins (Figs. 1B and 2C) strongly determine the overall fold by marking the ends of β-sheet strands and restricting the conformation of loop regions. In Tcad1, prolines 16, 18, 46, and 90 show a cis-peptide bond conformation. This is evident from strong NOE-correlations between the α-protons of the respective proline and the preceding residue and the observation of characteristic proline 13Cβ and 13Cγ chemical shifts (39). In addition, glycines 39, 41, 48, 57, 84, 89 as well as asparagine 12 have positive Φ-angles. Such cis-peptide bonds in conserved prolines and positive Φ-angles in conserved glycines and asparagines are also found in the crystal structures of N-cadherin (PDB code 1NCH; Pro-16, Pro-18, Pro-47, Gly-40, Gly-42, Gly-49, Gly-58, Gly-85, Asn-12), C-cadherin (PDB code 1L3W; Pro-18, Pro-47, Gly-40, Gly-42, Gly-49, Gly-58, Gly-85, Asn-12), and E-cadherin (PDB code 1FFS; Pro-18, Pro-
A notable feature of the Tcad1 structure are several residues (Val-21, Val-42, Asn-55, Asn-56, Val-69, Asn-83, Leu-87) with large ($^3J_{HNH}$) coupling constants characteristic for $\beta$-sheet like $\phi$-angles of $-90^\circ$ to $-130^\circ$. However, based on NOE-correlations, these residues adopt a helical structure with $\psi$-angles between $0^\circ$ and $-90^\circ$. Similar $\phi$- and $\psi$- angles are observed for the equivalent residues in N-, C- (except Asn-84) and E-cadherin.

In comparison to classical cadherins, the most interesting part of the Tcad1 structure is the region around isoleucine 2. In Tcad1, residues Ile-2 and Val-3 (strand A, Fig. 2A) form an

---

47, Gly-40, Gly-42, Gly-49, Gly-58, Gly-85, Asn-12). A notable feature of the Tcad1 structure are several residues (Val-21, Val-42, Asn-55, Asn-56, Val-69, Asn-83, Leu-87) with large ($^3J_{HNH}$) coupling constants characteristic for $\beta$-sheet like $\phi$-angles of $-90^\circ$ to $-130^\circ$. However, based on NOE-correlations, these residues adopt a helical structure with $\psi$-angles between $0^\circ$ and $-90^\circ$. Similar $\phi$- and $\psi$- angles are observed for the equivalent residues in N-, C- (except Asn-84) and E-cadherin.

In comparison to classical cadherins, the most interesting part of the Tcad1 structure is the region around isoleucine 2. In Tcad1, residues Ile-2 and Val-3 (strand A, Fig. 2A) form an
The side chain of Ile-2 in Tcad1 inserts into a pocket that is formed by residues equivalent to those lining the hydrophobic pocket for Trp-2 in classical cadherins (Fig. 3, B–D). Mostly hydrophobic interactions are found to residues Val-24, Asp-26, Arg-29, Phe-35, Val-77, Thr-79, Glu-88, and Val-91. Additional contacts involve the backbone of Val-25, Ser-27, Glu-78, Gly-89, and Pro-90 (Fig. 3, B, left side and C). In the structures of the C-, and E-cadherin, the conserved side chain indole and hydroxyl groups of Trp-2 and Tyr-36 donate hydrogen bonds to the backbone carbonyl groups of Asp/Glu-90 and Ser-26, respectively (Fig. 3B, right). These hydrogen bonds cannot be formed by the equivalent Ile-2 and Phe-35 in Tcad1 due to the absence of a hydrogen bond donor (Fig. 3B, left side). The structure of C-cadherin (Fig. 3, A and B, right side) revealed a further hydrogen bond between the side chains of Lys-8 and Gln-23 (19). Also, this hydrogen bond cannot be realized in Tcad1 because position 8 is occupied by a leucine.

It has been shown that strand-exchange during cadherin dimer formation depends on the formation of an intermolecular salt bridge between the N-terminal amino group and the side chain of Glu-89 (41). An equivalent glutamic acid is found at position 88 in T-cadherin. In Tcad1, the N-terminal nitrogen and the side chain oxygen atoms of Glu-88 may form an intramolecular salt bridge, because their distance is \( \approx 2.6 \) Å in 6 of the 20 final structures. However, the N terminus may also be involved in other ionic interactions (Fig. 3B, left side), i.e. the distance to the side chain oxygen atoms of the strongly conserved Asp-26 (Fig. 1C) is \( \approx 2.5 \) Å in all calculated structures and the distance to side chain oxygen atoms of the fully conserved Asp-28 is \( \approx 2.5 \) Å in 8 of 20 structures. These additional ionic interactions may thus stabilize the monomeric form and counteract the intermolecular interaction with Glu-88, which facilitates dimer formation in classical cadherins. Moreover, T-cadherin has a serine instead of an aspartate at position 1 (Fig. 1B). In classical cadherins this conserved negatively charged residue may further influence the monomer-dimer equilibrium, e.g. by repulsive intramolecular interactions with Glu-89.

It is revealing to compare the surface charge of monomeric Tcad1 (Fig. 3C) to other monomeric cadherin structures. Fig. 3D shows the surface charge of cad1 in monomeric M-Ecad12,
NMR Structure of the N-terminal Extracellular Domain of T-cadherin

A

B

C

D

T-cadherin (monomer)

C-cadherin (dimer)

T-cadherin

M-E-cadherin
which has an N-terminal extra methionine that abolishes its ability to form the strand-exchange dimer (22). In this structure, Trp-2 is inserted into the Trp-2 binding pocket on the same molecule. Hence, the surface of this molecule should closely mimic the surface of monomeric Ecad12 before it associates into the strand-exchange dimer. It is apparent from this electrostatic surface that strand-exchange is strongly favored by a high density of opposite charges across the entire contact surface around residue Trp-2. In contrast, the surface around Ile-2 in T-cadherin is more hydrophobic toward the bottom part of the molecule (C terminus) and rather acidic toward the top part. Thus, no favorable electrostatic interactions appear possible for the formation of a strand-exchange dimer.

Chemical Shift Changes upon Calcium Binding—To characterize the effect of calcium binding on the structure and the adhesive properties of Tcad1, we analyzed 15N-HSQC spectra of 0.2 mM Tcad1 at different calcium concentrations (supplemental Fig. S4). At 0.5 mM Ca2+, only weak shifts are observed, which are most pronounced for residues 67–70 in one of the calcium binding motifs. At Ca2+ concentrations of 5–10 mM, the equilibrium is completely driven to the calcium-bound form. Resonances of residues at the calcium binding sites (Glu-11–Asn-12, Leu-65—Ile-70, Ile-98—Asn-101) shift strongly (Fig. 4). Further, nearby residues show only weak to very weak displacements. No significant changes are observed for the N-terminal region around residue Ile-2 that would indicate structural rearrangements.

Calcium binding to E-cadherin significantly increases its affinity for homoassociation and its adhesiveness (23, 42). For Ecad12, homoassociation leads to distinct changes in NMR spectra (22), such that monomeric and dimeric forms can be clearly distinguished. Both calcium-free and -bound forms undergo dimerization. However, the respective dissociation constant (K_D) is much stronger (0.72 mM) in the presence of calcium than in its absence (10 mM) (22).

Addition of calcium to Tcad1 (0.2 mM) did not result in a decrease of the amide proton T_2 relaxation times (~18.5–22.5 ms), but made them overall more uniform (~22.5 ms). These data are consistent with a monomeric form of calcium-free and -bound Tcad1 and suggest that calcium binding results in compaction of the molecule due to reduced conformational freedom at the calcium binding sites. Moreover, a variation of the

**FIGURE 3.** Comparison of the binding pocket for residue 2 in different cadherins. A, in classical type I cadherins, the N-terminal extracellular domain (cad1) mediates cell-cell adhesion by forming a strand-swapped dimer, which is assumed to represent the trans contact (PDB ID 1L3W). Interactions between cadherin molecules from the same cell represent the cis contact. The right panel shows a ribbon representation of the cad1-mediated dimer of C-cadherin (PDB ID 1L3W) from two perspectives. Trp-2 (red) from one cad1 domain (yellow) binds into a hydrophobic pocket of a cad1 domain from a neighboring cadherin, which in vivo is presented from an opposing cell. B, detailed representation of the interactions in the binding pocket for residue 2 in monomeric T-cadherin (left) and dimeric C-cadherin (right). Hydrogen bonds are indicated by dotted black lines. Trp-2 is colored red, and the side chains of residues forming its binding pocket are colored blue. Residues 5 and 28 are shown in yellow and residues 8 and 23 in green. C and D, representation of the binding pocket for residue 2 in T-cadherin (C) and M-E-cadherin (D) in the context of the full molecule. M-E-cadherin (PDB ID 1FFS) is unable to form a strand-exchange dimer because of an additional N-terminal methionine. Residues important for homoassociation are shown in a space-filling mode on the left sides of C and D. The color coding is identical to B. The large conformational changes around residues 14–18 upon dimer formation are facilitated by Gly-15 (yellow). In T-cadherin, this position is occupied by a glutamine (Q15, yellow). The surface charge distributions (positive: blue, negative: red) of T-cadherin and M-E-cadherin are shown in the middle and right of C and D. The location of the three calcium binding sites is indicated by green circles. For M-E-cadherin, the N-terminal charge was calculated for D1. The putative cis-interaction site (see text) is indicated by a black circle. The M-E-cadherin structure (PDB ID 1FFS) is based on the mouse homologue and therefore has Glu instead of Asn (Fig. 1B) at position 86.
concentration of calcium-free Tcad1 (0.07–0.6 mM) did not induce any changes in the $^1$H-$^{15}$N HSQC spectra. This is in contrast to Ecad12 where such changes are clearly detectable even in the absence of calcium (22). $^{15}$N-relaxation data (supplemental Fig. S2) obtained on calcium-free Tcad12 (24.7 kDa) at a concentration of 0.86 mM yield an overall rotational correlation time of 11.7 ns. The latter is also consistent with a monomeric form. Thus the NMR evidence indicates that both Tcad12 and Tcad1 are monomeric in the absence of calcium up to at least millimolar protein concentrations. Because the addition of calcium at these protein concentrations does not give any evidence for homoassociation, we conclude that any putative association either in the presence or absence of calcium should be much weaker than for the case of calcium-free Ecad12 ($K_m$ 10 mM).

**EM and Solid-phase Binding Data for Tcad1–5 and Ecad1–5**—As the NMR data on Tcad1 and Tcad12 gave no evidence for dimerization, we additionally analyzed homophilic interactions of T-cadherin constructs containing the full ectodomain (cad1–5, Fig. 1A) by EM and solid-phase binding assays. To increase the local cadherin concentration and thereby to mimic the conditions on the cell surface, most of the used E- and T-cadherin constructs were fused at their C terminus to the coiled-coil trimerization domain of cartilage matrix protein (cmp in the construct name in Fig. 1A; a schematic representation of such a construct is depicted in supplemental Fig. S5A).

Calcium binding to classical cadherins results in characteristic changes in their appearance in electron micrographs. Binding of calcium at low concentrations (0.5–1 mM) stiffens the linkers between the five cadherin repeats and induces a characteristic curvature of the entire extracellular structure (23). The latter is also evident from the crystal structure of C-cadherin (Fig. 3A) (19). At higher calcium and protein concentrations, the electron micrographs of E-cadherin show ring-like and concatenated ring structures when ectodomains are tethered together by oligomerization domains (23). An analogous Tcad1–5cmp construct did not show the formation of such concatenated ring-like structures in electron micrographs under similar conditions (supplemental Fig. S5B). This further corroborates that T-cadherin has a much lower tendency for homodimerization than classical cadherins.

The adhesive capacity of different E- and T-cadherin constructs in the presence of calcium was further analyzed by solid-phase binding assays (Fig. 5). In these assays, various cadherin constructs (Ecad1–5cmp-H, Tcad1–5cmp-H, Tcad1Ecad2–5cmp-H, see Fig. 1A) were coated to the bottom of the reaction vial. These were reacted with Strept-tagged counterparts (Ecad1–5cmp-S, Tcad1–3-S, Tcad1–5cmp-S) added in solution. Subsequent binding of streptavidin-attached HRP to the Strept tag could be monitored by UV-spectroscopy based on the catalytic activity of HRP. The addition of Ecad1–5cmp-S to coated Ecad1–5cmp-H (Fig. 5A, open circles) resulted in a strong hyperbolic increase of the absorption of the ABTS substrate at 405 nm, which indicates strong homoassociation. Because of the specificity of cadherin interactions, no significant binding of Ecad1–5cmp-S to coated Tcad1–5cmp-H (Fig. 5A, black circles) or the domain exchange mutant Tcad1Ecad2–5cmp was observed (Fig. 5A, open triangles). When immobilized Tcad1–5cmp-H, Ecad1–5cmp-H, or Tcad1Ecad2–5cmp-H was incubated with increasing concentrations of monomeric Tcad1–3–S (up to 400 nM, Fig. 5B); also no binding was detected. Neither did Tcad1–5cmp-S react with coated Tcad1–5cmp-H, the chimera Tcad1Ecad2–5cmp-H, nor Ecad1–5cmp-H (Fig. 5C). Taken together, the solid-phase binding data give no evidence of homoaosociation of trimerized immobilized T-cadherin ectodomains to soluble T-cadherin in the submicromolar range. This is consistent with the absence of any NMR-detectable interactions of the Tcad1.

**FIGURE 5. Solid-phase binding assays with different E- and T-cadherin constructs (see Fig. 1A).** Coated proteins (50 nM) were His-tagged (-H) Ecad1–5cmp-H, Tcad1–5cmp-H, or Tcad1Ecad2–5cmp-H, soluble ligands (10–400 nM) were Strep-tagged (-S) Ecad1–5cmp-S (A), Tcad1–3–S (B), and Tcad1–5cmp-S (C). The assays were performed in the presence of 2 mM Ca$^{2+}$. Binding was monitored at 405 nm after incubation with 1 μg/ml HRP-conjugated streptavidin and ABTS as substrate. One typical experiment is depicted. Data points represent the mean value ± S.D. of four wells corrected by the negative control (unspecific binding to bovine serum albumin alone).
DISCUSSION

The N-terminal extracellular domain of classical type I cadherins mediates cell-cell adhesion by formation of a strand-swapped intercellular dimer. Trp-2 from one monomer binds thereby into a hydrophobic pocket presented by the equivalent domain of a cadherin molecule on a neighboring cell. The presented structural data of Tcad1 reveal how several features conserved in the amino acid sequences of T-cadherins from different organisms but distinct to those of other type I cadherins (Fig. 1) reduce its ability for dimerization. First, Tcad1 has an isoleucine instead of a tryptophan at position 2 and several of the residues forming the hydrophobic pocket for residue 2 have larger side chains (Tcad1: Val-24, Asp-26, Phe-35, Val-77, Thr-79, Gly-89, Val-91; Ecad1: Ile-24, Ser-26, Tyr-36, Ser/Ala-78, Ala-80, Asp/Asn/Glu-90, Met/Ile-92). Therefore the hydrophobic pocket for Ile-2 is smaller and has a different shape. The smaller van der Waals volume (43 Å³) of isoleucine (124 Å³) compared with tryptophan (163 Å³) is further expected to reduce the size of the surface area that is buried upon binding to the hydrophobic pocket. Moreover, because of the nature of their side chains, Ile-2 and Phe-35 can only make hydrophobic interactions but not the same type of hydrogen bonds observed for Trp-2 and Tyr-36 in E-cadherin. Second, in Tcad1 compared with classical cadherins the formation of a stable intramolecular β-sheet around Ile-2 may be facilitated by the presence of serine instead of proline at position 5. The ϕ- and ψ-angles of Ser-5 are approximately –150° and +150° whereas they are approximately –70° and +150° for Pro-5 in N- and E-cadherin. Prolines adopt typically ϕ-angles in the range of –40° to –100° (44). The β-sheet like ϕ-angle of Ser-5 in Tcad1 may therefore exert less conformational strain on the preceding N-terminal β-strand than Pro-5 in classical cadherins. Third, Tcad1 has a glutamine instead of a glycine at position 15. It has been described that the formation of the strand-exchange dimer in classical cadherins has the largest effect on the backbone conformation of residues 14–16 (20). Moreover the ψ-angles of residues 17–18 differ significantly between the closed monomer of M-E-cad (PDB ID 1FF5) and the dimer of mature E-cad12 (PDB ID 1Q1P). In the known structures of classical cadherins, Gly-15 has positive or largely negative ϕ-angles. Such ϕ-angles are typically not observed for glutamine. In Tcad1, the reduced backbone flexibility around Gin-15 (Fig. 3C, left and supplemental Fig. S2) is expected to hamper strand-exchange upon dimer formation by making T-cadherin less susceptible to conformational changes upon binding of calcium to the spatially close binding sites (Figs. 3C and 4). Fourth, studies with N-cadherin highlighted the relevance of an intermolecular salt-bridge between the N-terminal positively charged amide group and the negatively charged carboxylate of Glu-89 for dimer formation (41). Based on the observed distances in the calculated Tcad1 structures, ionic interactions between the N-terminal amide and Asp-26 as well as Asp-28 occur more often than with Glu-88 (Fig. 3B, left side). This may additionally stabilize the formation of a stable β-sheet around Ile-2 and lower the affinity for homoassociation. Fifth, the exchanges of Asp to Ser at position 1 and Lys/Arg to Val at position 25 change the surface charge distribution in the vicinity of residue 2. Whereas dimer formation in E-cadherin may be driven by favorable ionic interactions involving complementary surface patches (Fig. 3D, middle), such complementary charges are absent in T-cadherin (Fig. 3C, middle).

T-cadherin does not only lack many of the sequence features of classical cadherins, which are important for the trans interaction between the two cad1 repeats from opposing cells. T-cadherin also exhibits significant differences in the surface patch distal of position 2, which is thought to mediate the so-called cis contact with a cad2 repeat of a neighboring molecule on the same cell (Fig. 3A, left side). Overall, this surface region of Tcad1 (Fig. 3C, black circled region) has a similar shape but a different polarity compared with E- and C-cadherin. In classical cadherins, the first and the last residue of the HAV-motif (79–81) as well as Ser-37, X-55, and Asn/Ser-86 of cad1 (Fig. 1B) mediate interactions to residues Ile/Val-174 and Ala/Thr-176 on cad2 (supplemental Fig. S1). In Tcad1, the equivalent surface on cad1 is formed by an ETT motif (78–80) as well as Arg-36, Glu-54, and Lys-85. The surface on the second domain is formed by Ser-175 and Ala-177. However, two two-residue insertions (Thr-172–Val-173, Thr-183–Leu-184) and the substitution G178L are expected to significantly alter the surface characteristics of Tcad2 in this region. The increased polarity on cad1 and the presumably different shape of the surface on cad2 might therefore result in reduced complementarity and thereby weaker cis interactions.

In the current model of cadherin-mediated cell adhesion, the closed monomer, in which residue 2 binds into the hydrophobic pocket of the same protomer, competes with the strand-exchange dimer, where residue 2 binds into the hydrophobic pocket of a protomer on an opposing cell. Because the interactions in the monomeric and dimeric forms are very similar including the degree of buried surface area, strand swapping allows a binding reaction with a very high specificity but only millimolar affinity (20). Based on NMR titration measurements, binding of calcium decreases the $K_D$ for Ecad12 homoassociation from 10 to 0.72 mM (22). A higher $K_D$ because of a stabilization of the monomeric and/or a destabilization of the dimeric form would lower the number of adhesive contacts (20). Tcad1 did not show specific concentration-dependent NMR spectral changes that would indicate dimer formation in the presence or absence of calcium, and Tcad1–3 as well as Tcad1–5cmp showed no significant affinity for homoassociation in the solid-phase binding assays. Based on these data, the $K_D$ for T-cadherin homoassociation is estimated to be significantly larger than for Ca$^{2+}$-free Ecad12 (10 mM, Ref. 22). A higher stability of monomeric Tcad1 would be in agreement with the above-described structural differences to classical cadherins and with the observation that calcium-free T-cadherin is more resistant to proteolytic cleavage (28). The number of adhesion-mediating dimers should be highly dependent on concentration and increase quadratically with the cadherin surface density (20). The surface density of classical cadherins
can be controlled through interactions of their cytoplasmic domain with catenins and other signaling molecules, which direct them to the cell-cell contact sites (45, 46). T-cadherin does not have a cytosolic domain and localizes at lipid rafts as other GPI-anchored signaling proteins (47). This may be a further reason for the observed differences in mediating intercellular interactions.

The low adhesive capacity of T-cadherin is reminiscent of the behavior of Protocadherin-α. For both proteins, the low homophilic capacity coincides with the replacement of Trp-2 and several other residues important for cell-cell adhesion in classical cadherins. Protocadherin-α is only able to adhere to HEK293T cells upon activation of β1 integrin by Mn$^{2+}$ or a specific antibody (TS2/16), which induces binding of β1 integrin to Protocadherin-α (38). Early after the discovery of T-cadherin, it was suggested that lateral association with auxiliary molecules influences its clustering on the cell surface and thereby its ability to mediate intercellular interactions (28).

One may therefore speculate that similar to Protocadherin-α (38) interaction partners such as adiponectin (15), LDL (13, 16), or yet to be identified proteins are involved in cell-cell recognition by T-cadherin.

In summary, specific amino acid replacements in T-cadherin favor a closed monomeric form over the strand-exchange dimeric form of classical cadherins. These comprise the crucial I2W replacement, as well as other substitutions, which reduce the size of the binding pocket for Ile-2, modify the backbone conformation and flexibility around residues 5 and 15, and alter charge interactions around position 2. The binding of calcium induces significant spectral changes only around the calcium binding sites. Consistent with EM and solid-phase binding data, the observed spectral changes do not give any indication of homophilic interactions. Thus T-cadherin appears to be far less adhesive than classical cadherins and, consistent with its role in cell growth and migration, is likely to be involved in reversible and dynamic cell-cell adhesion-deadhesion.

Acknowledgments—We thank Therese Schulthess and Marco Rogowski for help with expression and purification as well as Thomas Niermann for providing the cDNA encoding for T-cadherin.

REFERENCES

1. Halbleib, J. M., and Nelson, W. J. (2006) Genes Dev. 20, 3199–3214
2. Lee, S. W. (1996) Nat. Med. 2, 776–782
3. Zhong, Y., Delgado, Y., Gomez, J., Lee, S. W., and Perez-Soler, R. (2001) Neuron 29, 483–498
4. Lee, S. W., Reimer, C. L., Campbell, D. B., Cheresh, P., Duda, R. B., and Ranscht, B. (2003) Faseb J. 19, 1737–1739
5. Ranscht, B., and Dours-Zimmermann, M. T. (1991) Neuron 7, 391–402
6. Doyle, D. D., Goings, G. E., Upshaw-Earley, J., Page, E., Ranscht, B., and Palfrey, H. C. (1998) J. Biol. Chem. 273, 6937–6943
7. Fothergill, L. M., and Nelson, W. J. (2006) J. Biol. Chem. 281, 33650–33663
8. Schubert, M., Labudde, D., Oschkinat, H., and Schmieder, P. (2002) Protein Sci. 11, 1013–1023
9. Schubert, M., Labudde, D., Oschkinat, H., and Schmieder, P. (2002) Protein Sci. 11, 1013–1023
10. Niermann, T., Kern, F., Erne, P., and Resink, T. (2000) Biochem. Biophys. Res. Commun. 276, 1240–1247
11. Koller, E., and Ranscht, B. (1996) J. Biol. Chem. 271, 30061–30067
12. Philippova, M., Ivanov, D., Tkachuk, V., Erne, P., and Resink, T. J. (2003) J. Biol. Chem. 278, 275–281
13. Kippen-Korgun, D., Osibow, K., Zoratti, C., Schraml, E., Greilberger, I., Kostner, G. M., Jurgens, G., and Graier, W. F. (2005) J. Cardiovasc. Pharmacol. 45, 418–430
15. He, W., Cowin, P., and Stokes, D. L. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10308–10313
16. Niermann, T., Kern, F., Erne, P., and Resink, T. (2000) Biochem. Biophys. Res. Commun. 276, 1240–1247
17. Philippova, M., Ivanov, D., Allenspach, R., Takuwa, Y., Erne, P., and Resink, T. (2005) Faseb J. 19, 588–590
Prisant, M. G., Richardson, J. S., and Richardson, D. C. (2003) *Proteins* **50**, 437–450
45. Nagafuchi, A., and Takeichi, M. (1988) *EMBO J.* **7**, 3679–3684
46. Lilien, J., and Balsamo, J. (2005) *Curr. Opin. Cell Biol.* **17**, 459–465
47. Philippova, M. P., Bochkov, V. N., Stambolsky, D. V., Tkachuk, V. A., and Resink, T. J. (1998) *FEBS Lett.* **429**, 207–210
48. Gouet, P., Robert, X., and Courcelle, E. (2003) *Nucleic Acids Res.* **31**, 3320–3323
49. Koradi, R., Billeter, M., and Wuthrich, K. (1996) *J. Mol. Graph* **14**, 51–55, 29–32
50. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) *J. Biomol. NMR* **8**, 477–486