Defining Interactions and Distributions of Cadherin and Catenin Complexes in Polarized Epithelial Cells

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Abstract. The cadherin/catenin complex plays important roles in cell adhesion, signal transduction, as well as the initiation and maintenance of structural and functional organization of cells and tissues. In the preceding study, we showed that the assembly of the cadherin/catenin complex is temporally regulated, and that novel combinations of catenin and cadherin complexes are formed in both Triton X-100–soluble and –insoluble fractions; we proposed a model in which pools of catenins are important in regulating assembly of E-cadherin/catenin and catenin complexes. Here, we sought to determine the spatial distributions of E-cadherin, α-catenin, β-catenin, and plakoglobin, and whether different complexes of these proteins accumulate at steady state in polarized Madin-Darby canine kidney cells. Protein distributions were visualized by wide field, optical sectioning, and double immunofluorescence microscopy, followed by reconstruction of three-dimensional images. In cells that were extracted with Triton X-100 and then fixed (Triton X-100–insoluble fraction), more E-cadherin was concentrated at the apical junction relative to other areas of the lateral membrane. α-Catenin and β-catenin colocalize with E-cadherin at the apical junctional complex. There is some overlap in the distribution of these proteins in the lateral membrane, but there are also areas where the distributions are distinct. Plakoglobin is excluded from the apical junctional complex, and its distribution in the lateral membrane is different from that of E-cadherin. Cells were also fixed and then permeabilized to reveal the total cellular pool of each protein (Triton X-100–soluble and –insoluble fractions). This analysis showed lateral membrane localization of α-catenin, β-catenin, and plakoglobin, and it also revealed that they are distributed throughout the cell. Chemical cross-linking of proteins and analysis with specific antibodies confirmed the presence at steady state of E-cadherin/catenin complexes containing either β-catenin or plakoglobin, and catenin complexes devoid of E-cadherin. Complexes containing E-cadherin/β-catenin and E-cadherin/α-catenin are present in both the Triton X-100–soluble and –insoluble fractions, but E-cadherin/plakoglobin complexes are not detected in the Triton X-100–insoluble fraction. Taken together, these results show that different complexes of cadherin and catenins accumulate in fully polarized epithelial cells, and that they distribute to different sites. We suggest that cadherin/catenin and catenin complexes at different sites have specialized roles in establishing and maintaining the structural and functional organization of polarized epithelial cells.

CADHERINS are a multifunctional family of transmembrane glycoproteins important in the morphogenesis of multicellular organisms (Takeichi, 1991; Kemler, 1992). During recognition and adhesion between cells, cadherins regulate homophilic, Ca^{++}-dependent interactions in epithelial cells. This initiates a cascade of events that leads to the structural and functional reorganization of cells, including formation of junctional complexes (tight junction, *zonula adherens*, desmosomes), organization of the actin cytoskeleton at the apical junctional complex, assembly of the membrane cytoskeleton, and development of membrane domains (Rodriguez-Boulan and Nelson, 1989; Nelson, 1992).

Recent studies have identified three cytoplasmic proteins, α-catenin, β-catenin, and plakoglobin, that bind noncovalently to the cytoplasmic domain of cadherins (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989). Formation of the cadherin/catenin complex is required for cadherin functions in cell–cell adhesion and cellular reorganization (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990). α-Catenin has sequence similarities to vinculin and is a candidate for linking

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the cadherin/catenin complex to the cytoskeleton (Herrenknecht et al., 1991; Nagafuchi et al., 1991). β-Catenin and plakoglobin are mammalian homologues of armadillo, a *Drosophila* protein involved in a signal transduction pathway leading to specification of segmental identity in the embryo (Riggleman et al., 1990; McCrea et al., 1991; Butz et al., 1992). Plakoglobin was originally identified as a component of desmosomes (Cowin et al., 1986).

Little is known about how interactions between cadherins at the cell surface of neighboring cells during cell recognition and adhesion lead to the reorganization of membrane and cytoskeletal proteins. Evidence has accumulated that other cytoplasmic proteins, including actin, fodrin, as well as *src* and *yes* kinases, also interact with the cadherin/catenin complex (Tsukita et al., 1993). These interactions may link the cadherin/catenin complex with the cytoskeleton and intracellular signaling pathways. In addition, recent studies have shown that cadherin/catenin interactions are modulated by the protooncogene Wnt-1 (Bradley et al., 1993; Hinck et al., 1994*b*), the mammalian homologue of the *Drosophila wingless* gene, which is part of the armadillo transduction pathway (for review see Nusse and Varmus, 1992).

From these observations, two minimal models of cadherin/catenin function can be postulated: a single cadherin/catenin complex that initiates a signaling pathway resulting in a cascade of independent intracellular events; alternatively, multiple cadherin/catenin complexes that have different distributions and regulate each of these events individually. We can discriminate between these models by analyzing the composition of cadherin/catenin complexes and their distributions in polarized epithelial cells.

In the preceding paper, we showed that assembly of newly synthesized E-cadherin with catenins in Madin-Darby canine kidney (MDCK)[1] epithelial cells is temporally regulated (Hinck et al., 1994*a*). Complexes of cadherin and catenins are present in the Triton X-100 (TX-100)–insoluble fraction of proteins. There also appear to be pools of α-catenin, β-catenin, and plakoglobin that are distinct from the cadherin/catenin complex. In this paper, we have examined the accumulation of these complexes and tested whether they have different spatial distributions. We used wide-field optical sectioning fluorescence microscopy in combination with three-dimensional image reconstruction to localize complexes, and chemical cross-linking to study the composition of the cadherin/catenin complexes. Our results show that there are multiple cadherin/catenin complexes and cadherin-independent pools of catenin complexes that have different distributions at the lateral membrane and throughout the cell, supporting the second model of cadherin/catenin function (see above). We suggest that the distributions of different complexes are related to specific functions in cell adhesion, signal transduction, and cellular reorganization.

Materials and Methods

General Methods and Reagents

Cell culture, immunoprecipitations and cross-linking were performed as described in the preceding paper (Hinck et al., 1994*a*). A monoclonal antibody against the extracellular domain of E-cadherin from MDCK cells (3G8) was the generous gift of Dr. Warren Gallin (University of Alberta, Alberta, Canada). Two different polyclonal antisera against the cytoplasmic portion of P-cadherin (Pcad1 and 2) were provided by Dr. Dietmar Vestweber (Max-Planck-Institut für Immunbiologie, Freiburg, Germany). Specific antibodies against each of the catenins (Hinck et al., 1994*b*) and the cytoplasmic domain of E-cadherin (Marrs et al., 1993) have been described previously.

Affinity Purification of Catenin Antibodies

The cognate peptides for the α-catenin, β-catenin, and plakoglobin antibodies were coupled to coupling gel (SulfoLink®; Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Antisera were incubated with the immobilized peptides. After washing the resins extensively with PBS, affinity-pure antibodies were eluted with 0.2 M glycine, pH 2.5. The pH of antibody containing fractions was adjusted to 7.5 with 1 M Tris, pH 8.0.

Immunoblotting

Immunoblotting was performed as described in the preceding paper (Hinck et al., 1994*a*). The antibodies were used at the following dilutions: α-catenin, 1:100; β-catenin, plakoglobin, and E-cadherin, 1:500; 3G8 (tissue culture supernatant), 1:100, P-cadherin 1 and 2, 1:200. Binding of the mouse monoclonal antibody 3G8 was detected by first incubating the membranes with a rabbit anti-mouse antiserum (Dako Corp., Carpinteria, CA) at a dilution of 1:100 for 1 h before incubation with ^{125}I-protein A.

Immunofluorescence Microscopy

Confluent monolayers of MDCK cells were maintained on collagen-coated Transwell polycarbonate filters (Transwell; Costar Corp., Cambridge, MA) for 8 d. Cells were extracted with CSK buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 10 μg/ml leupeptin, and 1 mM Pefabloc; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 15 min at 4°C and then fixed with 3.75% formaldehyde in PBS for 30 min. Alternatively, cells were fixed with 3.75% formaldehyde and then permeabilized with CSK buffer for 15 min at 4°C. Cells were blocked in PBS, 0.2% BSA, 1% normal goat serum for 1 h, and then incubated with a monoclonal antibody against E-cadherins (3G8, 1:100), and affinity-purified antibodies against α-catenin (1:200), β-catenin (1:500), or plakoglobin (1:750) in PBS, 0.2% BSA. After washing three times with PBS, 0.2% BSA, binding of the primary antibodies was detected with rhodamine-coupled goat anti-mouse (E-cadherin) and FITC-coupled goat anti-rabbit antibodies (catenin) (Boehringer Mannheim Biochemicals) diluted 1:100 in PBS, 0.2% BSA. When cells were fixed in −20°C methanol and stained as described above, the staining was similar to the staining of cells that were extracted with CSK buffer before fixation with formaldehyde (data not shown).

Three-dimensional images of immunostained epithelial monolayers were recorded using a Peltier-cooled charge-coupled device camera (Photometrics Ltd., Tucson, AZ) with a 1,340 × 1,037-pixel charge-coupled device chip (Kodak-Videk; Eastman Kodak Co., Rochester, NY). The camera was mounted on a fluorescence microscope workstation equipped with bandpass excitation and emission filters mounted on motorized wheels and a multiwavelength dichroic mirror (Chroma, Inc., Brattleboro, VT). All aspects of data collection were controlled by computer (SGI 4D-35; Silicon Graphics Corp., Mountain View, CA). The design and specifications of the system have been described previously (Hiraoka et al., 1991). Optical sections (512 × 512 pixels; effective pixel size = 0.0744 × 0.0744 μm) were recorded with a Plan ApoChromat 60x/NA1.4 lens (Olympus Corp., Lake Success, NY) at 0.2-μm intervals by changing the microscope focus with a computer-controlled motor (Nanomover; Melles Griot, Inc., Rochester, NY). Dual wavelength, three-dimensional images were recorded in a single focal series by alternating the appropriate excitation and emission filters for fluorescein isothiocyanate and tetramethylrhodamine at each focal plane. To correct for temporal fluctuations in illumination intensity caused by power instabilities in the mercury-arc lamp, the intensity of the lamp was directly measured using an avalanche photodiode–based photon-counting module (EG & G, Vaudreuil, Quebec, Canada). The out-of-focus information in these images was then removed using iterative, constrained, three-dimensional deconvolution, a technique that deblurs an image by moving out-of-focus intensity back to its originating point based on an empirical measure of the "point spread" function, which is the blurring of an image

1. *Abbreviations used in this paper*: CSK buffer, 50 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 10 μg/ml leupeptin, and 1 mM Pefabloc; DSP, dithiobis(succinimidylpropionate); MDCK, Madin-Darby canine kidney (cells); TX-100, Triton X-100.

caused by the limited resolution of the objective lens (Agard et al., 1989; Hiraoka et al., 1991). The volume rendered images of the three-dimensional data were calculated as previously described (Levoy, 1991).

Results

Three-dimensional Distributions of E-Cadherin and Catenins in Polarized Monolayers of MDCK Cells

The spatial distributions of E-cadherin, α-catenin, β-catenin, and plakoglobin in MDCK cells were analyzed by immunofluorescence using a wide-field optical sectioning microscope (Agard et al., 1989; Hiraoka et al., 1991). To ensure that the cellular junctions had formed completely and cells were fully polarized, MDCK cells were grown in confluent monolayers on Transwell filters for 8 d. We examined the distributions of both the TX-100–soluble and –insoluble fractions to correlate these studies with those in the preceding paper. The TX-100–insoluble fraction was examined by extraction of cells followed by fixation (Figs. 1–3, *Extracted*); the combination of TX-100–soluble and –insoluble fractions were examined in cells that were first fixed and then permeabilized (Figs. 1–3, *Total*). Cells were stained with both a mouse monoclonal antibody against E-cadherin, and affinity-purified rabbit polyclonal antibodies specific for either α-catenin (Fig. 1), β-catenin (Fig. 2), or plakoglobin (Fig. 3). Optical sections were recorded at 0.2-μm intervals from ≥2 μm below to 2 μm above the cell layer (total of 15–18 μm). Out-of-focus information was removed by a deconvolution algorithm, and the resulting images were displayed using a three-dimensional volume-rendering algorithm (Agard et al., 1989; Hiraoka et al., 1991; Levoy, 1991). Figs. 1–3 display three-dimensional images tilted by

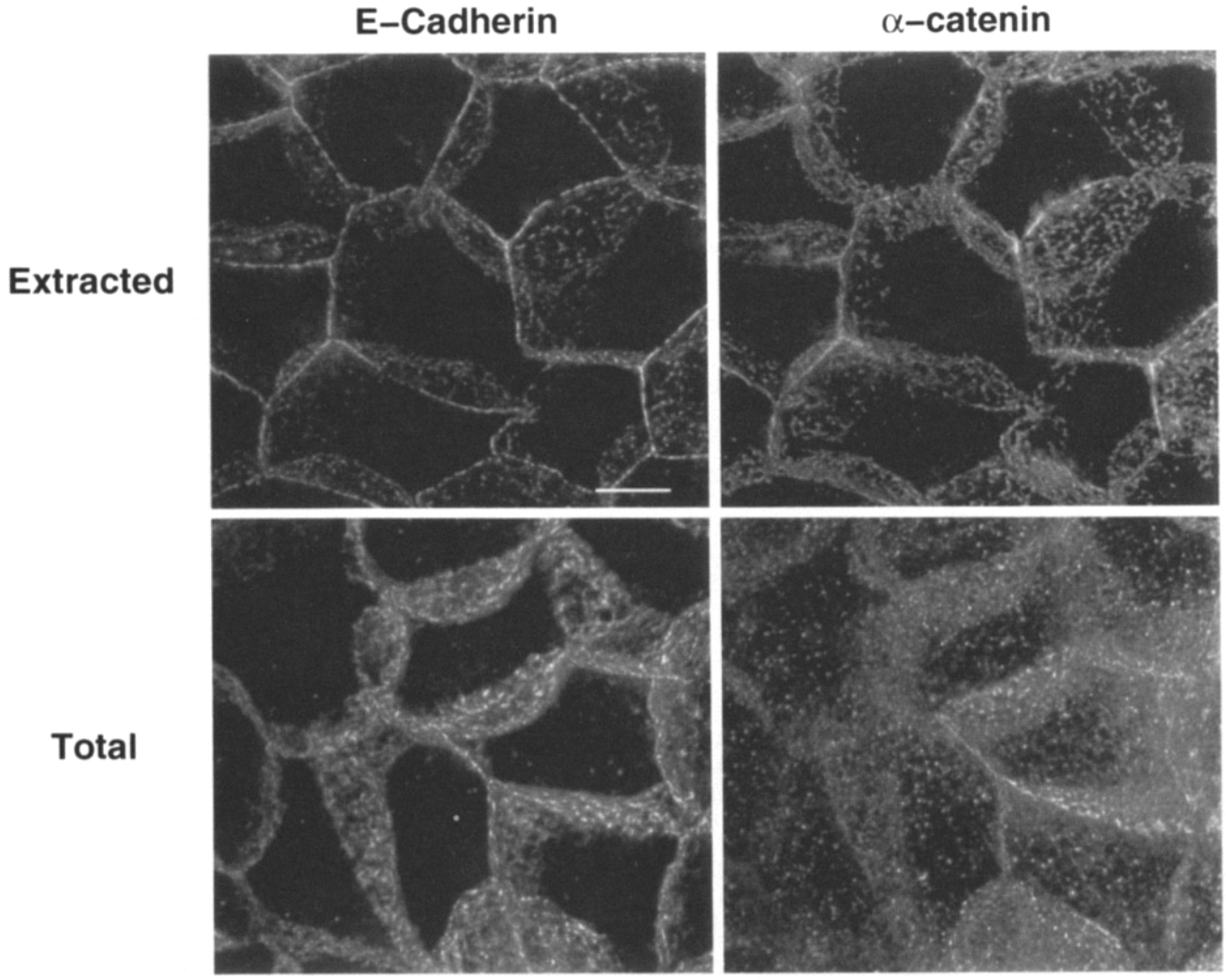


Figure 1. Double staining of MDCK cells for E-cadherin and α-catenin. Confluent monolayers of MDCK cells were established on collagen-coated Transwell filters and maintained for 8 d in high calcium media. Growth medium was replaced daily. The cells were washed with PBS, 0.5 mM $CaCl_2$, and either fixed with formaldehyde and extracted with CSK buffer (*Total*), or extracted with CSK buffer and then fixed with formaldehyde (*Extracted*). Cells were labeled with a mouse monoclonal antibody against E-cadherin and affinity-purified rabbit polyclonal antibodies against α-catenin followed by rhodamine-conjugated anti–mouse and fluorescein-conjugated anti–rabbit antibodies. Optical sections were recorded at 0.2-μm intervals. After removal of the out-of-focus information by deconvolution, three-dimensional images were generated using a volume rendering algorithm, and they are displayed rotated at a a 10–15° angle. Bar, 5 μm.

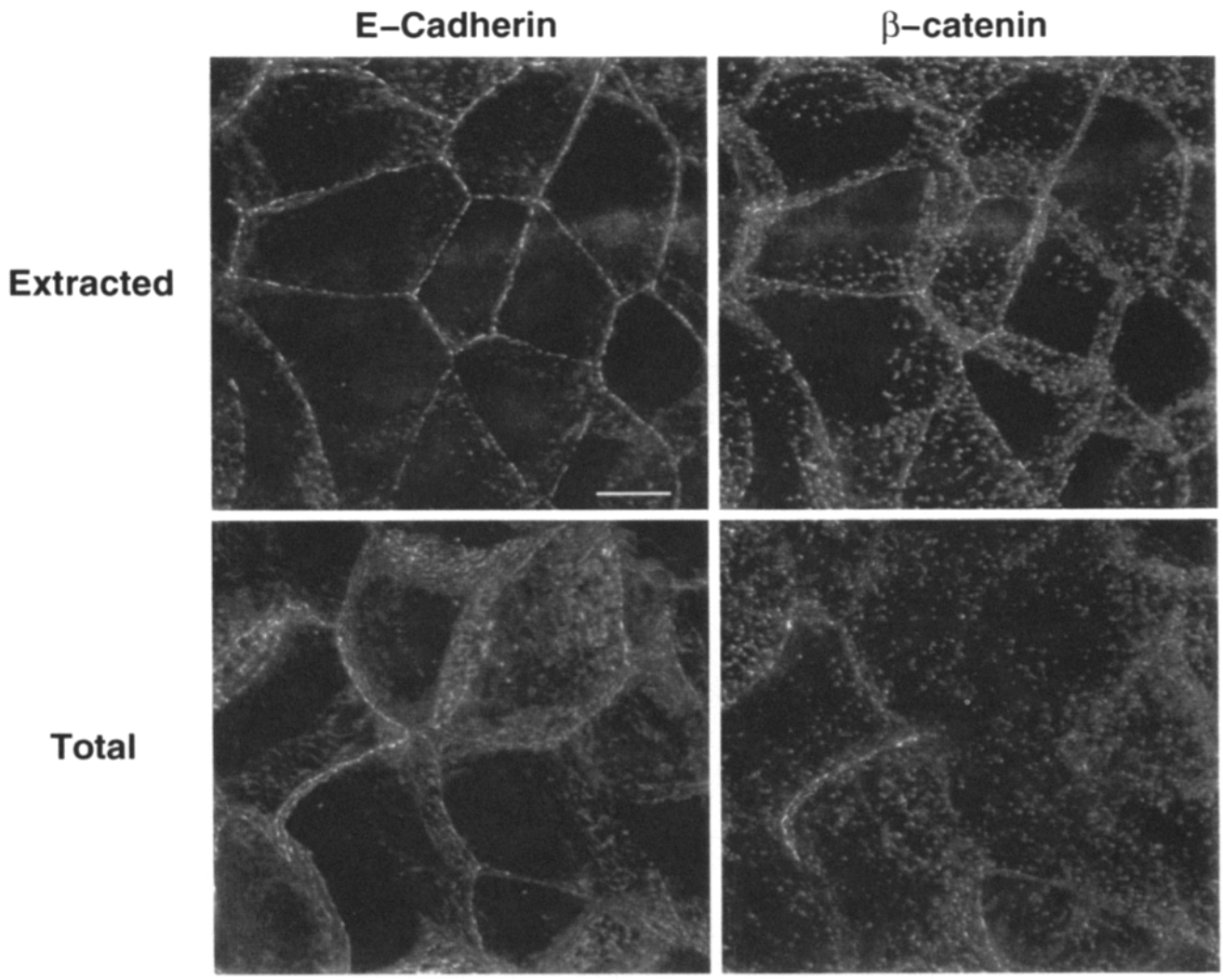


Figure 2. Double labeling of MDCK cells for E-cadherin and β-catenin. MDCK cells were treated as described in Fig. 1, but they were double stained with affinity-purified polyclonal antibodies against β-catenin and a mouse monoclonal antibody again E-cadherin.

10–15° to visualize the lateral membranes. The images are oriented with the apical membrane at the top and the basal membrane at the bottom. Extracted cells that were stained with antibodies to E-cadherin (*left*), and either of the three catenins (*right*) are shown in the top panels, and unextracted (*Total*) cells are shown in the bottom panels.

The degree of colocalization of E-cadherin and each of the three catenins is shown by the overlay of optical sections from cells stained with antibodies against E-cadherin and either α-catenin (*top*), β-catenin (*middle*), or plakoglobin (*bottom*) (Fig. 4). To illustrate the similarities and differences in the distributions of these proteins in extracted cells, individual optical sections are shown from the middle of the cell (*left panels*, 6–6.5 μm above the substratum) and at the apex of the lateral membrane (8.4–10 μm above the substratum), where the tight junction and the *zonula adherens* are located (apical junctional complex) (*right panels*). E-cadherin staining is shown in purple, catenin staining appears in green, and areas of overlapping staining are indicated in white.

In unextracted cells, E-cadherin is localized to the lateral membrane with little or no staining detectable at the basal or apical membranes. When three-dimensional images (Fig. 1, *Total*) and individual optical sections are examined (not shown), E-cadherin staining appears closely punctate at the lateral membrane. In extracted cells, both three-dimensional images (Fig. 1, *Extracted*) and individual optical sections (Fig. 4) show an openly punctate staining pattern at the lateral membrane. At the apical junctional complex, E-cadherin staining is almost continuous, and there appears to be an enrichment of E-cadherin in this area relative to other areas of the lateral membrane. The difference in E-cadherin staining between unextracted and extracted cells indicates that a higher proportion of E-cadherin is insoluble in TX-100 in the apical junctional complex than in the lateral membrane below the apical junctional complex.

In unextracted cells, α-catenin staining appears punctate throughout the cell and in the plane of the lateral membrane (Fig. 1, *Total*). In extracted cells, α-catenin staining is restricted to the lateral membrane in a punctate pattern (Fig. 1, *Extracted*). α-Catenin also appears to be enriched at the level of the apical junctional complex, similar to E-cadherin (Fig. 1, *Extracted*). In the area of the membrane below the apical junctional complex, some of the staining for α-catenin

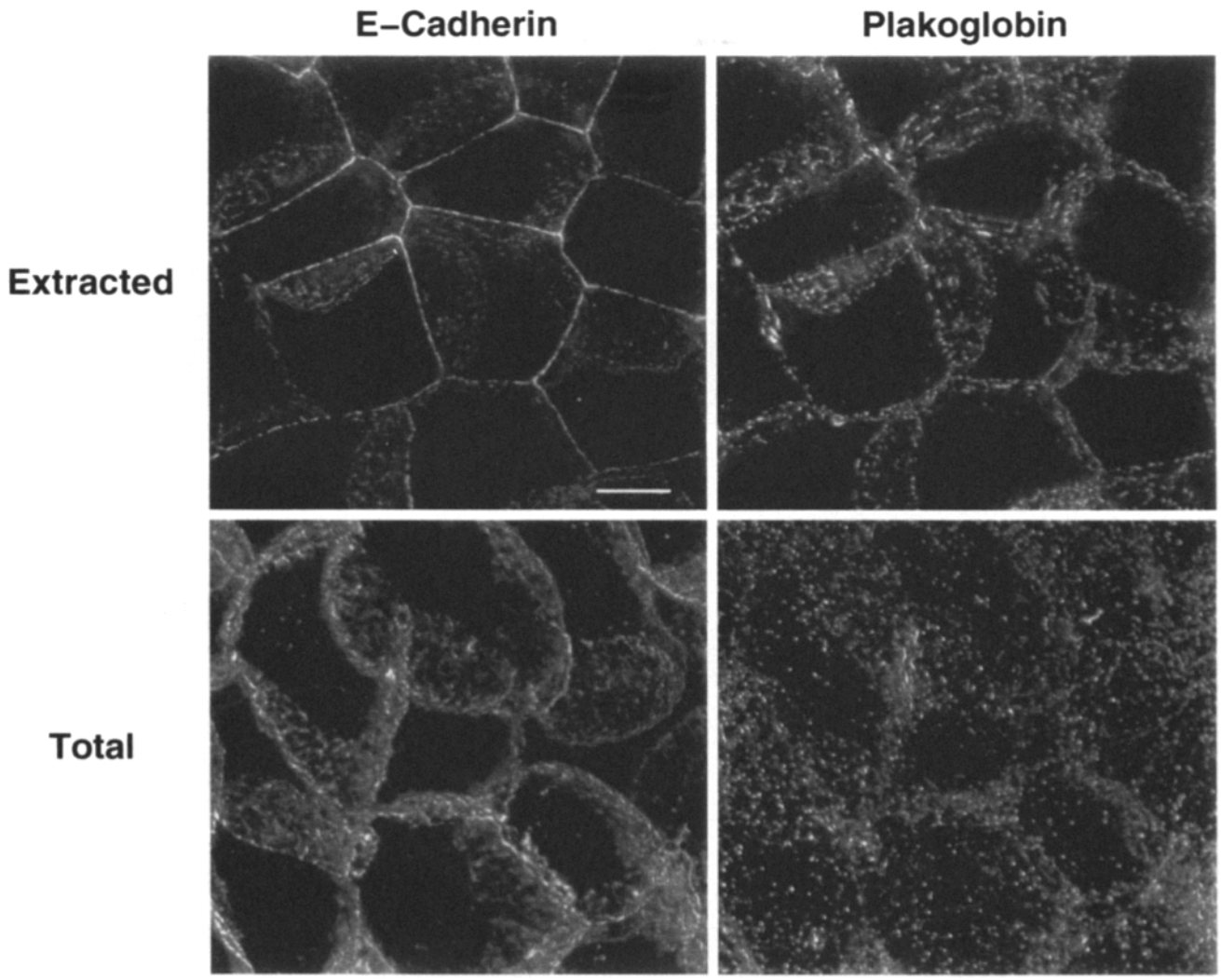


Figure 3. Double staining of MDCK cells for E-cadherin and plakoglobin. As in Figs. 1 and 2, but cells were double labeled with affinity-purified rabbit polyclonal antibodies against plakoglobin and a mouse monoclonal antibody against E-cadherin.

is coincident with that of E-cadherin (Fig. 4). However, other regions of the lateral membrane exhibit staining for either α-catenin or E-cadherin, indicating that in these cases both proteins are independently associated with the membrane (Fig. 4).

In unextracted cells, β-catenin staining is punctate throughout the cell and in the plane of the lateral membrane (Fig. 2, *Total*). In extracted cells, β-catenin staining is restricted to the lateral membrane in a punctate pattern. Some enrichment of β-catenin is also observed at the apical junctional complex, but this enrichment appears to be less than that of E-cadherin and α-catenin (Fig. 2, *Extracted*). As in the case of α-catenin, some areas of the lateral membrane exhibit patterns of β-catenin and E-cadherin staining that are similar, but staining for β-catenin that is different from E-cadherin staining is predominant (Fig. 4).

In unextracted cells, plakoglobin staining is punctate throughout the cell (Fig. 3, *Total*). In extracted cells, plakoglobin staining appears in a punctate pattern restricted to the lateral membranes. The sizes of the individual spots of plakoglobin staining are larger and less uniform than those of E-cadherin, α-catenin, and β-catenin (Fig. 3, *Extracted*). The punctate staining patterns of E-cadherin and plakoglobin do not overlap, and there appears to be no enrichment of plakoglobin at the apical junctional complex of extracted cells (Fig. 4).

In summary, we have found that in many cases, and particularly at the apical junctional complex, the spatial distributions of E-cadherin, α-catenin, and β-catenin in the lateral membrane are similar. However, our results show unequivocally that both α-catenin and β-catenin are present in regions of the lateral membrane that do not appear to contain E-cadherin, and that plakoglobin does not colocalize with E-cadherin in the lateral membrane of TX-100–extracted cells. Furthermore, α-catenin, β-catenin, and plakoglobin are present in a TX-100–soluble fraction that is distributed throughout the cell. These results, in combination with those presented in the preceding paper (Hinck et al., 1994*a*), strongly indicate that there are cadherin-independent pools of catenins. In the next series of experiments, we have sought

Middle
Top
E-cadherin
α-catenin
E-cadherin
β-catenin
E-cadherin
Plakoglobin

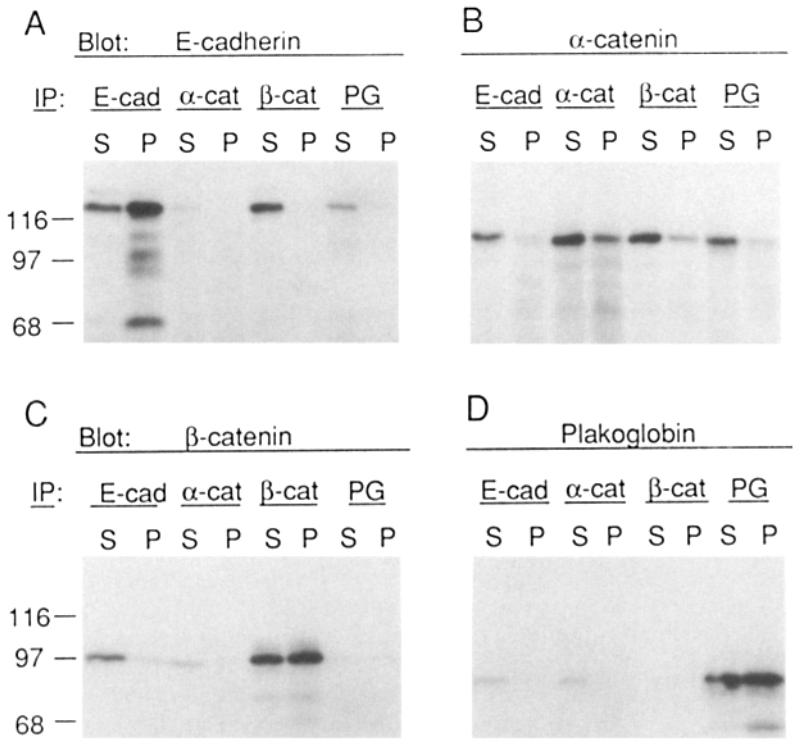


Figure 5. The composition of E-cadherin/catenin complexes in the TX-100–soluble and –insoluble pool at steady state. Confluent monolayers of MDCK cells were maintained on collagen-coated Transwell filters for 8 d. They were cross-linked with the reducible cross-linking reagents DSP and harvested in TX-100–containing lysis buffer. Soluble (*S*) and insoluble (*P*) fractions were immunoprecipitated (*IP*) with antibodies against E-cadherin (*E-cad*), α-catenin (*α-cat*), β-catenin (*β-cat*), and plakoglobin (*PG*). Each immunoprecipitate was subjected to SDS-PAGE and transferred to Immobilon-P membranes for immunoblotting (*Blot*) with the same panel of antibodies (*A*) E-cadherin immunoblot, (*B*) α-catenin immunoblot, (*C*) β-catenin immunoblot, and (*D*) plakoglobin immunoblot. Molecular mass standards (116, 97, and 68 kD) are indicated on the left.

to demonstrate biochemically the accumulation at steady state of cadherin/catenin complexes in the TX-100–insoluble fraction, and of cadherin-independent pools of catenins.

Protein Composition of Cadherin/Catenin Complexes and Cadherin-independent Catenin Complexes

The protein composition of cadherin/catenin complexes was examined in polarized MDCK cells with established cell contacts. We used the bifunctional cross-linking reagent dithiobis(succinimidylpropionate) (DSP) to preserve protein complexes in both the TX-100–soluble and –insoluble fractions. MDCK cells were grown in confluent monolayers on collagen-coated Transwell filters for 8 d, like those cells that were analyzed by high resolution immunofluorescence microscopy. Cells were incubated in the presence of DSP and extracted sequentially with buffers containing TX-100 and SDS to generate TX-100–soluble (S) and insoluble (P) fractions, as described in the preceding paper (Hinck et al., 1994*a*). Equal aliquots of each fraction were immunoprecipitated with E-cadherin (*E-cad*), α-catenin (*α-cat*), β-catenin (*β-cat*), or plakoglobin (*PG*) antibodies. Immunoprecipitates were reduced to reverse the cross-linking, separated by SDS-PAGE, transferred to Immobilon-P membranes, and probed with each of the four antibodies (Fig. 5).

Fig. 5 *A* reveals that >85% of E-cadherin is located in the TX-100–insoluble fraction; relatively little E-cadherin is detected in the E-cadherin immunoprecipitate from the TX-100–soluble fraction. E-cadherin is detected in the α-catenin, β-catenin, and plakoglobin immunoprecipitates from the TX-100–soluble fraction. Little E-cadherin is detected in either the α-catenin and β-catenin immunoprecipitates of the TX-100–insoluble fraction. No E-cadherin is detected in plakoglobin immunoprecipitates of the TX-100–insoluble fraction.

Fig. 5 *B* shows that α-catenin is present in the E-cadherin immunoprecipitate from both the TX-100–soluble and –insoluble fractions, but relatively more (85%) is detected in the TX-100–soluble fraction. Large amounts of α-catenin coimmunoprecipitate with β-catenin and plakoglobin from the TX-100–soluble fraction. α-Catenin is also detected in the β-catenin and plakoglobin immunoprecipitates from the TX-100–insoluble fraction.

Fig. 5 *C* shows that the fraction of β-catenin that is associated with E-cadherin is predominantly distributed in the TX-100–soluble fraction (84%). However, 60% of the total β-catenin is present in the TX-100–insoluble fraction. β-Catenin is detected in the α-catenin immunoprecipitate from the TX-100–soluble and –insoluble fractions although the amount is low. Little or no β-catenin is detected in the plakoglobin immunoprecipitate from either the TX-100–soluble or –insoluble fractions. The absence of β-catenin from these

Figure 4. Colocalization of E-cadherin and the catenins in extracted MDCK cells. Confluent monolayers of MDCK cells were maintained on collagen-coated Transwell filters for 8 d. Cells were extracted with TX-100–containing buffer and fixed with formaldehyde. They were stained with both mouse monoclonal antibodies against E-cadherin and affinity-purified rabbit polyclonal antibodies against α-catenin, β-catenin, or plakoglobin as indicated. To detect the primary antibodies, secondary antibodies coupled to rhodamine (E-cadherin) or FITC (catenins) were used. Staining for E-cadherin is indicated by purple, staining for catenins is shown in green, and overlapping areas are indicated by white. Individual optical sections from either the middle of the cells (6–6.5 μm above the substratum) or the apical junctional complex (8.4–10 μm above the substratum) are displayed.

immunoprecipitated complexes reflects the formation of mutually exclusive complexes containing either plakoglobin or β-catenin (PG IP), as shown also by Hinck et al. (1994*a*, 1994*b*).

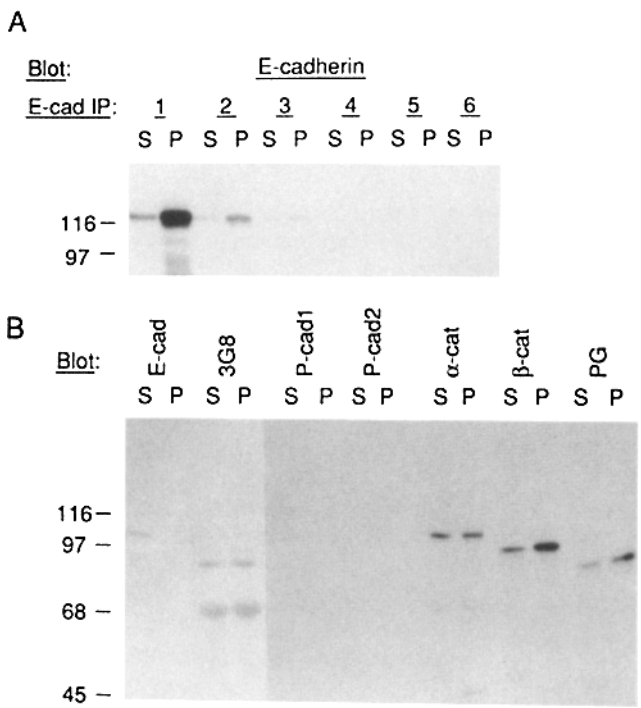
Fig. 5 *D* shows that 70% of plakoglobin is present in the TX-100–insoluble pool. Little plakoglobin is detected in the E-cadherin and α-catenin immunoprecipitates from the TX-100–soluble fraction, and no plakoglobin is present in the β-catenin immunoprecipitations from the TX-100–soluble and –insoluble fractions.

These biochemical studies confirm the existence of cadherin/catenin complexes in both the TX-100–soluble and –insoluble fractions. However, the experiment described above and those in the preceding paper (Hinck et al., 1994*a*) provide only indirect evidence for the existence of catenin complexes that are independent of cadherin. Therefore, we cleared cell extracts of E-cadherin and then examined the remaining proteins for complexes containing different combinations of catenins. MDCK cells were established in confluent monolayers on collagen-coated Transwell filters for 8 d. They were incubated with the reducible cross-linking reagent DSP and TX-100–soluble (S) and –insoluble (P) fractions were prepared. Each fraction was sequentially immunoprecipitated six times with the anticadherin antibody. This E-cadherin antibody (E-cad) was raised against the cytoplasmic domain of E-cadherin and recognizes the cytoplasmic domain of many cadherins (Marrs et al., 1993). To determine whether cadherin, and E-cadherin in particular, were quantitatively removed from these cell fractions, the depleted extracts were subjected to SDS-PAGE, transferred to Immobilon-P membranes, and probed with different cadherin antibodies: a polyclonal antibody to cadherin cytoplasmic domain (E-cad) (Marrs et al., 1993), a monoclonal antibody that specifically binds to the extracellular domain of E-cadherin (3G8), and antibodies raised against the intracellular domain of P-cadherin (P-cad1 and 2) that also crossreact with other cadherins (Vestweber, D., personal communication).

The first immunoprecipitation removed >90% of cadherins from the TX-100–insoluble fraction (Fig. 6 *A*); subsequent immunoprecipitations removed all of the remaining cadherins. A similar quantitative removal of cadherins from the TX-100–soluble fraction was performed. We did not detect any cadherins in the final cadherin immunoprecipitates from the TX-100–insoluble or –soluble fractions (Fig. 6 *A*). To ensure that the extracts were indeed cadherin depleted, they were probed with the panel of cadherin antibodies described above. Neither E- nor P-cadherin were detected (Fig. 6 *B*), demonstrating that the cell extracts were cleared of cadherins.

To determine if cadherin-depleted extracts contained catenins, they were probed with antibodies against α-catenin (*α-cat*), β-catenin (*β-cat*), or plakoglobin (*PG*) (Fig. 6 *B*). All three proteins were detected in both the TX-100–soluble and –insoluble protein fractions, indicating that pools of catenins exist independently of cadherin. To determine if these cadherin-independent catenins are associated with one another, we again performed immunoprecipitations followed by immunoblotting with different antibodies. Cadherin-depleted extracts were immunoprecipitated with antibodies against plakoglobin or β-catenin. These immunoprecipitates were probed with α-catenin antibodies (Fig. 6 *C*). Plakoglobin and β-catenin antibodies coimmunoprecipitate α-catenin in similar amounts from the TX-100–soluble and –insoluble fractions (Fig. 6 *C*).

Since cross-linking reactions are not always quantitative,



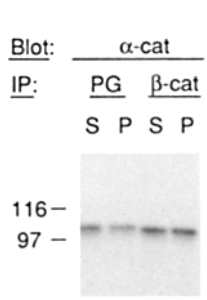


Figure 6. Catenins associate with each other in an E-cadherin–independent pool in the TX-100–soluble and –insoluble fractions. Confluent monolayers of MDCK cells were maintained on collagen-coated Transwell filters for 8 d. They were cross-linked with the reducible cross-linking reagent DSP and TX-100–soluble (*S*) and –insoluble (*P*) fractions were harvested. Each fraction was sequentially immunoprecipitated with antibodies against cadherin six times. (*A*) The immunoprecipitated material from each cycle (1–6) was subjected to SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with antibodies to cadherin to establish that cadherin had been removed. (*B*) One fraction of the remaining lysates was subjected to SDS-PAGE and immunoblotted with antibodies against E-cadherin (*E-cad* and *3G8*), P-cadherin (*P-cad 1* and *2*), α-catenin (*α-cat*), β-catenin (*β-cat*), and plakoglobin (*PG*). (*C*) Another fraction of the remaining lysates was immunoprecipitated (*IP*) with antibodies against plakoglobin (*PG*) or β-catenin (*β-cat*). The immunoprecipitated material was separated by SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted (*Blot*) with antibodies against α-catenin (*α-cat*). The identity of the lower molecular weight protein that is reactive with antibodies against E-cadherin in *B* is not known. The lower molecular weight proteins that are detected by immunoblotting extracts with the monoclonal antibody 3G8 against E-cadherin (*B*) are background bands that are nonspecifically bound by the rabbit anti–mouse secondary antibody used for the detection of the primary, mouse monoclonal antibody with ^{125}I-protein A (see Materials and Methods).

conclusions about the cadherin-independence of catenin complexes in the TX-100–insoluble fraction have to be drawn with caution. It is possible that cadherin-independent catenins detected in the TX-100–insoluble fraction were originally bound to a cadherin/catenin complex but were not covalently cross-linked to it so that they dissociated from the complex during the subsequent solubilization step. As a consequence, the amount of cadherin-independent catenins in the TX-100–insoluble fraction shown in Fig. 6 *B* may be higher than that originally present in the cell. However, we note that complexes of catenins are detected in the TX-100–insoluble fraction in this experiment (Fig. 6 *C*), demonstrating that catenins were cross-linked to each other (immunoprecipitations from the TX-100–insoluble fraction of non–cross-linked cells using catenin antibodies only contain the catenin against which the antibody is directed [Hinck et al., 1994*a*; Figs. 1 and 4]). In addition, the microscopy analysis, taken together with the biochemical experiments shown in this and the preceding paper (Hinck et al., 1994*a*), provide strong evidence for cadherin-independent pools of catenins in the TX-100–insoluble fraction. We conclude that there are catenin complexes independent of cadherin in the TX-100–soluble and –insoluble fractions.

Discussion

The cadherin/catenin complex is a key component in the initiation of cell–cell recognition and adhesion, as well as the elaboration of structural and functional organization in multicellular tissues and organs (Takeichi, 1991; Kemler, 1992). The complex transduces information from extracellular contacts made by cadherins to the cytoskeleton and intracellular signaling pathways (Nelson, 1992; Tsukita et al., 1993). We propose two minimal models of cadherin/catenin function: a single cadherin/catenin complex that initiates a signaling pathway resulting in a cascade of independent intracellular events; alternatively, multiple cadherin/catenin complexes that have different distributions and regulate each of these events individually. Based on previous studies, the cadherin/catenin complex at steady state is considered to be of a single type containing E-cadherin, α-catenin, β-catenin, and γ-catenin (plakoglobin) (Takeichi, 1991; Kemler, 1992). This view is based on the protein complex coimmunoprecipitated with E-cadherin from cells extracted with TX-100. The composition of the cadherin/complex in the TX-100–insoluble fraction has not been investigated until now (Hinck et al., 1994*a*). Previous studies also examined the distribution of E-cadherin in polarized epithelial cells, where it was shown to be enriched at the apical junctional complex (Boller et al., 1985). The distributions of α-catenin and β-catenin were not investigated. The availability of catenin-specific antibodies has allowed us to analyze cadherin/catenin complexes independently of cadherin. High resolution microscopy using these antibodies provides a detailed, comparative analysis of protein distributions. Our results demonstrate that there are multiple cadherin/catenin and catenin complexes that have different subcellular distributions.

Multiple Cadherin/Catenin Complexes at Steady State in MDCK Cells

In MDCK cells, we detect cadherin/catenin complexes at steady state that contain E-cadherin and α-catenin, and either β-catenin or plakoglobin. This result demonstrates that there are mutually exclusive associations of β-catenin and plakoglobin with E-cadherin (see also Hinck et al., 1994*a*, Fig. 1). Since β-catenin and plakoglobin share ~66% sequence identity (McCrea et al., 1991), they may bind to the same site on E-cadherin. However, there are differences in the distribution of these complexes in cells. We found that the E-cadherin/β-catenin complex, but not the E-cadherin/plakoglobin complex, is present in the TX-100–insoluble fraction (Figs. 4 and 5). Since formation of stable cell–cell contacts correlates with TX-100 insolubility (McNeill et al., 1993), we suggest that E-cadherin/plakoglobin complexes do not participate in the formation and maintenance of stable, E-cadherin–mediated cell–cell contacts in MDCK cells. Additionally, plakoglobin and E-cadherin do not colocalize in the TX-100–insoluble fraction (Fig. 3, *Extracted*, and Fig. 4). The lack of E-cadherin/plakoglobin complexes in the TX-100–insoluble pool may represent dissociation of plakoglobin from E-cadherin and exchange with β-catenin (see also Hinck et al., 1994*a*). Alternatively, the E-cadherin/plakoglobin complex may be degraded more rapidly than the E-cadherin/β-catenin complex, resulting in the accumulation of only the latter in the TX-100–insoluble fraction. Note that plakoglobin is principally involved in cell adhesion through desmosomes (Fig. 7) (Cowin et al., 1986). It is possible that binding of plakoglobin to desmosomal cadherins (desmoglein, desmocollin) is more stable than plakoglobin binding to E-cadherin. Differences in the affinity of plakoglobin and β-catenin for different cadherins may be responsible for sorting of these proteins to either the *zonula adherens* (E-cadherin/β-catenin) or *macula adherens* (desmoglein/plakoglobin), as discussed below (Fig. 7).

Complexes containing α-catenin and plakoglobin or β-catenin, but not E-cadherin were detected in cadherin-depleted cell extracts. Microscopy demonstrated that E-cadherin–independent pools of α-catenin and β-catenin are localized in the lateral membrane below the apical junctional complex of TX-100–extracted cells. In addition, α-catenin, β-catenin, and plakoglobin are distributed throughout the cell in the TX-100–soluble pool. These data confirm the existence of cadherin-independent pools of catenins that were identified in the preceding paper (Hinck et al., 1994*a*). The fact that these pools of catenins accumulate at steady state shows that they are not assembly intermediates or incorrectly processed proteins. Possible functions of these complexes will be discussed below.

Different Spatial Distributions of Cadherin/Catenin Complexes and Functions in Cellular Organization

Microscopy defined the distributions of cadherin/catenin and catenin complexes in both the TX-100–soluble and –insoluble fractions. There are three principal locations: throughout the cell, along the lateral membrane below the apical junctional complex, and at the apical junctional complex (Fig. 7). We propose that these distributions are related to specific functions.

All three catenins were distributed throughout the cell interior in patterns that were different from that of E-cadherin (Fig. 7). These intracellular pools of catenins were completely extracted from cells with TX-100. Biochemically, we

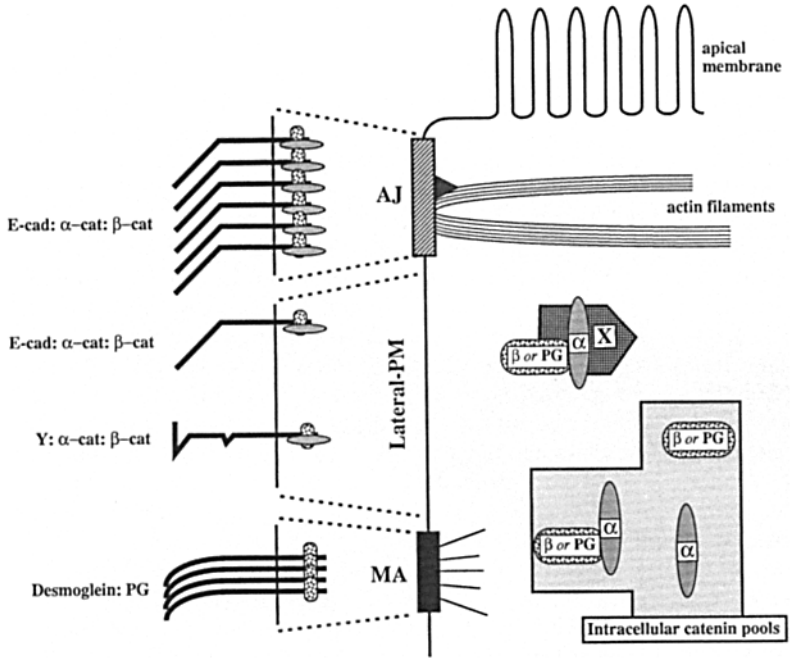


Figure 7. Subcellular distribution of different cadherin/catenin complexes in completely polarized MDCK cells. Complexes containing E-cadherin, α-catenin and β-catenin are distributed throughout the lateral plasma membrane (*Lateral-PM*). In TX 100–extracted cells, the concentration of these complexes is highest at the apical junctional complex (*AJ*, *shaded box*). This region contains the *zonula adherens* and the *zonula occludens*. Also shown is the belt of actin filaments at the apical junctional complex. The triangle depicted at the apical junctional complex represents actin accessory proteins, as well as tyrosine kinases that are concentrated at this site (see text). Both α-catenin and β-catenin bind to the lateral membrane independently of E-cadherin in the TX-100–insoluble fraction. They associate with unknown membrane proteins, denoted by "Y." We cannot formally exclude the possibility that α-catenin and β-catenin associate with Y individually. Plakoglobin does not associate with E-cadherin in the TX-100–insoluble fraction, but it is found in desmosomes (*macula adherens*, *MA*) in association with desmosomal cadherins like desmoglein. The association of plakoglobin with E-cadherin in the TX-100–soluble fraction is not shown here because the localization of these complexes was not investigated. In the TX-100–soluble fraction, α-catenin is free or bound to either β-catenin or plakoglobin with or without cadherin. Catenin complexes also associate with other cytosolic proteins denoted by "X." One candidate for X is the APC protein (see text).

showed that these pools contain α-catenin/β-catenin and α-catenin/plakoglobin complexes. It is also possible that pools of individual catenins are present. We showed by sucrose gradient analysis of proteins extracted from cells with TX-100 that a portion of α-catenin is not associated with either E-cadherin, plakoglobin, or β-catenin; this may represent an individual pool of α-catenin (Hinck et al., 1994*a*). We propose two functions for these intracellular pools of catenins. First, intracellular pools of catenins may provide reservoirs for the exchange and assembly of the catenins. In the preceding paper, we proposed that these reservoirs participate in regulating the assembly of cadherin/catenin complexes (Hinck et al., 1994*a*). Second, intracellular pools of catenins may bind to other proteins (Fig. 7). We do not know if these cadherin-independent pools of catenins are cytosolic, or whether they are associated with cytoskeletal components or membrane organelles. It is interesting to note that the product of a tumor suppressor gene, APC, forms a cytosolic complex with α-catenin and β-catenin that is independent of cadherin (Rubinfeld et al., 1993; Su et al., 1993). Such interactions may play a role in sequestering catenins to prevent them from binding to cadherins, or linking other proteins to signaling pathways common to cadherins.

A second location for E-cadherin/catenin, and catenin complexes is the lateral membrane (Fig. 7). In addition to roles in maintaining cell–cell contacts, we suggest that each of these complexes play at least one other role. The cadherin/catenin complex provides a link between cell adhesion molecules, the cytoskeleton, and other integral membrane proteins that is important in generating polarized distributions of proteins to the lateral plasma membrane. For example, Na^+/K^+-ATPase is restricted to the lateral membrane upon induction of E-cadherin–mediated cell adhesion. The common link between the cadherin/catenin complex and Na^+/K^+-ATPase is the membrane cytoskeleton, which is composed of ankyrin and spectrin (Nelson et al., 1990). When linkage of the cadherin/catenin complex to the cytoskeleton is lost, Na^+/K^+-ATPase remains uniformly distributed over the cell surface (McNeill et al., 1990). In addition, the cadherin-independent pools of catenins may bind directly to other membrane proteins (Fig. 7). In the preceding paper, we demonstrated by chemical cross-linking that another five as yet unidentified proteins are part of the cadherin/catenin complex (Hinck et al., 1994*a*). Recently, other membrane proteins have been identified in association with catenins, including desmosomal cadherins (Korman et al., 1989) and the EGF receptor (Näthke, I. S., L. Hinck, J. Papkoff, and W. J. Nelson, unpublished result). Linkage of catenins to these membrane proteins, which are located on the lateral membrane of polarized epithelial cells (Brändli et al., 1991; Wollner et al., 1992), may play a role in regulating receptor distribution through binding of these proteins to the cytoskeleton, similar to a mechanism for localizing Na^+/K^+-ATPase to the lateral membrane (see above). In addition, since growth factor receptors are involved in transducing mitogenic responses, their association with catenins may also indicate a role for catenins in this signal transduction pathway (see also Hinck et al., 1994*a*).

The highest concentration of E-cadherin, α-catenin, and β-catenin in the TX-100–extracted cell is in the region of the apical junctional complex. There are several reasons for the concentration of cadherin/catenin complexes at this location. The apical junctional complex contains the tight junction that requires cadherin-mediated cell adhesion and actin filament organization for formation and regulation (Madara, 1987). The apical junctional complex is also the site for organization of a circumferential ring of actin filaments (Fig. 7) (Wessels et al., 1971). It has been proposed that actin fila-

ments and the apical junctional complex are important in morphogenetic movements of cells through a "purse string" type contractile action (Wessels et al., 1971). Several actin accessory proteins have been identified in the apical junctional complex, where it is thought that they play a role in actin filament organization (Tsukita et al., 1992, 1993). E-cadherin/catenin complexes that localize to the apical junctional complex may interact directly or indirectly with these molecules to regulate the assembly of the cytoskeleton at this location and maintain the structural integrity at sites of cell–cell interactions during changes in actin filament organization.

Members of the *src* family of protein kinases, c-*src* and c-*yes*, are also localized at the apical junctional complex (Fig. 7) (Tsukita et al., 1991; Tsukita et al., 1993). The mechanism of localization and function of these tyrosine kinases are unknown. One possibility is that they regulate the function of the E-cadherin/catenin complex (Matsuyoshi et al., 1992; Behrens et al., 1993). Overexpression of v-src leads to a decrease in cell adhesion and correlates with an increase in the level of tyrosine phosphorylation of the proteins in the cadherin/catenin complex, particularly β-catenin (Matsuyoshi et al., 1992; Behrens et al., 1993). We do not know whether these tyrosine kinases are activated by another protein to phosphorylate the cadherin/catenin complex, or if the cadherin/catenin complex itself can activate *src* kinases. Together, these observations indicate that, in addition to its structural role, the apical junctional complex has important regulatory functions. It is interesting to note that the enrichment of E-cadherin and catenins at the apical junctional complex is only observed in cells that have been growing in confluent monolayers for >5 d. In cells grown in confluent monolayer for ≤5 d, the distribution of E-cadherin, α-catenin and β-catenin is still uniform throughout the lateral membrane of TX-100–extracted cells (Näthke, I. S., and J. R. Swedlow, unpublished data). Lateral membrane localization of cadherins and catenins may be related to cell adhesion and organization of membrane proteins that are initiated immediately after cell contact and recognition. Complete development of the apical junctional complex may require longer and is involved in specialized structural and regulatory functions of the epithelium.

In summary, cadherin-mediated cell–cell adhesion initiates a cascade of intracellular events that leads to the structural and functional reorganization of cells, including: formation of junctional complexes, organization of the actin cytoskeleton at the apical junctional complex, assembly of the membrane cytoskeleton, and development of membrane domains (Rodriguez-Boulan and Nelson, 1989; Nelson, 1992). We speculated that there is either a single cadherin/catenin complex involved in initiating all of these events, or that there are multiple complexes and each complex initiates a single event. Our data show that there are multiple cadherin/catenin and catenin complexes that have different distributions in the cell.

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