Lateral clustering of the adhesive ectodomain: a fundamental determinant of cadherin function

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Background: Classical cadherin-based cellular adhesion is mediated by a multi-component protein complex that links the adhesive binding activity of the cadherin ectodomain to the actin cytoskeleton. Despite the importance of cadherins in morphogenesis and development, we know very little about how cells determine and alter cadherin adhesive strength. In this study, we sought to identify specific cellular mechanisms that modulate cadherin function by studying adhesion between cells transfected with *Xenopus* C-cadherin mutant molecules and substrata coated with the purified ectodomain of C-cadherin.

Results: Using the FKBP–FK1012 protein oligomerization system, we found that forced clustering, in cells, of cadherin mutants lacking the cytoplasmic tail significantly increased cellular adhesive strength. Therefore, redistribution of the adhesive binding sites of cells into clusters can influence adhesion independently of other protein interactions mediated by the cadherin cytoplasmic tail. Furthermore, cells transfected with full-length C-cadherin demonstrated dynamic changes in adhesion over time that correlated with clustering but not with changes in the surface expression of C-cadherin or in the composition of the cadherin–catenin complex. The cytoplasmic tail was, however, necessary for clustering of wild-type cadherin.

Conclusions: These studies directly demonstrate a fundamental role for lateral clustering in cadherin function. The distribution of cadherin binding sites presented at the cell surface, a cellular property which is regulated by the cadherin cytoplasmic tail, is an important mechanism which modulates cellular adhesion independently of cytoskeletal activity or signalling.

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Background

Cadherin-based cellular adhesion is a critical determinant of tissue architecture in developing and adult metazoan organisms [1–3]. Cadherins participate in processes as diverse as cohesion and gastrulation movements of the early embryo [4,5], tissue segregation [2], epithelia formation [1,2,6], and the preservation of tissue architecture in developed organisms [7]. The strength of adhesive interactions between cells is a fundamental parameter of cadherin function [8]. Changes in adhesive strength, regulated by environmental signals, such as growth factors [9,10], appear to be necessary for dynamic cellular movements to occur and for tissue patterning to be maintained once established. Cells must therefore be able to support a range of cadherin adhesive strengths that are determined by cellular and environmental signals. Ultimately, such dynamic modulation of cadherin function must occur through changes in the molecular mechanisms which determine cadherin adhesive strength.

Despite recent progress in defining the molecular interactions between cadherins and other molecules [1–3], relatively little is known about how adhesion is modulated. Classical cadherins (which include E-cadherin, N-cadherin, P-cadherin and *Xenopus* C-cadherin) are single-pass integral membrane proteins which function as multi-protein complexes: the cadherin ectodomains mediate homophilic adhesive binding [2,11], while the cytoplasmic tails associate with cytoplasmic proteins (catenins) that can interact with the actin cytoskeleton [3,12]. It is consequently possible that adhesive strength might be altered by changes in cadherin expression, in cadherin binding affinity, in the distribution of cadherin binding sites presented on the cell surface, in the quantity or composition of the cadherin–catenin complex, or in the interaction between this complex and the cytoskeleton. Of these possibilities, change in the distribution of cadherin binding sites has often been invoked, and it has commonly been proposed that clustering may play a fundamental role in regulating cellular adhesion on the basis of the observed accumulation of cadherins at cell–cell adherens junctions [13,14]. Furthermore, it has been suggested that cooperative, lateral interactions between cadherin ectodomains may be sufficient to support strong adhesion [15], implying that the distribution of cadherin binding sites presented at the cell surface might be a

fundamental mechanism by which clustering strengthens adhesion.

Junctional assembly is a complex process, however, which entails events as diverse as the localized concentration of adhesive binding sites at the surface, cytoskeletal reorganization, and accumulation of potential cell signalling molecules. To date, it has been impossible to identify specific mechanisms that modulate adhesion during junctional assembly or clustering. Yet to understand the regulation of adhesion, it is essential to discriminate amongst the possible mechanisms and to identify those that mediate dynamic modulation of adhesive strength. In this study, we sought to address this problem using both purified proteins and cultured cells transfected with *Xenopus* C-cadherin. We present direct experimental evidence that endogenous cadherins can cluster and modulate adhesion in response to exogenous purified C-cadherin ectodomain. Importantly, forced clustering in cells of the ectodomain alone significantly increases adhesive strength. Therefore, the surface distribution of adhesive binding sites is a fundamental, distinct mechanism by which lateral clustering modulates cadherin adhesive strength, independently of any other adhesive contributions mediated by the cadherin cytoplasmic tail and its associated proteins.

Results

In order to analyze the molecular determinants of cadherin adhesive strength, we sought an experimental system in which clustering of the adhesive ectodomain of C-cadherin could be induced in cells, independently of any potential protein interactions associated with the cadherin cytoplasmic tail. We reasoned that this approach would allow us specifically to test for a role for the clustering of cadherin adhesive sites at the cell surface, as opposed to any other cellular events mediated by the complete cadherin–catenin complex during clustering or junctional assembly.

To do this, we used the recently described FKBP–FK1012 oligomerization system [16,17]. FK1012 is a cell-permeant divalent derivative of FK506 that can induce the oligomerization of proteins containing multiple repeats of the FK506-binding protein, FKBP12. Accordingly, we designed a chimeric protein (ECFK) consisting of the ectodomain and predicted transmembrane region of C-cadherin, fused to three tandem repeats of FKBP12 (Fig. 1a). Oligomerization of the intracellular FKBP repeats by FK1012 would be predicted to induce clustering of the ectodomains of ECFK. Chinese hamster ovary

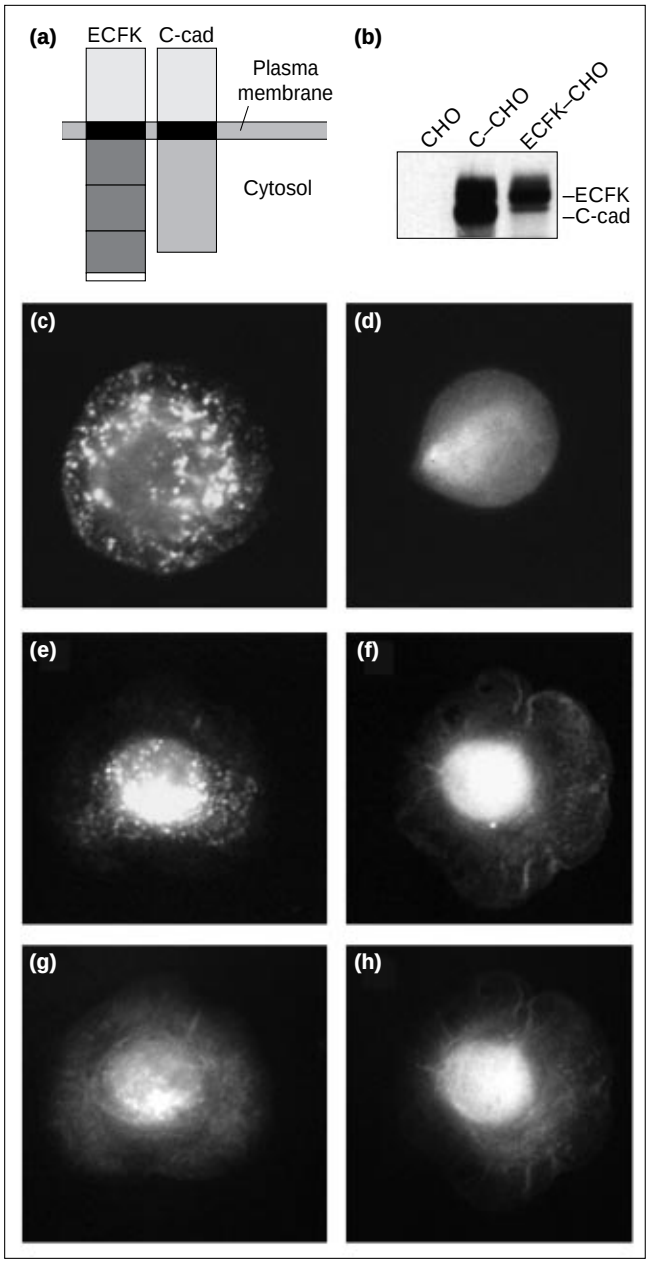


Figure 1

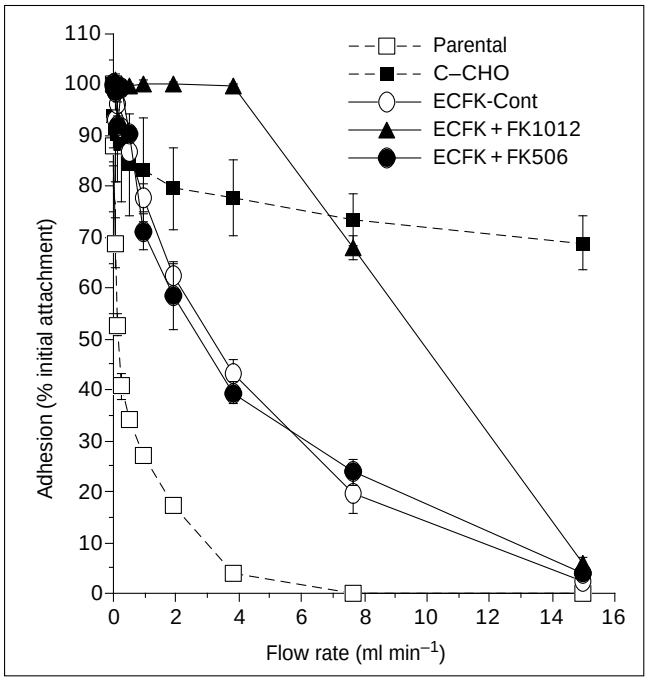
FK1012 induces clustering of the cadherin–FKBP12 chimeric protein, ECFK. (a) ECFK is a chimera of the ectodomain (light grey) and transmembrane region (black) of C-cadherin (C-cad) fused to 3 tandem repeats of the FK506-binding protein, FKBP12 (dark grey), and a Flag-epitope tag at the carboxyl terminus (white). (b) Comparison of protein expression in parental (CHO) and stably transfected cell lines expressing C-cadherin (C–CHO) or EFCK (EFCK–CHO). (c–f) Clustering of ECFK is induced specifically by FK1012. The cellular distribution of ECFK was compared after prior exposure to either (c,e) FK1012 (1 μM for 12 h) or (d,f) FK506M (2 μM for 12 h). Cells were isolated by trypsinization in the presence of Ca^{2+} and plated onto glass coverslips coated with either (c,d) CEC1-5 or (e,f) poly-L-lysine, allowed to adhere for 60 min, then fixed and stained for the Flag-epitope tag of ECFK. In the presence of FK1012, cells showed prominent clusters of staining for ECFK when plated on either (c) CEC1-5 or (e) poly-L-lysine. (d,f) Only diffuse staining for ECFK was seen in the presence of FK506M, irrespective of the substrate used. (g,h) Cells in (e,f) were also stained for F actin using FITC–phalloidin. F-actin staining was predominantly diffuse, irrespective of whether ECFK was clustered.

cells stably transfected with ECFK (ECFK–CHO) expressed a polypeptide of approximately 140 kD in size that reacted with antibodies to both the ectodomain of C-cadherin (Fig. 1b) and the Flag-epitope tag of the cytoplasmic FKBP repeats (data not shown). This molecular weight is consistent with the predicted contribution of three FKBP12 repeats to the size of the truncated component of the mature cadherin molecule.

In order to determine if clustering of ECFK could be induced by FK1012, we used a quantitative experimental system to study cadherin-specific adhesive interactions [18]. This assay uses the adhesive ectodomain of C-cadherin, expressed as a secreted, soluble recombinant protein (CEC1-5), as a substrate for cell attachment. ECFK–CHO cells, grown under standard conditions, were incubated with FK1012 (1 μM), then isolated and plated onto CEC1-5. As shown in Figure 1c, staining for the Flag-epitope tag revealed a clearly clustered distribution of ECFK at the cell–substrate interface. Clustered staining of ECFK at the free surfaces of cells treated with FK1012 was also detected using antibodies directed against the ectodomain of C-cadherin (data not shown). In contrast, cells incubated in medium alone (data not shown) or exposed to the monovalent parent drug FK506M (2 μM; Fig. 1d), which does not induce oligomerization [16], showed only diffuse staining for ECFK. Clustering of ECFK depended specifically upon the drug used, but was not dependent upon the specific substrate to which cells adhered: clustering of ECFK was also induced by FK1012 (Fig. 1e), but not by FK506M (Fig. 1f), when cells were plated onto poly-L-lysine.

Induction of clustering by FK1012 considerably increased the strength of adhesion between ECFK–CHO cells and CEC1-5, measured as the resistance to detachment using a sensitive laminar-flow assay [18]. In this assay, cells were infused into glass capillaries coated with CEC1-5, allowed to attach under stasis for 10 minutes, then exposed to progressively increasing buffer flow rates (Fig. 2); ECFK–CHO cells, incubated in medium alone, adhered to CEC1-5 with adhesive strengths that were greater than those of parental CHO cells, but significantly less than those of C–CHO cells — cells expressing similar levels of wild-type C-cadherin (Fig. 1b). This is consistent with our recent demonstration that mutant cadherin molecules lacking the cytoplasmic tail retain adhesive binding activity, but do not adhere as strongly as full-length cadherins [18]. FK1012 significantly increased the adhesion of ECFK–CHO cells to CEC1-5 (Fig. 2). Indeed, over most of the range of the assay, ECFK–CHO cells incubated with FK1012 adhered to CEC1-5 as strongly as, or more strongly than, did C–CHO cells. Only at the highest flow rates did the adhesion of ECFK–CHO cells incubated with FK1012 fall below the adhesion of C–CHO cells. No change in adhesion was observed when ECFK–CHO cells were incubated with monovalent FK506 (Fig. 2). Therefore, forced clustering of only the cadherin ectodomain can significantly increase adhesion, independently of any specific protein interactions mediated by the cadherin cytoplasmic tail.

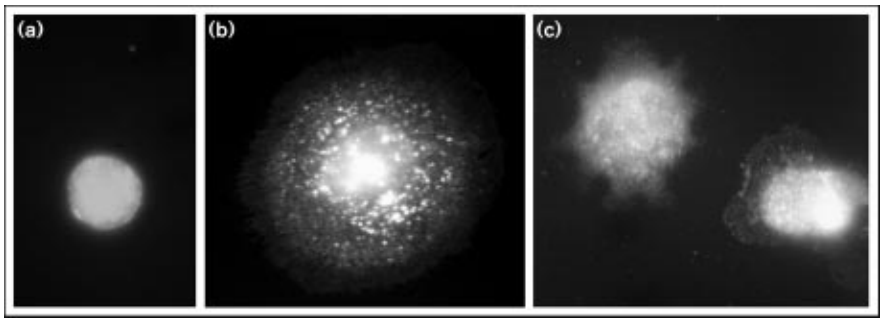
Figure 2



FK1012-induced clustering increases adhesion supported by the chimeric protein ECFK. ECFK–CHO cells were incubated under standard conditions either in medium alone (ECFK-Cont), FK1012 (1 μM for 12 h; ECFK + FK1012) or in FK506 (2 μM for 12 h; ECFK + FK506), then isolated in the presence of Ca^{2+}, infused into glass capillaries coated with CEC1-5, and allowed to attach for 10 min in the presence of the appropriate drugs. Adhesive strength was measured as the resistance to progressively increasing laminar-flow rates. ECFK–CHO cells incubated in buffer alone showed adhesive strengths intermediate between those of parental CHO cells (parental) and CHO cells stably transfected with C-cadherin (C–CHO). Adhesiveness of ECFK–CHO cells was significantly increased by the prior exposure to FK1012, but not to FK506.

Although neither FK506 derivatives nor FKBP12 are known to interact with the cytoskeleton, it has been suggested that clustering of integral membrane proteins might non-specifically induce reorganization of the actin cytoskeleton and perhaps thereby influence adhesion [19]. However, ECFK–CHO cells co-stained with FITC–phalloidin showed uniform, predominantly diffuse F-actin staining in the presence of either FK1012 (Fig. 1g) or FK506M (Fig. 1h), although ECFK clustering occurred only in the presence of FK1012 (Fig. 1e,f). In particular, there was no apparent concentration of F-actin staining in the regions of ECFK clusters (compare Fig. 1g with Fig. 1e). Therefore, in this system, clustering of ECFK

Figure 3



Ligand-specific clustering of C-cadherin in response to CEC1-5. C–CHO cells were isolated by trypsinization in the presence of Ca^{2+} and plated onto glass coverslips coated with either (a,b) CEC1-5 or (c) poly-L-lysine. After (a) 10 min and (b,c) 60 min, cells were fixed, permeabilized and stained for cadherin. (a) After 10 min, cells had attached, but remained rounded and showed diffuse cadherin staining at the cell–substrate contact zone. (b) After 60 min, cells plated onto CEC1-5 had spread and displayed prominent clusters of cadherin staining at the basal surfaces in contact with the substrate. (c) In contrast, cells plated on poly-L-lysine also spread after 60 min but displayed predominantly diffuse cadherin staining.

appeared to occur without any change in actin cytoskeletal organization that was detectable at the level of the light microscope.

Our findings indicated that forced clustering of only the cadherin ectodomain could significantly modulate adhesive strength. We next sought evidence that clustering of the physiological, full-length cadherin molecule could participate in dynamic modulation of adhesive strength in our experimental system. The adhesion of C–CHO cells to substrata coated with CEC1-5 allowed us to readily assess the distribution of cadherins in the plane of the plasma membrane by immunofluorescent staining using an antibody directed against the conserved cytoplasmic tail of classical cadherins. We found that, over a period of 45–60 minutes, C–CHO cells attached to and spread upon glass coverslips coated with CEC1-5 (Fig. 3). After 10 minutes, when cells had attached but remained rounded, cadherin staining was diffusely distributed on the cell surface (Fig. 3a). By 60 minutes, however, spread cells showed prominent focal patches of cadherin staining at the basal surfaces in contact with CEC1-5, as well as some more diffuse staining at free margins (Fig. 3b). In contrast, cadherin staining remained largely diffuse in cells plated for 60 minutes onto poly-L-lysine (Fig. 3c) or fibronectin (data not shown), despite similar degrees of spreading. Therefore, the adhesion of C–CHO cells to CEC1-5 was accompanied by the apparent ligand-specific clustering of cadherin molecules into patches at the cell surface.

Cadherin clustering correlated with significant changes in the strength of adhesion between C–CHO cells and CEC1-5 (Fig. 4a): using C–CHO lines expressing different levels of cadherin (Fig. 4b), we found that the resistance to detachment in the laminar-flow assay increased with the level of cadherin expression, confirming that adhesive strength can be altered by differences in protein expression [20]. For both cell lines, adhesive strength was also significantly influenced by the duration that cells were initially allowed to attach to the substrate (Fig. 4a); adhesive strength was consistently increased after 30–60 minutes attachment compared with 10 minutes attachment. This was particularly noticeable for the C–CHO3 line, which expresses relatively low levels of C-cadherin, whose adhesive strength was only marginally greater than that of parental cells after 10 minutes, but substantially increased after 60 minutes (Fig. 4a).

Although we have previously shown that C–CHO cells bind specifically to CEC1-5 [5], it was formally possible that, with prolonged attachment, secretion of matrix proteins might contribute to the changes in adhesive strength seen over time. Minor increases in adhesion were seen in parental CHO cells allowed to attach to CEC1-5 for 60 minutes, but these were largely inhibited by the RGD-containing peptide GRGDTP (in single-letter amino-acid code; data not shown), which inhibits integrin-mediated adhesion [21,22]. The peptide GRGDTP had no effect on the adhesion between C–CHO cells and CEC1-5, however, at any time of attachment (data not shown), providing strong evidence that cadherin-mediated adhesion was the major adhesive interaction involved in the temporal strengthening of adhesion between C–CHO cells and CEC1-5.

The temporal strengthening of adhesion occurred without any detectable change in cadherin expression or in the composition of the cadherin–catenin complex; over the 60 minute time course during which C–CHO cells displayed clear strengthening of adhesion to CEC1-5, we could detect no change in total cellular cadherin levels or in the proportion of cadherin at the cell surface, as determined by accessibility to digestion by extracellular trypsin (Fig. 4c). At all times the majority of cadherin was digested by trypsin in calcium-free conditions, whereas in the presence of 1 mM Ca^{2+}, which renders cadherins resistant to trypsin [23], the levels of cadherin detected were similar to those in total cell lysates. Therefore, the majority of C-cadherin appeared to be at the cell surface,

and this did not change during the 60 minute attachment periods of our experiments. As shown in Figure 4d, C-cadherin immunoprecipitates contained protein bands recognized by antibodies directed against β-catenin and α-catenin. There was, however, no detectable change with time in the levels of cadherin-associated catenin in cells attached to CEC1-5 for 0–60 minutes (Fig. 4d). Taken together, these findings support a strong correlation between adhesive strengthening and clustering of wild-type C-cadherin in response to CEC1-5.

Discussion

In this study, we sought to identify specific cellular and molecular mechanisms capable of determining and modulating cadherin adhesive strength. Our findings directly demonstrate a fundamental role for lateral clustering in cadherin function. They further indicate that the distribution of adhesive binding sites presented at the cell surface is a major, distinct mechanism by which clustering of classical cadherins alters adhesion, independently of any separate adhesive contributions either from the association of these cadherins with the cytoskeleton or from other cytoplasmic interactions.

Although lateral clustering has often been invoked as a general mechanism to modulate adhesion [20,24,25], clear experimental evidence for this hypothesis has previously been difficult to obtain. A role for clustering was first inferred from correlations between changes in cellular phenotype (compaction or spreading) and the assembly of adhesive cell junctions (zonula adherens and focal adhesions, respectively) [13,26]. More recently, quantitative adhesion assays and cells expressing specific adhesion molecules have been used to document correlations between clustering and changes in adhesive strength for a number of adhesion molecules, including integrins [24] and cadherins [20]. Indeed, in the present study, we found that dynamic temporal changes in adhesive

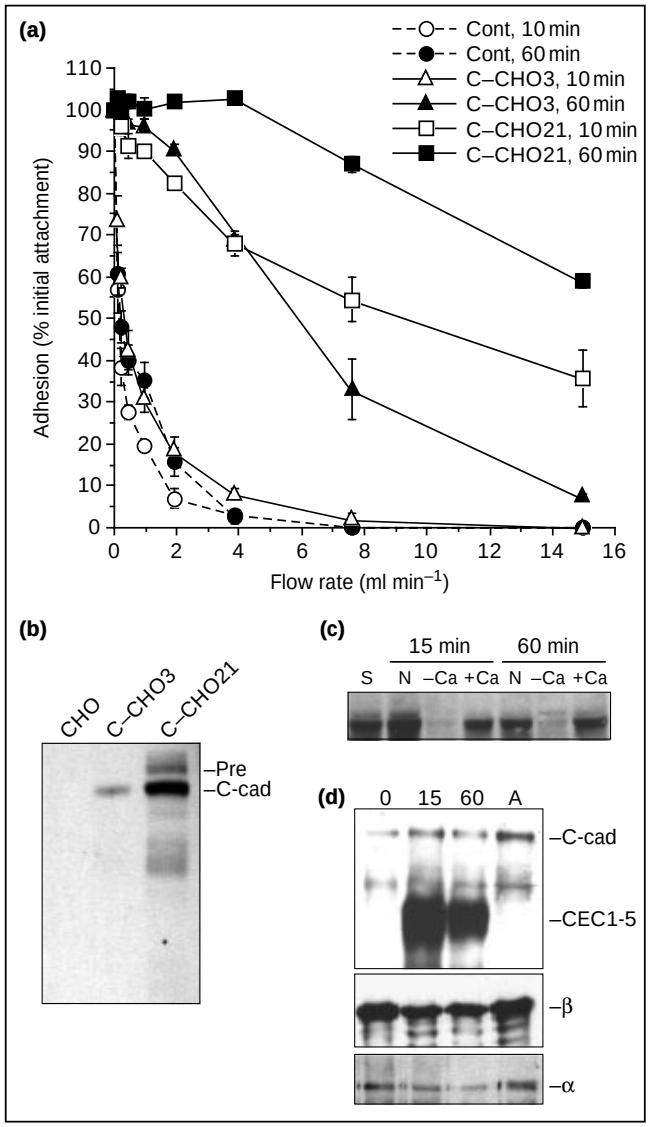


Figure 4

Temporal strengthening of cadherin-based adhesion. (a) The adhesion between C–CHO cells and substrata coated with CEC1-5 depends upon the duration of attachment to substrate. Parental CHO cells (Cont) or CHO cells expressing low (C–CHO3) and high (C–CHO21) levels of C-cadherin were allowed to attach for 10–60 min to glass capillaries coated with CEC1-5, then assayed for adhesive strength. All assays were performed in the presence of the RGD peptide, GRGDTP (1 mg ml^{-1}), to inhibit possible integrin-based adhesion. Adhesion of both C–CHO3 and C–CHO21 cells was significantly increased when cells were allowed to attach for 60 min at stasis compared with 10 min. All data are expressed as mean ± SEM (n = 3). (b) Expression of C-cadherin in parental and stably transfected CHO cells. C–CHO21 cells expressed approximately 10 times the amount of C-cadherin (C-cad) as C–CHO3 cells; a smaller amount of the cadherin precursor (Pre) was also detectable in C–CHO21 cells. (c) Surface expression of C-cadherin in C–CHO cells adhering to CEC1-5. C–CHO cells, freshly isolated by trypsinization in the presence of Ca^{2+}, were allowed to attach for 15 min or 60 min to dishes coated with CEC1-5, cells were then exposed to trypsin (0.01% for 30 min) in the presence of either 1 mM Ca^{2+} (+Ca) or 2 mM EDTA (–Ca). The reactions were stopped by the addition of soy bean trypsin inhibitor (10 mg ml^{-1}, Sigma), and cells lysed in NP40 lysis buffer. Control lysates were prepared from freshly isolated cells not plated onto CEC1-5 (S) and from cells plated onto CEC1-5, but not exposed to further trypsin (N). Equal quantities of protein were separated by SDS–PAGE, transferred to nitrocellulose, and probed with the monoclonal antibody 6B6 directed against the ectodomain of C-cadherin. Total levels of cadherin in NP40 lysates of cells adhering to CEC1-5 but not exposed to trypsin (N) did not change significantly between 15 and 60 min, nor was there any significant change over this time in the amount of cadherin digested in the absence of extracellular calcium. (d) Composition of the cadherin–catenin complex in C–CHO cells adhering to CEC1-5. Lysates from confluent cultures of C–CHO cells (A), or freshly isolated C–CHO cells plated onto CEC1-5 for 0–60 min, were immunoprecipitated with a polyclonal antibody directed against CEC1-5. Western blots of the cadherin immunoprecipitates were probed for C-cadherin, β-catenin and α-catenin. The C-cadherin immunoblots recognized both full length C-cadherin (C-cad) and a 90 kD band consistent with CEC1-5. There was no difference in the levels of C-cadherin, α-catenin (α) or β-catenin (β) detected in the immunoprecipitates.

strength not only correlated with clustering of C-cadherin, but occurred without any detectable change either in the surface expression of C-cadherin, or in the composition of the cadherin–catenin complex — two alternative mechanisms that have been postulated to regulate cadherin-based adhesion [20,27]. Despite these striking correlations, however, it was impossible in these and similar studies [20,24] to discriminate between a possible contribution of clustering and other events associated with the complex process of junction assembly, in particular cytoskeletal activity and intracellular signalling, which can influence cell morphology independently of adhesion itself.

We sought to circumvent this analytic problem by using the FKBP–FK1012 system to force oligomerization of the cadherin ectodomain alone, independently of any other protein interactions that might be mediated by the cadherin cytoplasmic tail. Recently, we found that tailless truncation mutants of C-cadherin retain adhesive activity that is considerably less than that of native cadherins, but is detectable using sensitive adhesion assays [18]. Consistent with this, the diffusely distributed ECFK chimeric protein supported detectable, but weak, adhesion. Significantly, however, forced clustering of ECFK by the oligomerizing agent FK1012 induced a considerable increase in adhesive strength to levels similar to those supported by the wild-type cadherin over all but the highest flow rates used in our assays. Therefore, lateral clustering alone was sufficient to substantially increase the weak adhesion supported by the diffusely distributed ectodomain alone. This indicates that, although interactions with the cytoskeleton may also influence cadherin adhesive strength [20,28–31], the redistribution of adhesive binding sites presented at the cell surface is a fundamental, distinct, mechanism by which clustering of wild-type cadherins strengthens adhesion.

Although our study does not address the physical basis of adhesive strengthening, it is attractive to postulate that clustering increases adhesive strength by distributing stresses across multiple bonds, which must be broken simultaneously for cell detachment to occur [32,33]. Clustering may also increase the avidity of ligand binding by increasing the local concentration of adhesion receptors; these possibilities can be best discriminated between when biophysical assays for cadherin binding affinity are available. By either of these non-exclusive mechanisms, a range of adhesive strengths may be generated depending on the degree of clustering of adhesion molecules at the cell surface. Indeed, the observation that compaction of mouse embryo blastomeres coincides with the accumulation of E-cadherin in junctions [13], strongly suggests that cells are capable of regulating the surface distribution of cadherins.

Our findings also provide insight into the specific contributions of the ectodomain and cytoplasmic tail in determining cadherin adhesive strength. The observation that cadherin mutants lacking the cytoplasmic tail mediate only weak adhesion [34,35] has led to the inference that cytoplasmic interactions, particularly those with the actin cytoskeleton, are likely to be the principal determinants of adhesive strength. Despite this, the ectodomain mediates specific adhesive binding and retains adhesive activity [11,18]. Furthermore, it has recently been proposed that cooperative interactions between dimeric cadherin ectodomains might convert the weak binding activity of the ectodomain into strong cellular adhesion; in the most extreme interpretation of this 'zipper' model, which is based on interpretation of the crystal structure of a fragment from the ectodomain of N-cadherin [15], the ectodomain alone might drive self-assembly of cadherins into clusters or junctions.

Although our findings support the notion that redistribution of the adhesive ectodomain into clusters or junctions can fundamentally increase cadherin adhesive strength, it is important to note that, in the absence of FK1012, ECFK remained diffusely distributed on the surfaces of cells despite being plated onto substrata coated with CEC1-5, capable of inducing clustering of the full-length cadherin molecule. Therefore, the ectodomain of C-cadherin alone did not appear to be sufficient to drive clustering; instead, the cytoplasmic tail appears to be necessary for the wild-type cadherin molecule to cluster. This suggests a working model in which the cytoplasmic tail may contribute to strong cadherin-based adhesion by supporting clustering of the adhesive binding sites presented at the cell surface, perhaps by regulating the ability of the cadherin to diffuse in the plane of the membrane. Furthermore, in contrast to self-assembly of cadherin ectodomains, clustering mediated by the cytoplasmic tail provides a mechanism by which intracellular signals could be transduced to generate the physiological range of adhesive strengths that cells appear to support [9].

Conclusions

The present findings provide the first direct evidence that lateral clustering constitutes a fundamental determinant of cadherin function. The distribution of adhesive binding sites presented at the cell surface is a major, discrete mechanism by which clustering modulates adhesion, independently of any separate adhesive contributions from cytoskeletal activity or other cytoplasmic events. This is likely to be a general mechanism to regulate adhesion, insofar as other adhesion molecules have been observed to accumulate in junctions or clusters. These findings therefore provide an empirical basis for further analysis of the molecular mechanisms that regulate the function of cadherins and other adhesion molecules in developing and adult tissues.

Materials and methods

Drugs

FK1012 and FK506 were synthesized as previously reported [16].

Plasmid construction and cell lines

For expression of C-cadherin in mammalian cells, the cDNA encoding full-length *Xenopus* C-cadherin (7B3; [36]) was excised from Bluescript with *Eco*R1 and subcloned into either the expression vector pEE14 (for C–CHO21 cells) or the vector pcDNA3 (Stratagene, La Jolla, California; for C–CHO3 cells).

The C-cadherin–FKBP12 chimeric protein ECFK was constructed as follows. DNA encoding the transmembrane region of C-cadherin, from the *Hin*dIII site of the ectodomain to the conserved amino acid residues KRKK found at the transmembrane–cytoplasmic junction, was isolated using the polymerase chain reaction (PCR), and ligated into the Bluescript (SK) cloning vector. The PCR primers were 5′-GTGGTGGATAT CGAAAAGCTTGTCGGTGGT-3′ and 5′-TTCTTTTTCCATATGCACA ACCTTCTTTCTCTTCAG-3′. An *Nde*1 cloning site was introduced at the 3′ end of the PCR fragment for cloning purposes. An *Nde*1–*Eco*R1 fragment from plasmid pBJ5-MZF3E$_{Flag}$ encoding three tandem FKBP12 repeats and a carboxy-terminal Flag-epitope tag was then ligated to the 3′ end of the DNA encoding the cadherin transmembrane region. This resulted in the spacer amino-acid sequence KRKKVVH-MQTLAPRVE between the predicted cadherin transmembrane region and the first FKBP repeat of the encoded ECFK. The DNA encoding the ectodomain of C-cadherin was then restored by insertion of a *Kpn*1–*Hin*dIII fragment from the plasmid pBS-7B3. All PCR fragments and ligation sites were checked by sequencing using the Sequenase II kit. The ECFK-coding region was then cloned into the mammalian expression vector pEE14 at the *Eco*R1 site.

CHO cells were transfected using lipofectin, and stable cell lines were selected using either G418 selection for plasmids based on the pcDNA3 expression vector or methionine sulfoximine (Sigma Chemical Co., St. Louis, Missouri) selection for plasmids based on the pEE14 vector. For all the experiments described, stable cell lines were grown under standard conditions for 48 h, then isolated by incubation with crystalline trypsin (0.01% w/v) in Hanks balanced salt solution containing 1 mM $CaCl_2$ (HBSS/Ca^{2+}; at 37°C for 10 min), washed and resuspended in HBSS/Ca^{2+}. For experiments involving FK1012 or FK506, cells were incubated with the drug overnight and resuspended in HBSS/Ca^{2+} containing the same concentration of the appropriate drug.

Protein purification and laminar-flow adhesion assays

CEC1-5 was purified from conditioned media as reported previously [18]. The modified laminar-flow assay was performed as described previously [18]. In brief, glass capillaries (1 mm internal diameter) were coated overnight with CEC1-5 (10 μg ml^{-1}) and non-specific binding sites blocked with 10 mg ml^{-1} BSA. Cells were grown and isolated as described previously, infused into capillaries and allowed to attach under stasis for 10–60 min, then exposed to progressively increasing flow rates of buffer (150 mM NaCl, 20 mM Hepes, 1 mM $CaCl_2$, pH 7.4). The number of cells attached to the substrate in a microscope field were counted at 1 min intervals and data normalized to the number of cells present in the field before infusion of buffer. All assays were conducted in the presence of the RGD peptide, GRGDTP (1 mg ml^{-1}; Sigma).

Western blotting and immunoprecipitations

To assess the expression of cadherins and ECFK, cells were extracted in 1% NP40 extraction buffer (1% NP40, 10 mM Hepes, 150 mM NaCl, 1.5 mM EDTA, pH 7.4, supplemented with protease inhibitors as described previously [9]) and equal amounts of protein separated by SDS–PAGE, transferred to nitrocellulose, and probed with the monoclonal antibody 6B6, directed against the ectodomain of C-cadherin [9].

To assess the composition of the cadherin–catenin complex, cells were extracted in 1% NP40 lysis buffer, and aliquots of NP40-soluble supernatants containing equal quantities of protein were then immunoprecipitated with a polyclonal antibody directed against CEC1-5, and collected with Protein-A–sepharose beads. Beads were washed in 1% NP40 buffer and boiled in SDS–Laemmli buffer containing DTT (50 mM). Equal quantities of the cadherin immunoprecipitates were separated by SDS–PAGE, transferred to nitrocellulose, and probed using the monoclonal antibody 6B6 directed against C-cadherin, and monoclonal antibodies directed against β-catenin and α-catenin (Transduction Laboratories).

Surface expression of C-cadherin was assessed by its sensitivity to digestion with extracellular trypsin. Cells were exposed to trypsin (0.01%; 30 min) in the presence of 1 mM Ca^{2+} or in the presence of 2 mM EDTA. The reactions were stopped by addition of soy bean trypsin inhibitor (10 mg ml^{-1}, Sigma), cells collected by centrifugation and lysed in 1% NP40 lysis buffer. Equal quantities of protein were separated by SDS–PAGE, transferred to nitrocellulose, and probed with the monoclonal antibody 6B6 directed against the ectodomain of C-cadherin.

Immunofluorescence microscopy

Glass coverslips were coated by incubation overnight at 4°C with CEC1-5 (10 μg ml^{-1}) or poly-L-lysine (10 μg ml^{-1}), then blocked with BSA (10 mg ml^{-1}) for 1–2 h (room temperature). Isolated cells were allowed to adhere to glass coverslips for various periods of time then fixed (in PBS containing 3% paraformaldehyde, for 30 min at 4°C), permeabilized (using 0.25% Triton X-100, for 10 min at room temperature), and then incubated with primary antibodies (16 h at 4°C) and TR-conjugated secondary antibodies (Molecular Probes, Eugene, Oregon; 1 h at room temperature). C-cadherin was stained with a polyclonal antibody directed against the cytoplasmic tail of E-cadherin ([37]; a kind gift from J. Marrs) used at a dilution of 1:100; the Flag-epitope tag of ECFK was stained using the anti-Flag M2 monoclonal antibody (20 μg ml^{-1}; Kodak IBI). For double-labelling studies, FITC–phalloidin (40 μM; Sigma) was added with the secondary antibody in the final incubation step. Specimens were examined with a Zeiss Axioskop equipped with plan-APOCHROMAT ×63 and ×100 objectives. Images were photographed using Kodak Elite 400 film, and processed using Adobe Photoshop.

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