Mutations affecting transmembrane segment interactions impair adhesiveness of E-cadherin

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SUMMARY

Lateral clustering of E-cadherin molecules is required for the adhesive properties of this cell-cell adhesion molecule. Both the extracellular domain and the cytoplasmic region of E-cadherin were previously reported to contribute to lateral clustering, but little is known about a role of the transmembrane domain in this respect. Following our previous findings indicating self-assembly of artificial transmembrane segments based on leucine residues, we asked whether the leucine-rich transmembrane segment of E-cadherin participates in lateral clustering. Here, we demonstrate that its transmembrane domain self-assembles as analyzed using the ToxR reporter system. Certain point mutations within the transmembrane domain markedly reduced self-assembly. To study whether the same point mutations also affect E-cadherin-mediated adhesion in vivo, wild-type and mutant E-cadherin cDNAs were transfected into Ltk− cells. Indeed, cell aggregation assays revealed significantly reduced adhesiveness when mutations had been introduced which disrupted transmembrane segment interaction. In control experiments, cell-surface expression, interaction with catenins and the cytoskeleton as well as trypsin-resistance of the protein were unaffected. These data suggest that interactions between the transmembrane segments are important for the lateral association of E-cadherin molecules required for cell-cell adhesion.

Key words: E-cadherin, Transmembrane segment, Lateral clustering, Cell adhesion

INTRODUCTION

Cadherins comprize a family of homophilic, Ca2+-dependent, single-span transmembrane cell-cell adhesion molecules that have a crucial function in tissue formation and maintenance during development and in adult organisms (Gumbiner, 1996; Huber et al., 1996a; Larue et al., 1994; Riethmacher et al., 1995). The transmembrane segments (TMSs) and cytoplasmic tails of classical cadherins are those domains that exhibit the highest homology among different family members. The tails are associated with a set of cytoplasmic proteins, the catenins (α-, β-, γ-catenin) that connect cadherins to actin (Kemler, 1993). Strong cadherin-mediated cell adhesion is dependent on the correct formation of the cadherin-catenin complex. Cells mutant in one of the catenins show impaired cell adhesion, as exemplified by a number of cancer cell lines (Bullions et al., 1997; Kawanishi et al., 1995; Shimoyama et al., 1992). Recent results indicate that the C-terminal amino acids of cadherins may also be involved in the association of the cadherin-catenin complex with the cytoskeleton (Finnemann et al., 1997). p120ctn, a major src-kinase substrate (Reynolds et al., 1994), the recently identified IQGAP1, a target of small GTPases (Kuroda et al., 1998) and the adaptor protein Shc (Xu et al., 1997) were also found associated with the cytoplasmic domain and are assumed to play a role in the regulation of E-cadherin mediated cell adhesion.

Cadherins are engaged in two different types of self-interaction, homophilic interactions between cadherin molecules localized on opposing cells and lateral interactions within the same cell membrane (Angres et al., 1996; Brieher et al., 1996; Yap et al., 1997a). The selectivity of homophilic binding appears to be specified by the N-terminal extracellular domains of cadherins (Nose et al., 1990). Ultrastructural data revealed that cadherin molecules accumulate in adherens junctions and thus suggested lateral interactions between cadherins on the cell surface (Vestweber et al., 1987). The crystal structures of the amino-terminal domains of N- and E-cadherins revealed non-covalent interactions which were suggested to contribute to homophilic binding and to lateral interactions (Nagar et al., 1996; Shapiro et al., 1995). Indeed, mutations introduced into the interfaces of these N-terminal domain dimers were shown to abolish adhesion presumably by blocking lateral interaction (Tamura et al., 1998), although a recently published new X-ray structure indicates that this might...
be due to abrogated trans-interactions (Pertz et al., 1999). Similarly, previous ultrastructural studies with recombinant E-cadherin ectodomains that were artificially clustered by genetic fusion to the assembly domain of rat cartilage oligomeric matrix protein, showed that the distal ends representing the N-terminal part of E-cadherin were found to be connected (Tomschy et al., 1996). Upon lateral interaction, the cadherin adhesion complexes of contacting cells are assumed to interact (Tomschy et al., 1996) and to coalesce into punctate aggregates parallel to binding to the actin cytoskeleton (Adams et al., 1996, 1998).

In addition to the extracellular domain, the juxtamembrane region of the cytoplasmic tail was recently reported to affect lateral clustering, possibly via intermediate binding proteins (Ozawa and Kemler, 1998; Yap et al., 1998). On the other hand, a role of the single TMS in cadherin adhesiveness has, to our knowledge, not yet been described.

It is well known that specific interactions between TMSs are important for folding and/or oligomerization of many integral membrane proteins (Lemmon and Engelman, 1994). For efficient side-chain packing, the interacting TM-helices frequently assume positive (arkin et al., 1994; Pinto et al., 1997) or negative (MacKenzie et al., 1989) packing angles. Previously it has been shown that these TM helix-helix interfaces displaying positive packing angles within crystallized membrane proteins form by mutual packing of hydrophobic residues arranged in a characteristic heptad repeat motif which is reminiscent of soluble leucine-zipper interaction domains (Cohen and Parry, 1990; Langosch and Heringa, 1998; Rees et al., 1989). Confirming the concept of membrane-spanning leucine zippers, we have found that an oligo-leucine sequence as well as a heptad repeat motif of leucines self-assemble in membranes as well as in detergent solution. A database search with degenerate versions of this heptad pattern of leucines identified TMSs from a wide variety of functionally different proteins. These included members of different cadherin families. Based on the homology of cadherin TMSs to this leucine motif, we predicted that they may also self-assemble and thereby support the lateral interactions between cadherin molecules in the plasma membrane of adhesive cells (Gurezka et al., 1999).

Here, we show that the isolated mouse E-cadherin TMS indeed self-assembles in membranes and that self-assembly is suppressed by certain point mutations. The same TMS mutations introduced into full-length E-cadherin strongly reduced its adhesive function in eukaryotic cells suggesting that TMS interactions may contribute to lateral interaction.

MATERIALS AND METHODS

ToXR plasmids

The parental pToXR construct was made by ligating a synthetic oligonucleotide cassette consisting of oligonucleotides 5'-CTAGCG-GGATCCTCGCCCTGCTGATTCGCTCTGTCTGGGTTCTGCTTTCT-3’ and 5'-GATCAGAACAGCAGACAGACAGAGAATGCAGAACAGGCGAGGATCCCG-3’. The SacI/NheI fragment was cloned into pKSII. The resulting plasmid expressing the maltose binding protein (MBP) was verified by DNA sequencing. The MBP was cloned into the SacI/NheI fragment of pShuttle to generate pShuttleMBP. The resulting plasmid was verified by DNA sequencing. The MBP was cloned into the SacI/NheI fragment of pShuttle to generate pShuttleMBP. The resulting plasmid was verified by DNA sequencing.

ToXR activity assays

Transcription activation was determined upon expression of the ToXR constructs in the indicator strain FHK12 in several independent experiments as described (Langosch et al., 1996). In addition, 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the cultures to enhance the dynamic range of the β-galactosidase activities (in Miller units, MU, means ± s.d.) elicited by the different constructs (Gurezka et al., 1999).

Western blotting was done as described with an antiserum recognizing the MalE moiety of the constructs (Langosch et al., 1996).

Site-directed mutagenesis of E-cadherin

A SacI fragment of pl31NU encoding full-length mouse E-cadherin was cloned into pKSII to generate pKUS. For mutagenesis a Xhol/SacI fragment was cloned into pKSII. The resulting plasmid pKUS was used for the following mutagenesis experiments performed as described (Stappert et al., 1992). The primer 5’-GGTGGTGGGAATCAGGCTCTCAATGCGAAGACATCGTACG-3’ containing the tag sequence necessary for subsequent PCR amplification was used for the primer extension reaction. To replace specific amino acids, the following mutagenic primers were used: 83/2 5’-AACAGCTAGGAGCAGCGGCCGCCAGGAATCAGGCGGCCG-3’; 84/2 5’-CCGGATACGCATCGGCAGGACGGAGGC-3’; 1570A 5’-AAACAGCTAGGAGCAGCGGCCGCAATGCGAAGACATCGTACG-3’; 1570P 5’-AACAGCTAGGAGCAGCGGCCGCAATGCGAAGACATCGTACG-3’ and 1570P 5’-AACAGCTAGGAGCAGCGGCCGCAATGCGAAGACATCGTACG-3’. The mutated strand was selectively amplified by PCR with Pwo DNA-polymerase (Boehringer Mannheim, Germany) using the primer 5’-GGTGGTGGGAATCAGGCTCTCAATGCGAAGACATCGTACG-3’ and 3’ primer. PCR products were digested with Xhol/SacI and cloned into pKSII vector. New Smal or ApaI sites introduced with oligonucleotides 83/2 or 84/2, respectively, were used to identify the mutants which were verified by sequencing. Ndel/NheI fragments encompassing the point mutations were subsequently cloned into pKUS after deletion of the wild-type Ndel/NheI fragment. The SacI fragments of these plasmids then was reintroduced into the SacI digested starting plasmid pl31NU resulting in the mutagenized mouse E-cadherin expression vectors pl31NU83 and pl31NU84. pl31NU1570A and pl31NU1570P were generated by ligation of the Xhol/NheI fragment of the mutagenized PCR products into Xhol/NheI digested pl31NU and mutants were identified and verified by sequencing.

DNA transfection

Purified plasmid DNAs were introduced into mouse Ltk− cells by calcium-phosphate precipitation as described (Huber et al., 1996b). After 2 weeks of G418 (1 mg/ml) selection, surviving cells were picked and propagated at 0.2 mg/ml G418. The E-cadherin expression level of obtained clones was analyzed by fluorescence activated cell scans (FACS) and Western blotting. Cultures to enhance the dynamic range of the β-galactosidase activities (in Miller units, MU, means ± s.d.) elicited by the different constructs (Gurezka et al., 1999).

Immunoprecipitation and immunofluorescence

[35S]Met/Cys metabolic labeling, Triton X-100 solubilization and immunoprecipitations with 2 μg anti-gp84 antibodies (Kasper et al., 1995) were done as described (Huber et al., 1997). For
immunofluorescence, cells were grown on collagen-coated coverslips and fixed in methanol for 10 minutes at −20°C. After blocking in PBS, 1% (v/v) FCS, cells were incubated with anti-gp84 (5 μg/ml), anti-E-cadherin antibody (0.5 μg/ml) (C20820, Transduction Laboratories, USA) or anti-β-catenin antibody (5 μg/ml) (Transduction Laboratories, USA), followed by incubation with the appropriate DTAF-conjugated secondary antibody (Dianova, Germany). Western blotting was done as described (Huber et al., 1997). To analyze the trypsin resistant 84 kDa fragment of E-cadherin, cells were trypsinized in HBS, 2 mM CaCl2, 0.1% (w/v) trypsin and incubated for 2 hours at 37°C under constant agitation. Subsequently, cells were pelleted by centrifugation and the supernatants were subjected to immunoprecipitation with anti-gp84 antibody.

Cell aggregation assays
For cell aggregation assays, 10⁶ cells plated as single cells and grown over night were washed with HBS, 2 mM CaCl₂ and dissociated with HBS, 2 mM CaCl₂ containing 0.01% (w/v) trypsin for 10 minutes at 37°C. After washing with a 1:1 mixture of HBS and DMEM containing 5% (v/v) FCS, cells were resuspended in 1 ml of this mixture in the presence of 5 μg/ml DNase I. Cells were allowed to aggregate for 45 minutes at 37°C with a constant rotation of 70 rpm. The extent of cell aggregation was determined by counting the number of particles and expressed according to the method of Nagafuchi and Takeichi (1988) by the index (N₀–Nₜ)/N₀, where N₀ is the number of particles at the beginning of incubation and Nₜ is the number of particles counted after incubation. Aggregation assays with independent clones yielded comparable data.

RESULTS
Self-assembly of the E-cadherin TMS
A remarkable feature of different cadherin TMSs is the prevalence of leucine residues. Previously, we showed that an oligo-leucine sequence or a heptad repeat motif of leucines grafted onto an alanine host sequence (LLAALLALLA)ALLALLALL) function as artificial TMSs which self-assemble via mutual packing of the leucine side-chains. Based on the partial homology of the leucines within this motif and cadherin TMSs, we predicted that the latter may be involved in lateral assembly of cadherin polypeptides on the surface of eukaryotic cells (Gurezka et al., 1999). Here, we initially examined the potential self-assembly of the mouse E-cadherin TMS using the ToxR transcription activator system. This reporter system is based on an engineered version of the ToxR protein that is anchored by a single TMS of choice within the inner membrane of *Escherichia coli* where it is thought to exist in a monomer/dimer equilibrium. The dimeric form binds to the cholera toxin promoter thus activating expression of a downstream lacZ reporter gene which therefore indicates self-assembly in the membrane (Fig. 1A) (Kolmar et al., 1995). We previously established this system as a reliable tool to study TMS interactions using the structurally well characterized glycophorin A TMS dimer for reference (Brosig and Langosch, 1998; Langosch et al., 1996).

Expressed in the context of the ToxR protein, the leucine-rich part of the E-cadherin TMS elicited significant transcription activation (905±224 MU, mean ± s.d.) which was similar to that elicited by the leucine heptad motif described above (929±186 MU; see: Gurezka et al., 1999). To disrupt self-assembly, we introduced several point mutations. Proline residues are known to introduce kinks into TM-helices (von Heijne, 1991) and are thus predicted to affect their self-assembly. Therefore, we initially substituted four consecutive residues covering one full turn of a presumed α-helix individually to proline. These mutations reduced the signal by an average of 42% (Fig. 1B,C). This disruptive effect of the proline mutations could be enhanced by simultaneously mutating the N- or C-terminal neighbours of proline 571 to

![Fig. 1. Self-assembly of the cadherin TMS in membranes.](image-url)

(A) Functional organization of ToxR chimeric proteins. The cytoplasmic ToxR domain is linked via the E-cadherin TMS (CAD) to the periplasmic MalE moiety. Upon dimerization, ToxR binds to the cholera toxin (ctx) promoter thus initiating lacZ transcription in the indicator cells. MalE, maltose binding protein; OM, outer membrane; IM, inner membrane. (B) Amino acid sequences of the mouse E-cadherin TMS and the mutants derived thereof. Dots represent wild-type residues. (C) Various levels of transcription activation determined upon expressing the constructs in the reporter strain FHK12 indicate sequence-specific TMS assembly in the membrane. The bars represent mean specific β-galactosidase activities calculated from the number of data points given for each construct; error bars denote standard deviation. (D) Western blot of transformed FHK12 cells demonstrates similar expression levels of the different 65 kDa proteins. The order of samples corresponds to that in C. Residues are numbered according to Ringwald et al. (1987).
Expression of E-cadherin TMS mutants in Ltk\(^{-}\) cells

To assess whether the E-cadherin TMS is important for the adhesive properties of E-cadherin, mutations affecting TMS self-assembly efficiently (I570G/L571P and L571P/L572G), moderately (I570P) or not measurably (I570A) in our ToxR reporter assays were introduced into full-length E-cadherin. Wild-type and mutant E-cadherin cDNAs were transfected into Ltk\(^{-}\) cells, a fibroblast cell line devoid of endogeneous cadherin (Nagafuchi and Takeichi, 1988). Stable clones were isolated and clonal cells expressing comparable levels of wt or mutant E-cadherins (I570G/L571P, L571P/L572G, I570P, I570A) were isolated by fluorescence-activated cell sorting (FACS) and magnetic cell separation.

To ascertain that the TMS mutations did not impair cellular localization, association with catenins or the overall structure of E-cadherin, we performed a number of control experiments. Surface expression of mutant E-cadherin was verified by indirect immunofluorescence. Anti-gp84 antibodies recognizing the extracellular domain gave similar cell surface staining for wt and mutant E-cadherins (Fig. 2). In stainings with anti-\(\beta\)-catenin antibodies, \(\beta\)-catenin colocalized with E-cadherin at the plasma membrane (data not shown).

Since association with cytoplasmic catenins is essential for E-cadherin function, formation of cadherin-catenin adhesion complexes was analyzed by co-immunoprecipitation experiments. E-cadherin from [\(^{35}\)S]Met/Cys labeled cell lysates adjusted to equal amounts of total protein was precipitated with the anti-gp84 antibody. Proteins with apparent molecular masses of 102 kDa, 88 kDa and 82 kDa co-precipitated with comparable amounts of wt or mutant E-cadherins (I570G/L571P, L571P/L572G, I570P, I570A) were isolated by fluorescence-activated cell sorting (FACS) and magnetic cell separation.

To confirm that mutant E-cadherins behave identical to wt protein in this respect, the detergent-soluble and -insoluble fractions were compared by immunoblotting. No difference in the distribution of the cadherin-catenin complex could be detected in cells expressing wt or mutant E-cadherins (Fig. 4).
To address the structural integrity of the mutant E-cadherins, we examined their resistance to tryptic digestion in the presence of Ca²⁺. Extensive tryptic digestion (0.1% trypsin in the presence of 2 mM Ca²⁺ for 2 hours) generated a specific soluble and stable 84 kDa fragment which was immunoprecipitated from the supernatants of cells expressing either wt or mutant E-cadherin (Fig. 5). Since this fragment is a diagnostic marker for the correct native structure of the extracellular domain (Ozawa and Kemler, 1990), we exclude that the TMS mutations affect the E-cadherin extracellular domain structure. No proteolysis of E-cadherin was detectable in cells treated with 0.01% trypsin in the presence of 2 mM Ca²⁺, the standard conditions used for cell dissociation prior to the cell aggregation assays (data not shown).

**Cell aggregation assays**

From the biochemical analyses described above, it was concluded that the TMS mutations had no detectable effect on several characteristic features of E-cadherin. To test whether the TMS mutations influence the adhesive properties of E-cadherin, cell aggregation assays were performed. To this end, single cell suspensions of the different clonal cell lines were generated by trypsinization and allowed to aggregate for 45 minutes under constant agitation. The number of particles corresponding to the sum of single cells and cell aggregates was counted before and after the incubation period. Based on these numbers, the aggregation index was calculated as specified in Materials and Methods. Importantly, Ltk⁻ cells expressing E-cadherins with mutations disrupting self-assembly showed a marked decrease in adhesiveness with an about 40%-60% lowered aggregation index (I570G/L571P, 0.40±0.16; L571P/L572G, 0.62±0.04; I570P, 0.57±0.12; means ± s.d., n=4) as compared to cells expressing wt protein (0.95±0.04) or the non-disruptive mutant I570A (0.92±0.03) (Fig. 6A). In accordance with particle counting, visual examination confirmed that aggregates of cells characterized by reduced aggregation indices were significantly reduced in size (Fig. 6B). Untransfected cells did not aggregate to a significant degree. These results demonstrate that the adhesive properties of E-cadherin TMS point mutants are correlated to the levels of TMS self-association analyzed by the ToxR system.

**DISCUSSION**

E-cadherin-mediated cell-cell adhesion is crucial for epithelial cell differentiation and tumor progression. In this context it is important to understand the molecular mechanisms influencing the adhesive properties of E-cadherin. Adhesive strength is regulated by the levels of cadherin expression, the proper association of cadherins with the cytoskeleton mediated by the catenins, the homophilic binding affinities of cadherins and/or the lateral clustering of cadherin molecules on the cell surface (for review see: Yap et al., 1997b). Lateral clustering of cadherin molecules is a prerequisite for strong adhesion (Angres et al., 1996; Brieger et al., 1996; Yap et al., 1997a). Previous reports indicated that the N-terminal ectodomain supports lateral interaction of N- and E-cadherins (Nagar et al., 1996; Pertz et al., 1999; Shapiro et al., 1995; Tamura et al., 1998; Tomschy et al., 1996). Also, a juxtamembrane region of the cytoplasmic domain was implicated in lateral clustering of cadherins, although controversial results were obtained in
studies with different cadherin family members. VE-cadherin lacking the COOH-terminal 82 residues (Navarro et al., 1995) and C-cadherin retaining the juxtamembrane 94 residues (Yap et al., 1998) were reported to exhibit cell adhesive activity whereas the adhesiveness of E-cadherin lacking the C-terminal 37 residues (Ozawa and Kemler, 1998) was impaired. In this study, we investigated the relevance of the TMS for the adhesive function of mouse E-cadherin on the molecular and cellular level. Based on our previous observation showing that artificial and natural leucine-rich TMSs tend to self-assemble (Gurezka et al., 1999), we had predicted a contribution of the TMS to lateral interaction between E-cadherin molecules. Using the ToxR reporter system we indeed found that the E-cadherin TMS self-assembles in a membrane environment. The identity of the residues forming the E-cadherin TMS interface is currently not clear. When the TMS sequence is aligned to the repeated heptad \(abcdefg\) pattern characteristic of leucine zippers (Langosch and Heringa, 1998; Lupas, 1996), the majority of its leucine residues align with the \(a, d, e,\) and \(g\) positions forming the interfaces of these helix-helix interaction domains. Assuming \(\alpha\)-helicity of the E-cadherin TMS, we speculate therefore that its self-assembly is mediated by a leucine zipper type of side-chain packing. This type of TM-helix-helix interaction has also been described to account for self-assembly of other single-span membrane proteins like phospholamban (Arkin et al., 1994; Simmerman et al., 1996) and the M2 proton channel (Pinto et al., 1997).

To examine the functional relevance of E-cadherin TMS-TMS interactions, we compared the effects of different TMS mutations on TMS self-assembly with their effects on adhesiveness of full-length E-cadherin in cell-culture. We found that only those mutations disrupting TMS self-assembly reduced cell-cell adhesiveness. In control experiments, we ascertained, that the TMS mutants behaved like their wild-type control in several aspects known to potentially influence adhesiveness. Cell surface expression of mutant and wt E-cadherins was comparable thus excluding that differences in protein levels accounted for reduced adhesiveness. Association of catenins with the cytoplasmic domain and the identical distribution of
E-cadherin molecules within the detergent-soluble and -insoluble fraction indicated that the association of the cadherin-catenin complex with the actin cytoskeleton was not affected by the TMS mutations. Furthermore, trypsin resistance suggested that the TMS mutations do not indirectly influence the structure of the extracellular domain. Thus, we ascribe reduced adhesiveness to a decrease in lateral clustering caused by the TMS mutations. We can, however, presently not exclude that the TMS mutations extend subtle effects on the conformation of the extracellular domains and/or interfere with a signalling function of E-cadherin.

An E-cadherin-GFP fusion protein was recently employed in a high resolution tracking study of E-cadherin during the formation of cell-cell contacts (Adams et al., 1998). This study defines three stages of contact formation. At initial sites of cell-cell contacts, E-cadherin is clustered into puncta and may be connected to the actin cytoskeleton reducing its mobility within the cell membrane. In a second stage, the E-cadherin clusters increase in size and form adhesion plaques at the ends of developing contacts. The coordinated reorganization of actin cables in stage three maximizes the contact area between two cells and strengthens contacts. Within the framework of this model, we assume that interactions between E-cadherin TMSs are involved in stage one where initial E-cadherin clusters have to be formed. Therefore, the TMS may represent a domain acting in concert with the previously described interaction domains. A direct demonstration of an effect of the TMS mutations on lateral assembly within the Ltk− cell membrane was not feasible since the complex of E-cadherin molecules in Ltk− cells is refractory to chemical crosslinking and dissociates upon detergent solubilization and sucrose gradient analysis as also demonstrated in earlier reports (Ozawa and Kemler, 1992; Ozawa and Kemler, 1998). This is in contrast to recent observations made in A431 cells, where lateral dimers of E-cadherin or E-cadherin-desmoglein chimeric molecules could be demonstrated by sucrose gradient centrifugation or immunoprecipitation experiments (Chitaev and Troyanovsky, 1998; Norvell and Green, 1998). We assume that this is due to a different nature of the two cell lines. Human epidermoid carcinoma A431 cells exhibit typical adhesive and cytoskeletal structures of epithelial cells, whereas Ltk− cells are of fibroblast origin and may miss some components that stabilize E-cadherin clusters. Conceivably, therefore, the effect of TMS mutations may be more pronounced in Ltk− cells. Recently, Takeda et al. (1999) reported that E-cadherin dimers in EL-cells can be chemically cross-linked. We applied this technology to compare our E-cadherin constructs but were not able to detect a specific crosslinking product using different concentrations of crosslinker including those reported (Takeda et al., 1999).

Our results may provide an alternative explanation for observations made with N-cadherin truncation mutants (Kintner, 1992; Hermiston and Gordon, 1995). Upon overexpression in the presence of endogenous full-length cadherin, mutants lacking the extracellular domain had a dominant-negative effect on adhesiveness. This was originally attributed in part to competitive binding to catenins via the cytoplasmic domains (Kintner, 1992). Recent data, however, show that these constructs reduce expression of endogenous catenins by decreasing their metabolic stability independent of competition for catenins (Nieman et al., 1999; Troxell et al., 1999). Furthermore, it was shown that a cytoplasmically localized cadherin cytoplasmic domain had no dominant-negative effect (Nieman et al., 1999; Troxell et al., 1999). Thus, Troxell et al. postulated a mechanism sensing the cadherin levels within a cell. Since the dominant-negative fragments included the TMS (Kintner, 1992; Hermiston and Gordon, 1995), we speculate in light of our present data that they may interfere with the self-assembly of endogenous cadherin TMSs and thus disturb the formation of functional lateral clusters in adherens junctions. This may affect trans-interactions and/or decrease metabolic stability possibly by influencing the E-cadherin recycling dynamics (Le et al., 1999). Interestingly, T-cadherin, a cadherin family member anchored to the plasma membrane through a glycosylphosphatidylinositol moiety mediates calcium-dependent, homophilic cell adhesion (Vestal and Ranscht, 1992) and was reported to be located in plasma membrane ‘rafts’ (Doyle et al., 1998). Two recent reports demonstrated clustering of glycosylphosphatidylinositol-anchored proteins in membrane microdomains (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998). Thus, T-cadherin may be clustered in the cell membrane by restriction to microdomains whereas in the case of E-cadherin the TMS may be required for lateral clustering within the membrane. We envision two potential mechanisms describing how the TMS may contribute to the formation of lateral cadherin clusters on cell surfaces. TMS-TMS interactions may co-operate with the N-terminal interaction domain in lateral dimer formation (Fig. 7A) and/or cluster preformed lateral dimers (Fig. 7B). At present we cannot distinguish between these two mechanisms and do not want to exclude that both mechanisms are active.

Taken together, these data support the concept that lateral clustering of cadherin molecules in the lipid bilayer of cell membranes is important for adhesiveness. Co-operative self-interactions of different E-cadherin domains appear to be involved in this process and thus might underly modulation of the cadherin adhesion system during different stages in development and tissue formation. Disturbance of this fine-tuned system could be an initial step leading to cell invasiveness as found in certain forms of cancer.

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