Intracellular Domain of Desmoglein 3 (Pemphigus Vulgaris Antigen) Confers Adhesive Function on the Extracellular Domain of E-Cadherin without Binding Catenins

Joo-Young Roh and John R. Stanley

Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract. For the extracellular (EC) domain of E-cadherin to function in homophilic adhesion it is thought that its intracytoplasmic (IC) domain must bind α- and β-catenins, which link it to the actin cytoskeleton. However, the IC domain of pemphigus vulgaris antigen (PVA or Dsg3), which is in the desmoglein subfamily of the cadherin gene superfamily, does not bind α- or β-catenins. Because desmogleins have also been predicted to function in the cell adhesion of desmosomes, we speculated that the PVA IC domain might be able to act in a novel way in conferring adhesive function on the EC domain of cadherins.

To test this hypothesis we studied aggregation of mouse fibroblast L cell clones that expressed chimeric cDNAs encoding the EC domain of E-cadherin with various IC domains. We show here that the full IC domain of PVA as well as an IC subdomain containing only 40 amino acids of the PVA intracellular anchor (IA) region confer adhesive function on the E-cadherin EC domain without catenin-like associations with cytoplasmic molecules or fractionation with the cell cytoskeleton. This IA region subdomain is evolutionarily conserved in desmogleins, but not classical cadherins.

These findings suggest an important cell biologic function for the IA region of desmogleins and demonstrate that strong cytoplasmic interactions are not absolutely necessary for E-cadherin–mediated adhesion.

THE originally described or classical cadherins, the prototypic example of which is E-cadherin, function as homophilic, calcium-dependent adhesion molecules (45, 46). These are widely distributed in epithelial as well as non-epithelial cells and are thought to be crucial in cell–cell adhesion and interactions in development thereby contributing to tissue architecture and formation. Their importance is also underscored by the correlation of their loss of function with metastatic potential of certain epithelial cancers (3, 47).

Cadherins are integral membrane glycoproteins that have a transmembrane domain that spans the membrane once (4). The EC amino-terminal domain can be subdivided into four homologous subdomains and a fifth subdomain closest to the cell membrane. The intracytoplasmic (IC)[1] domain can be subdivided into an highly charged intracellular anchor (IA) subdomain, just inside the cell, and a so-called IC cadherin segment (ICS) (see Fig. 1).

The crucial function of the EC domain of cadherins in mediating calcium-dependent cell adhesion has been demonstrated by both antibody interference with function as well as by site-directed mutagenesis. For example, the monoclonal antibodies ECCD-1 and DECMA-1, which are directed against the amino-terminal and carboxy-terminal extracellular (EC) domain, respectively, block E-cadherin–mediated adhesion (33, 36). Mutagenesis of one of the calcium binding sites of E-cadherin abrogates its adhesive function (35). Finally, mutagenesis of the cleavage site of the precursor polypeptide which prevents its proper processing to the mature form, also interferes with adhesive function (37). These mutagenesis studies suggest that proper EC conformation may be important for correct function.

However, not only is the EC domain of cadherins crucial for proper function, but, perhaps surprisingly, so is their IC domain (29). In fact, the IC domains of various classical cadherins are the most highly conserved domains within this family (10, 29). These domains are known to bind cytoplasmic molecules called catenins (34, 51). Co-precipitation experiments have demonstrated that cadherins bind to at least three molecules, α-, β-catenin and plakoglobin (19, 24, 34, 40, 51). α- and β-catenins are thought to link the cadherins to the actin cytoskeleton (14, 27, 28, 34, 38). This associa-

Address all correspondence to J. R. Stanley.

Dr. Stanley's present address is Department of Dermatology, University of Pennsylvania, 211 CRB, 415 Curie Blvd., Philadelphia, PA 19104. Tel.: (215) 898-3240. Fax: (215) 573-2033.

1. *Abbreviations used in this paper*: CMV, cytomegalovirus; EC, extracellular; Ecad, E-cadherin; HCMF, Hepes-buffered, calcium- and magnesium-free Hank's balanced salt solution; IA, intracellular anchor; IC, intracytoplasmic; ICS, intracytoplasmic cadherin segment; PCR, polymerase chain reaction; PVA, pemphigus vulgaris antigen.

tion has been demonstrated by showing that a certain proportion of cadherins associate with the nonionic detergent-insoluble cytoskeleton. Differential detergent extraction of cadherins has also shown that β-catenin is most tightly bound to the IC domain of cadherins, and that α-catenin may provide the link to the actin cytoskeleton (38).

Various studies have demonstrated that the binding of the IC domain of cadherins to the catenins is correlated with the functional ability of the EC domain (27, 28, 38). Thus, cadherins that have truncated IC domains which can no longer bind the catenins, do not function to aggregate cells, even though the EC domain is expressed properly on the cell surface. Loss of as few as 37 amino acids from the carboxy terminus of the E-cadherin IC domain results in loss of the ability to bind catenins and mediate adhesion (27, 38). Similarly, cancer cells that have normal E-cadherin expression, but are genetically deficient in α-catenin expression, cannot aggregate (13, 42). Furthermore, expression of α-catenin in these cells through cDNA transfection allows them to aggregate. Thus, in these cells even full-length E-cadherin does not inherently have the ability to mediate adhesion without interaction with catenins.

These types of studies have led to a generally accepted principle of cadherin function: cadherins must bind catenins inside the cell for them to function properly outside the cell. This intracellular binding is thought to confer adhesive function because of linkage of cadherins to the cytoskeleton and/or because clustering of the cadherin molecules increases the avidity of their interactions. However, in spite of this dogma, there is some evidence that, in special situations, the EC domain of cadherins might retain some biological activity (9). For example, the EC domain of E-cadherin in one study (50), but not another (11), could interfere with E-cadherin–mediated cell–cell adhesion. The EC domain of N-cadherin, when immobilized on a dish, can also demonstrate some biological activity in promoting retinal cell attachment and neurite outgrowth (39), but in this case the attaching cells had intact N-cadherin presumably with proper cytoskeletal linkage. A chimeric molecule consisting of the EC domain of E-cadherin (L-CAM) and the transmembrane/IC domain of N-CAM is able to function in adhesion, perhaps because the N-CAM IC domain binds different cytoskeletal components than that of E-cadherin (16). Finally, T-cadherin, which is anchored to the membrane by a glycosyl phosphatidylinositol group and thus presumably does not bind the cytoskeleton through interaction with catenins, can mediate rather weak cell–cell adhesion (48). However, the EC domain of T-cadherin lacks several properties of classical cadherins (i.e., E-cadherin, N-cadherin, and P-cadherin).

Desmogleins form a subfamily of the cadherin supergene family (8, 20, 30, 49). Desmogleins and classical cadherins are homologous in amino acid sequence both in their EC domains as well as in the ICS of their IC domain. However, the desmogleins have a longer IC region than do classical cadherins (Fig. 1). This carboxy-terminal subdomain contains a desmoglein-specific repeating amino acid motif, and is, therefore, termed the intracellular repeat region. Whereas the classical cadherins localize to adherens junctions (which associate with actin microfilaments), the desmogleins are found in desmosomes (into which insert keratin or other intermediate filaments). Two of the desmogleins, Dsgl and Dsg3, are autoantigens in the autoimmune blistering skin diseases pemphigus foliaceus and pemphigus vulgaris, respectively (43). In fact, because Dsg3 was discovered as the target of autoantibodies in patients it is also termed PVA (2). These desmogleins have been shown to bind plakoglobin, but not α- or β-catenin, in epithelial cells (21, 40, 41).

Although the function of desmogleins has not been clearly elucidated, they are speculated to function in cell adhesion because: (*a*) they are members of the cadherin gene superfamily; (*b*) autoantibodies against them cause loss of cell adhesion; (*c*) they localize to the desmosome, a cell adhesion structure. We speculated, therefore, that the IC domain of desmogleins might function in conferring adhesion without binding α- and β-catenin and without interaction with the actin cytoskeleton. To test this hypothesis we expressed cDNA encoding a chimeric molecule consisting of the EC domain of E-cadherin and the IC domain of PVA in L cells, mouse fibroblasts that contain α- and β-catenin but no endogenous cadherins, and tested for adhesive function as well as cytoplasmic and cytoskeletal interactions.

Materials and Methods

Construction of Chimeric cDNAs in Eukaryotic Expression Vectors

β-actin promoter-driven eukaryotic expression vectors with cDNA encoding mouse E-cadherin (pBATEM2) and the neomycin resistance gene (pBATneo) were kindly provided by Masatoshi Takeichi (31). EcadPV cDNA (see Fig. 1) was constructed by amplifying the EC domain of E-cadherin by polymerase chain reaction (PCR) with primers containing EcoRV (5′ primer) and Eco0109 (3′ primer) restriction sites on their 5′ ends. The transmembrane and IC domains of PVA were obtained by Eco0109 and NotI digestion of the previously cloned cDNA (2). These products were cloned into the EcoRV/NotI multiple cloning site of pcDNAI/Amp (Invitrogen Corp., San Diego, CA), a eukaryotic expression vector with a cytomegalovirus (CMV) promoter. Dideoxy DNA sequencing revealed no PCR mutation in the EC domain of E-cadherin in this construct.

EcadPVΔ320 cDNA (see Fig. 1) was constructed by PCR amplification of the EcadPV cDNA with the 5′ primer at the 5′ end of the cDNA and the 3′ primer upstream of the 3′ end so that the final product encoded a 320-amino acid-truncated IC domain. This PCR product was subcloned into the CMV promoter-driven eukaryotic expression vector pcDNA3 (Invitrogen).

EcadΔ135 was constructed as previously described (as pBATEM24) (27).

Transfection and Cloning of L cells

L cells (CCL 1.1 American Type Tissue Culture Collection, Rockville, MD) were grown in Dulbecco-modified Eagle's minimal essential medium/Ham's F12 (GIBCO-BRL, Bethesda, MD), supplemented with 10% fetal calf serum, in one-well tissue culture chamber slides (Nunc, Inc., Roskilde, Denmark). Transfection with 3 μg EcadPV or EcadΔ135, each with 0.3 μg pBATneo or with 3 μg of EcadPVΔ320 and lipofectamine (GIBCO-BRL) was performed according to the manufacturer's directions. After 6 h, fresh medium was added. 48 h after transfection, cells were transferred to a 100 mm dish and cultured in medium containing 400 μg/ml G418 (GIBCO-BRL). After 12–14 d, G418-resistant clones were isolated with cloning rings. Immunofluorescence for cell surface expression of the E-cadherin EC domain was used to determine which clones to further characterize.

The Ecad clone used for these studies has been described previously (1).

Immunofluorescence and Flow Cytometric Analysis of L Cell Clones

To detect the EC domain of E-cadherin on the surface of L cell clones we performed immunofluorescence on living cells with ECCD-2, a monoclonal anti-E-cadherin antibody kindly provided by Masatoshi Takeichi (Kyoto University, Kyoto, Japan), and an isotype-matched rat IgG2a (Pharmingen, San Diego, CA), as previously described (44). ECCD-2 was used as undiluted hybridoma culture supernatant. A 1:100 dilution of a 1 mg/ml rat IgG2a (Pharmingen) was used as a negative control. A fluorescein-conju-

gated F(ab')$_2$-goat anti-rat IgG (Tago Inc., Burlingame, CA) diluted 1:40 was used to detect these antibodies.

We also used the same antibodies to estimate the amount of cell surface expression of the E-cadherin ectodomain by flow cytometric analysis of living cells, as previously described (1). In this case 5×10^5 cells in 200 μl were incubated with 10 μl of ECCD-2 hybridoma culture supernatant or 0.1 μg of rat IgG2a isotype control.

Immunoprecipitation

Immunoprecipitation of nonionic detergent extracts of radiolabeled L cell clones was performed essentially as described (1). Clones were radiolabeled for 4 h with [^{35}S]methionine/cysteine in methionine- and cysteine-free Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The supernatant of the detergent lysate was collected after centrifugation at 100,000 *g* for 30 min. Lysate containing 30×10^6 cpm per immunoprecipitate was first preabsorbed at 4°C with 25 μl of normal rat serum for 2 h, and then twice with 25 μl of protein G sepharose 4 fast flow (Pharmacia, Piscataway, NJ) for 1–2 h each. The preabsorbed supernatant was immunoprecipitated with ~1–2 μg of ECCD-2 (purified from hybridoma culture supernatant with protein G) or rat IgG2a isotype control for 2 h, followed by 25 μl of protein G suspension for 1 h. Immunoprecipitates were washed five times with 0.5% Triton X-100 in TBS (150 mM NaCl in 10 mM Tris-HCl, pH 7.4), then eluted with sample buffer containing dithiothreitol and resolved on 6% tris-glycine SDS gels (Novex, San Diego, CA).

Immunoblotting

Transfected L cell clones were extracted with sample buffer containing dithiothreitol. After incubation at 100°C for 2 min, samples from equivalent cell numbers were electrophoresed on 8% tris-glycine SDS gels, and then electrophoretically transfered to nitrocellulose (Novex). Dr. Jackie Papkoff (Sugen, Inc., Redwood City, CA) kindly provided rabbit anti-mouse β-catenin serum and rabbit anti-mouse plakoglobin serum (12), which were used for immunoblotting at 1:1,000 and 1:500, respectively, according to previously reported procedures (2). Antibodies were diluted in 5% powdered milk in 0.05% Tween-20 in TBS, and blots were washed with 0.05% Tween-20 in TBS. The bound rabbit antibody was detected with a 1:500 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Tago), and then developed (BCIP/NBT system; Bio-Rad Laboratories).

Cell Aggregation Assays

Single cell suspensions of L cell clones were obtained by incubation at 37°C for 20 min with 0.01% trypsin (T-8003; Sigma Chemical Co., St. Louis, MO) in 10 mM Hepes-buffered, calcium- and magnesium-free Hank's balanced salt solution (HCMF; Biofluids Inc., Rockville, MD) with either 1 mM $CaCl_2$ (T/C condition; 0.01% trypsin in 1 mM $CaCl_2$) or 1 mM EDTA (T/E condition; 0.01% trypsin in 1 mM EDTA). Trypsin was neutralized with 2 vol HCMF containing 0.05% soybean trypsin inhibitor (GIBCO-BRL), 10% fetal calf serum, and 10 μg/ml DNAse I (GIBCO-BRL). Cells were washed with the same trypsin-neutralizing solution, and then dispersed into single cell suspension of 3×10^5 cells per 3 ml of HCMF containing 1% bovine serum albumin, 10 μg/ml DNAse I, and 1 mM $CaCl_2$ or 1 mM EDTA. Each 3 ml of these cells was added to wells of six-well tissue culture plates (Falcon 3046; Becton Dickinson Labware, Mountain View, CA) which had been previously coated at 37°C for 2 h with 2% bovine serum albumin in TBS. The cells were incubated on a rotary shaker (80 rpm) at 37°C for 20 min to 1 h, and then observed and photographed and/or fixed by adding glutaraldehyde from a 70% stock solution (Polysciences Corp., Warrington, PA) to a final concentration of 2% before counting particles with a Coulter Counter (model Z_B; Coulter Electronics, Hialeah, FL). The degree of aggregation was calculated by the formula $(N_0 - N_{60})/N_0) \times 100\%$ where N_0 and N_{60} are the particle number at time 0 and 60 min, respectively (27).

To study inhibition of E-cadherin aggregation, cells were aggregated in 24-well plates (Costar Corp., Cambridge, MA) with a 1:100 dilution of ECCD-1 (53) or, as a control, PCD-1 (32) ascites (from nude rat). Hybridomas producing both antibodies were kindly provided by M. Takeichi.

Determination of Fractionation of Ecad and EcadPV with the Detergent-insoluble Cytoskeleton

Nonionic detergent extracts of L cell clones (soluble fraction) and SDS extracts of the resulting pellet (insoluble fraction) were subjected to immunoblotting as previously described (27) with the following minor modifications. 3×10^6 cells were extracted with 100 μl of 2.5% Nonidet P-40 in HCMF to obtain the soluble fractions. The insoluble pellet was extracted with 200 μl of 1× SDS gel sample buffer (Novex). Immunoblotting was performed as described above except that membranes were blocked with 3% gelatin in TBS and stained with a 1:25 dilution in 0.1% Tween-20 of a 35× concentrated ECCD-2 hybridoma supernatant. ECCD-2 was detected with alkaline phosphatase-conjugated goat anti-rat IgG (Tago).

Computer Analysis

The GAP and PileUp program from the University of Wisconsin Genetics Computer Group software (7) on a VAX computer were used to determine homologies among desmogleins and cadherins.

Results and Discussion

Cloning of L Cells Expressing the EC Domain of E-Cadherin Together with Various IC Domains

Eukaryotic expression vectors were constructed with cDNA encoding the EC domain of E-cadherin and either the complete or truncated E-cadherin IC region or the complete or truncated IC region of PVA (Dsg3). These vectors were transfected, along with a neomycin resistance gene, into L cells, mouse fibroblasts that do not normally express cadherins. L cell clones stably expressing these constructs were selected in G418-containing growth medium.

The proteins encoded by these constructs are shown in Fig. 1. Clones expressing full-length E-cadherin (Ecad) were used as a positive control for calcium-dependent cell aggregation assays (26). Clones expressing E-cadherin minus the carboxy-terminal 135 amino acids (EcadΔ135) were used as negative controls for each aggregation assay to rule out non-specific aggregation. It is known that this truncated molecule does not bind catenins, does not organize with the actin cytoskeleton, and does not mediate calcium-dependent aggregation (27). Although we arbitrarily chose EcadΔ135 as a functional negative control for our adhesion assay, it should be noted that truncations of the E-cadherin carboxy terminus as small as 50 or 37 residues have been reported by three different research groups to also cause complete loss of the ability of E-cadherin to mediate aggregation in this system (16, 27, 38).

Clones expressing chimeric molecules containing the EC domain of E-cadherin and the transmembrane and IC do-

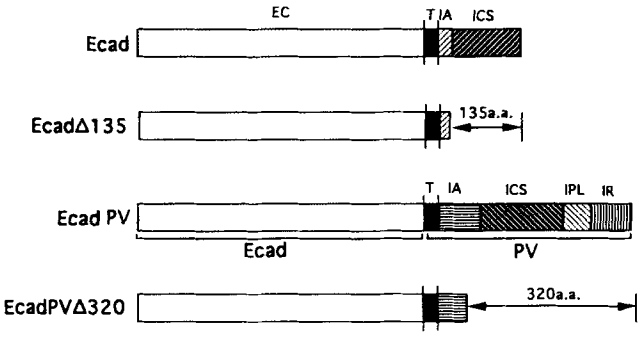


Figure 1. Proteins expressed by L cell clones transfected with cDNAs. *EC*, extracellular domain of E-cadherin; *T*, transmembrane region; *IA*, intracellular anchor; *ICS*, intracytoplasmic cadherin segment; *IPL*, intracytoplasmic proline-rich linker; *IR*, intracytoplasmic (desmoglein) repeating unit. *PV*, region of chimeric EcadPV molecule from PVA; *Ecad*, region from E-cadherin.

main of PVA (EcadPV) were used to determine if the IC domain of PVA could substitute for that of E-cadherin in conferring adhesive function on the EC domain of E-cadherin. Finally, clones expressing EcadPV minus the carboxy-terminal 320 amino acids (EcadPVΔ320) were used to determine which subdomains of the IC region of PVA might be important in conferring function on the EC domain of E-cadherin.

Detection of Cell Surface Expression of the Ectodomain of E-Cadherin on Ecad, EcadPV, and EcadΔ135 Clones

L cell clones described above were first screened by immunofluorescence with ECCD-2, a monoclonal anti-E-cadherin antibody. Clones with positive cell surface expression were selected for further study. There was no clear cut difference in the pattern of immunofluorescence staining between Ecad and EcadPV clones (Fig. 2, *A* and *B*). E-cadherin, in both cases, tended to be concentrated at cell–cell borders, but was also seen on the free surface of cells. However, EcadΔ135 clearly showed a different pattern with more diffuse cell surface staining and more rounded cells (Fig. 2 *C*). Control neomycin-transfected L cells (neoβ) did not show any staining with ECCD-2 (Fig. 2 *D*).

For aggregation studies we wanted to use clones that expressed about similar amounts of the ectodomain of E-cadherin on their cell surface. Therefore, we used flow cytometric analysis to roughly quantitate and compare clones for cell surface expression of the EC domain of E-cadherin. Flow cytometric analysis was done at the same time as aggregation studies because, interestingly, we noted that with increasing time in culture EcadPV clones tended to display lower cell surface expression of the E-cadherin ectodomain (whereas Ecad clones had stable expression). Fig. 3 *A* shows that Ecad, EcadΔ135, and EcadPV clones had comparable surface expression of the E-cadherin ectodomain. These are the same clones used for aggregation in Fig. 5 *A* (see below).

The EcadPV Chimeric Molecule Does Not Show Tight Association with α- or β-Catenins in L Cells

Full length desmogleins, including PVA, do not bind α- or β-catenins in epithelial cells (40, 41). We wanted to rule out the possibility that the chimeric EcadPV molecule binds these catenins in these L cell clones. ECCD-2 was used to precipitate EcadPV and Ecad from metabolically radiolabeled proteins extracted with nonionic detergent from the corresponding L cell clones (Fig. 4). As expected, α- and β-catenin were co-precipitated with Ecad. However, with the same conditions, these catenins were not co-precipitated with EcadPV. Two independently isolated EcadPV clones were immunoprecipitated with the same result.

In keratinocytes and other epithelial cells the IC domain of PVA binds plakoglobin (21). Since fibroblasts may contain small amounts of plakoglobin (5, 17), we wanted to deter-

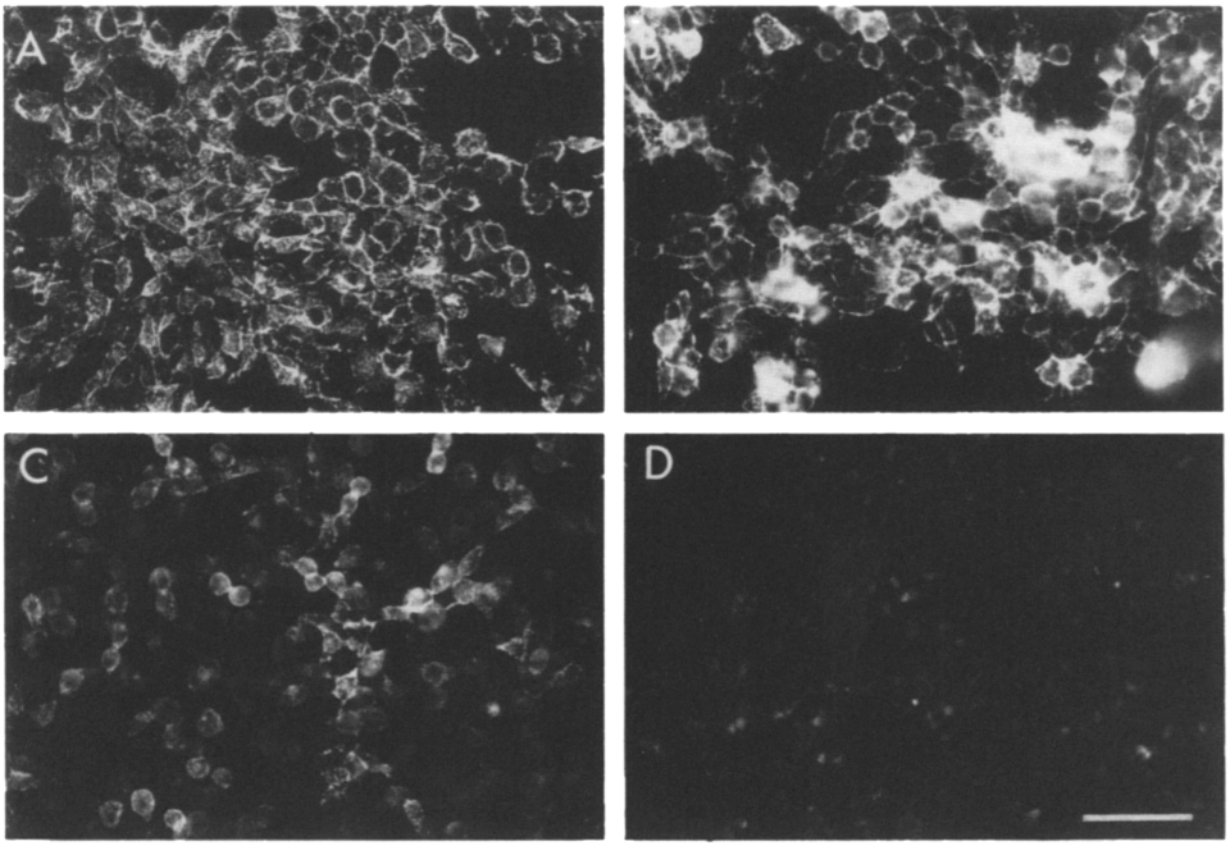


Figure 2. Immunofluorescence of L cell clones with anti-E-cadherin antibody, ECCD-2. (*A*) Ecad clone. (*B*) EcadPV clone. (*C*) EcadΔ135 clone. (*D*) neoβ clone. Bar, 100 μm.

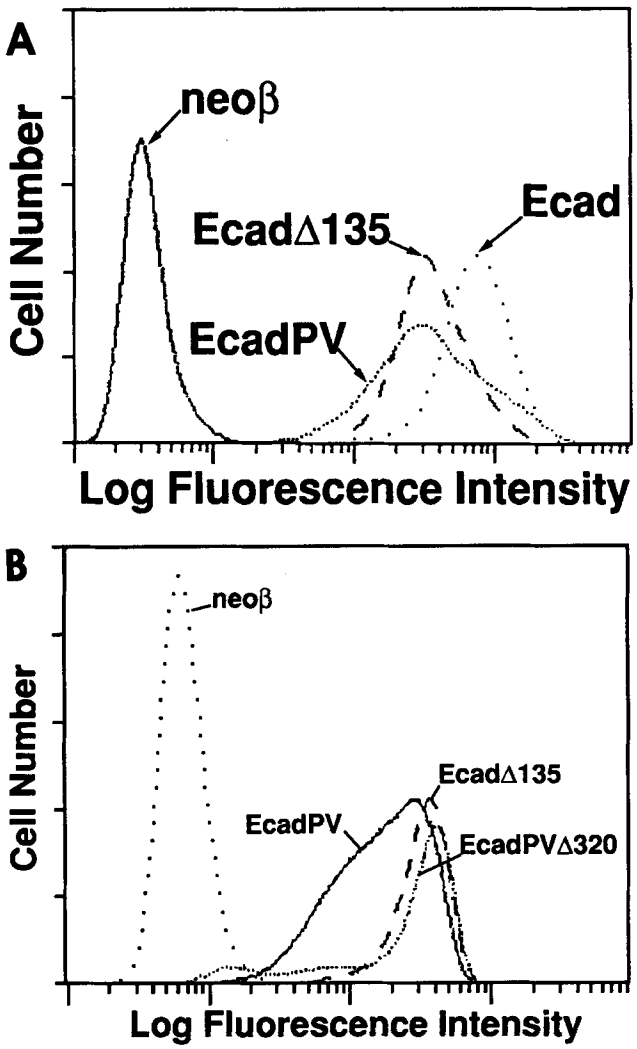


Figure 3. Flow cytometric analysis of L cell clones stained with ECCD-2.

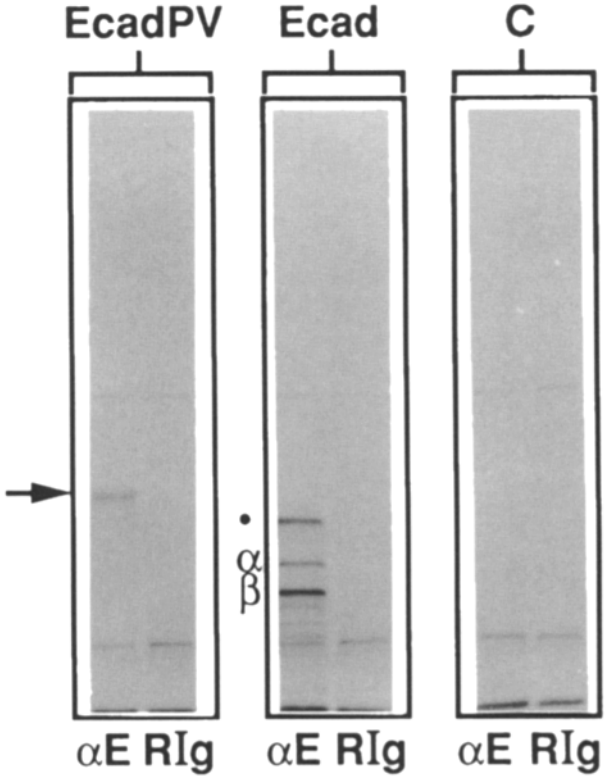


Figure 4. Immunoprecipitation of nonionic detergent extracts of L cell clones. EcadPV and Ecad indicate that extracts of these L cell clones were precipitated. *C* refers to a neoβ control clone. *αE*, ECCD-2; *RIg*, rat antibody isotype control. Arrow shows EcadPV chimeric molecule. Note that α- and β-catenin are not co-precipitated with EcadPV, but are with Ecad (indicated by dot).

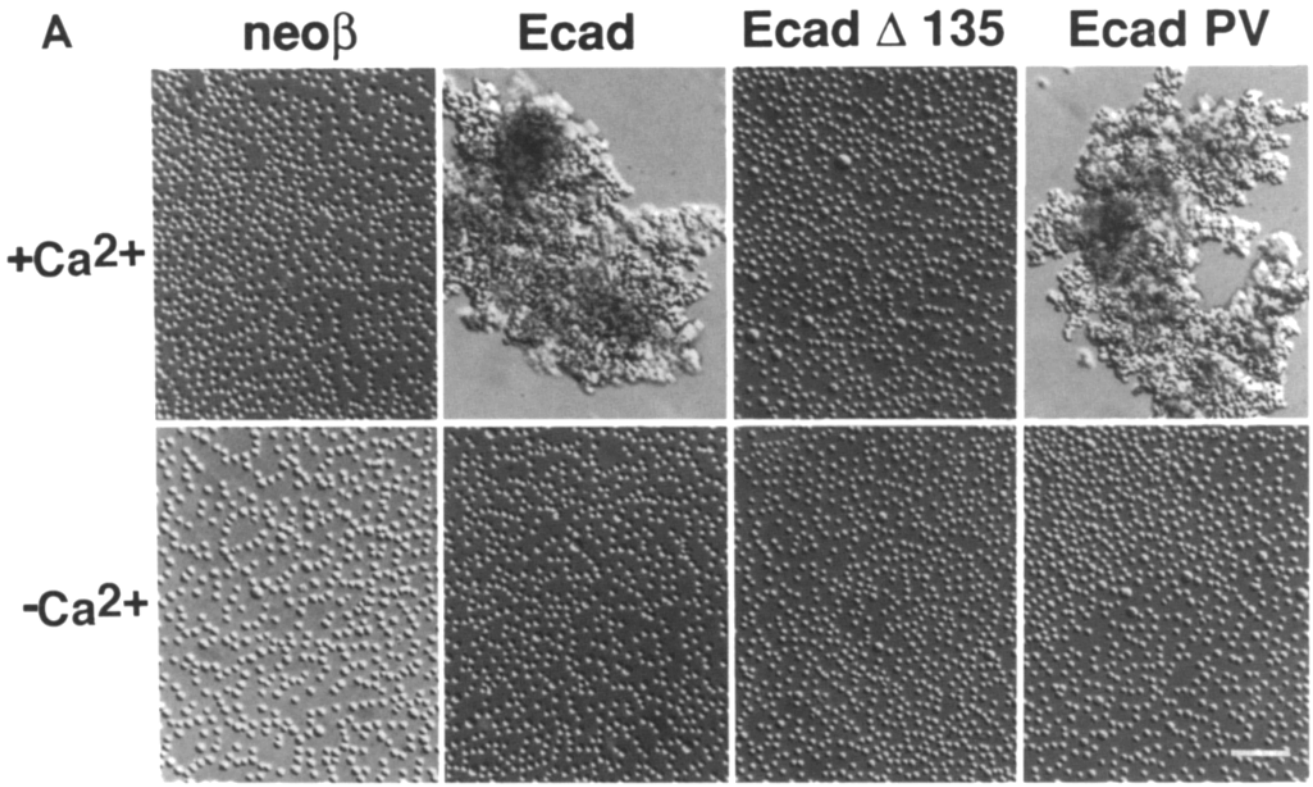
mine if our chimeric EcadPV molecule bound plakoglobin in these L cells. Although, as shown in Fig. 4, we did not usually detect plakoglobin co-precipitated with EcadPV, with long exposures of these gels there was a faint band in the molecular weight range of plakoglobin, suggesting that there may be a small amount of plakoglobin in these cells or that its association with EcadPV is weak.

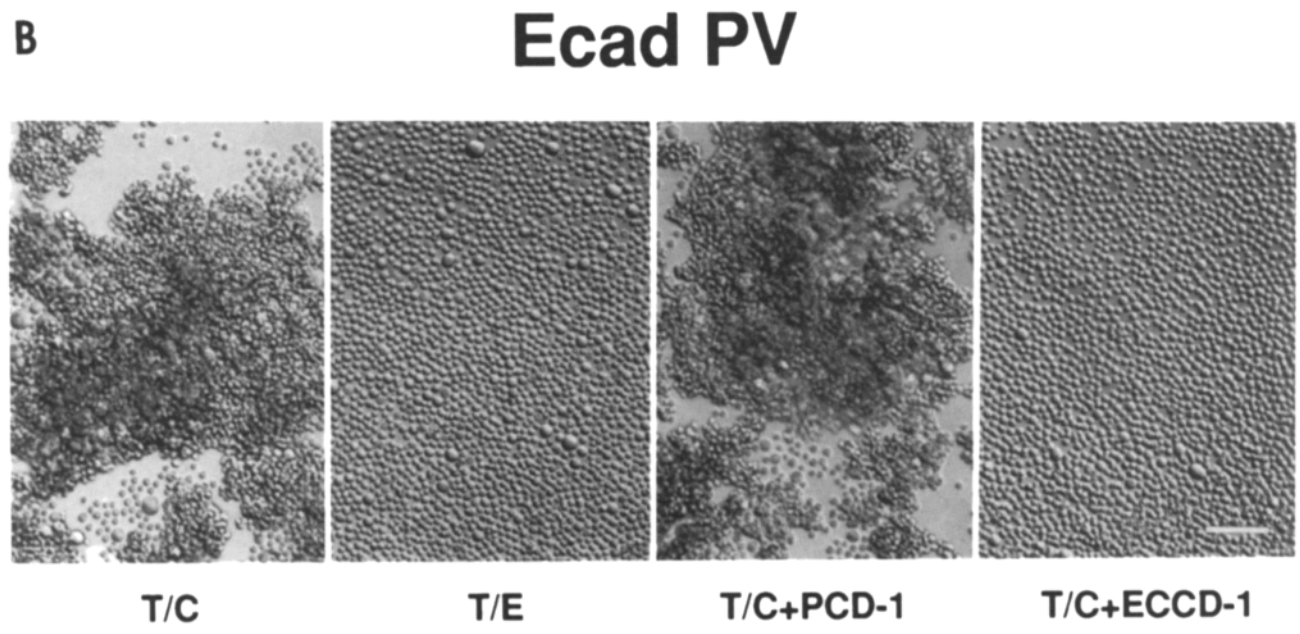
All the above data indicated that we had established EcadPV clones that expressed the EC domain of E-cadherin in about the same amounts as Ecad clones, but that, unlike Ecad, the EcadPV chimeric molecule did not bind α- or β-catenins and, with the possible exception of slight plakoglobin binding, did not show a strong association with other cytoplasmic molecules in these cells. We could then ask the question whether EcadPV could mediate aggregation of these cells as does full-length, authentic, E-cadherin. In other words we could test the generally accepted dogma that E-cadherin must bind cytoplasmic α- and β-catenins and/or the cytoskeleton to function in adhesion.

EcadPV L Cell Clones Aggregate with the Same Properties as Do Ecad Clones

To test aggregation, we made single cell suspension of L cell clones with T/C and let cells aggregate with gentle swirling at 37°C for 20–60 min in the presence or absence of 1 mM calcium, as previously described (26). Fig. 5 *A* shows that, as expected (because cadherin aggregation is calcium dependent), the Ecad clone aggregated well in the presence, but not in the absence of calcium, whereas the neoβ clone, without any cadherin expressed, did not aggregate under either condition. Also, as previously reported (27), the EcadΔ135 clone did not aggregate either with or without added calcium (Fig. 5 *A*). However, the EcadPV clone aggregated strikingly with, but not without, calcium (Fig. 5 *A*). As with the Ecad clone, the EcadPV clone showed such marked aggregation that it could be seen grossly as large clumps in the tissue culture dish. Aggregation can be semi-quantitated by counting number of particles before and after aggregation. Ecad and EcadPV clones showed 86 and 72% aggregation, respectively, whereas neoβ and EcadΔ135 showed less than 2% aggregation. The aggregation assays shown in Fig. 5 *A* were performed simultaneously with, and correspond to, the flow cytometric data in Fig. 3 *A*. In addition, four different EcadPV clones showed similar striking aggregation.

To further show that EcadPV aggregation was indeed cadherin mediated we wanted to show that it would not take place after cells were harvested with T/E. A well-established





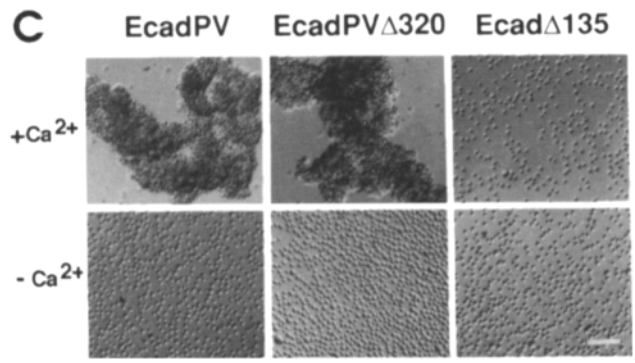


Figure 5. Aggregation assays of L cell clones. (*A*) Ecad and Ecad PV show calcium-dependent aggregation, whereas neoβ and Ecad-Δ135 do not. (*B*) EcadPV aggregation is destroyed by T/E, but not T/C, and is inhibited by ECCD-1, but not control antibody PCD-1. (*C*) EcadPVΔ320 shows calcium-dependent aggregation to the same degree as EcadPV. EcadΔ135 shows no aggregation. Bar, 150 μm.

property of the EC domain of E-cadherin is that it is resistant to degradation by T/C, independent of whether its IC domain binds catenins, but sensitive to degradation by T/E (6, 15, 27, 45, 52). Fig. 5 *B* shows that EcadPV cells only aggregate after T/C, but not T/E, incubation.

Finally, we showed that the aggregation of EcadPV cells, like Ecad cells, was inhibited by ECCD-1, a monoclonal antibody known to block E-cadherin function (53), but not by the irrelevant monoclonal antibody PCD-1, against P-cadherin (32) (Fig. 5 *B*).

Unlike Ecad, EcadPV Does Not Partition with the Cytoskeletal Fraction of L Cells

It is thought that one reason E-cadherin must bind to α- and β-catenins in order to function is that these catenins might provide a linkage to the actin cytoskeleton (27, 34). This linkage of E-cadherin to the cytoskeleton has been classically assayed in L cell transfectants by extracting the cells with nonionic detergent, then subjecting the soluble and insoluble (cytoskeletal) fraction to immunoblotting for E-cadherin (25, 27, 34). In this assay, binding of catenins by E-cadherin correlates with partition of a fraction of the E-cadherin with the detergent insoluble cytoskeleton. On the other hand, E-cadherin that is truncated in its IC domain and no longer binds catenins, no longer fractionates with the cytoskeleton. Therefore, it has been speculated that catenins confer adhesive function on E-cadherin by providing binding to the cytoskeleton.

This speculation suggests that one reason EcadPV might function in adhesion is that the PVA IC domain might interact directly with the cytoskeleton. To test this possibility, we assayed EcadPV and Ecad L cell clones with the standard assay described above (Fig. 6). The nonionic detergent soluble and insoluble fractions were subjected to immunoblotting with ECCD-2. We used the Ecad L cell clone as a positive control in this assay to confirm that a fraction of the E-cadherin did partition with the nonionic detergent insoluble cytoskeleton. In contrast, unlike E-cadherin, EcadPV was totally soluble with nonionic detergent, and none was detected in the cytoskeletal fraction. Therefore, we conclude that EcadPV does not show the same type linkage to the cytoskeleton as does E-cadherin.

Only the IA Subdomain of PVA, Which Is Highly Conserved in Desmogleins, Is Necessary to Confer Function on the EC Domain of E-Cadherin

We next wanted to determine if the ICS subdomain PVA is necessary to confer function on the EC region of E-cadherin. The ICS subdomain of Dsg1 (23) as well as PVA (unpublished observation) is necessary for plakoglobin binding, therefore, this subdomain would be necessary for activating the EC domain of E-cadherin if a small amount of plakoglobin binding with EcadPV is important. The ICS region of PVA and human E-cadherin also share 44% homology and, therefore, this cadherin-like segment might substitute in some other way for that of authentic E-cadherin in conferring function. We, therefore, created the cDNA construct encoding EcadPVΔ320, which deletes the entire PVA IC domain except for about half of the IA region (Fig. 1). Surprisingly, L cell clones expressing this construct (Fig. 3 *B*) showed striking calcium-dependent aggregation to the same degree as EcadPV transfected clones (Fig. 5 *C*), both about 80%. In addition, immunoprecipitation of EcadPVΔ320 from these clones showed no co-precipitated molecules, even after long exposure of the gels.

Another way of determining if the IC region of EcadPVΔ320 interacts with catenins is to determine their steady-state levels in these clones, because it has been suggested that plakoglobin and β-catenin are stabilized in cells by their interactions with the IC domain of cadherins (12, 22). We, therefore, determined steady-state levels of β-catenin and plakoglobin by immunoblotting extracts of Ecad, EcadΔ135, and EcadPVΔ320 clones (Fig. 7). Both molecules were at much higher levels in Ecad cells, than in either EcadΔ135

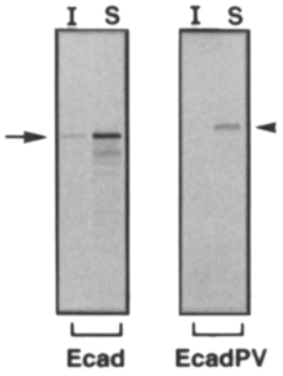


Figure 6. Fractionation of Ecad and EcadPV with the nonionic detergent soluble (*S*) and insoluble (*I*) fractions of L cell clones. Part of Ecad fractionates with the insoluble fraction, but EcadPV is completely extracted in the soluble fraction, suggesting it does not associate strongly with the nonionic detergent-insoluble cytoskeleton.

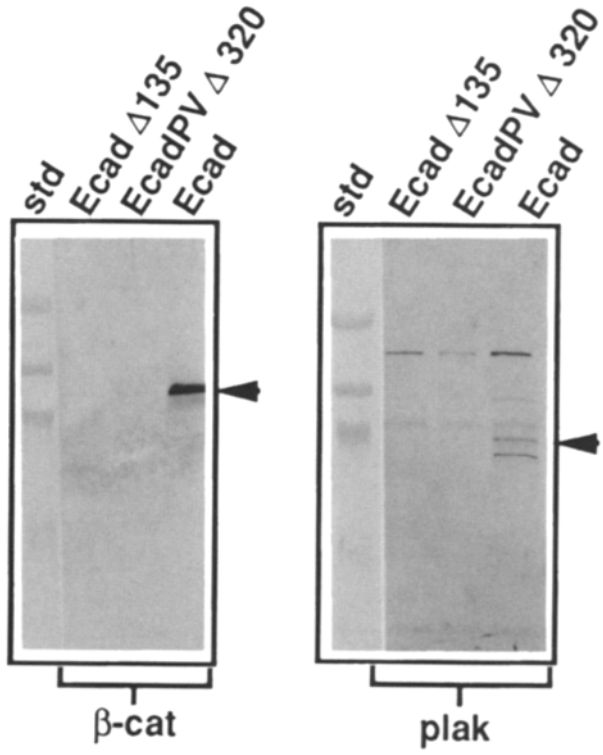


Figure 7. Immunoblots of β-catenin (*β-cat*) and plakoglobin (*plak*) in extracts of Ecad, EcadPVΔ320, and EcadΔ135 clones. Arrow indicates β-catenin, arrowhead indicates plakoglobin. Other bands in the plakoglobin immunoblot are not identified, but the band just below plakoglobin may be a breakdown product. Note that β-catenin and plakoglobin are stabilized only in the Ecad clones. Std indicates molecular weight standards (205, 116, 80, 49 kD).

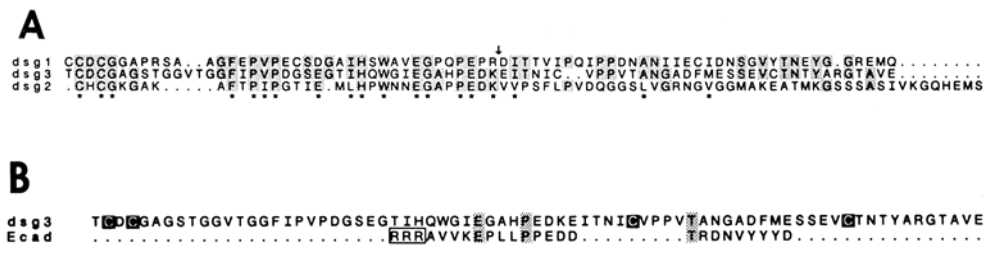


Figure 8. Amino acid homologies among the IA regions of Dsg1, Dsg2, and Dsg3 (*A*), and between these regions of Dsg3 and E-cadherin (*B*). (*A*) Dsg1, Dsg2, and Dsg3 show marked homology. Shading indicates identical amino acids between at least two of the desmogleins. Asterisk (*) indicates the three desmogleins have identical or chemically homologous amino acids at that position. Amino acids to the right (carboxy-terminal) of the vertical arrow were truncated in the EcadPVΔ320 construct. Note that the major homology in the IA region was preserved in EcadPVΔ320. (*B*) As illustrated by Dsg3 and E-cadherin (*Ecad*), desmogleins and classical cadherins show little homology in the IA region for several reasons: (*a*) the IA region of cadherins is much shorter than that of desmogleins; (*b*) there are few identical amino acids when the IA regions are aligned (*shaded*); (*c*) classical cadherins lack cysteines (*reverse font*) in this subdomain; (*d*) Cadherins have positively charged amino acids (*boxed*) just inside the membrane, whereas desmogleins have cysteines in conserved locations in this area.

or EcadPVΔ320 cells, demonstrating that the IC domains of these later two cell types do not stabilize β-catenin or plakoglobin. This data is consistent with the immunoprecipitation data which show little, if any, interaction of EcadPVΔ320 with catenins or plakoglobin.

These results show that a minimum of 40 amino acids of the IA region of PVA are sufficient to confer function on the EC region of E-cadherin, therefore, suggesting a potentially important function for this region of the IC domain of PVA. The IA region of E-cadherin does not fulfill this function because truncated constructs of E-cadherin containing the entire IA region do not mediate aggregation (16, 27, 38). In fact, removal of only 37 residues from the carboxy terminus of E-cadherin results in complete loss of function (27, 38).

If there is an important cell biologic function in the IA region of PVA, it might be expected to be conserved in amino acid sequence among desmogleins. On the other hand, since it presumably does not fulfill this function in classical cadherins it would not be expected to be conserved between desmogleins and classical cadherins. This is indeed the case. Fig. 8 *A* shows a high degree of amino acid homology in the IA subdomains of Dsg1, Dsg2, and Dsg3, especially in the 40 amino acids preserved in EcadPVΔ320. There is no significant homology between this subdomain of desmogleins and that of E-cadherin (Fig. 8 *B*).

We tried to determine if we could eliminate 26 of these remaining 40 amino acids from the IA subdomain by making an EcadPVΔ346 construct, but clones derived expressing this construct were very unstable, and even in the initially isolated clones they were heterogeneous for cell surface expression of the E-cadherin ectodomain.

In conclusion, then, using the same methods previously shown to demonstrate α- and β-catenin binding and cytoskeletal association of classical cadherins, we show here that neither property is detectable in an EcadPV or EcadPVΔ320 chimeric molecule, yet these molecules function in cell–cell aggregation. We conclude that E-cadherin is able to function in adhesion without tight α- or β-catenin and/or cytoskeletal association. Since the PVA IC region has been shown to bind to plakoglobin, it could be argued that this cytoplasmic interaction might activate the EC domain of E-cadherin. However, little plakoglobin is co-precipitated with EcadPV and none is detectable in co-precipitates of EcadPVΔ320. In addition, steady-state plakoglobin levels are stabilized by E-cadherin (presumably by association of plakoglobin with the E-cadherin IC domain), but not by EcadPVΔ320. Finally, the EcadPVΔ320 construct does not contain the ICS region known to be critical for plakoglobin binding by desmogleins.

These data suggest an important function for the highly conserved IA cytoplasmic subdomain of desmogleins. We speculate that the most likely function of this region might be to cluster the molecules in the membrane. Clustering might be an important factor in increasing the efficiency of E-cadherin aggregation, as has previously been suggested (18). For desmoglein function, clustering might be an important factor in desmosome formation. In any case, the observations presented here suggest that future studies focusing on the IA region of desmogleins are likely to demonstrate important cell biologic functions.

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