Recognition of E-cadherin by Integrin αEβ7

REQUIREMENT FOR CADHERIN DIMERIZATION AND IMPLICATIONS FOR CADHERIN AND INTEGRIN FUNCTION*

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We have investigated the importance of dimerization of E-cadherin in the heterophilic adhesive interaction between E-cadherin and integrin αEβ7. Dimerization of cadherin molecules in parallel alignment is known to be essential for homophilic adhesion and has been attributed to Ca2+-dependent interactions in the domain 1-2 junction or to cross-intercalation of Trp2 from one molecule to the other. We have disrupted either or both of these proposed mechanisms by point mutations in E-cadherin-Fc and have tested the modified proteins for αEβ7-mediated cell adhesion. Prevention of Trp2 intercalation had no adverse effect on integrin-mediated adhesion, whereas disruption of Ca2+ binding permitted adhesion but with reduced efficiency. Both modifications in combination abolished recognition by αEβ7. In EGTA, αEβ7 adhered to wild type E-cadherin but not to the Trp2 deletion mutant. Independent evidence that the mutations prevented either or both mechanisms for dimerization is presented. The data show that dimerization is required for recognition by αEβ7 and that it can take place by either of two mechanisms. Implications for the roles of the αE and β7 integrin subunits in ligand binding and for Trp2 and Ca2+ in the assembly of cadherin complexes are discussed.

E-cadherin is a homophilic cell adhesion molecule expressed by epithelial cells. It is a type 1 transmembrane protein that contains five Ig-like extracellular domains maintained in a rodlike conformation by interdomain calcium atoms. In addition to its adhesive activity, E-cadherin has an important role as a cell signaling molecule and through both activities serves to maintain the integrity of epithelia (1). E-cadherin is also a counterreceptor for one member of the integrin family, the mucosal T cell integrin αEβ7 (2, 3). This heterophilic adhesive interaction is thought to play a part in the retention of lymphocytes in or near mucosal epithelia (4). Recently, much attention has been focused on the molecular mechanisms for the two types of adhesion. A model has been proposed for homophilic adhesion in which adjacent cadherin molecules on the cell surface dimerize in parallel alignment (cis-dimer) and interdigitate with dimers on an opposing cell to form a zipper-like assembly of multiple low affinity contacts (5). Homophilic adhesion, heterophilic adhesion and cadherin dimerization depend mainly on contact sites on the extracellular, N-terminal domain EC1.

The mechanism for cadherin dimerization is not yet fully understood. X-ray crystallographic studies on cadherin domain 1 or domains 1 and 2 together have suggested two alternative mechanisms. Shapiro et al. (5) observed that domain 1 of N-cadherin formed cis-dimers by cross-intercalation of Trp2 into a hydrophobic pocket in its neighboring domain. In contrast, Nagar et al. (6) found that E-cadherin domains 1 and 2 dimerize through calcium-dependent hydrogen bonding in the interdomain junction. Pertz et al. (7) obtained similar results and showed that Trp2 docked into the hydrophobic pocket of its own domain rather than providing a bridge to its neighbor. The crystal structure of N-cadherin domains 1 plus 2 failed to reveal a cis-interaction by either mechanism (8). Support for Ca2+ dependence of dimerization has come from observation of purified recombinant cadherin molecules by electron microscopy (7), atomic force microscopy (9), and physical measurements of cadherin domains in solution (10). Investigations into the mechanism of cadherin dimerization on the cell surface have given a different picture. Cadherin complexes have been isolated from cell lysates and analyzed by sedimentation or electrophoresis. In this situation, Trp2 was shown to be essential for cis-dimer formation (11-14), and Ca2+ was required for the formation (15) but not the stability (12, 13, 16) of the dimers. It is not known whether the two proposed mechanisms for dimerization could operate simultaneously, but the crystal structures of ECAD1,2 (6, 7) mitigate against this possibility. Although the precise roles of Trp2 and interdomain calcium are not fully understood, it is widely accepted that both are required for homophilic adhesion and that cis-dimerization is an essential part of the process.

Integrin αEβ7 has been shown to bind to the distal surface of domain 1 and the contact site has been mapped in detail. Karecla et al. (17) showed that Glu31 in the solvent-exposed CD loop was critical for recognition by the integrin. Recently, this observation has been confirmed and extended to identify a role also for the FG loop located nearby (18). Higgins et al. (19) demonstrated that these loops provide a contact surface for the A-domain of the αE integrin subunit. Thus, the acidic side chain of Glu31 docks into the MIDAS site of the αE A domain and Phe238 from the A-domain is accommodated in a hydrophobic cleft between the BC and FG loops of E-cadherin. The MIDAS site on the integrin β7 subunit is also required for ligand binding (19, 20) and would be expected to engage an acidic side chain from the ligand or from the αE subunit, but this has not been identified.

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The present report examines the importance of cis-dimerization in the N-terminal domain of E-cadherin for heterophilic adhesion to αEβ7. The two proposed mechanisms for dimer formation have been investigated. We have prepared a series of E-cadherin-Fc fusion proteins in which amino acid substitutions have been made either in the N terminus, to prevent Trp2 cross-intercalation, or in the junction between domains 1 and 2, to prevent co-ordination of one or more of the three calcium atoms present. The two categories of modification are referred to as “single” mutations or, when combined, as “double” mutations. The recombinant proteins have been tested for their ability to support αEβ7-mediated cell adhesion and to dimerize. None of the amino acid substitutions would be expected to have any direct effect on the contact site on E-cadherin, which engages the integrin. The results show that cadherin dimerization is essential for adhesion to the integrin and provide a new perspective on the relative importance of Trp2 and the EC1-EC2 interdomain junction for the formation of cis-dimers.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies

The mouse T cell hybridoma MTC-1 was grown in RPMI 1640 supplemented with 10% FCS and 5 ng/ml recombinant transforming growth factor-β2. Under these conditions, the cells expressed high levels of αEβ7. The mouse gut epithelial cell line CMT93, which expresses E-cadherin on the cell surface, was grown in Dulbecco’s modified Eagle’s medium plus 10% FCS. The rat monoclonal antibodies ECCD-2, which recognizes E-cadherin on the cell surface, was grown in Dulbecco’s modified Eagle’s medium plus 10% FCS. The mouse T cell hybridoma MTC-1 was grown in RPMI 1640 supplemented with 10% FCS and 5 ng/ml recombinant transforming growth factor-β2. Under these conditions, the cells expressed high levels of αEβ7. The mouse gut epithelial cell line CMT93, which expresses E-cadherin on the cell surface, was grown in Dulbecco’s modified Eagle’s medium plus 10% FCS. The rat monoclonal antibodies ECCD-2, which recognizes E-cadherin on the cell surface, was grown in Dulbecco’s modified Eagle’s medium plus 10% FCS and 5 ng/ml recombinant transforming growth factor-β2. Under these conditions, the cells expressed high levels of αEβ7.

E-cadherin-Fc Constructs

The extracellular region of mouse E-cadherin, excluding the signal sequence and propeptide was fused to human IgG1 Fc using the vector signal plg-Tail (R & D Systems), which incorporates a CDS3 signal sequence. The construct introduced three additional amino acids (Met, Asp, and Leu) to the N terminus of fully processed, mature E-cadherin. The extracellular region extending up to, but not including, the transmembrane domain was fused to the complete IgG1 Fc hinge. cDNA for the extracellular region of E-cadherin was prepared by polymerase chain reaction using primers introducing Bgl II and Pfu 8414830650 via goat anti-Fc as described for enzyme-linked immunosorbent assay. Beckman Instruments equipped with 12-mm Epon double sector cells in an An-60 Ti rotor. The proteins were dissolved in 20 mM Tris/HCl, pH 7.9, 100 mM NaCl, 2 mM CaCl2. The protein concentration was adjusted to 0.5 mg/ml for sedimentation equilibrium runs and to 0.15 mg/ml for the sedimentation velocity scans. Sedimentation coefficients were corrected to standard conditions (water, 20 °C). Sedimentation equilibrium runs were carried out from 8000 to 30,000, depending on molecular mass. Apparent molecular masses were evaluated from ln A versus r² plots, where A is the absorbance and r is the distance from the rotor center.

Cell Adhesion Assays

Integrin-mediated Adhesion—E-cadherin-Fc fusion proteins were coated to a 96-well enzyme-linked immunosorbent assay plate (Costar; 3590) via goat anti-Fc as described for enzyme-linked immunosorbent assays. MTC-1 cells were labeled with BCECF-AM (Molecular Probes catalog no. B-3051) by incubation in 1 ml of serum-free RPMI containing 5 μg/ml BCECF-AM for 30–40 min at 37 °C. The cells were then washed and resuspended at 5 × 10^5/ml in assay medium, HBSS (Sigma) containing 1.25 mM Ca^2+ and 0.8 mM Mg^2+ plus 1% FCS. The cells were then activated by the addition of phorbol 12-myristate 13-acetate (15 ng/ml) 5 × 10^5 cells were added to each well in a total volume of 200 μl. The plate was centrifuged for 2 min at 16 × g to gently sediment the sample. After incubation for 5 min at 37 °C the wells were then removed by gentle pipetting, and the attached cells were quantitated using a fluorescence plate reader. For assays conducted in the absence of Ca^2+, MTC-1 cells were suspended in Hanks’ balanced saline solution plus 0.1% bovine serum albumin, activated with phorbol 12-myristate 13-acetate, and then washed free of Ca^2+ and transferred to Hanks’ balanced saline solution lacking Ca^2+ and Mg^2+.

Preparation of E-cadherin-Fc Fusion Proteins

COS7 cells were transfected using a modification of the DEAE-dextran method (24). After overnight recovery in Dulbecco’s modified Eagle’s medium plus 10% FCS, the cells were cultured in Dulbecco’s modified Eagle’s medium plus 1% FCS for 4–6 days. The medium was then harvested, cleared of cell debris by centrifugation at 1500 × g, and then centrifuged at 50,000 × g for 1 h. Sterile supernatants were stored at 4 °C. A representative set of E-cadherin-Fc fusion proteins (WT, MDL, WV, Ca23, WV + Ca23, and MDL + Ca23) were analyzed by gel filtration fast protein liquid chromatography on a Superdex-200HR 10/30 column equilibrated in 50 mM Hepes, pH 7.5, 150 mM NaCl, 0.05% Tween 20 to verify that they were monodisperse. The double mutant, Ca23 + WV, was susceptible to aggregation on long term storage, so particular care was taken to ensure that all experiments were conducted with monodispersed material. The double mutant was also subjected to N-terminal sequencing to ensure that no degradation had taken place. E-cadherin-Fc proteins were purified from culture supernatant by elution from protein A-Sepharose using 0.1 M glycine/HCl or 50 mM citrate, pH 3.0, followed by immediate neutralization in Tris and restoration of Ca^2+ to 2 mM. Purity was verified by SDS-polyacrylamide gel electrophoresis. Concentrations were determined by absorbance at 290 nm.

Enzyme-linked Immunosorbent Assay

The concentration of E-cadherin-Fc in culture supernatants was determined by enzyme-linked immunosorbent assay using purified E-cadherin-Fc as a standard. 96-well plates were coated overnight with affinity-purified goat anti-human Fc at 5 μg/ml in PBS. Dilutions of the E-cadherin-Fc fusion protein were incubated on the plate for 1 h at room temperature, and their binding was detected by a similar incubation with biotinylated goat anti-human Fc (Jackson ImmunoResearch Laboratories), followed by avidin-horseradish peroxidase (Sigma). BINDING of rat monoclonal antibodies DECMA-1 and ECCD-2 (both at 1 μg/ml) to E-cadherin-Fc was detected using horseradish peroxidase-labeled goat anti-rat IgG (Chemicon). For titration of sheep antibodies to the BC loop and the ββ domain, E-cadherin-Fc proteins were immobilized using rabbit anti-human Fc (Pierce), and binding of the antibodies was detected with a mouse monoclonal antibody to sheep IgG (Sigma). With both antibodies, reactivity was specifically inhibited by their respective peptides.

Analytical Ultracentrifugation

Sedimentation equilibrium and sedimentation velocity experiments were performed at 20 °C using a Beckman Optima XL-A analytical ultracentrifuge (Beckman Instruments) equipped with 12-mm Epon double sector cells in an An-60 Ti rotor. The proteins were dissolved in 20 mM Tris/HCl, pH 7.9, 100 mM NaCl, and 2 mM CaCl2. The protein concentration was adjusted to 0.5 mg/ml for sedimentation equilibrium runs and to 0.15 mg/ml for the sedimentation velocity scans. Sedimentation coefficients were corrected to standard conditions (water, 20 °C). Sedimentation equilibrium runs were carried out from 8000–17,000 rpm, depending on molecular mass. Apparent molecular masses were evaluated from ln A versus r² plots, where A is the absorbance and r is the distance from the rotor center.
with 0.1% bovine serum albumin, 1 mM EGTA, and 10 mM Mg2+. For experiments involving antibody-mediated inhibition of adhesion, purified IgG antibodies (DECMA-1, ECCD2, M290) were present in the adhesion assay at 10 μg/ml (DECMA-1 and ECCD2) or 2.5 μg/ml (M290). Adhesion results are expressed as mean percentage of cells adhering in three or four replicate wells ± S.E.

Homophilic Adhesion—E-cadherin-Fc fusion proteins were attached to assay plates as described above. CMT93 cells were dissociated by chelation (nonenzymatic cell dissociation solution; Sigma) and maintained in suspension in Dulbecco’s modified Eagle’s medium plus 10% FCS at 37 °C for 20 min. They were then transferred to Hank’s balanced saline solution plus 1% FCS, and 8 × 10^5 cells were added to each well. The plates were then incubated at 37 °C for 30 min. Nonadherent cells were washed away, and residual adherent cells were quantitated by measuring acid phosphatase activity (25).

Cleavage with Factor Xa

1–2 μg of E-cadherin-Fc bound to 10 μl of protein A-Sepharose was suspended in 50 μl of 100 mM NaCl, 2 mM CaCl2, 20 mM Tris/HCl, pH 8.0. 1 μg of factor Xa (1 μl at 1 mg/ml, New England Biolabs) was added and incubated at room temperature for 5 h. Released cadherin fragments were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using DECMA-1. Residual IgFc on the beads was released with SDS sample buffer and analyzed by Western blotting using anti-Fc.

RESULTS

Production and Characterization of Wild Type and Mutant E-cadherin-Fc Proteins—Table I lists the two categories of E-cadherin mutations: calcium loss mutants and N-terminal modifications that affect Trp2 intercalation. A third group of E-cadherin-Fc molecules was prepared in which the two types of mutation were combined (double mutants). Analytical ultracentrifugation experiments were performed to determine molecular masses and sedimentation coefficients of wild type E-cadherin-Fc and the double mutant, WV + Ca2,3. These proteins represent the two most extreme situations with respect to possible conformational differences. The sedimentation coefficients of the wild type and mutant were closely similar (Table II), which implies that their fractional coefficients were also similar. Because the shapes proposed for these molecules are complex, these data do not distinguish between the presence or absence of cis-dimerization of cadherin chains within the Fc fusion proteins. Sedimentation equilibrium experiments for molecular mass determinations were performed at 17,000 rpm and 8000 rpm. At 17,000 rpm, both wild type and mutant proteins were monodisperse (Table II). In contrast, sedimentation of the wild type protein at a lower speed, 8000 rpm, showed the presence of a predominant species with an apparent molecular mass of 441 kDa, which is likely to represent E-cadherin-Fc molecules joined in pairs by a low affinity adhesive trans-interaction. Conversely, the double mutant, which would not be expected to be active in homophilic adhesion, gave a heterogeneous mixture of species at 202 and 400 kDa. Although it is not possible to quantify precisely the two subpopulations, we conclude that the ability of the mutant protein to form trans-interactions was greatly impaired. We emphasize that the concentration of E-cadherin-Fc proteins used in adhesion assays was ~100-fold lower than that used for ultracentrifugation, and under these circumstances all of the preparations were monodisperse.

Table I

| Amino acid changes | Direct consequences | Abbreviation |
|--------------------|--------------------|--------------|
| Addition of Met, Asp, and Leu to N terminus | Structural change likely to prevent Trp2 docking | MDL |
| W220/V3A | Prevention of Trp2 docking | WV |
| D67A | Removal of a ligand for Ca1 in domain 1–2 junction | Ca1 |
| Q101A/N102A | Removal of ligand for Ca2 and Ca3 in domain 1–2 junction | Ca2,3 |
| D134A | Removal of ligand for Ca3 in domain 1–2 junction | Ca3 |

Table II

| Sedimentation Coefficients and Molecular Masses of E-cadherin-Fc Proteins—WT and the double mutant E-cadherin-Fc WV + Ca2,3 determined by analytical ultracentrifugation |
|-----------------|-----------------|-----------------|
| Protein | Sedimentation coefficient S_{20,w} | Molecular mass |
| E-cadherin-Fc WT | 6.3 | 198\textsuperscript{b} 441\textsuperscript{c} |
| E-cadherin-Fc WV + Ca2,3 | 6.6 | 210,202/400\textsuperscript{a} |

\textsuperscript{a} The proteins were analyzed at a concentration of 0.5 mg/ml.

\textsuperscript{b} Rotor speed of 17,000 rpm.

\textsuperscript{c} Rotor speed of 8000 rpm.

α\textsubscript{E}β\textsubscript{7} Integrin Interacts with E-cadherin Dimers

α\textsubscript{E}β\textsubscript{7} dependent Adhesion of MTC-1 Cells to Wild Type or Mutant E-cadherin-Fc Fusion Proteins—Fig. 1a illustrates the basic parameters of the integrin-mediated adhesion assay and shows adhesion of MTC-1 cells to wild type E-cadherin-Fc adsorbed to an assay plate via anti-human Fc. Adhesion was completely inhibited by the anti-mouse E-cadherin antibody ECCD2 and by the α\textsubscript{E} specific antibody M290, which recognizes the α\textsubscript{E} A-domain. The antibody DECMA-1, specific for E-cadherin domain 4, had no effect on integrin-mediated adhesion. Mutant proteins were then compared with wild type for their ability to support α\textsubscript{E}β\textsubscript{7}-mediated adhesion (Fig. 1b). The MDL and WV mutants had no effect on adhesion; nor did these two modifications in combination. The mutations Ca1, Ca2,3, and Ca3 supported adhesion but with reduced efficiency. In contrast, double mutants, in which modifications to the N terminus were combined with the Ca2,3 loss mutations, failed to support adhesion. Fig. 1c shows that all types of mutation completely abrogated homophilic adhesion of CMT93 cells to the E-cadherin-Fc proteins. To gain a more complete picture of the effects of the mutations on α\textsubscript{E}β\textsubscript{7}-mediated adhesion, some of the mutant proteins were titrated in the integrin-dependent adhesion assay (Fig. 2). The titration curve given by the WV mutant was identical to that of wild type E-cadherin-Fc (Fig. 2a). In contrast, the mutants Ca2,3 and Ca3 supported adhesion less efficiently and titrated to background levels of adhesion at higher coating levels than those observed with wild type E-cadherin (Fig. 2b). Ca1 behaved similarly (Fig. 2c). These results are taken from separate experiments, and the differences in adhesion to wild type E-cadherin reflect interassay variation commonly experienced in cell adhesion tests of this type, due mainly to variation in the levels of α\textsubscript{E}β\textsubscript{7} expressed by the MTC-1 cells. Our results with mutants that adversely affect Ca2,3 coordination are broadly consistent with an earlier report that α\textsubscript{E}β\textsubscript{7} can adhere to E-cadherin-Fc in the absence of Ca2,3 (26). We have observed that Ca2,3-independent adhesion of wild type E-cadherin could be obtained by activating the integrin with 10 mM Mg2+. Prestimulation of the cells with phorbol 12-myristate 13-acetate before adhesion in the presence of EGTA maximized the adhesion obtained. Fig. 3 compares integrin-mediated adhesion with wild type E-cadherin Fc and with the WV mutant, in the presence of EGTA and 10 mM Mg2+. Approximately 50% of MTC-1 cells adhered to wild type E-cadherin, but adhesion to the WV mutant was negligible.

\textsuperscript{2} P. Kilshaw, unpublished results.
This result is consistent with the view that intercalation of Trp2 provides a mechanism for dimer formation when Ca\textsuperscript{2+} is lacking. Taken together, the results from cell adhesion tests suggest that cis-dimerization of E-cadherin by either mechanism is required for recognition by \(\alpha_E\beta_7\) but that dimerization mediated by Ca\textsuperscript{2+} in the EC1-EC2 junction gives the preferred conformation.

Antibodies to Domain 1 Peptides Distinguish Monomers from Dimers—Although recognition of E-cadherin dimers by \(\alpha_E\beta_7\) provides a plausible explanation for our results, there remained a less likely alternative possibility that Ca\textsuperscript{2+} in the EC1-EC2 junction, or Trp2 in the \(\beta\) strand could each mediate distant effects on the conformation of the BC loop that would influence integrin binding. We have addressed this issue through the use of two antisera to peptides in domain 1, one antiserum to a peptide spanning the BC loop and a second to part of the \(\beta\) strand (Fig. 4). The amino acid sequence of the latter lies in a cleft at the interface between the two juxtaposed EC1 domains and, in dimeric E-cadherin, would be inaccessible to antibodies. The antiseras were tested against wild type or mutant proteins in the presence of varying levels of Ca\textsuperscript{2+} or in EGTA. Fig. 5 shows that the two antiseras behaved similarly; reactivity of anti-BC is shown in Fig. 5a, and reactivity of anti-\(\beta\) is shown in Fig. 5b. The WT and Ca\textsubscript{2,3} proteins were not recognized by peptide-specific antibodies regardless of the presence or absence of Ca\textsuperscript{2+}. In contrast, the double mutant (WV + Ca2,3), in which both mechanisms for dimerization were absent, was recognized by both antibodies under all conditions. The behavior of the WV mutant in these assays depended on the level of Ca\textsuperscript{2+}. No binding was detected in the presence of 1.25 mm or 0.5 mm Ca\textsuperscript{2+}, but, when the concentration was reduced to 0.125 mm, weak reactivity was just detectable, and in Ca\textsuperscript{2+}-free Tris or EGTA, antibody binding was maximal. The similar behavior of the two antibodies can be explained by the inability of either of them to recognize dimeric E-cadherin. The \(\beta\) epitope was selected specifically to be inaccessible in the dimer, but the epitope on the BC loop is exposed. Molecular modeling with cadherin dimers and Fab fragments (not shown) strongly suggests that access by an IgG molecule to the BC loop would be compromised in the dimer by the close proximity of the two EC1 domains.

Conformational Changes in E-cadherin-Fc Proteins—a Factor Xa cleavage site was introduced into wild type and five mutant E-cadherin Fc proteins, immediately distal to the three disulfide bonds of the Ig hinge, to allow release of the extracellular domains of E-cadherin. The presence of this site had no effect on the functional activity of the proteins in heterophilic or homophilic adhesion tests nor on reactivity with our peptide-specific antibodies. All of the preparations were shown to be monodisperse by fast protein liquid chromatography. We observed striking, consistent differences in the digestion products of the mutant Fc fusion proteins following treatment with factor Xa. The pattern correlated with the ability of the mutants to support \(\alpha_E\beta_7\)-mediated adhesion and to form dimers. Fig. 6a shows a Western blot of the digestion products seen with our panel of mutants. Introduction of the cleavage site into WT E-cadherin caused factor Xa to release an 86-kDa fragment corresponding to the EC1–EC5. A similar pattern was seen with the MDL and WV mutants. In contrast, the Ca2,3 mutant gave a closely spaced double band at \(\sim 170 \) kDa in

![Cell adhesion to E-cadherin-Fc proteins.](http://www.jbc.org/)
addition to the expected product at 86 kDa. The double mutants, WV/H11001Ca2,3 and MDL/H11001Ca2,3, gave a single band at 170 kDa and the expected 86-kDa product. Upon reduction, all digestion products were resolved at 86 kDa (Fig. 6b). These results demonstrate that factor Xa cleaved some of the mutants at nonspecific sites near the Ig hinge, in close proximity to the expected position, to give a 170-kDa disulfide-bonded product. Analysis of the residual material remaining on the Sepharose beads after digestion (i.e. the remaining IgFc) gave a 50-kDa band before reduction (Fig. 6c) and a 25-kDa band after reduction (Fig. 6d), demonstrating that the aberrant cleavage sites were within the Ig hinge and were flanked on either side by disulfide bonds. The patterns of cleavage shown in Fig. 6 were seen in all experiments performed and with separate preparations of the E-cadherin-Fc proteins. We attribute the altered pattern of cleavage to major conformation changes in the proteins, specifically to the introduction of greater segmental flexibility and dynamic freedom in the hinge region caused by failure of cis-dimer formation by the double mutants. In the case of the Ca2,3 mutant, which we argue is dimerized by Trp2 cross-intercalation, the loss of Ca2+ at the EC1-EC2 junction would introduce freedom of movement between EC1 and EC2 (27), and, because the other domain junctions in the cadherin molecule are relatively rigid, this increased freedom would be transmitted to the Ig hinge. In this case, two closely spaced aberrant factor Xa cleavage sites were present. In effect, loss of interdomain Ca2+ or failure of dimerization at the level of domain 1 causes the molecule to flex more at the hinge. Cleavage by factor Xa is acting as a sensitive indicator of conformational change. Nonspecific cleavage by factor Xa is known to occur in regions of high flexibility and at lysyl residues (28, 29); both criteria are fulfilled in the circumstances of our experiments. Cumulative evidence from the present results and from previous studies on the role of Ca2+ and Trp2 in dimer formation suggests that our E-cadherin-Fc proteins adopt the conformations proposed in Fig. 7.

**DISCUSSION**

The primary aim of this study was to determine whether cis-dimerization of E-cadherin is required for recognition by αEβ7. We chose E-cadherin-Fc proteins for the analysis because the disulfide bonds of the Ig hinge, in close proximity to the expected position, to give a 170-kDa disulfide-bonded product. Upon reduction, all digestion products were resolved at 86 kDa (Fig. 6b). These results demonstrate that factor Xa cleaved some of the mutants at nonspecific sites near the Ig hinge, in close proximity to the expected position, to give a 170-kDa disulfide-bonded product. Analysis of the residual material remaining on the Sepharose beads after digestion (i.e. the remaining IgFc) gave a 50-kDa band before reduction (Fig. 6c) and a 25-kDa band after reduction (Fig. 6d), demonstrating that the aberrant cleavage sites were within the Ig hinge and were flanked on either side by disulfide bonds. The patterns of cleavage shown in Fig. 6 were seen in all experiments performed and with separate preparations of the E-cadherin-Fc proteins. We attribute the altered pattern of cleavage to major conformation changes in the proteins, specifically to the introduction of greater segmental flexibility and dynamic freedom in the hinge region caused by failure of cis-dimer formation by the double mutants. In the case of the Ca2,3 mutant, which we argue is dimerized by Trp2 cross-intercalation, the loss of Ca2+ at the EC1-EC2 junction would introduce freedom of movement between EC1 and EC2 (27), and, because the other domain junctions in the cadherin molecule are relatively rigid, this increased freedom would be transmitted to the Ig hinge. In this case, two closely spaced aberrant factor Xa cleavage sites were present. In effect, loss of interdomain Ca2+ or failure of dimerization at the level of domain 1 causes the molecule to flex more at the hinge. Cleavage by factor Xa is acting as a sensitive indicator of conformational change. Nonspecific cleavage by factor Xa is known to occur in regions of high flexibility and at lysyl residues (28, 29); both criteria are fulfilled in the circumstances of our experiments. Cumulative evidence from the present results and from previous studies on the role of Ca2+ and Trp2 in dimer formation suggests that our E-cadherin-Fc proteins adopt the conformations proposed in Fig. 7.
together, possibly by a leucine zipper-type mechanism, and have proposed that this mechanism acts in concert with cis-interactions at domain 1 to stabilize the dimer. At low Ca\(^{2+}\) concentrations, other ectodomains may also be involved (33). We have focused specifically on cis-interactions at domain 1. The mutations in the N-terminal region of domain 1 and in the Ca\(^{2+}\) binding sites in the EC1-EC2 junction were selected to investigate the two proposed mechanisms for dimerization. We extended the N terminus by three amino acids, MDL, because this would be expected to interfere with the cross-intercalation of Trp2 between domain pairs (5) or docking into its own domain (7). As predicted, results with this mutant were identical to those with the WV mutant and are consistent with previous reports that correct cleavage of the N terminus (34) and the presence of Trp2 (12, 13) are both essential for cadherin function. Mutations of amino acids coordinating Ca1, Ca2,3, or Ca3 were selected by reference to two published crystal structures (6, 7) and a previous report (35) that the Ca3 mutant (Asp134\(^{3}\)Ala) prevents homophilic adhesion. The Ca2,3 mutant (Q101A/N102A) removed Gln 101, which is thought to play a key role in hydrogen bonding between the domain pair (6). The binding site for Ca1 was targeted because it is thought to have a lower affinity for Ca\(^{2+}\) than the other two sites (7, 27) and to be required for homophilic adhesion but not dimer formation (7). Our results with Ca1 were, however, similar to those with the other two calcium loss mutants. Calcium binding has not yet been measured empirically in these mutants, so it is not absolutely certain that we achieved selectivity for particular calcium atoms; loss of a single calcium ligand may have destabilized the junctional region as a whole.

Prevention of Trp2 intercalation alone was shown to have no effect on integrin-mediated adhesion, and loss of one or more calcium atoms in the EC1-EC2 junction permitted E\(^{-}\)/mediated adhesion but with reduced efficiency. The two types of modification in combination completely prevented adhesion, which implies that integrity of one or the other mechanism for dimerization was required. A reciprocal relationship between Trp2 and interdomain Ca\(^{2+}\) was confirmed by the observation that integrin-mediated adhesion in the presence of EGTA was abrogated by the removal of Trp2.

Binding of peptide antibodies specific for the BC loop or the B strand strongly supports our contention that dimers can form by either mechanism. The antibodies bound to double mutants, which should not form dimers, but did not bind to wild type or single mutants in which dimerization would be expected. Experiments with the Trp2 replacement mutant, WV, demonstrated that the Ca2\(^{2+}\)-dependent mechanism required Ca\(^{2+}\) concentrations higher than 125 \(\mu\)M. At this level, the first indication of dissociation into monomers was detected.

Attempts to monitor dimerization by chemical cross-linking experiments with E-cadherin-Fc have been unsuccessful for technical reasons. Similarly, electron microscopic observations on the proteins have been confounded by disruption of the dimeric conformation during the preparative procedures. Nev-

**FIG. 5.** Reactivity of E-cadherin-Fc proteins with two peptide antisera in varying concentrations of calcium. a, antiserum to the BC loop; b, antiserum to the B strand. Antibody binding tests were performed in EGTA (C), Ca\(^{2+}\)-free TBS (D), 0.125 mM Ca\(^{2+}\) (F), 0.5 mM Ca\(^{2+}\) (A), or 1.25 mM Ca\(^{2+}\) (B). Both antisera recognized the cadherin monomer only.


Figure 6. Cleavage of E-cadherin-Fc proteins with factor Xa reflects conformational changes in the IgFc hinge. A factor Xa cleavage site (FXa) was introduced into the N-terminal side of the IgFc hinge in our panel of proteins. The products released after digestion with factor Xa were separated by SDS-polyacrylamide gel electrophoresis under nonreducing (a) or reducing (b) conditions and were detected by Western blotting with DECA-1. WT E-cadherin-Fc lacking a factor Xa site was not cleaved by factor Xa (left-hand track). In the other proteins, the band at ~86 kDa represents E-cadherin EC1-EC5. The products at ~170 kDa are paired EC1-EC5 units joined together by disulfide bonds within the IgFc hinge. The paired EC1-EC5 units became separated upon reduction (b). The result shows that aberrant cleavage occurred in E-cadherin-Fc proteins in which dimerization via EC1 had failed or was maintained by Trp2 only. Residual IgFc remaining after release of EC1-EC5 was separated under nonreducing (c) or reducing (d) conditions and detected by blotting with anti-Fc; products were present at 50 and 25 kDa, respectively, representing disulfide-bonded or single Fc chains. This result confirms that aberrant cleavage occurred at sites within the Ig hinge, flanked on either side by disulfide bonds. The high MW bands in c and d represent small amounts of residual intact or partially digested E-cadherin-Fc remaining on the beads.

Figure 7. Proposed structures of the wild type and mutant E-cadherin-Fc proteins. The model on the left shows the Ca\(^{2+}\)-dependent dimer, which has a relatively firm structure with little flexibility at the EC1-EC2 junction. The center model shows the Trp2-dependent dimer, which has lost rigidity at the EC1-EC2 junction when Ca\(^{2+}\) is lacking here and therefore is permitted flexibility in the directions of the arrows. The third model has lost the capacity to dimerize and shows free flexibility at the Ig hinge. All models would be expected to show some flexibility also at right angles to the plane of the paper. Positions of the factor Xa cleavage site and the three disulfide bonds are shown.

Nevertheless, our studies with factor Xa proteolysis have provided additional evidence for major conformational changes consistent with the presence of absence of dimers. Generation of nonspecific cleavage products in the IgFc hinge using factor Xa can be adequately explained by differences in flexibility at the Ig hinge related to dimerization at the level of EC1. To our knowledge, this is a novel use of a restriction peptidase to monitor conformational changes in a protein.

Another laboratory recently reported that dimerization of E-cadherin was unnecessary for either \(\alpha_E\beta_7\)-mediated adhesion or homophilic adhesion (36). The authors observed that unmodified E-cadherin-Fc fusion protein that had been reduced and alkylated in the Ig hinge and was assumed to be monomeric functioned normally in either type of assay. The interpretation is, however, flawed, because in these circumstances strong noncovalent interactions between the CH3 domains of the paired Fc regions would have maintained the dimeric structure (37, 38).

**Implications for Integrin Function**—In principal, a dimeric ligand could either bind two separate integrin molecules by monovalent interactions or one integrin molecule in a bivalent manner. Recent studies on the crystal structure of the ICAM-1 dimer suggest that monovalency is likely in the interaction with LFA-1 (39). The critical Glu\(^{34}\) integrin contact residues in the ICAM-1 dimer are located on outward facing surfaces on opposite sides of the molecule a maximum distance apart, about 4.2 nm (39). In contrast, the two Glu\(^{31}\) residues that lie side by side on the top surface of dimeric E-cadherin and are recognized by \(\alpha_E\beta_7\) are more closely spaced, about 2.4 nm apart. A convincing model has recently been proposed for the interaction between the A-domain of the \(\alpha_E\) integrin subunit and the top surface of E-cadherin domain 1, involving docking of E-cadherin Glu\(^{31}\) into the \(\alpha_E\) MIDAS site (19). It is doubtful whether two \(\alpha_E\) A-domains could dock simultaneously onto the E-cadherin dimer. Additional steric constraints imposed by the \(\beta\)-propeller of the \(\alpha\)-subunit and the presence of the \(\beta_7\) subunit in the intact integrin would make this stoichiometry highly unlikely. An alternative explanation is that contact residues from each component of the EC1 domain pair are recognized by a single \(\alpha_E\beta_7\) molecule. If so, the spatial relationships between the exposed acidic side chains on the top surfaces of the two cadherin molecules would be critical for integrin binding. The Ca\(^{2+}\)-dependent dimer would present the paired recognition sites in a relatively firm structure. By contrast, the Trp2-dependent dimer would permit freedom of movement of the two EC1 domains relative to one another, which would compromise correct spacing of the contact sites. This could explain why integrin binding was less efficient in the Ca\(^{2+}\) loss mutants.

It is clear from mutagenesis studies (19) and from epitope mapping of blocking antibodies (20, 40) that the A-like domain of the \(\beta_7\) subunit, especially the MIDAS cleft within it, is essential for cadherin recognition. It is possible that the A-like domain of the \(\beta_7\) subunit may dock to an acidic side chain on the top surface of one component of the dimer, while the \(\alpha_E\) A-domain engages Glu\(^{31}\) from the other component.
Direct evidence for this is not yet available. It has been proposed that, in general, the integrin β-subunit A-like domain functions to regulate ligand-binding by the A-domain of the α-subunit and, in integrins that lack an α-subunit A-domain, directly participates in ligand binding (41). Structural models have been proposed for both situations (41, 42). Recent experiments in which the α9-A-domain has been locked by mutagenesis into an “open” conformation have shown that the ligand binding activity of LFA-1 is attributable entirely to the α chain A-domain and that the β subunit performs a regulatory role (43, 44). In suggesting ligand engagement by both α and β subunits, we envisage that α9 may be a special case. Why should this be so? The α9β7 integrin is unusual in two respects. First, expression is normally confined to intraepithelial lymphocytes in the gut, a population of highly cytotoxic, potentially self-reactive, effector T cells specialized for a local tissue-specific function (45). Second, only one ligand, E-cadherin, has been identified for α9β7. Recently, weak cadherin-independent binding to an unidentified ligand on intestinal microvascular endothelial cells has also been reported (46), but the physiological significance of this is not clear. Tightly restricted ligand-binding specificity may be necessary for α9β7, because it is important that potentially autoreactive mucosal T cells be retained in their correct location. Ligand recognition requiring ligand contact by both integrin subunits would provide a mechanism for ensuring more precise specificity than that seen in other integrins. A recent report that α9-deficient mice develop an inflammatory skin disorder (47) is consistent with the idea that correct tissue compartmentalization of these effector T cells is important.

Two Mechanisms for Cis-dimerization—Evidence on the role of Ca2+ in cis-dimerization is complex and partly conflicting. The average dissociation constant for calcium binding on intestinal microvascular endothelial cells has also been reported (46), but the physiological significance of this is not clear. Tightly restricted ligand-binding specificity may be necessary for α9β7, because it is important that potentially autoreactive mucosal T cells be retained in their correct location. Ligand recognition requiring ligand contact by both integrin subunits would provide a mechanism for ensuring more precise specificity than that seen in other integrins. A recent report that α9-deficient mice develop an inflammatory skin disorder (47) is consistent with the idea that correct tissue compartmentalization of these effector T cells is important.

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Recognition of E-cadherin by Integrin $\alpha_E$\beta_7: REQUIREMENT FOR CADHERIN DIMERIZATION AND IMPLICATIONS FOR CADHERIN AND INTEGRIN FUNCTION

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