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T-cadherin structures reveal a novel adhesive binding mechanism

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Abstract

Vertebrate genomes encode nineteen “classical” cadherins and about a hundred non-classical cadherins. Adhesion by classical cadherins depends on binding interactions in their amino terminal EC1 domains, which swap N-terminal β -strands between partner molecules from apposing cells. However, strand swapping sequence signatures are absent from non-classical cadherins, raising the question of how these proteins function in adhesion. Here we show that T-cadherin, a GPI-anchored cadherin, forms dimers through an alternative non-swapped interface near the EC1-EC2 calcium binding sites. Mutations within this interface ablate the adhesive capacity of T-cadherin. These non-adhesive T-cadherin mutants also lose the ability to regulate neurite outgrowth from T-cadherin expressing neurons. Our findings reveal the likely molecular architecture of the T-cadherin homophilic interface, and reveal its requirement for axon outgrowth regulation. The adhesive binding mode employed by T-cadherin may also be used by other non-classical cadherins.

INTRODUCTION

Cadherins are a large family of cell surface transmembrane proteins that mediate intercellular adhesion in vertebrates and invertebrates¹⁻³. Sequence analysis reveals numerous cadherin subfamilies^{4,5}, including the “classical” cadherins whose biological roles in cell recognition, development, and tissue homeostasis have been well characterized. Nineteen classical cadherins, six members of the type I and thirteen members of the type II subfamily, are conserved in vertebrate genomes^{4,5}. Type I cadherins are typically expressed broadly in

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Author contributions CC determined and refined all the crystal structures; FB produced all the wild-type recombinant proteins; NZ performed the neurite outgrowth assays and surface biotinylation; HCV performed the cell aggregation studies; PK performed the SPR experiments; GA performed the AUC experiments; OH and JB prepared mutant proteins; XJ helped in crystallographic data collection and refinement; SP and JV performed bioinformatic analysis; BR designed and analyzed cell-based experiments, TMJ helped write the manuscript, BH and LS designed experiments, analyzed data, and wrote the manuscript.

Accession codes Protein Data Bank: Coordinates for T-cadherin regions mouse EC1-2, chicken EC1-2, mouse EC1, chicken EC1, and *X. Laevis* EC1, have been deposited with accession codes 3K5R, 3K5S, 3K6F, 3K6I, and 3K6D, respectively.

epithelia, whereas type II cadherins have more finely-grained expression patterns often restricted to the nervous system and vasculature⁶⁻⁸. Numerous non-classical cadherins have been characterized, including the gene-clustered protocadherins⁹, flamingo-like cadherins with receptor-like seven-helix transmembrane regions¹⁰, and very large cadherins like cadherin-23 which forms “rope-like” helical structures between adjacent stereocilia of acousticolateral hair cells^{4,5,11}.

Insight into the structure and function of cadherins has been acquired through studies of classical cadherins, which are all single-pass class I transmembrane proteins with adhesive ectodomains. Their mature ectodomains are composed of five tandem β -sandwich fold “extracellular cadherin” domains¹², termed EC1 to EC5. Three Ca^{2+} ions bind at each of the linker regions between successive EC domains^{13,14}, and rigidify the interdomain connections¹⁵. Cell adhesion mediated by classical cadherins depends on the binding between cadherin extracellular domains presented on the surfaces of apposing cells and is regulated through intracellular association with β - and α -catenins which affect the dynamics of the actin-based cytoskeleton^{16,17}.

Structural aspects of the homophilic adhesive interactions of classical cadherins have been revealed in crystallographic studies of ectodomain regions from both type I^{13,14,18-21} and type II²² subfamilies. These crystal structures show two-fold symmetrical dimers whose interfaces involve residues belonging exclusively to the amino-terminal membrane-distal EC1 domains. In each classical cadherin adhesive interface, the N-terminal β -strand (the A*-strand) of each protomer juts out and inserts one (type I) or two (type II) conserved Trp side chains into the hydrophobic core of the partner EC1 domain. The formation of an interface based on strand-swapping is an example of the more general phenomenon of “3D domain-swapping”, an oligomerization mechanism that results in low affinity binding even for protein-protein interfaces with large surface area²³. Although both type I and type II classical cadherins employ a strand swap binding mechanism, type I interfaces are formed exclusively by “swapped” elements, whereas type II cadherin interfaces also include large regions of interaction that are not swapped²². Sequence analysis suggests that desmosomal cadherins also employ a strand swapped interface⁵. Strikingly, however, the sequence determinants of strand swapping are absent from most other cadherins, including all invertebrate cadherins, the gene-clustered protocadherins, flamingos, and other non-classical cadherins, suggesting that they exploit different binding mechanisms.

Classical cadherins of both subfamilies are often co-expressed with a variant non-classical cadherin family member, truncated (T-) cadherin²⁴, for which a single gene is present in each vertebrate genome^{8,25-28}. T-cadherin is unusual among cadherins in that it lacks transmembrane and cytoplasmic regions, and instead is attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) moiety. T-cadherin is, nevertheless, a close phylogenetic relative of classical cadherins, sharing a common ectodomain organization and high sequence similarity²⁴ (mouse T-cadherin is 46% identical to mouse N-cadherin over the mature ectodomain). Yet T-cadherin differs in that it lacks a Trp-containing A*-strand which appears to be a common element in all cadherin domains that strand-swap⁵. Instead T-cadherin has an Ile residue in place of the key strand-swap anchor residue Trp 2. Despite this notable difference, expression of T-cadherin in CHO and L-cells confers calcium-dependent homophilic adhesion^{25,29}. NMR structural studies of a T-cadherin EC1 protein revealed a cadherin fold, but this protein was monomeric and thus could not provide insight into the mechanism of adhesion³⁰.

Understanding of the functional roles of T-cadherin is still incomplete. In vivo, axons that express T-cadherin avoid regions demarcated by T-cadherin expression³¹. In vitro, T-cadherin-positive neurons extend stunted neurites with a convoluted morphology on

monolayers of cells transfected with T-cadherin, contrasting with more extended axon patterns exhibited on naïve cell substrates³². A recent study using ectopic expression and RNA interference in developing chick embryos provides direct evidence for T-cadherin's role as a negative guidance cue for extending motor axons (HCV and BR, unpublished data). In contrast to the inhibitory effects of T-cadherin, classical cadherins typically promote axon outgrowth, although the mechanisms underlying this phenomenon also remain undetermined³. T-cadherin is expressed on vascular endothelial cells³³, and affects pathology-induced neovascularization *in vivo*³⁴. In this latter context, however, T-cadherin seems to act as a binding protein for the adipocyte-derived hormone adiponectin³⁵.

To gain insight into the divergent biological properties of non-classical cadherins we undertook a structural, biophysical, and functional analysis of T-cadherin. Crystallographic results show that the T-cadherin dimer interface, unlike that of classical cadherins, does not involve strand swapping. Rather, T-cadherin pairs dimerize at a site between the EC1 and EC2 domains, close to the Ca²⁺ binding region. This configuration, which we now refer to as an 'X-dimer' because the dimeric assembly of elongated molecules resembles the shape of an 'X', is topologically identical to dimers found for E-cadherin proteins containing mutations that prevent strand swapping (see accompanying manuscript). Mutations designed to disrupt the X-interface in T-cadherin ablate molecular dimerization, homophilic cell adhesive capacity, and the effects of T-cadherin on neurite extension. By contrast, mutations of T-cadherin regions that correspond to the strand swapping elements classical cadherins have no effect on T-cadherin binding. These findings reveal a novel molecular mechanism for homophilic adhesion, and demonstrate that the recognition of T-cadherin molecules on one cell by those from neighboring cells is critical for effects on neurite outgrowth.

RESULTS

Single-domain EC1 fragments from T-cadherin are monomeric

We determined the crystal structures of EC1 domain fragments of T-cadherins from three vertebrate species: chicken, mouse, and *Xenopus laevis*. Each of these structures crystallized in distinct crystal forms (Table 1) but revealed highly similar molecular structures comprising a typical cadherin domain topology consisting of seven β -strands forming two sheets, with two of the strands connected by a "pseudo- β -helix", a structural element present in type I but not type II cadherins (Fig. 1A).

Unlike crystal structures of EC1 domains from classical cadherins, which form dimers with symmetrically exchanged A*-strands between partner molecules, chicken T-cadherin EC1 appears monomeric, with the entire A*-strand integrated into the main body of its own protomer. Ile2, which aligns with the critical anchor residue, Trp2, of classical cadherins, is accommodated in a hydrophobic pocket lined by aliphatic (Val24, Val77, and Val79) and aromatic (Phe35) side chains (Fig. 1B). This pocket is similar to the Trp2 acceptor pocket of classical cadherins but, in the T-cadherin structure, contacts are intramolecular rather than intermolecular. Similar conformations are observed in the crystal structures of mouse and *X. laevis* T-cadherin EC1, each determined at 1.8Å resolution (Fig. 1A). We analyzed the buried surface area as well as hydrophobic and polar contacts in these three T-cadherin structures to look for assemblies that could represent functional dimers. No significant interfaces were found between molecules related by crystallographic or non-crystallographic symmetry, nor was a conserved pattern of interaction discovered among the structures from the different species. Moreover, analytical ultracentrifugation results showed that each T-cadherin EC1 domain was monomeric in solution (data not shown), differing from classical cadherin EC1 domains which behave as dimers. We conclude that all the three T-cadherin EC1 structures represent monomers which, unlike equivalent fragments of classical cadherins, do not form strand-swapped dimers.

Ca²⁺-bound T-cadherin EC1-2 fragment is a non-swapped dimer

To investigate the potential role of other EC domains in T-cadherin homophilic interactions, we determined the 2.9 Å crystal structure of an EC1-2 ectodomain fragment of chicken T-cadherin. In the crystal structure, shown in Fig. 2A, both the EC1 and EC2 domains display the usual β -sandwich fold typical of cadherin domains, and three Ca²⁺ ions coordinated in the conserved interdomain calcium-binding site. The EC1 domain of this structure is virtually identical to those determined in the single-domain structures presented above, with a root mean square deviation (rmsd) of less than 1 Å for main-chain atoms.

The crystallographic asymmetric unit of the EC1-2 structure contains two molecules, which come together to form an X-shaped two-fold symmetric dimer which buries 2173 Å² of solvent accessible surface. The contact area of the dimer is centered on the interdomain linker region (residues 99-104, which present 447.6 Å² of buried surface area upon dimerization), but also involves both EC1 and EC2 domains (buried surface areas of 926.0 Å² and 799.4 Å², respectively). Contacts across EC1 domains are formed through two hydrophobic patches, mainly involving residues from the N-terminal A-strand (Fig. 2E). The first of these includes side chain interactions between residues Leu3, Thr5, and Ile25, and is located near the “top” of EC1. The second locus of EC1-EC1 interactions is found toward the “bottom” of EC1 and involves side chains from Leu8, Pro10, and Ile98. The “core” of the X-dimer, around the juxtaposed calcium binding sites, is characterized by hydrophobic contacts mediated by the side chains of residues that emanate from the EC2 domains: Met198, Val203, and Leu205 (Fig. 2C). These numerous hydrophobic contacts are likely to play an important role in stabilization of the X-dimer.

Additionally, polar and ionic interactions are detected within or flanking the “core” region of the X-dimer. First, in the center of the “core” region, linker residues Asp99 and Gln100 of each protomer, which also coordinate Ca²⁺, are hydrogen-bonded to each other across the dimer interface. Flanking the “core” region, Arg14 from the EC1 domain of each protomer forms hydrogen bonds with the backbone carbonyl groups from Pro137 and Thr139 in the partner EC2 domain. Remarkably, the relative positioning of Arg14 and Asp140 of the opposite EC2 domain, even though not a canonical salt bridge, strongly suggests that they form one, at least transiently, during regular molecular dynamic motions. In addition, Arg104 is hydrogen bonded to the backbone of Asp202 from the partner EC2 domain (Fig. 2C).

The T-cadherin X-shaped dimer described here closely resembles previously observed EC1-2 dimers found in E-cadherin crystal structures derived from constructs with extra residues at their N-termini (rmsd ~1.9 Å for 363 core alpha-carbons) (Fig. 2B)^{14,19} which are unable to strand swap^{14,18,36}. The T-cadherin and these E-cadherin X-dimer interfaces are topologically identical, and of remarkably similar size (2173 vs. 2170.7 Å² buried surface area) and chemical character (Figs. 2C and D). In each case, a large hydrophobic area is buried upon dimerization, and probably plays an important role in dimer stabilization. In both cases, hydrophobic contacts established along the length of the EC1 domain involve residues from the N-terminal A-strand (Fig. 2E). Within the “core” region, both structures show interactions involving the side chains of hydrophobic residues emanating from the EC2 domain. The flanking edges of the interface are demarcated by similar polar interactions: in the T-cadherin structure where the Arg14 → Asp140 and Arg104 → Asp202 interactions correspond to well-formed salt bridges (Lys14 → Asp138, and Arg105 → Glu199) in the E-cadherin X-dimer structure.

Structure of a Ca²⁺-free T-cadherin EC1-2 ectodomain fragment

Both T-cadherin and classical cadherins require the presence of calcium to mediate adhesion^{25,29}. However, structures of multi-domain cadherins have not been determined in the absence of calcium. We therefore determined the 2.0 Å crystal structure of a mouse T-cadherin EC1-2

fragment (Fig. 3) crystallized in a Ca^{2+} -free form by the inclusion of citrate in the crystallization experiment. Citrate behaves as a calcium chelator, and thus no Ca^{2+} ions are found in this structure. Although no substantial differences are observed between individual domains in the Ca^{2+} -free structure and the equivalent ones in the Ca^{2+} -bound EC1-2 structure presented above, we find that the relative orientation between domains and the quaternary structure are drastically altered.

In the Ca^{2+} -free T-cadherin structure, the EC1 and EC2 domain of the T-cadherin monomer fold onto each other, creating a clamshell-like structure. A relatively small ($927 \text{ \AA}^2$) intramolecular interface is found between the EC1 and EC2 domains, with the interface formed mainly by van der Waals contacts. Few specific interactions, such as hydrogen bonds or salt bridges, are formed. It appears, therefore, that this intramolecular interface is due to crystal packing effects. Nevertheless our observations imply that the absence of Ca^{2+} leaves six unbalanced negative charges on the calcium-coordinating residues in the interdomain region, and permits much greater flexibility of the linker region. Ca^{2+} -dependent rigidity of interdomain regions of classical cadherins has been demonstrated previously by NMR analyses of Ca^{2+} -free E-cadherin³⁷, and electron microscopic images have also shown the “collapse” of rigid E-cadherin ectodomains upon Ca^{2+} removal¹⁵. The T-cadherin EC1-2 Ca^{2+} -bound and Ca^{2+} -free structures now provide an atomic-level view of the effects of Ca^{2+} in the stiffening of the cadherin ectodomain. Importantly, T-cadherin EC1-2 fragments do not associate as dimers in the Ca^{2+} -free structure or in solution (see below). Taken together, the Ca^{2+} -bound and Ca^{2+} -free T-cadherin EC1-2 structures illustrate how removal of Ca^{2+} can prevent formation of the dimer interface found in the Ca^{2+} -bound form, which requires a rigid interdomain linker and the appropriate orientation of the EC1 and EC2 domains.

In summary, the crystallographic studies of T-cadherin ectodomain fragments presented here show that T-cadherin is similar in overall structure to classical cadherins, but differs markedly in the EC1 domain region. These differences prevent binding through strand-swapping, the primary adhesive binding mode for classical cadherins. Instead, T-cadherin EC1-2 forms a Ca^{2+} -dependent X-shaped dimer that involves interaction between elements near the Ca^{2+} binding regions of partner T-cadherin molecules.

These structural observations raise the question of whether the crystallographically-observed X-shaped dimer corresponds to the structure utilized by T-cadherin to mediate Ca^{2+} -dependent cell adhesion. To address this question, we employed biophysical and cellular assays with wild-type and site-directed mutant proteins to determine whether T-cadherin ectodomain dimerization in solution and in a cellular context depends on structural elements of the X-dimer.

T-cadherin binding affinities and design of non-binding mutants

To determine whether the crystallographically observed X-dimer is relevant to T-cadherin molecular interactions in solution, we first carried out binding affinity experiments with wild type T-cadherin domains using analytical ultracentrifugation (AUC) as well as surface plasmon resonance (SPR). AUC measurements (Supplementary Fig. 1) showed a monomer/dimer equilibrium for wild-type protein, with $K_D = 41.4 \pm 1.7 \mu\text{M}$, in the presence of Ca^{2+} (Table 2). This value is in the range found for classical cadherin ectodomains, including C- and E-cadherins whose AUC-determined binding affinities have been reported as $64 \mu\text{M}$ and $80 \mu\text{M}$, respectively^{38,39}. As expected, the addition of EDTA prevented dimer formation (data not shown). For SPR assays, wild-type T-cadherin EC1-2 was covalently immobilized on a Biacore CM5 chip surface to measure its interaction with single-domain EC1 and two-domain EC1-2 T-cadherin fragments. T-cadherin EC1 did not bind, in either the presence or absence of Ca^{2+} , to the derivatized surface carrying the two-domain fragment (Fig. 4A). The binding of T-cadherin EC1-2 to the derivatized chip was observed in the presence of Ca^{2+} , but no interactions were evident when EDTA was included (Fig. 4B).

We next used the T-cadherin X-dimer structure as a basis for designing mutants intended to disrupt dimerization. As shown in Fig. 2C, the relative position of residues Arg14 and Asp140 suggests that they can, at least transiently, form a salt-bridge that will stabilize the interface between the two protomers. To disrupt this interaction, we produced two single and two double T-cadherin EC1-2 mutants: R14E, R14S, R14E D140S, and R14S D140S. These mutant proteins were purified to homogeneity, and exhibited the same elution profile in size exclusion chromatography, and the same solubility as the wild-type protein. In SPR experiments using chips coated with wild-type T-cadherin EC1-2, these mutants either failed to bind to the immobilized cadherin (R14E, and R14E D140S), or bound at a level at least five-fold reduced from wild-type protein (R14S, and R14S D140S) (Fig. 4C). These SPR data support the conclusion, derived from the three-dimensional crystal structures, that both the EC1 and the EC2 domains are necessary for T-cadherin dimerization, and strongly suggests that the dimer interface observed in the crystal mediates T-cadherin dimerization in solution.

Binding is unaffected by mutations in the “strand swapping” region

Despite the evidence supporting T-cadherin interaction through the X-dimer interface, including the dimeric crystal structure, lack of strand swapping found for EC1 structures, and diminution of binding by X-dimer mutants in SPR experiments, it remains possible that T-cadherin could engage in strand swap binding as classical cadherins do. To test this possibility, we prepared T-cadherin EC1-2 proteins with mutations either in the X-dimer interface or the potential “strand swapping region”, and assessed the effects of these mutations on homodimerization by AUC (Fig. 5). As can be seen in Fig. 5A, the introduction into T-cadherin of mutations known to interfere with classical cadherin strand swapping have no significant effect on T-cadherin homodimerization. These mutations include extension of the N-terminus by two amino acids, MR, and alanine mutation of I2, which corresponds to the Trp2 anchor residue of classical cadherins. Furthermore, T-cadherin containing an extra glycine residue at the N-terminus, the form for which the dimeric structure was determined, shows identical homodimerization behavior to wild-type T-cadherin (Fig. 5A). By contrast, mutations in the strand swapping region of classical cadherins such as E-cadherin interfere significantly with homodimerization (Fig. 5B). Wild-type E-cadherin dimerizes with $K_D=98.6 \pm 15.5 \mu\text{M}$, but extension of the N-terminus by the residues MR weakens binding to $K_D=257.5 \pm 17.5 \mu\text{M}$. Mutation of the Trp2 swapping-anchor residue weakens binding even more potently to $K_D=916 \pm 47 \mu\text{M}$. These experiments (Table 2) clearly distinguish the behavior of T-cadherin from that of classical cadherins when challenged with mutations in the strand swapping region, and strongly suggests that T-cadherin does not engage in strand swap binding.

In contrast to strand dimer mutations, a mutation targeting the X-dimer interface in T-cadherin (R14E) strongly inhibited homodimerization in AUC experiments, causing the mutant to be monomeric in solution (Figure 5C). This is in agreement with the SPR data reported above (Fig. 4C). Homodimerization could be partially restored by introduction of a second mutation (D140K), which together with R14E represents a “charge reversal” of the wild-type salt bridge. We reasoned that this double mutation would, at minimum, reduce the electrostatic repulsion between mutated Glu14 and Asp140 in the X-dimer interface. This double mutant (R14E D140K) dimerized in solution, albeit with a lower affinity ($149 \pm 17 \mu\text{M}$) than wild-type protein (Fig. 5C).

X-dimer interface mutants disrupt Ca^{2+} -dependent cell aggregation

To determine whether the crystallographically-observed X-dimer interface is involved in T-cadherin mediated cell adhesion, we investigated the effects of dimer-disrupting mutations on the function of full-length T-cadherin in a cellular context. Previous studies have shown that CHO cells over-expressing T-cadherin form aggregates in Ca^{2+} -containing medium²⁵. To assess the role of the X-interface in cell adhesion, we generated stable Chinese hamster ovary

(CHO) cell lines that constitutively expressed either wild-type or mutant full-length T-cadherins, and used them in cell aggregation assays.

Expression of wild-type and mutant T-cadherin on the membrane surface of stably transfected CHO cells was confirmed by immunostaining. Expression levels for cell lines producing each T-cadherin X-dimer mutant appeared identical to that of wild-type T-cadherin (Supplementary Fig. 2). Staining was located on the cell surface, as a ring around the cell in a single confocal optical section. The immunofluorescence signal was punctate in distribution, consistent with the possibility that T-cadherin may localize to discrete lipid micro-domains due to its GPI anchoring.

We performed a series of cell aggregation experiments to assess the effect of X-dimer disrupting mutants on T-cadherin mediated cell adhesion (Fig. 6). Non-transfected parental CHO cells express no detectable cadherin protein, and thus exhibit very little background aggregation in the presence of Ca^{2+} (Supplementary Fig. 2 and 3). In contrast, CHO cells transfected with wild-type T-cadherin form large aggregates under the same conditions (Fig. 6; Vestal et al., 1992). None of the four T-cadherin mutant cell lines induced cell aggregation in the presence of Ca^{2+} . Thus, a single amino acid mutation at R14 is sufficient to abrogate T-cadherin-based cellular aggregation, consistent with the binding affinity measurements reported in the previous section. These data suggest that the homophilic interaction between CHO cells transfected with T-cadherin is indeed mediated by the interface identified in the crystal structure.

X-dimer interface mutants fail to inhibit neurite outgrowth *in vitro*

In addition to its cell adhesive activity, T-cadherin has been implicated in the control of axon growth and guidance^{31,32,40,41}. We used the mutants identified above to determine whether X-dimer engagement is also necessary to elicit the regulatory effects of T-cadherin on neurite outgrowth. Neurite outgrowth from motor neurons is robust when grown on a monolayer of naïve CHO cells, but stunted when the neurons are grown on a monolayer of CHO cells that stably express T-cadherin³². To determine whether structural elements of the X-dimer are necessary for T-cadherin mediated inhibition of neurite outgrowth, we assessed the extension of motor axons when presented with an X-dimer incompetent R14S D140S substrate.

Neurite outgrowth assays were performed with ES cell-derived motor neurons⁴² grown on naïve, T-cadherin expressing, and R14S D140S T-cadherin expressing CHO cell monolayers. Surface expression of wild-type and mutant T-cadherins in stably transfected CHO cells was verified by surface biotinylation (Supplementary Fig. 3A). The ES cell-derived motor neurons express T-cadherin, as shown by Western blotting analysis with an anti-T-cadherin antibody (Supplementary Fig. 3B). Immunostaining with the same antibody demonstrated that the majority of ES cell-derived motor neurons express T-cadherin on their surfaces (data not shown). ES cell-derived motor neurons cultured on naïve CHO cell monolayers for ~18h grew long axons (Figs. 7A and B). In agreement with previous reports³², a ~50% reduction in axonal length was observed when motor neurons were cultured on CHO cells expressing wild-type T-cadherin (Figs. 7A and B). In contrast, neurite lengths of motor neurons grown on CHO cells expressing the R14S D140S T-cadherin mutant were similar to that observed on naïve control cells (Fig. 7A and B). Thus, the T-cadherin mediated inhibitory effect on neurite length is abolished by a mutation that disrupts the T-cadherin X-dimer interface.

Nevertheless, since ES cell derived motor neurons express wild-type T-cadherin, the question remains whether homophilic interaction between the neuron- and CHO cell-presented T-cadherin is required for effects on neurite outgrowth. To address this question, we performed similar experiments using spinal neurons dissected from wild-type and T-cadherin knockout mice³⁴. Dissociated neurons were cultured on monolayers of CHO cells expressing either

wild-type T-cadherin, or one of the four dimer interface mutants, with naïve CHO cells used as controls. After 21 h the cultures were fixed and immunostained using the neuron specific β III Tubulin antibody (TUJ-1). Neurite lengths were measured and compared in each condition.

Spinal neurons isolated from wild-type mice showed a ~50% reduction of neurite length on wild-type T-cadherin substrates in comparison with parental non-transfected CHO control cells. However, neurons isolated from T-cadherin knock-out mice extended axons to a similar length as neurons presented with control substrates (Fig. 7C). Neurons from wild-type mice grown on substrates expressing each of the dimer interface mutants also showed neurite lengths similar to neurons grown on control substrates (Fig. 7C). Together, these results provide evidence that T-cadherin-mediated neurite outgrowth inhibition relies on the ability to form *trans* dimers using the non-swapped X-interface identified in the crystal structure.

DISCUSSION

Mechanisms of classical cadherin adhesion have been understood mainly through the strand-swap model of binding between cadherin EC1 domains^{13,18,22,43}. However, the vast majority of cadherins lack the sequence determinants of strand swap binding⁵. The structures of T-cadherin presented here define a binding mechanism for a non-classical cadherin that lacks the signatures of strand swapping. Our results show that T-cadherin engages in intercellular recognition through a homophilic interface that is distinct from that of classical cadherins. Cell-based experiments *in vitro* show that binding through this interface is required for cell adhesion, and that T-cadherin mediated inhibition of neurite outgrowth depends on the homophilic engagement of T-cadherin.

The novel adhesive interface of T-cadherin, designated here the “X-interface”, joins two elongated EC1-2 molecules through a region near their Ca^{2+} binding sites to form a tetrahedral, X-like, form. A number of the Ca^{2+} liganding residues participate in contacts that span this interface. The X-interface buries approximately 2200 Å², significantly more than the type I cadherins strand swapped interfaces (between 1600 Å² and 1800 Å²), and slightly less than that of type II cadherins (around 2700 Å²). Yet these interfaces differ in character: while the X-interface is formed exclusively by surface interactions with shallow features, the strand swap interface is centered on the insertion of hydrophobic Trp side chains into the hydrophobic core of the cadherin binding mate. Despite a far less intimate association than in strand-swapped cadherin dimers, the ~25 μM K_D binding affinity of the T-cadherin X-dimer is somewhat tighter than has been reported for both E-^{18,39,44} and C-cadherins³⁸. This apparent paradox probably has its basis, in part, in the idea that domain swap binding leads to lowering of interaction affinities²³, but it may also be due to the removal of charged groups, including the bound Ca^{2+} ions, from the vicinity of the solvent environment. In fact, both sets of binding affinities are relatively weak compared to protein-protein interfaces that bury comparable amounts of surface area²³.

The similarity in dimerization affinities between T-cadherin and classical cadherins is consistent with the ability of T-cadherin to mediate cell adhesion. The cell aggregation assays reported here confirm previous work²⁵ showing that T-cadherin promotes cell-cell adhesion despite the absence of a cytoplasmic or transmembrane domain. Moreover, the inhibition of adhesion by mutants designed to disrupt dimerization suggests that the X-interface represents the molecular interaction through which T-cadherin mediates adhesion.

The effects we observe on motor axon patterning on mutant and wild type T-cadherin substrates, taken as a whole, can best be explained by the formation of *trans* X-dimers between T-cadherin molecules. Our findings show that T-cadherin-mediated neurite responses depend on substrate T-cadherin molecules competent to form X-dimers, and the presence of T-cadherin

on neuronal surfaces. Further, mutations that disrupt X-dimer formation interfere with T-cadherin-mediated cell adhesion, a process that depends on intermolecular interactions between molecules from contacting cells. Thus, X-dimer formation between T-cadherin on neurons and in the environment provides the simplest model consistent with our data, although other explanations, including X-dimer formation in *cis*, or contributions from unidentified accessory molecules cannot be excluded as alternative possibilities.

It is striking that E-cadherin fragments with extra N-terminal residues that cannot strand-swap form binding interfaces essentially identical to the T-cadherin X-interface^{14,19}. This raises the possibility that the X-dimer may play a functional role in classical cadherins as well as for T-cadherin. Our findings suggest that in classical cadherins the X-like interface could be engaged transiently as an intermediate before the mature strand-swapped interface forms, perhaps contributing to the kinetics of strand swapping (see accompanying manuscript by Harrison *et al.*⁴⁵).

The X-dimer identified here for T-cadherin represents a “second” cadherin adhesive interface. The “first” adhesive interface – the strand swap interface – was revealed in structural studies on classical cadherins, which dimerize through swapping the N-terminal A* strand, including the anchor residue Trp2 which is conserved in all classical EC1 domains^{13,18,20}. Sequence- and structure-based analyses reveal that there are determinants of strand swapping that are unique to classical and desmosomal cadherin EC1 domains⁵. These include the conserved Trp2 which is present only in EC1, and a shorter “hinge” region between the A* and A strands which may induce strain in the monomer conformation, thus favoring strand swapping. Except for the EC1 domains of classical and desmosomal cadherins, these features are absent in all cadherin domains found in vertebrates and invertebrates⁵. This suggests that most cadherin domains will not dimerize through strand exchange. T-cadherin has the same number of residues in the A*/A strand region as do classical cadherins but the conserved Trp2 is replaced by Ile. This alone may inhibit swapping, but other factors may also play a role.

Database sequence analyses suggest that the majority of cadherins, including protocadherins, T-cadherin, and all cadherins from invertebrates, are likely to bind through a mechanism that does not involve strand swapping⁵, raising the question of whether the adhesive mechanism found for T-cadherin will be predictive of the nature of interactions for these cadherins. We note that the calcium binding regions, which contribute structural elements to the adhesive interface of T-cadherin, are among the most highly conserved in sequence, even in highly-divergent non-classical cadherins^{4,5}. Future studies will be required to address whether other non-classical cadherins mediate binding via interfaces related to the X-dimer.

METHODS

Crystallographic methods

We expressed and purified proteins as described in Supplementary Methods. We grew crystals with the vapor diffusion method by mixing equal volumes of protein solution and crystallization buffer at 4°C. The crystallization conditions were as follows (added cryoprotectants are indicated in parentheses): mouse T-cadherin EC1: 0.2M ammonium sulfate, 20% (w/v) PEG 3350, pH 6.0 (30% [v/v] glycerol); chicken and *Xenopus laevis* T-cadherin EC1 crystallized in the same conditions: 50mM Zn acetate, 20% (w/v) PEG 3350 (35% [v/v] glycerol or 25% [v/v] ethylene glycol); mouse T-cadherin EC1-2: 50% saturated ammonium sulfate, 33mM Na citrate pH 5.6 (15% [v/v] glycerol); chicken T-cadherin EC1-2: 20% (w/v) MPD, 15% (w/v) PEG 400, 50mM Na acetate pH 5.5 (35% [v/v] glycerol). We obtained two phasing derivatives of the mouse T-cadherin EC1-2 crystals by soaking either in mother liquor containing 1mM methylmercury (II) chloride for 1 hour, or in cryoprotectant solution supplemented with 0.5 M NaBr for 30 seconds prior to flash freezing. Complete

datasets from crystals frozen at 100K were collected at the SGX-CAT beamline facilities (ID-31) at the Advanced Photon Source (mouse T-cadherin EC1-2), or at the National Synchrotron Light Source at Brookhaven National Laboratory, beamlines X4C (mouse T-cadherin EC1) and X29 (chicken T-cadherin EC1 and EC1-2, and *Xenopus laevis* T-cadherin EC1). We processed and scaled the data with the HKL2000 suite (mouse T-cadherin EC1 and EC1-2), XDS (chicken T-cadherin EC1), or Mosflm and Scala (*Xenopus laevis* T-cadherin EC1 and chicken T-cadherin EC1-2). We collected all data sets at 0.9791Å wavelength, except the bromine experiment which we collected at 0.9202Å.

We obtained initial low-resolution phases for the mouse T-cadherin EC1-2 crystal structure by SIR/AS analysis of the methyl mercury derivative using site positions determined with the program Solve⁴⁶. These phases were then utilized to locate the positions of ordered bromine atoms in anomalous-difference Fourier maps. We performed phase calculations utilizing both the mercury and bromine sites, using Sharp⁴⁷ to generate experimental electron density maps. After solvent flattening using Resolve⁴⁸, models of the individual mouse T-cadherin EC1 and EC2 domains were fit into the experimental maps and fitted manually using the program Coot⁴⁹. We performed structure refinement with CNS⁵⁰ and Refmac⁵¹. We solved all other crystal structures by molecular replacement with the program Phaser⁵² using the refined mouse T-cadherin EC1 or EC2 domain structures as independent search models. Rounds of manual model fitting in Coot were alternated with refinement using Refmac (all structures) and CNS (mouse and chicken T-cadherin EC1). Ramachandran angles were in the most favored regions for 87.3%, 79.0%, 92.6%, 93.8%, and 87.7% for the mouse EC1-2, chicken EC1-2, mouse EC1, chicken EC1, and *X. laevis* EC1 structures, respectively. Crystallographic statistics are summarized in Table 1.

Cell aggregation and neurite outgrowth assays

We performed short term aggregation assays essentially as described previously except incubation time was 45 minutes²².

We prepared spinal neuron primary cultures from E14 wildtype (C57/BL6, Harlan–Sprague–Dawley, Indianapolis, IN) and T-cadherin knockout mice³⁴. We removed spinal cords from 4-6 mouse embryos and dissociated cells in 30U mL⁻¹ Papain for 45 minutes at 37°. We performed neurite outgrowth assays essentially as described⁵³ by depositing $\sim 7.5 \times 10^4$ dissociated neurons onto confluent monolayers of CHO-FLP cells transfected with wildtype mouse T-cadherin, or T-cadherin having single or double point mutations in 8-well chamber slides (Nunc). We incubated cultures for 21 hours at 37° in 50% DMEM/F-12 and 50% Neurobasal media (Invitrogen) supplemented with 2% (v/v) horse serum, 1× B27, 1× N2 (Invitrogen), 100pg mL⁻¹ GDNF, 10ng/ml CNTF, and 1ng/ml BDNF (Sigma). We then fixed cultures for 30 minutes with 2% (w/v) PFA. Neurons and neurites were observed using immunohistochemistry with antibodies against Tuj-1 (Covance Research Products). We measured neurites using ImageJ with identical contrast and brightness settings for each condition. N=3 independent experiments, we measured 200-600 neurites for each condition for each experiment. Statistical analyses of each neuron genotype consisted of one-way ANOVA and Tukey post-test, using Prism.

Embryonic stem cell-derived motor neuron cultures

We derived ES cells from Hb9-GFP transgenic mice and differentiated into motor neurons as described previously⁴². Briefly, ES cells were grown for 2 days to form embryoid bodies and then treated with 1μM retinoic acid (Sigma) and 1μM sonic hedgehog agonist (Hh-Ag1.3, Curis Inc.) for 5 days to induce motor neuron differentiation. We then dissociated cells using papain (Worthington) and cultured for 24 hrs in 1:1 Advanced DMEM/F12 : Neurobasal, supplemented with 1X B27, 2mM L-Glutamine, and 5ng mL⁻¹ GDNF. We fixed cultures after

21 hours and immunostained with anti GFP antibody (Molecular Probes) for neurite outgrowth assay as described above.

Cell surface immunostaining

Twenty-four hours after plating, we incubated living cells in primary antibody diluted in L-15 medium + 8 mM glucose for 30 min at room temperature. We first washed cells two times with L-15 medium + 8 mM glucose and then incubated the cells with secondary antibody (Cy3-conjugated anti-rabbit) for 20 min at room temperature. Following fixation in 4% (v/v) paraformaldehyde for 10 min at 4°C, we washed the cells three times in cold 1X PBS and then we mounted the cells in Vectashield mounting medium and analyzed using confocal microscopy. Surface localization of cadherin was confirmed using a cell surface biotinylation assay (Supplementary Methods).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structures of T-cadherin EC1 domains. (A) Superposed ribbon diagrams from crystal structures of mouse, chicken, and *X. laevis* T-cadherins. (B) Expanded view of A-strand region. The residue highlighted in yellow, Ile2, aligns with the strand swap anchor tryptophan residue of classical cadherins. Side chains are shown for residues with contacts less than 4 Å from Ile2 side chain.

Figure 2.

Structures of X-dimer form chicken T-cadherin, and comparison with the mutant E-cadherin¹⁴ 1EDH X-dimer structure. (A) Ribbon diagram of T-cadherin X-dimer, with bound calcium ions shown as green spheres; (B) X-dimer from E-cadherin N-terminal extension mutant; Close-up view showing atomic-level interactions in the T-cadherin interface (C), and the E-cadherin interface (D). Interactions are described in the main text. Asn12, Asp102, Asp103, Ser138 and Leu143 are buried in the T-cadherin X-dimer interface but do not participate in identifiable specific interactions. Residues Arg14 and Asp140, underlined in (C), were mutated to disrupt the T-cadherin interface (see text). (E) Comparison of EC1 domain interface regions of the T-cadherin X-dimer with the X-dimer and strand-swapped dimer of E-cadherin (from Haussinger *et al* 2004).

Figure 3.
Calcium-free structure of mouse T-cadherin EC1-EC2. Side chains within the calcium-binding region are shown.

Figure 4.

SPR analysis of T-cadherin interactions. (A) Binding of a T-cadherin EC1 protein (blue trace) and T-cadherin extracellular domains 1-2 (red trace) at 100 μM each to T-cadherin EC1-2 captured to the sensor surface. The signals were normalized to account for the different masses of the two proteins. Buffer injections are shown in black. (B) Interaction of 100 μM T-cadherin EC1-2 with T-cadherin EC1-2 tethered to the chip surface in the presence of 3 mM CaCl₂ (shown in orange) and 3 mM EDTA (shown in green). The buffer injections are represented as black traces. (C) Interactions of T-cadherin EC1-2 mutants R14E, R14E D140S, R14S and R14S D140S with sensor-bound T-cadherin EC1-2. For each experiment, binding was tested at a concentration range 100-0.78 μM (identical concentrations were used for each protein tested). The responses for all mutants have been scaled to the responses of the wild type EC1-2 domain, in cases where no binding is observed (e.g. R14E and lower concentrations of R14S) the traces collapse together at zero response.

Figure 5.

Sedimentation equilibrium AUC profiles of T-cadherin and E-cadherin wild-type and mutant proteins. (A) Mutations designed to inhibit strand swapping have no effect on the monomer-dimer equilibrium observed for mouse T-cadherin EC1-2. (B) Strand swapping mutations substantially lower the affinity of homodimerization of mouse E-cadherin EC1-2 fragments. (C) Mutation of the X-dimer interface in T-cadherin yields a monomeric profile in AUC; homodimerization is partially restored by D140K mutation (see main text).

Figure 6.

Cell aggregation experiments show that the X-interface is required for adhesion. Non-transfected CHO cells and T-cadherin transfectants in the presence of EDTA show no aggregation. Wild-type T-cadherin transfectants show robust aggregation in the presence of calcium, which is ablated by mutations within the X-interface. Scale bar 300 μ m.

Figure 7.

T-cadherin mediated inhibition of neurite outgrowth depends on the X-interface. (A) Fluorescence micrographs of HB9:GFP ES cell-derived motor neurons cultured on either naïve CHO cells, T-cadherin expressing CHO cells, or X-interface mutant T-cadherin expressors. (B) Mean neurite outgrowth length of ES cell-derived motor neurons cultured on naïve, wild-type, or mutant T-cadherin expressing CHO cells. (C) Mean neurite outgrowth length of cultured mouse spinal motor neurons from wild-type and T-cadherin knockout animals, cultured on naïve, wild-type, or mutant T-cadherin expressing CHO cells.

crystallographic statistics

Table 1

A. Data processing and refinement									
Dataset	Mouse T-cad EC1-2 native	Mouse T-cad EC1-2 MM	Mouse T-cad EC1-2 Br	Chicken T-cad EC1-2	Mouse T-cad EC1	Chicken T-cad EC1	X. laevis T-cad EC1		
Data processing									
Space group and unit cell	H32 107.7 226.7	H32 107.7 226.4	H32 108.6 227.3	P2 ₁ 2 ₁ 2 ₁ 55.8 79.2 105.2	P6 ₂ 50.4 122.5	P2 ₁ 2 ₁ 2 ₁ 44.15 44.93 56.67	14 ₁ 22 78.91 62.58		
Molecules per asymmetric unit	2	2	2	2	2	1	1		
Resolution limit (Å)	2.0	2.4	2.7	2.9	1.8	1.1	1.8		
Unique reflections	34355	19932	14544	10854	16137	42837	9439		
Redundancy	11.1	11.2	22.2	6.3	10.8	5.7	13.4		
Completeness (%) (highest resolution shell)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	99.9 (99.9)	99.3 (99.9)	99.8 (97.3)	99.9 (100.0)		
Average I/σ(I) (highest resolution shell)	31.4 (11.6)	38.5 (9.8)	47.3 (12.9)	5.3 (2.3)	45.2 (18.0)	11.7 (6.4)	11.0 (3.6)		
Rmerge (%) (highest resolution shell)	6.7 (27.3)	5.9 (27.3)	6.9 (27.4)	12.1 (30.5)	4.8 (12.5)	11.7 (18.6)	3.9 (20.9)		
Refinement									
Rwork (%)	21.3	--	--	21.3	16.8	14.2	22.7		
Rfree (%)	25.9	--	--	29.4	22.6	15.8	28.4		
Rmsd bonds (Å)	0.02	--	--	0.007	0.009	0.015	0.017		
Rmsd angles (°)	2.1	--	--	1.2	1.4	2.0	1.9		
Protein atoms	3356	--	--	3330	1538	760	760		
Water molecules	195	--	--	27	221	354	102		
Average B (Å ²) (protein atoms)	29.6	--	--	33.5	21.0	7.6	30.8		
Average B (Å ²) (water molecules)	30.0	--	--	18.7	31.0	26.6	33.6		

Table 2

Dissociation constants (K_D) from equilibrium AUC analyses of T-cadherin and E-cadherin fragment homodimerization

Protein	Nature of the mutation	K_D (μM)
T cadherin EC1-2 wild-type (WT)	wild type	41.4 ± 1.7
T cadherin EC1-2 MR extension	strand-swapping mutants	34.1 ± 6.3
T cadherin EC1-2 I2A		37.1 ± 4.1
T cadherin EC1-2 G extension		16.5 ± 0.80
T cadherin EC1-2 R14E	X-dimer mutants	monomer
T cadherin EC1-2 R14E/D140K (charge reversal rescue mutant)		149 ± 17
E cadherin EC1-2 wild-type (WT)	wild type	98.6 ± 15.5
E cadherin EC1-2 MR extension	strand-swapping mutant	257.5 ± 17.5
E cadherin EC1-2 W2A		916 ± 47