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Dynamics of Cadherin/Catenin Complex Formation: Novel Protein Interactions and Pathways of Complex Assembly

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Abstract. Calcium-dependent cell–cell adhesion is mediated by the cadherin family of cell adhesion proteins. Transduction of cadherin adhesion into cellular reorganization is regulated by cytosolic proteins, termed α-, β-, and γ-catenin (plakoglobin), that bind to the cytoplasmic domain of cadherins and link them to the cytoskeleton. Previous studies of cadherin/catenin complex assembly and organization relied on the coimmunoprecipitation of the complex with cadherin antibodies, and were limited to the analysis of the Triton X-100 (TX-100)–soluble fraction of these proteins. These studies concluded that only one complex exists which contains cadherin and all of the catenins. We raised antibodies specific for each catenin to analyze each protein independent of its association with E-cadherin. Extracts of Madin-Darby canine kidney epithelial cells were sequentially immunoprecipitated and immunoblotted with each antibody, and the results showed that there were complexes of E-cadherin/α-catenin, and either β-catenin or plakoglobin in the TX-100–soluble fraction. We analyzed the assembly of cadherin/catenin complexes in the TX-100–soluble fraction by [^{35}S]methionine pulse-chase labeling, followed by sucrose density gradient fractionation of proteins. Immediately after synthesis, E-cadherin, β-catenin, and plakoglobin cosedimented as complexes. α-Catenin was not associated with these complexes after synthesis, but a subpopulation of α-catenin joined the complex at a time coincident with the arrival of E-cadherin at the plasma membrane. The arrival of E-cadherin at the plasma membrane coincided with an increase in its insolubility in TX-100, but extraction of this insoluble pool with 1% SDS disrupted the cadherin/catenin complex. Therefore, to examine protein complex assembly in both the TX-100–soluble and –insoluble fractions, we used [^{35}S]methionine labeling followed by chemical cross-linking before cell extraction. Analysis of cross-linked complexes from cells labeled to steady state indicates that, in addition to cadherin/catenin complexes, there were cadherin-independent pools of catenins present in both the TX-100–soluble and –insoluble fractions. Metabolic labeling followed by chase showed that immediately after synthesis, cadherin/β-catenin, and cadherin/plakoglobin complexes were present in the TX-100–soluble fraction. Approximately 50% of complexes were titrated into the TX-100–insoluble fraction coincident with the arrival of the complexes at the plasma membrane and the assembly of α-catenin. Subsequently, >90% of labeled cadherin, but no additional labeled catenin complexes, entered the TX-100–insoluble fraction. These results indicate that catenins either interact with cadherin complexes synthesized at different times, or there was exchange of catenins between labeled and unlabeled cadherin complexes. In addition, cross-linking revealed additional proteins associated with catenin complexes. We propose a model in which the assembly of cadherin/catenin complexes is regulated by the exchange of catenins with cadherin-independent and cadherin-bound pools of proteins.

CALCIUM-dependent cell–cell adhesion proteins are morphoregulatory molecules that play important roles in the structural and functional organization of cells in tissues and organs of multicellular organisms (Takeichi, 1991). Previous studies showed that homotypic interactions between cadherins on adjacent epithelial cells lead to cell–cell adhesion, as well as the rapid development of structural and functional polarity. However, little is known about the mechanisms involved in transducing the signal of extracellular contacts between cadherin molecules into cellular reorganization. Candidates for key regulatory molecules are the proteins that bind the cadherin cytoplasmic domain (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989).

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Initial studies showed that deletion of the cytoplasmic domain of cadherins results in an adhesion-defective molecule that does not bind to the cytoskeleton (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990). An adhesion-deficient phenotype also results from the overexpression of the cadherin cytoplasmic domain in *Xenopus* embryos; this creates a dominant negative mutant that competes with endogenous cadherin for catenins (Kintner, 1992). Biochemical analysis showed that a limited number of cytoplasmic proteins coimmunoprecipitate with cadherins when cells are extracted in the presence of Triton X-100 (TX-100)[1] (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989). The most prominent of these cytoplasmic proteins have apparent molecular weights of 102, 94, and 86 kD, and are termed α-, β-, and γ-catenin, respectively (Ozawa et al., 1989). Recently, α-catenin (Herrenknecht et al., 1991; Nagafuchi et al., 1991) and β-catenin (McCrea et al., 1991) were cloned, and γ-catenin was identified as plakoglobin (Knudsen and Wheelock, 1992; Peifer et al., 1992; Piepenhagen and Nelson, 1993).

α-Catenin exhibits 30% sequence identity to vinculin (Herrenknecht et al., 1991; Nagafuchi et al., 1991), a protein involved in linking membrane proteins to the cortical cytoskeleton at sites of cell-substratum and cell-cell contact (Bendori et al., 1989). The expression of α-catenin is required for cell-cell adhesion. Cell lines that are deficient in α-catenin do not adhere, but cell adhesion can be restored by transfection of α-catenin cDNA (Hirano et al., 1992). β-Catenin and plakoglobin are homologous to *armadillo*, a *Drosophila* segment polarity gene (Peifer and Wieschaus, 1990; McCrea et al., 1991; Butz et al., 1992). Plakoglobin was originally identified as a major component of desmosomes (Cowin et al., 1986). While the function(s) of these *armadillo* protein family members is unknown, modulation of their interactions with cadherins affects the strength of cell-cell adhesion. For example, posttranslational modification of β-catenin by $pp60^{v\text{-}src}$ phosphorylation leads to a decrease in the strength of adhesion and a concomitant increase in cell migration (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993). In contrast, expression of the Wnt-1 protooncogene in mammalian cells leads to increased steady-state levels of β-catenin and plakoglobin, the stabilization of the cadherin/β-catenin complex, and a concomitant increase in the strength of cell-cell adhesion (Bradley et al., 1993; Hinck et al., 1994). Together, these studies indicate that catenins play a central role in the regulation of cadherin function in cell adhesion, as well as in the linkage of cadherins to the cytoskeleton and intracellular machinery involved in structural and functional organization of the cell.

To elucidate the multiple effects of cadherin-mediated cell-cell adhesion on cellular structure and function, it is important to define protein-protein interactions involved in the assembly and function of the cadherin/catenin complex. Much of the information on assembly has been obtained using E-cadherin antibodies to coimmunoprecipitate a cadherin/catenin complex from cell extracts under nondenaturing conditions. Based on these analyses, it has been proposed that one molecule of α-catenin and at least one molecule of β-catenin and plakoglobin simultaneously bind to a single cadherin molecule (Ozawa et al., 1989; McCrea and Gumbiner, 1991; Shore and Nelson, 1991; Takeichi, 1991; Kemler, 1992; Ozawa and Kemler, 1992; Grunwald, 1993). β-Catenin appears to bind to E-cadherin most tightly (McCrea and Gumbiner, 1991), and this complex forms immediately after the synthesis of E-cadherin (McCrea and Gumbiner, 1991; Ozawa and Kemler, 1992). α-Catenin associates with the cadherin/β-catenin complex later in the secretory pathway (Ozawa and Kemler, 1992). These data suggest a static model where subunit disassembly and exchange within the complex do not occur. In addition, the technical necessity of maintaining noncovalent cadherin/catenin interactions has limited previous analyses to complexes solubilized from cells in buffers containing TX-100. It is anticipated that the TX-100–insoluble fraction of this complex is important in cadherin function because cell-cell adhesion requires interaction of the cadherin/catenin complex with the cortical cytoskeleton (Hirano et al., 1987; Nagafuchi and Takeichi, 1988; Ozawa et al., 1990; Balsamo et al., 1991; Ozawa and Kemler, 1992). This interaction correlates with an increase in resistance of cadherin to extraction with nonionic detergents (Hirano et al., 1987; Nagafuchi and Takeichi, 1988; Shore and Nelson, 1991).

To examine the synthesis, distribution, and complex assembly of catenins independently of their coimmunoprecipitation with cadherin, we generated specific antibodies directed against α-catenin, β-catenin, and plakoglobin. In addition, we examined the TX-100–insoluble fraction of cadherin/catenin complexes using a protein cross-linking approach that permits extraction of the proteins under denaturing conditions without disruption of the complex. Our results demonstrate that the assembly of the cadherin/catenin complex in Madin-Darby canine kidney (MDCK) epithelial cells is dynamic. The multiple steps of assembly and the exchange of proteins in the complex may represent potential targets for regulation.

Materials and Methods

Growth and Maintenance of Cells

MDCK cells were maintained in DME/FBS as described previously (Nelson and Veshnock, 1987). For experiments in which the medium is switched from low calcium medium (LCM; 5 μM) to high calcium medium (HCM; 1.8 mM), cells were grown for 2 d at low cell density. Cells were trypsinized and plated at confluent density in LCM (2.5×10^6) on collagen-coated polycarbonate filters (Transwell filters, 0.45 μm; Costar Corp., Cambridge, MA) and grown for 8 h. Cell-cell contact was induced synchronously across the monolayer by raising the Ca^{++} concentration of the growth medium to 1.8 mM (HCM). For all other experiments, cells were trypsinized and plated at confluent density on collagen-coated polycarbonate filters in HCM and grown for 8 d.

Antibodies

The antibodies directed against E-cadherin (E-cad) (Marrs et al., 1993), α-catenin (α-cat), β-catenin (β-cat), and plakoglobin (PG) have been described previously (Hinck et al., 1994). The α-cat antibody was concentrated from serum by a 50% ammonium sulfate precipitation.

Metabolic Labeling

Cultures were preincubated in labeling medium (DME/FBS/minus methionine) for 20 min. The medium was removed, and 250 μCi of [^{35}S]methionine was added to the basolateral side of the Transwell filter in a total vol-

1. *Abbreviations used in this paper*: α-cat, α-catenin; β-cat, β-catenin; DSP, dithiobis(succinimidylpropionate); E-cad, E-cadherin; HCM, high calcium medium; LCM, low calcium medium; MDCK, Madin-Darby canine kidney (cells); PG, plakoglobin; TX-100, Triton X-100.

ume of 100 μl of labeling media; 1 ml of labeling media was added to the apical compartment. The cells were pulse-labeled for 15 min, rinsed 2× in chase medium (DME/FBS/10,000-fold excess unlabeled methionine), and chased for the indicated times. For the 16-h label, 1 ml of overnight labeling medium (DME/FBS/10% methionine) containing 250 μCi of [^{35}S]methionine was added to the basolateral side of the Transwell filter; 1 ml of overnight labeling medium was added to the apical compartment. The medium was replaced every 4 h with medium containing fresh label. For the determination of molecular ratios, the number of methionines per protein was determined based on sequences submitted to Genbank (murine mature E-cadherin, 10; murine α-catenin, 28; murine β-catenin, 30; human plakoglobin, 26).

Cell Extraction and Immunoprecipitation

Cells were rinsed 2× in PBS and solubilized in CSK buffer (50 mM NaCl, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 300 mM sucrose, 1.2 mM PMSF, 10 μg/ml leupeptin) for 20 min at 4°C on a rocking platform. The cells were scraped from the filter with a rubber policeman and sedimented in a microfuge (Beckman Instruments, Inc., Fullerton, CA) for 10 min. The soluble supernatant was collected. The cell pellet was triturated in 100 μl SDS immunoprecipitation buffer (15 mM Tris, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% SDS), incubated at 100°C for 10 min, and then diluted to 1 ml with CSK buffer. Extracts were preincubated with 10 μl preimmune serum and 50 μl of pansorbin cells (Calbiochem, La Jolla, CA) before immunoprecipitation with specific antibodies. For the multiple immunoprecipitations with E-cad, α-cat, β-cat, and PG antibodies at each time point, a single extract was divided into equal volume aliquots.

Antibodies were prepared for immunoprecipitation by preincubation of 10 μl of serum with 50 μl of a 50% slurry of protein A–Sepharose (Pharmacia LKB Biotechnology, Alameda, CA) for 1 h at 4°C, followed by 1× wash with PBS and resuspension to 50 μl in PBS. This slurry was added to the soluble and insoluble fractions, and incubated for 2 h at 4°C on a rocking platform. The samples were washed sequentially with high stringency buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% TX-100, 1% Na-deoxycholate, 0.1% SDS, 120 mM NaCl, 25 mM KCl), underlaid with 10% sucrose and pelleted; high salt buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% TX-100, 1% Na-deoxycholate, 0.1% SDS, 1 M NaCl); and low salt buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA).

Gel Electrophoresis

Protein samples were incubated in 75 μl of SDS sample buffer for 10 min at 100°C before separation in a SDS-7.5% polyacrylamide gel (Laemmli, 1970). For metabolically labeled samples, the gel was immersed in Amplify as directed by the manufacturer (Amersham International, Amersham, United Kingdom), dried under a vacuum, and exposed to x-ray film (XAR-5; Eastman Kodak, Rochester, NY) at −80°C. The gels were analyzed using a scanning densitometer (ScanJet IIc; Hewlett-Packard Co., Palo Alto, CA) and Image Software (National Institutes of Health, Bethesda, MD). The molecular weight standards are β-galactosidase (M_r 116,000), phosphorylase b (M_r 97,000), and bovine serum albumin (M_r 68,000).

Immunoblotting

Immunoprecipitates were separated by SDS-PAGE and then electrophoretically transferred to membranes (Immobilon P; Millipore Corp., Bedford, MA). The membranes were blocked in gelatin wash buffer (GWB; 15 mM Tris-HCl, pH 7.5, 120 mM NaCl, 5 mM NaN_3, 1 mM EDTA, 0.1% Tween-20, 0.1% gelatin) containing 5% milk, followed by incubation with antibodies (E-cad, β-cat, and PG, 1:500; α-cat, 1:100) in gelatin wash buffer for 2 h. Antibody incubation was followed by extensive washing in gelatin wash buffer, incubation with ^{125}I-protein A (0.1 μCi/ml) (Du Pont/NEN, Boston, MA) for 1 h, extensive washing in gelatin wash buffer, and exposure to x-ray film (XAR-5; Eastman Kodak) at −80°C.

Sucrose Density Gradient Fractionation

Cell cultures were harvested and the TX-100–soluble fraction was generated (see above). Linear sucrose gradients (10–20%) were overlaid with the TX-100 fraction of cell extracts and centrifuged in a rotor (SW60Ti; Beckman Instruments, Inc.) at 60,000 rpm for 5 h at 4°C, as previously described (Nelson et al., 1990). Gradients were fractionated from bottom to top into 19 fractions. Two gradients were run for each time point, and the fractions were collated before they were split into equivalent aliquots for immunoprecipitation. The S value marker proteins are catalase (11.35 S), aldolase (7.35 S), and bovine serum albumin (4.6 S).

Chemical Cross-linking

Cell cultures were rinsed 2× with PBS, and 1 ml of PBS containing 200 μg/ml dithiobis(succinimidylpropionate) (DSP) (Pierce Chemical Co., Rockford, IL) was added to the apical and basolateral compartments, and incubated for 20 min at room temperature on a rocking platform. The DSP was prepared as a 100× stock in DMSO and diluted immediately before use. The cell cultures were rinsed and then incubated for 5 min at room temperature in quenching buffer (PBS/50 mM glycine, pH 7.4). Cells were harvested as described above, except that the cytoskeleton contained 20 mM glycine, pH 7.4. The immunoprecipitated complexes were separated in a 4% nonreducing gel and, similar to the results of Ozawa and Kemler (1992), two very diffuse bands with apparent molecular masses of 190–225 and 320–370 kD were observed (data not shown). These protein bands were completely reduced by the following experimental protocol: incubation of the immunoprecipitated complexes in sample buffer (20% β-mercaptoethanol) at 100°C for 10 min and addition of 10 μl of 1 M DTT to the gel lanes before protein separation.

Results and Discussion

Characterization of Catenin-specific Antibodies and Defining the Composition of the Cadherin/Catenin Complex

Monospecific antipeptide antibodies were generated against unique sequences of either α-catenin (α-cat antibody), β-catenin (β-cat antibody), or plakoglobin (PG antibody). On Western blots, each antibody reacted with a single protein with the appropriate molecular mass (102 kD, α-catenin; 94 kD, β-catenin; 86 kD, plakoglobin), and did not cross-react with the other catenins (Fig. 1). The specificity of these antibodies was demonstrated by blocking protein reactivity with cognate peptides (Hinck et al., 1994; data not shown). An antibody raised against the cytoplasmic domain of E-cadherin (E-cad antibody) was described previously (Marrs et al., 1993).

An initial characterization of the composition of the cadherin/catenin complex was performed by sequential immunoprecipitation and immunoblotting with E-cad, α-cat, β-cat, and PG antibodies (Fig. 1). To ensure that only newly synthesized complexes are analyzed, and not protein complexes involved in previous cell contacts, cells were grown for 2 d at very low density and without cell–cell contacts (Nelson and Veshnock, 1987). Confluent monolayers of MDCK cells were subsequently established on Transwell filters in DME/FBS containing 5 μM Ca^{++} (LCM). Under these conditions, cells do not form cell–cell contacts because the calcium-dependent cadherin molecule is inactivated; raising the Ca^{++} concentration of the growth media to 1.8 mM Ca^{++} (HCM) results in rapid and synchronous induction of intercellular contact across the monolayer (Nelson and Veshnock, 1987). This experimental protocol allows the examination of de novo assembly of proteins into junctions (Pasdar and Nelson, 1988).

4 h after the induction of cell–cell contact, the cells were extracted in cytoskeleton buffer containing TX-100, generating TX-100–soluble (S) and –insoluble (P) fractions of total cellular protein (Pasdar and Nelson, 1988). The TX-100–insoluble fraction was extracted by boiling in buffer containing 1% SDS; this disrupts the cadherin/catenin complexes. Proteins solubilized in buffers containing TX-100 or 1% SDS

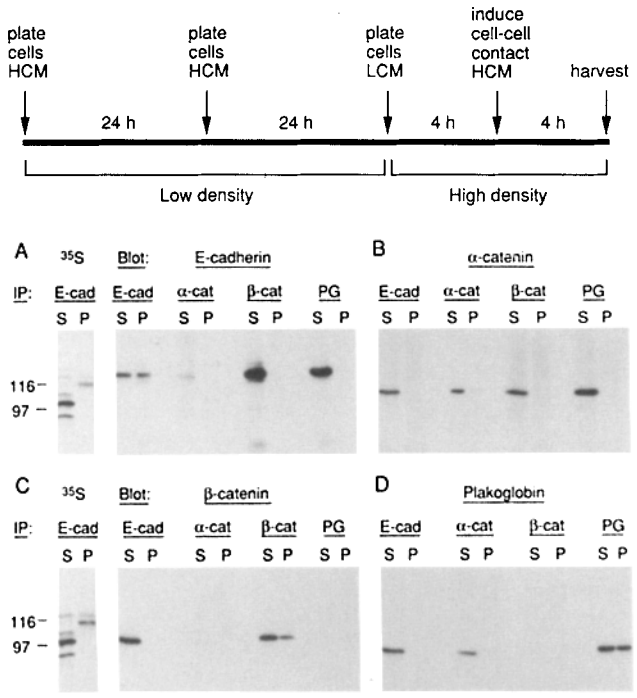


Figure 1. Characterization of catenin-specific antibodies and defining the composition of the cadherin/catenin complex. MDCK cells were rendered contact naive by consecutive platings at low density. The cells were then plated at confluent density in LCM, and the media was switched to HCM for 4 h as outlined in the schematic time line. The cells were extracted in buffers containing TX-100, generating TX-100–soluble (*S*) and –insoluble (*P*) fractions. The TX-100–insoluble fraction was solubilized in 1% SDS, 100°C. Each fraction was immunoprecipitated with E-cad, α-cat, β-cat, and PG antibodies (*IP*). The immunoprecipitates were separated in a 7.5% SDS-PAGE and were then electrophoretically transferred to Immobilon-P membranes. The membranes were probed with E-cad (*A*), α-cat (*B*), β-cat (*C*), and PG (*D*) antibodies (*Blot*). Note that those lanes immunoblotted with the same antibody used to immunoprecipitate the extract, contain one fifth the amount of sample. To show cadherin and the associated catenins for reference, MDCK cells were metabolically labeled with [^{35}S]methionine for 4 h, and extracts were prepared. The TX-100–soluble (*S*) and –insoluble (*P*) fractions were immunoprecipitated with E-cad antibody (*A* and *C*, ^{35}S).

are designated TX-100 soluble (S) and TX-100–insoluble (P), respectively. Equal aliquots of extract were immunoprecipitated with each antibody. The immunoprecipitates were washed under high stringency conditions in buffers containing 1 M NaCl and 0.1% SDS, which were used previously to investigate cadherin/catenin complex formation (Shore and Nelson, 1991). The immunoprecipitates were separated by 7.5% SDS-PAGE, transferred to Immobilon-P membranes, and probed with the immunoprecipitating antibody, as well as with each of the other antibodies. Note that lanes immunoblotted with the same antibody used for immunoprecipitation contain one fifth as much sample as the others. For reference, MDCK cells were metabolically labeled with [^{35}S]methionine for 4 h, extracts were prepared, and the TX-100–soluble (S) and –insoluble (P) fractions were immunoprecipitated with the E-cad antibody (Fig. 1, *A* and *C*; E-cad, ^{35}S). Immunoprecipitation from the TX-100–soluble fraction shows a complex of proteins that is coimmunoprecipitated and includes the other members of the cadherin/catenin complex. Based on immunogenic reactivity, electrophoretic mobility in SDS-polyacrylamide gels, and complex formation with E-cadherin, proteins of molecular mass 102, 94, and 86 kD are designated as α-catenin, β-catenin, and plakoglobin, respectively. In addition, proteins of 135-, 120-, and 115-kD polypeptides are specifically recognized by the E-cad antibody (Fig. 1, *A* and *C*; E-cad ^{35}S, *S*, 120 kD; *P*, 120 and 115 kD; see also Fig. 4 *A*), and they represent the precursor, mature, and intermediate forms of E-cadherin, respectively (Shore and Nelson, 1991).

E-cadherin and α-catenin immunoblots show that both of these proteins are detected in complexes immunoprecipitated with each of the catenin antibodies from the TX-100–soluble fraction (Fig. 1, *A* and *B*). In contrast, the β-catenin and plakoglobin immunoblots show that β-catenin is not detected in the plakoglobin immunoprecipitate (Fig. 1 *C*) and vice versa (Fig. 1 *D*). Thus, these results show that E-cadherin does not form a single complex with all three catenins. Instead, the E-cadherin/α-catenin complex associates with either β-catenin or plakoglobin. The accompanying paper provides insight into the steady state distribution and potential significance of these distinct complexes (Näthke et al., 1994). It is noteworthy that only a relatively small amount of E-cadherin and β-catenin are detected in the α-catenin immunoprecipitates compared to the other catenin immunoprecipitates (Fig. 1, *A* and *C*). This suggests that the α-catenin antibody inefficiently recognizes α-catenin in association with the other proteins; however, pulse/chase (data not shown) and chemical cross-linking (see Figs. 4 *B* and 5 *B*) studies show that the antibody is capable of coimmunoprecipitating complexes. A second possibility is that there is a large pool of E-cadherin–independent α-catenin that titrates the antibody (see Figs. 2 and 3). This would result in a mixed immunoprecipitate containing both cadherin/catenin complexes and cadherin independent complexes.

In the TX-100–insoluble fraction, complexes were disrupted by boiling in 1% SDS. As a result, the antibodies immunoblotted their cognate proteins only in samples that were immunoprecipitated with the same antibody (e.g., E-cad IP/E-cad blot). The results show that E-cadherin, β-catenin, and plakoglobin are distributed between the TX-100–soluble and –insoluble fractions (Fig. 1, *A*, *C*, and *D*), but there appears to be little or no α-catenin in the TX-100–insoluble fraction (Fig. 1 *B*).

This experiment demonstrates several features of the cadherin/catenin complex: (*a*) individual catenins can be positively identified in the cadherin/catenin complex; (*b*) proteins of the cadherin/catenin complex are distributed between the TX-100–soluble and –insoluble fractions; and (*c*) there are two different cadherin/catenin complexes. Thus, the availability of catenin-specific antibodies provides the first opportunity to analyze the synthesis, complex assembly, distribution, and fate of each protein independently of their noncovalent interaction with cadherin.

Three types of experiments were performed to investigate the composition and assembly of the cadherin/catenin complex in MDCK epithelial cells: (*a*) separation of TX-100–soluble proteins in sucrose density gradients to examine the de novo assembly of cadherin/catenin complexes at the

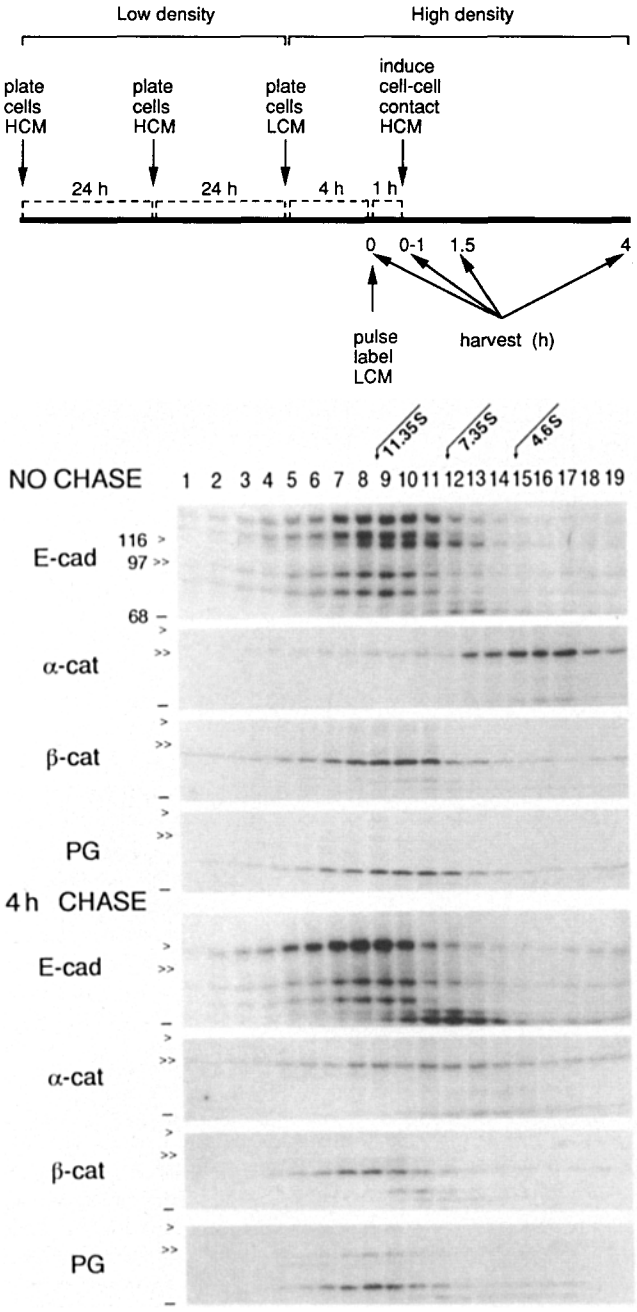


Figure 2. Sucrose gradient fractionation of TX-100–soluble cadherin/catenin complexes; distinguishing free and assembled populations of α-catenin. MDCK cells were rendered contact naive by consecutive platings at low density. The cells were then plated at confluent density in LCM, the cultures were pulse labeled with [^{35}S]methionine for 15 min (*NO CHASE*) and chased in the absence of [^{35}S]methionine for 1 h (*0–1-h chase*). The media was switched to HCM and the chase was continued for 1.5 h (*1.5-h chase*) and 4 h (*4-h chase*). This protocol is represented in a schematic time line. At each time point, cell extracts were prepared and the TX-100–soluble pool was fractionated on a 10–20% linear sucrose gradient. 19 fractions were collected from the bottom (fraction 1) to the top (fraction 19) and equivalent aliquots of each fraction were immunoprecipitated with E-cad, α-cat, β-cat, and PG antibodies. The immunoprecipitates were subjected to 7.5% SDS-PAGE followed by fluorography.

time of induction of cell–cell contacts; (*b*) [^{35}S]methionine pulse-chase labeling followed by chemical cross-linking to examine the assembly of cadherin/catenin complexes in the TX-100–soluble and –insoluble fractions in fully polarized cells; and (*c*) steady-state labeling with [^{35}S]methionine followed by chemical cross-linking to analyze the composition of cadherin/catenin complexes in the TX-100–insoluble fraction of fully polarized cells. Each experiment involves analysis of protein complexes by immunoprecipitation with E-cad, α-cat, β-cat, or PG antibodies. The results are discussed at the end of each section in a synopsis. A new model of assembly pathways for the cadherin/catenin complex is proposed in Fig. 6 *A*. Fig. 6 *B* also provides a table for cross-referencing results and conclusions concerning specific steps in assembly of cadherin/catenin complexes.

Sucrose Gradient Fractionation of TX-100–soluble Cadherin/Catenin Complexes: Distinguishing Free and Assembled Populations of α-Catenin

Confluent monolayers of MDCK cells were established in LCM and pulse labeled with [^{35}S]methionine in LCM for 15 min. Replicate cultures were extracted without a chase period (Figs. 2 and 3, *NO CHASE*), or after 1-h chase in LCM (Fig. 2, *0–1 h*), or after an additional 1.5-h (Fig. 3, *1.5 h*) or 4-h chase (Figs. 2 and 3, *4 h*) in HCM to induce cell–cell contacts. Solubilized protein fractions were separated in 10–20% linear sucrose gradients. 19 fractions were collected from each gradient. Each fraction was divided into four equal aliquots that were immunoprecipitated with E-cad, α-cat, β-cat, or PG antibodies. The autoradiograms of each immunoprecipitate for 0- (no chase) and 4-h chase are shown in Fig. 2, and the results from the densitometric analysis of these autoradiograms, as well as those of 0–1- and 1.5-h chases (autoradiograms not shown), are plotted in Fig. 3.

Immunoprecipitation of each fraction at time 0 with antibodies directed against E-cadherin shows that [^{35}S]methionine-labeled precursor (135-kD) and mature (120-kD) forms of E-cadherin cosediment in fractions 6–12 with an apparent S value of 10–13 (Figs. 2 and 3, *NO CHASE*, *E-cad*). The sedimentation rate of the intermediate form of E-cadherin (115 kD) is slightly slower (fractions 8–13) than that of either the precursor or mature forms. [^{35}S]methionine-labeled β-catenin and plakoglobin are coimmunoprecipitated with E-cadherin in a complex, and they exhibit a sedimentation profile identical to that of the precursor and mature forms of E-cadherin. Little or no [^{35}S]methionine-labeled α-catenin is coimmunoprecipitated in the E-cadherin/β-catenin or E-cadherin/plakoglobin complex at time 0. Immunoprecipitation of fractions from the same gradient with antibodies directed against either β-catenin or plakoglobin show a peak of [^{35}S]methionine-labeled protein between fractions 7–14 that overlaps but is broader than the peak fractions containing the precursor, intermediate, and mature forms of E-cadherin (Figs. 2 and 3, *NO CHASE*, β-cat, and *PG*).

At time 0, immunoprecipitation of sucrose gradient fractions with α-catenin antibodies reveals that [^{35}S]methionine-labeled α-catenin has a sedimentation profile that is very different than those of the E-cadherin/β-catenin and E-cadherin/plakoglobin complexes (Figs. 2 and 3, *NO CHASE*, *α-cat*). α-Catenin sediments with a peak distribution between

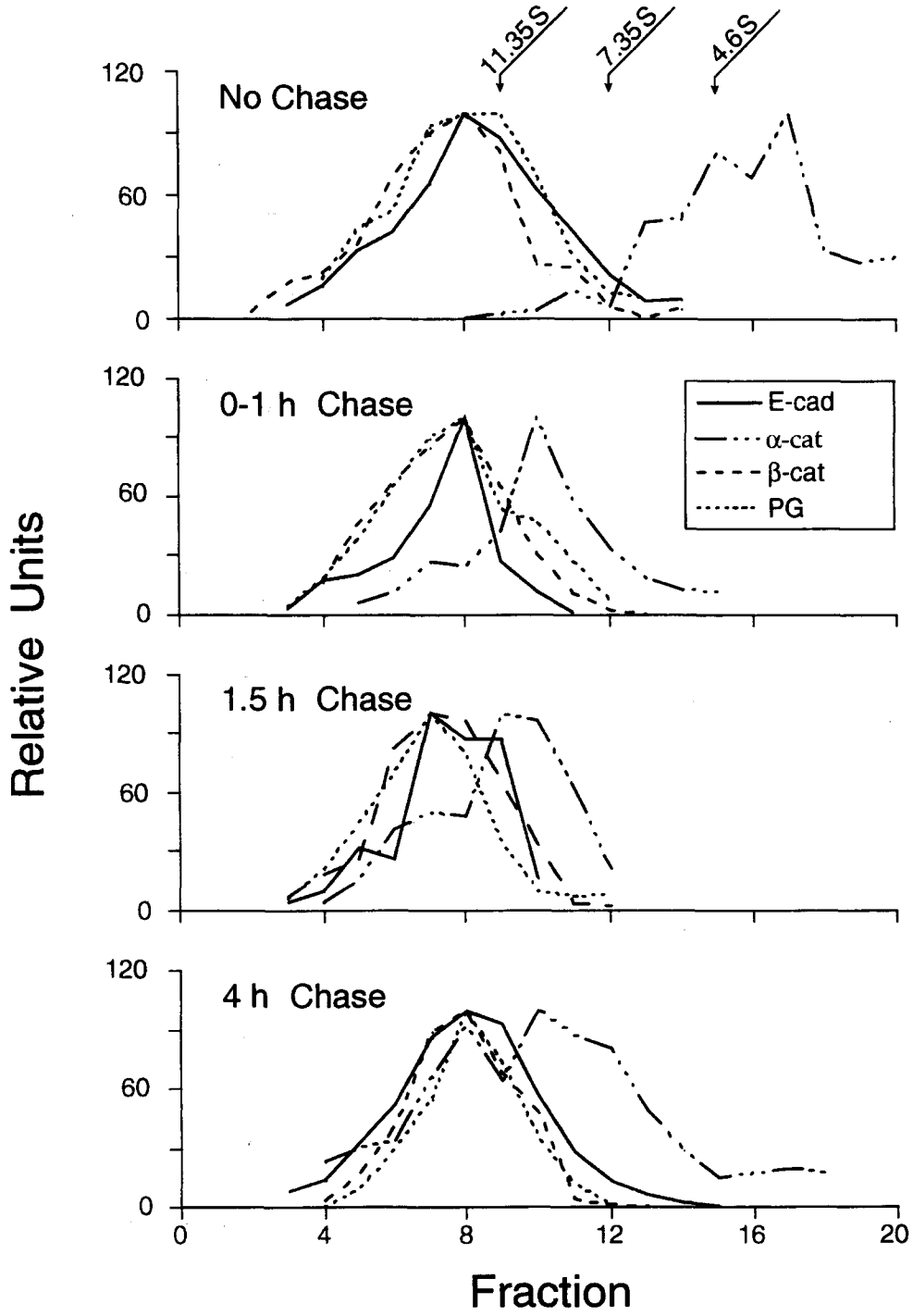


Figure 3. Sucrose gradient sedimentation profiles of the labeled E-cadherin, α-catenin, β-catenin, and plakoglobin proteins immunoprecipitated from sucrose density gradient fractions. MDCK cell cultures were pulse-labeled with [^{35}S]methionine for 15 min and chased in the absence of [^{35}S]methionine for the indicated time periods (see Fig. 2). At each time point, cell extracts were prepared and treated as described in Fig. 2. The autoradiograms from two separate experiments were quantified by scanning densitometry, and the relative abundance of each protein in each fraction (relative units) is plotted against fraction number.

fractions 13 and 19 with an apparent S value of 3.5. Little or no [^{35}S]methionine-labeled α-catenin sediments in fractions containing the E-cadherin/β-catenin or E-cadherin/plakoglobin complex. To determine whether α-catenin is cosedimenting with unlabeled E-cadherin, the immunoprecipitates were transferred to Immobilon-P membranes and probed with E-cad antibody. No unlabeled E-cadherin sedimented in fractions 13–19 with newly synthesized α-catenin (data not shown).

The distributions of proteins in the sucrose gradient after a 1-h chase in LCM (0–1 h) and a subsequent 1.5-h chase in HCM are illustrated in Fig. 3. The E-cadherin/β-catenin and E-cadherin/plakoglobin complexes cosediment in a sharper peak than those extracted from cells at time 0 (Fig. 3, *0–1 h*, *E-cad*, *β-cat*, and *PG*). There is a significant change in the sedimentation of the population of α-catenin (Fig. 3, *0–1 h*, *α-cat*). Although the overall sedimentation profile of α-catenin is distinct from that of the E-cadherin complex, a small portion of α-catenin is now detected in the fractions containing the E-cadherin/β-catenin and E-cadherin/plakoglobin complexes, and a large portion of α-catenin sediments faster than that extracted at time 0 (fractions 9–14 compared to

fractions 13–19). Similar distributions of these proteins are detected after a further chase period of 1.5 h in HCM (Fig. 3, *1.5 h*).

After a 4-h chase in HCM, immunoprecipitation of sucrose gradient fractions with E-cadherin antibody resolves complexes containing mature E-cadherin/β-catenin and E-cadherin/plakoglobin (Figs. 2 and 3, *4 h, E-cad*). The sedimentation rate of cadherin complexes at 4 h is faster (apparent S value of 14–16) than that of the complexes extracted at time 0 (apparent S value of 10–13). Immunoprecipitation of each fraction with β-catenin or plakoglobin antibodies shows that the sedimentation profile of these proteins overlaps that of E-cadherin (Figs. 2 and 3, *4 h, β-cat*, and *PG*). At present, the reason that the E-cadherin/β-catenin and E-cadherin/plakoglobin complexes sediment faster after a 4-h chase is unknown, but may reflect the fact that α-catenin is now assembled into the complexes. Under the conditions of high stringency immunoprecipitation (McCrea and Gumbiner, 1991), we detect only a small amount of [^{35}S]methionine-labeled α-catenin coimmunoprecipitating with E-cadherin after 4 h of chase (Figs. 2 and 3, *4 h, E-cad*). However, direct analysis of the sedimentation profile of [^{35}S]methionine-labeled α-catenin reveals that α-catenin sediments in a broad distribution (fractions 5–15) (Figs. 2 and 3, *4 h, α-cat*). Densitometric scanning of the autoradiograms resolves the α-catenin band into two subpopulations. One of these subpopulations sediments with an apparent S value of 14–16 and cosediments with the E-cadherin/β-catenin and E-cadherin/plakoglobin complexes. The other subpopulation sediments with an apparent S value of 9, distinctly different from the pools of E-cadherin/β-catenin and E-cadherin/plakoglobin.

Synopsis. Immediately after synthesis, [^{35}S]methionine-labeled E-cadherin/β-catenin and E-cadherin/plakoglobin complexes cosediment in the sucrose gradient, and these proteins are immunoprecipitated with E-cadherin antibodies (Figs. 2 and 3, *NO CHASE, E-cad*). Analysis of [^{35}S]methionine-labeled β-catenin and plakoglobin distributions in the sucrose gradient using specific antibodies indicates that the majority of each protein cosediments with E-cadherin (Figs. 2 and 3, *NO CHASE, β-cat*, and *PG*). Note, however, that the overlap is not perfect. This may reflect E-cadherin–independent pools of β-catenin and plakoglobin that are in homo-oligomeric complexes or in complexes with proteins that are as yet unidentified.

In contrast, [^{35}S]methionine-labeled α-catenin initially sediments in fractions of the sucrose gradient that are distinct from those of the E-cadherin/β-catenin and E-cadherin/plakoglobin complexes (Figs. 2 and 3, *NO CHASE, α-cat*). During the subsequent chase period, the sedimentation profile of α-catenin changes, and a portion of α-catenin cosediments with the complex of E-cadherin/β-catenin or E-cadherin/plakoglobin. The timing of the assembly of α-catenin into the E-cadherin complex is consistent with the arrival of [^{35}S]methionine-labeled E-cadherin at the cell surface (Shore and Nelson, 1991).

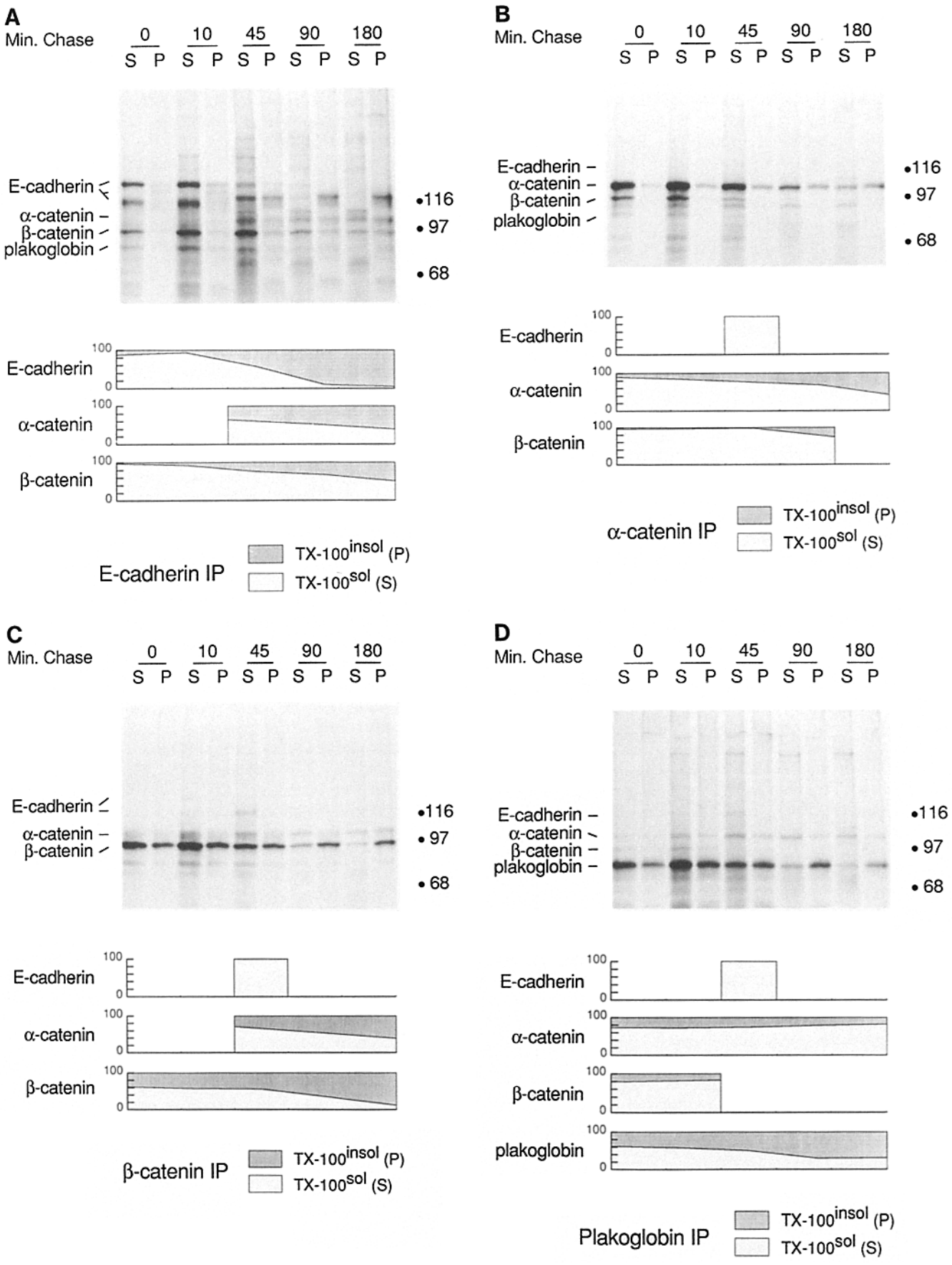
During the course of the pulse-chase analysis, we distinguished three populations of α-catenin. Immediately after synthesis, we detected a population that sediments with an apparent S value of 3.5 (Figs. 2 and 3, *NO CHASE, α-cat*). We suggest that this population constitutes monomeric α-catenin. Subsequently, after the chase, we detected two populations of α-catenin; a population (apparent S value of 14–16 S) that cosedimented with the E-cadherin, β-catenin, and plakoglobin complex; and an intermediate population (apparent S value of 9 S) that was distinct from the cadherin complex (Fig. 2, *4 h, α-cat*; Fig. 3, *0–1, 1.5, 4 h, α-cat*). The intermediate population of α-catenin has sedimentation characteristics different from that of both the putative monomeric population and the cadherin complex. We suggest that α-catenin in this pool either has a different conformation, is associated with the other catenins or itself (homo- or hetero-oligomers), or is associated with unidentified proteins.

Cross-linking Reveals Dynamic Assembly of the Cadherin/Catenin Complex in the TX-100–insoluble Fraction

E-cadherin, α-catenin, β-catenin, and plakoglobin were detected in the TX-100–insoluble fraction in the experiment represented in Fig. 1 (*P*), but we were unable to determine whether they were in a complex. Conditions required to solubilize the TX-100–insoluble cell residue (1% SDS at 100°C) caused dissociation of these complexes, allowing only isolation of individual proteins with specific antibodies. Therefore, in the following series of experiments, the pulse-chase analysis was done in conjunction with chemical cross-linking before protein extraction to investigate TX-100–insoluble complexes (Fig. 4).

Furthermore, we wanted to examine the nature of the cadherin-independent pool of α-catenin that was revealed in the experiment shown in Figs. 2 and 3, where cell–cell contact was induced during the course of the pulse-chase analysis. It is possible that this pool represents protein that is unassembled because of the absence of cell–cell contact and is present only before de novo junction formation. We analyzed the kinetics of the cadherin/catenin complex assembly before the induction of cell–cell contact (in LCM), during the formation of cell–cell contacts (LCM to HCM), and in the presence of established cell–cell junctions (HCM). The association of the catenins with cadherin appears similar under all of these conditions (Hinck, L., and I. S. Näthke, unpublished results). In the following series of experiments, we analyzed cross-linked cadherin/catenin complexes in cells with extensive cell–cell contacts.

MDCK cell monolayers were established on Transwell filters and grown for 8 d. Cells were pulse labeled with [^{35}S]-methionine for 15 min (time 0), and were then chased in the presence of excess, unlabeled methionine for 10, 45, 90, and 180 min. At each time point, cells were cross-linked, and extracts were prepared, divided into equal aliquots, and immunoprecipitated with E-cad (Fig. 4 *A*), α-cat (Fig. 4 *B*), β-cat (Fig. 4 *C*), or PG (Fig. 4 *D*) antibodies as previously described. The relative proportions of each protein in the TX-100–soluble and –insoluble fractions are presented in graph form. Note that the absolute amount of plakoglobin in an E-cadherin immunoprecipitate is difficult to determine because two-dimensional gel electrophoresis resolves the plakoglobin band into two proteins identified as plakoglobin and the protease-sensitive extracellular domain of E-cadherin (Piepenhagen and Nelson, 1993; Barth, A., unpublished results). Therefore, quantitative analysis of plakoglobin was limited to plakoglobin immunoprecipitates.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TX-100sol (S)

E-Cadherin. Fig. 4 *A* shows cross-linked protein complexes immunoprecipitated with E-cadherin antibodies during the chase period. Immediately after the chase, at time 0, 98% of [^{35}S]methionine-labeled E-cadherin is detected in the TX-100–soluble fraction (Fig. 4 *A*, *0 min*, *S*); approximately equal amounts of E-cadherin precursor (135 kD) and intermediate forms (115 kD) are detected. Significantly more [^{35}S]methionine-labeled β-catenin than plakoglobin is detected in the cross-linked cadherin complex; no [^{35}S]methionine-labeled α-catenin is present. At this time, the TX-100–insoluble fraction contains very little or no [^{35}S]methionine-labeled E-cadherin (Fig. 4 *A*, *0 min*, *P*).

After a 10-min chase, >90% of [^{35}S]methionine-labeled E-cadherin, β-catenin, and plakoglobin are present in the TX-100–soluble fraction (Fig. 4 *A*, *10 min*, *S*), but there is a small amount (<10%) of [^{35}S]methionine-labeled E-cadherin precursor (135 kD), β-catenin, and plakoglobin present in the TX-100–insoluble fraction (Fig. 4 *A*, *10 min*, *P*). After 45 min of chase, most of [^{35}S]methionine-labeled E-cadherin in the Triton-100–soluble fraction is in the mature form (120 kD) (Fig. 4 *A*, *45 min*, *S*). At this time, ~40% of [^{35}S]methionine-labeled E-cadherin is detected in the TX-100–insoluble fraction (Fig. 4 *A*, *45 min*, *P*). Analysis of the catenins, cross-linked in the complex and coimmunoprecipitated with E-cadherin, shows that α-catenin is detected for the first time, and that 36% of α-catenin, 24% of β-catenin, and 32% of plakoglobin are present in the TX-100–insoluble E-cadherin complex at this time (Fig. 4 *A*, *45 min*, *P*).

After a 90-min chase, the composition of the cross-linked E-cadherin/catenin complex has changed dramatically. More than 90% of [^{35}S]methionine-labeled E-cadherin is detected in the TX-100–insoluble fraction (Fig. 4 *A*, *90 min*, *P*). In direct contrast, only 45% of E-cadherin–associated α-catenin and 30% of E-cadherin–associated β-catenin are in the TX-100–insoluble fraction; plakoglobin is no longer detectable (Fig. 4 *A*, *90 min*, *P*). The precipitating antibody is E-cadherin and, therefore, the presence of labeled catenins in the TX-100–soluble fraction indicates the presence of unlabeled E-cadherin. This distribution of [^{35}S]methionine-labeled E-cadherin and catenins between these two fractions is maintained after a 180-min chase.

α-Catenin. Immunoprecipitation of cross-linked cell extracts at time 0 with α-cat antibodies reveals that 90% of the [^{35}S]methionine-labeled α-catenin is in the TX-100–soluble fraction (Fig. 4 *B*, *0 min*, *S*). The immunoprecipitated complex also contains [^{35}S]methionine-labeled β-catenin, a trace amount of plakoglobin, but no E-cadherin. We did not detect labeled β-catenin or plakoglobin in the complex after 45 and 10 min, respectively. During the chase, the relative amount of α-catenin in TX-100–insoluble fraction increases from 10% of the total amount at time 0 to 60% at 180 min (Fig. 4 *B*, *0–180 min*, *P*).

β-Catenin. Immunoprecipitation of the cross-linked cell extracts at time 0 with β-cat antibody reveals 60% of [^{35}S]-methionine-labeled β-catenin and a small amount of plakoglobin in the TX-100–soluble fraction (Fig. 4 *C*, *0 min*, *S*). At 45 min, E-cadherin is also detected in the TX-100–soluble fraction in the β-catenin immunoprecipitate. However, the detection of [^{35}S]methionine-labeled E-cadherin and plakoglobin is transient; by 90 min of chase, neither protein is present (Fig. 4 *C*, *90 min*, *S*). At early time points, a diffuse protein band that migrated slower than β-catenin was detected. It resolved into a discrete band at 45 min that has an electrophoretic mobility similar to that of α-catenin, which is clearly present in both the TX-100–soluble and –insoluble fractions at this time (Fig. 4 *C*, *45 min*, *S* and *P*). The relative amount of α-catenin in the β-catenin immunoprecipitates from both the TX-100–soluble and –insoluble fractions remains approximately constant. 40% of β-catenin is present in the TX-100–insoluble fraction at time 0 (Fig. 4 *C*, *0 min*, *P*). The relative amount of β-catenin in TX-100–insoluble fraction increases from 44% at 45 min to 88% at 180 min.

Plakoglobin. Immunoprecipitation of cross-linked cell extracts at time 0 with PG antibody reveals that 60% of [^{35}S]methionine-labeled plakoglobin is present in the TX-100–soluble fraction (Fig. 4 *D*, *0 min*, *S*). Small amounts of newly synthesized α- and β-catenin are also present in this fraction, but little or no α-catenin or β-catenin are present in the TX-100–insoluble fraction at this time (Fig. 4 *D*, *0 min*, *P*). After a 10-min chase, increased amounts of α- and β-catenin are detected in both the TX-100–soluble and –insoluble fractions (Fig. 4 *D*, *10 min*, *S* and *P*). After a 45-min chase, [^{35}S]methionine-labeled β-catenin is no longer detected in either the TX-100–soluble or –insoluble fractions (Fig. 4 *D*, *45 min*, *S*, and *P*). [^{35}S]methionine-labeled E-cadherin is detected in the TX-100–soluble complex at 45 min, but by 90 min of chase, it is no longer detected in the complex (Fig. 4 *D*, *45–90 min*, *S*). The relative amount of α-catenin in the plakoglobin immunoprecipitates from the TX-100–soluble and –insoluble fractions remains approximately constant. During the chase, the relative amount of plakoglobin in TX-100–insoluble fraction increases from 38% at time 0 to 70% at 90 and 180 min (Fig. 4 *D*, *0–180 min*, *P*).

Synopsis. The analysis of cadherin/catenin complex assembly using chemical cross-linking has revealed novel aspects of the assembly pathway. First, protein complexes formed between [^{35}S]methionine-labeled and unlabeled proteins (i.e., synthesized before or after the pulse of [^{35}S]methionine) can form (Fig. 4 *A*, *45 min*, *S* and *P*), indicating that assembly of the E-cadherin/catenin complex is not synchronous although the turnover rate of each protein is similar (data not shown). It is possible that [^{35}S]methionine-labeled α- and β-catenin exchange from [^{35}S]methionine labeled to unlabeled E-cadherin. Alternatively, [^{35}S]methionine-labeled

Figure 4. Synthesis and assembly of the cross-linked E-cadherin/catenin complexes immunoprecipitated with E-cad, α-cat, β-cat, and PG antibodies from the TX-100–soluble and –insoluble fractions of MDCK cells. MDCK cell cultures, grown for 8 d at confluent density, were pulse labeled with [^{35}S]methionine for 15 min (0) and chased in the absence of [^{35}S]methionine for 10, 45, 90, and 180 min. At each time point, the cultures were subjected to chemical cross-linking before preparation of cell extracts. Equivalent aliquots of the TX-100–soluble (*S*) and –insoluble fractions (*P*) were immunoprecipitated with E-cad (*A*), α-cat (*B*), β-cat (*C*), and PG (*D*) antibody. The immunoprecipitates were subjected to 7.5% SDS-PAGE followed by fluorography. The relative proportion of each protein in the TX-100–soluble and –insoluble fractions are presented in graph form.

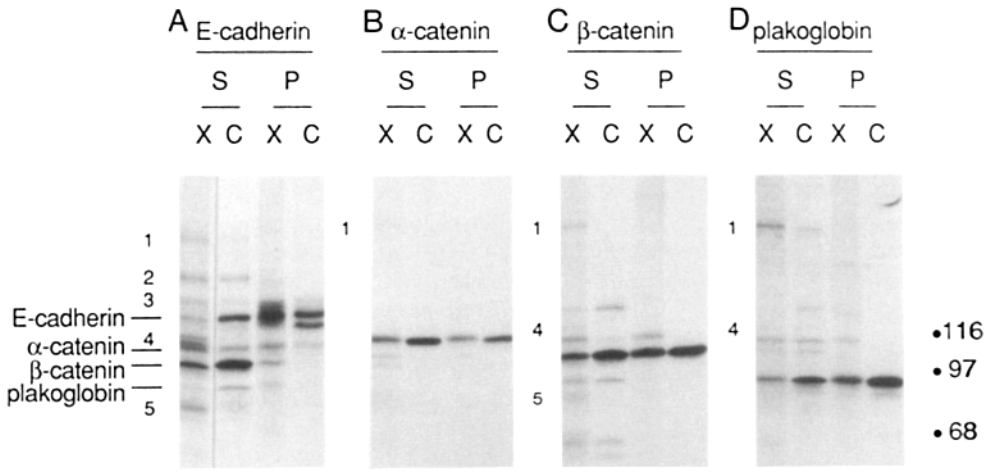


Figure 5. Comparison of chemically cross-linked and control cadherin/catenin complexes isolated from the TX-100–soluble and –insoluble fractions of MDCK cells. Replicate MDCK cell cultures, grown for 8 d at confluent density, were labeled with [^{35}S]methionine for 20 h. At each time point, cell cultures were subjected either to chemical cross-linking (*X*) or to control treatment (*C*), extracts were prepared and equivalent aliquots of the TX-100–soluble (*S*) and –insoluble fractions (*P*) were immunoprecipitated with E-cad, α-cat, β-cat, and PG antibodies. The immunoprecipitates were subjected to 7.5% SDS-PAGE followed by fluorography. E-cadherin, α-catenin, β-catenin, and plakoglobin protein bands are indicated. Unknown protein bands are also indicated: band *1*, p180; band *2*, p150; band *3*, p135 (E-cadherin precursor); band *4*, p110; band *5*, p70. The length of time the autoradiogram was exposed to x-ray film depended on the immunoprecipitating antibody: E-cad, 28 h; α-cat and PG, 14 h; and β-cat, 8 h.

α- and β-catenin may bind unlabeled E-cadherin during the initial assembly of the complex.

Second, these results indicate that portions of total β-catenin and plakoglobin exist independent of an association with E-cadherin. At time 0, [^{35}S]methionine-labeled E-cadherin and catenins are not immunoprecipitated with the E-cadherin antibody from the TX-100–insoluble fraction (Fig. 4 *A*, *0 min*, *P*). In contrast, when the same extracts are immunoprecipitated with β-cat or PG antibodies, significant proportions of [^{35}S]methionine-labeled β-catenin and plakoglobin are detected in the TX-100–insoluble fraction (Fig. 4, *C* and *D*, *0 min*, *P*). As suggested above, this may indicate that [^{35}S]methionine-labeled catenins are titrated into the TX-100–insoluble fraction, where they bind unlabeled E-cadherin. However, it is important to note that there is little E-cadherin present in β-catenin or plakoglobin immunoprecipitates from the TX-100–insoluble fraction of cells labeled to steady-state and cross-linked (see Fig. 5, *C* and *D*, *P*, *X*; see also Näthke et al., 1994). Therefore, these results signify the presence of E-cadherin–independent pools of β-catenin and plakoglobin. These pools of catenins may play a role in the exchange or nonsynchronous assembly of proteins in the cadherin/catenin complex (see Fig. 6).

Third, these experiments give insight into the potential for heterodimerization between the two *armadillo* family members. Immunoprecipitation of the cell extracts with plakoglobin antibody reveals a complex containing newly synthesized plakoglobin, β-catenin, and α-catenin present in both the Triton X-100–soluble and –insoluble fractions at time 0 and 10 min (Fig. 4 *D*, *0–10 min*, *S* and *P*). However, this complex is transient; by 45 min, newly synthesized β-catenin is no longer detected. Similar data are presented in the β-catenin immunoprecipitate (Fig. 4 *C*). The β-catenin/plakoglobin complex is unstable; it is only detected in chemically cross-linked cells. We demonstrated in Fig. 1 that only complexes containing either β-catenin or plakoglobin accumulate at steady state. Taken together, our results show that complexes containing β-catenin and plakoglobin may form. However, these complexes are transient and unstable. They do not contain E-cadherin and they do not accumulate at steady state.

Fourth, the 45-min chase period is a critical point in the assembly of the E-cadherin/catenin complex. At this time, labeled α-catenin enters the E-cadherin/β-catenin or E-cadherin/plakoglobin complex, and labeled E-cadherin begins to titrate into the TX-100–insoluble fraction (Fig. 4 *A*, *45 min*, *S* and *P*). This time is coincident with the time required for newly synthesized E-cadherin to reach the plasma membrane (Shore and Nelson, 1991).

Comparison of Cadherin/Catenin Complexes Isolated from Cross-linked and Control MDCK Cells

E-cadherin–independent pools of catenins could be present in preassembled catenin complexes, associated with other as yet unidentified proteins or unassociated in free pools. To further investigate proteins coimmunoprecipitated with the cadherin/catenin complexes, we labeled cells to steady state with [^{35}S]methionine and examined both the Triton X-100–soluble and –insoluble fractions. Confluent monolayers of MDCK cells were established on Transwell filters and grown for 8 d. Cells were labeled with [^{35}S]methionine for 20 h; since the average half-life of the proteins is ~4 h (data not shown), >96% of proteins in the cadherin/catenin complex should be labeled. After labeling, cells were incubated in buffer containing the reversible cross-linking reagent DSP for 20 min at room temperature (Fig. 5). Control reactions were performed in a parallel set of cultures using identical buffer without DSP (control). Cell extracts were prepared, and equal aliquots of each extract were immunoprecipitated with E-cad, α-cat, β-cat, or PG antibodies as described previously.

Fig. 5 shows proteins in complexes that had been cross-linked and then sequentially extracted from cells with 0.5% TX-100 (*TX-100 soluble*, *S*) and then 1% SDS (*TX-100 insoluble*, *P*); control samples (*C*) are shown next to cross-linked samples (*X*). In addition to previously identified components

of the cadherin/catenin complex, we identified polypeptides p180 (*1*), p150 (*2*), p135 (*3*), p110 (*4*), and p70 (*5*) that are cross-linked to E-cadherin and/or one of the catenins; p135 is the E-cadherin precursor (data not shown). These proteins bind the cadherin/catenin complex specifically and are not precipitated with preimmune sera (data not shown).

E-Cadherin. Immunoprecipitation of cross-linked, TX-100–solubilized proteins with E-cadherin antibody reveals the following proteins: p180 (*1*), p150 (2), p135 (*3*), p110 (*4*), and p70 (*5*), the mature form of E-cadherin, α-catenin (the protein band appears as a doublet), β-catenin, and plakoglobin (Fig. 5 *A*, *S*, *X*). Of these proteins, there is little or no p180, p135, p110, or p70 present in the control, TX-100–soluble extract (Fig. 5 *A*, S, C). Comparison of the molecular ratios of E-cadherin/α-catenin/β-catenin in the TX-100–soluble fraction between cross-linked (1:0.7:0.6) and control extracts (1:0.1:0.5) indicates that α-catenin is removed from the cadherin/catenin complexes that are not cross-linked (*C*) under the stringent wash conditions used during immunoprecipitation (McCrea and Gumbiner, 1991). Immunoprecipitation of the cross-linked, TX-100–insoluble fraction with E-cadherin antibodies also reveals a complex of proteins: E-cadherin, α-catenin, and β-catenin (Fig. 5 *A*, *P*, *X*). Of these proteins, only the intermediate and mature forms of E-cadherin are immunoprecipitated from the control, TX-100–insoluble extract (Fig. 5 *A*, *P*, *C*).

α-Catenin. Immunoprecipitation of the cross-linked, TX-100–soluble extract with the α-cat antibody reveals the following proteins: p180 (*1*), α-catenin and β-catenin (Fig. 5 *B*, *S*, *X*); only α-catenin is present in the control, TX-100–soluble fraction (Fig. 5 *B*, *S*, *C*). The molecular α-catenin/β-catenin ratio was not calculated because of the large excess of α-catenin. Immunoprecipitation of either the cross-linked or control TX-100–insoluble cell extracts with α-cat antibody shows that only α-catenin is immunoprecipitated (Fig. 5 *B*, *P*, *X* and *C*). The electrophoretic mobility of α-catenin is slightly slower in the cross-linked extracts compared to the control cell extracts.

β-Catenin. Immunoprecipitation of the cross-linked, TX-100–soluble extract with the β-cat antibody reveals the following proteins: p180 (*1*), p110 (*4*), p70 (*5*), E-cadherin, α-catenin, β-catenin, and plakoglobin (Fig. 5 *C*, *S*, *X*). Of these proteins, E-cadherin, β-catenin, plakoglobin, and a trace amount of α-catenin are immunoprecipitated with the β-cat antibody from the control, TX-100–soluble extract (Fig. 5 *C*, *S*, *C*). Immunoprecipitation of the cross-linked, TX-100–insoluble fraction shows that α-catenin and β-catenin are present (Fig. 5 *C*, *P*, *X*); β-catenin is immunoprecipitated from the control, TX-100–insoluble fraction (Fig. 5 *C*, *P*, *C*). Note that the small amount of plakoglobin detected in the β-cat immunoprecipitate from the TX-100–soluble fraction was discussed in the synopsis to Fig. 4.

Plakoglobin. Immunoprecipitation of the cross-linked, TX-100–soluble extract with the PG antibody reveals the following proteins: p180 (*1*), p110, α-catenin, p96, and plakoglobin (Fig. 5 *D*, *S*, *X*). The 96-kD protein binds nonspecifically to the antibody; it is not blocked when the antibody is preabsorbed with cognate peptide (data not shown). In addition to these proteins, labeled E-cadherin is also coimmunoprecipitated from the control extract (Fig. 5 *D*, *S*, *C*), although it is not immunoprecipitated in the cross-linked complex; the reason for this is unknown. A complex of proteins containing α-catenin and plakoglobin is immunoprecipitated with PG antibody from the cross-linked, TX-100–insoluble fraction of this extract (Fig. 5 *D*, *P*, *X*); only plakoglobin is immunoprecipitated from the control, TX-100–insoluble fraction (Fig. 5 *D*, *P*, *C*). The predominant protein identified in these immunoprecipitates is plakoglobin, and the remaining proteins are minor components.

Synopsis. Labeling cells to steady state followed by chemically cross-linking cells before extraction allows us to draw conclusions about the protein composition of the cadherin/catenin complex in both the TX-100–soluble and –insoluble fractions.

This cross-linking experiment demonstrates, for the first time, the presence of several different cadherin and catenin complexes in the TX-100–insoluble fraction: E-cadherin, α-catenin, and β-catenin (Fig. 5 *A*, *P*, *X*); α-catenin and β-catenin (Fig. 5 *C*, *P*, *X*); and, α-catenin and plakoglobin (Fig. 5 *D*, *P*, *X*). The identification of these cadherin and catenin complexes in the TX-100–insoluble fraction is important. First, formation of stable cell–cell contacts results in the appearance of a pool of E-cadherin at the contact site, which is TX-100–insoluble (McNeill et al., 1993). Second, immunofluorescence studies of E-cadherin distributions in polarized monolayers of cells revealed that a significant pool of protein is located on the lateral (cell–cell contact) membranes and is TX-100–insoluble (Shore and Nelson, 1991; Näthke et al., 1994). Third, loss of catenin binding to the cytoplasmic domain of cadherin inhibits formation of the TX-100–insoluble pool of these proteins (Nagafuchi and Takeichi, 1988).

In a study by Ozawa and Kemler (1992), the E-cadherin/α-catenin/β-catenin ratio was reported to be 1:3:1.5; based on the methionine content of each protein; this translates into a molecular ratio of 1:1:0.5. In our studies, we confirmed that the molecular ratio of E-cadherin:β-catenin is 1:0.5 in the TX-100–soluble fraction in the presence or absence of chemical crosslinking (Fig. 5 *A*, *S*, *X* and *C*). This ratio may indicate that a single β-catenin binds two cadherins simultaneously, or it may reflect the presence of a pool of E-cadherin not bound to β-catenin. Some of the E-cadherin may be associated with plakoglobin, but our results indicate that this amount is very small and cannot make up the difference in the E-cadherin/β-catenin ratio. The possibility that E-cadherin is not bound to β-catenin is supported by morphological evidence that is presented in the accompanying paper and shows that the subcellular distributions of E-cadherin and β-catenin do not overlap in some sites (Näthke et al., 1994). A pool of cadherin that is not bound to catenins may not be functional, or it may provide only weak adhesivity since catenin binding is important for linkage of cadherin to the cytoskeleton (Hirano et al., 1987; Nagafuchi and Takeichi, 1988; Ozawa et al., 1990; Ozawa and Kemler, 1992).

Chemical cross-linking preserved interactions between E-cadherin and α-catenin in the TX-100–soluble fraction, resulting in a greater amount of α-catenin immunoprecipitating with E-cad antibody from the cross-linked (1:0.7) (Fig. 5 *A*, *S*, *X*) compared to the control, non–cross-linked TX-100–soluble fraction (1:0.1) (Fig. 5 *A*, *S*, *C*). Since α-catenin appears to bind E-cadherin through its association with β-catenin (Jou, T.-Z., and W. J. Nelson, unpublished result), the reported ratio of 1:1:0.5 for E-cadherin/α-catenin/β-catenin (Ozawa and Kemler, 1992) could be interpreted as evi-

dence for the binding of two α-catenins to each β-catenin. In our investigation, the stoichiometry of E-cadherin/α-catenin/β-catenin is 1:0.7:0.5. One interpretation of this ratio is that one α-catenin binds one β-catenin; the extra α-catenin in the complex may bind plakoglobin, which we did not quantitate for technical reasons (see Fig. 4). Alternatively, there may be different populations of β-catenin that bind either one or two α-catenins, depending on subcellular localization and function. The precise interactions and stoichiometry between proteins of the complex is unknown and under investigation.

This cross-linking experiment also provides another strong indication that a portion of β-catenin exists independent of an association with E-cadherin. The molecular ratio of E-cadherin/β-catenin in a β-cat immunoprecipitate of the TX-100–soluble fraction is 1:6 in the cross-linked and 1:2 in the control fraction (Fig. 5 *C*, *S*, *X*, and *C*). This is compared to an E-cadherin immunoprecipitate where the molecular ratio of E-cadherin/β-catenin in the cross-linked and control, TX-100–soluble fractions is 1: ~0.5 (Fig. 5 *A*, *S*, *X*, and *C*). This demonstrates that in the TX-100–soluble fraction more β-catenin is immunoprecipitated with β-cat antibody than is coimmunoprecipitated with E-cad antibody. Therefore, more β-catenin is present in the extract than can be accounted for in the E-cadherin immunoprecipitate. Since proteins were labeled to steady state, we can exclude the possibility that complexes contain both labeled and unlabeled proteins. When the total amount of α- and β-catenin immunoprecipitated with their respective antibodies are compared to the amount of these proteins coimmunoprecipitated with E-cadherin, we calculate that as much as 50% of these catenins are not directly associated with E-cadherin (note that the exposure time of the E-cadherin autoradiogram in Fig. 5 is two and four times longer than those for α- and β-catenin, respectively).

We identified at least five proteins that are cross-linked to the E-cadherin dependent and/or independent pools of catenins. Some of these proteins are also coimmunoprecipitated when cells are not cross-linked, but cross-linking increases the amount in the complex presumably by preserving their interaction with the E-cadherin/catenin complex. The identity of these proteins is unknown and will be the focus of future studies.

Conclusion: A New Model of Cadherin/Catenin Complex Assembly

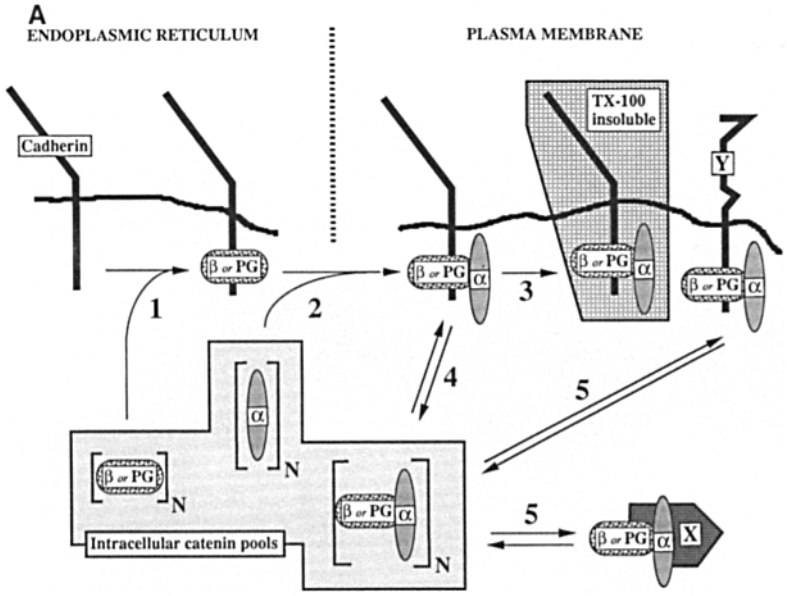
Previous studies of the assembly and protein composition of the cadherin/catenin complex suggested that assembly of the complex was coordinated with the synthesis of the proteins, and that a single complex existed that contains each of the catenins bound to a cadherin (Ozawa et al., 1989; McCrea and Gumbiner, 1991; Shore and Nelson, 1991; Takeichi, 1991; Kemler, 1992; Ozawa and Kemler, 1992; Grunwald, 1993). Our present studies have benefited from the use of antibodies specific to each of the proteins of the cadherin/catenin complex, and from the use of chemical cross-linking to examine the assembly and composition of the cadherin/catenin complex in the TX-100–insoluble fraction. A primary advantage has been the ability to analyze the catenins independently of the requirement to coimmunoprecipitate them with cadherin. The results of this study have revealed that more than one E-cadherin/catenin complex is assembled; assembly of the complexes in both the TX-100–soluble and –insoluble fractions is dynamic; cadherin-independent pools of catenins are present; and different pools of either cadherin/catenins or catenin complexes are exchanged. Each of these points and the supporting experimental evidence were discussed above in the synopsis of each section of the results. Based on these new insights, we propose a model of the assembly pathway of protein complexes involving catenins (Fig. 6 *A*; Fig. 6 *B* provides cross-reference for specific assembly steps and the supporting data).

We suggest that the critical components of the cadherin-catenin assembly pathway(s) are pools of α-catenin, β-catenin, and plakoglobin that are in excess of the pool of cadherin. Our analysis indicates that ≥50% of α- and β-catenin and plakoglobin exist in cadherin-independent pools of protein. Our results indicate that the catenin pools are composed of either "free" pools of each protein, or complexes of α-catenin/β-catenin and α-catenin/plakoglobin. These pools were detected in cells before cell–cell contact and in cells with extensive cell–cell contact. Therefore, we suggest that these catenin pools are not present simply for de novo assembly of junctions, but rather that they play a critical role in regulating complex formation with cadherins and other proteins. In the accompanying paper, we show that cadherin-independent pools of catenins are localized in both the lateral membrane and throughout the cell, and we directly demonstrate the existence of E-cadherin independent α-catenin/β-catenin and α-catenin/plakoglobin complexes (Näthke et al., 1994).

The first step in assembly of the cadherin/catenin complex is the assembly of either β-catenin or plakoglobin with newly synthesized cadherin (Fig. 6, step *1*: see data in Figs. 1 and 5). The second assembly step involves incorporation of α-catenin into the E-cadherin/β-catenin or E-cadherin/plakoglobin complex and this occurs concomitantly with the time of arrival of the complex at the plasma membrane (Fig. 6, step *2*: see data in Figs. 2–4). At present, we do not know the mechanism involved in the temporal regulation of α-catenin assembly into the cadherin complex. We suggest that α-catenin assembly at this time may ensure that cadherin is only functional at the plasma membrane. The third step in complex assembly is the titration from the TX-100–soluble to the TX-100–insoluble fraction (Fig. 6, step *3*: see data in Figs. 4 and 5). For cadherin, this occurs concomitantly with arrival at the plasma membrane and the loading of α-catenin (Fig. 4 *A*). At present, we do not know the mechanism(s) involved in the appearance of the cadherin/catenin complex in the TX-100–insoluble fraction. One possibility is that the complex becomes incorporated into the actin-based cytoskeleton, coincident with the association of α-catenin. It is noteworthy that α-catenin has homology to vinculin and protein 4.1 (Herrenknecht et al., 1991; Nagafuchi et al., 1991), both of which are known to mediate the interaction of membrane proteins with the actin cytoskeleton (Burridge et al., 1988; Bennett, 1990).

Once assembly of the cadherin/α-catenin/β-catenin or cadherin/α-catenin/plakoglobin complex is completed at the plasma membrane, we suggest that there is exchange of α-catenin, β-catenin, and plakoglobin proteins between the pool bound to cadherin and a cadherin-independent pool in the cytosol. It is possible that exchange occurs between pools of individual proteins, or between "preassembled" α-cate-

A



B

Step	Process	*Figure*/Experiment
1	Mutually exclusive association of β-catenin or plakoglobin with E-cadherin	*Fig. 1*: immunoprecipitation, immunoblot *Fig. 5*: steady state label, cross-link
2	Association of α-catenin with cadherin/catenin complexes concommitant with the arrival of E-cadherin at the cell surface	*Fig. 2 & 3*: pulse chase, sucrose gradient *Fig. 4*: pulse chase, cross-link
3	TX-100 insolubility of cadherin/catenin complexes	*Fig. 4*: pulse chase, cross-link *Fig. 5*: steady state label, cross-link
4	Presence of free pools of α- and β-catenin	*Fig. 2 & 3*: pulse chase sucrose gradient *Fig. 5*: steady state label cross-link (stoichiometric anlysis)
	Exchange between free pools of α-/β-catenin complexes with E-cadherin/catenin complexes	*Fig. 4*: steady state label, cross-link
5	Association of α-catenin, β-catenin or plakoglobin with membrane and/or cytosolic proteins other than E-cadherin	*Fig. 5*: steady state label, cross-link

Figure 6. Model of cadherin/catenin complex assembly. (*A*) Cadherin/catenin complexes are assembled by first loading either β-catenin or plakoglobin onto the newly synthesized cadherin (step *1*). α-Catenin (α) is loaded concomitant with the time of arrival of the cadherin/β-catenin or cadherin/plakoglobin complex at the plasma membrane (step *2*). The complex is titrated from the TX-100–soluble to –insoluble (*stippled box*) fraction (step *3*). There is exchange between the E-cadherin bound pools of the α-catenin, β-catenin, and plakoglobin with "free" pools of individual proteins, or with preassembled α-catenin/β-catenin or α-catenin/plakoglobin complexes (step *4*). The cadherin independent pool of catenin can also bind to other proteins on the membrane (protein Y) (step *5*) and in the cytosol (protein X) (step *5*). (*B*) Cross-referencing between putative steps in the assembly pathway of cadherin/catenin complexes and the supporting data derived from individual figures.

nin/β-catenin and α-catenin/plakoglobin complexes (Fig. 6, step *4*: see data in Fig. 4). This is supported by the fact that newly synthesized catenins bind to a pool of cadherin that was not synthesized at the same time (Fig. 4 *A*). In addition, our studies of cadherin/catenin complex assembly in other cell types shows that following initial binding to cadherin, β-catenin can subsequently dissociate from the complex (Hinck et al., 1994).

We suggest that a critical aspect of the assembly process is the regulation of the size of the catenin pools. Our previous results showed that Wnt-1 may play a role in regulating β-catenin but not α-catenin pool size in mammalian cells. Wnt-1, the product of a protooncogene, is a secreted glycoprotein that functions in regulating cell growth and adhesion (Nusse and Varmus, 1992). We demonstrated that the expression of Wnt-1 increased the size of the pools of β-catenin and plakoglobin, resulting in stabilized assembly of β-catenin with cadherin (Hinck et al., 1994). One explanation of this result is that the increased size of the catenin pool shifts the equilibrium between the bound and "free" pools of β-catenin towards assembled β-catenin/cadherin complexes. The mechanism of Wnt-1 regulation of catenin pool size is unknown, but preliminary results show that it occurs at a posttranscriptional level. One possibility is that β-catenin and plakoglobin stability is regulated by phosphorylation. Wnt-1 expression, however, had little or no effect on the size of the α-catenin pool (Hinck et al., 1994), indicating that other factors regulate the size of this pool independently of the β-catenin and plakoglobin pools.

There is increasing evidence that catenins bind to other proteins in addition to cadherins (Fig. 6, step *5*, see data in Fig. 5). In our cross-linking experiments, we showed at least five additional proteins were incorporated into the cadherin/catenin or catenin complexes (Fig. 5). Although the identification and characterization of these proteins is under investigation, there is evidence that they may include other members of the cadherin superfamily in the desmosome (Korman et al., 1989), the EGF receptor (Hinck, L., and I. S. Näthke, unpublished result), and the product of the APC gene, a protein that contributes to the progression of colorectal tumorigenesis (Rubinfeld et al., 1993; Su et al., 1993; Troxell, M., and W. J. Nelson, unpublished result).

Catenins bind to cadherins and to other proteins, some of which play roles in the regulation of cell proliferation (see above). We suggest that control of the equilibrium between "free" and bound pools of catenins could play a critical role in regulating cellular responses to extracellular signals for cell–cell adhesion and cell proliferation. We propose that a shift in the equilibrium towards assembly of catenins with cadherins could increase cell–cell adhesion. In contrast, a shift in the equilibrium towards assembly of catenins with growth factor receptors could facilitate the transduction of signals for cell proliferation. If these shifts in the equilibrium of catenin binding are coupled to each other, then upregulation of one of the responses could result in a commensurate downregulation of the other response. Thus, the balance between adhesion and proliferation will determine whether the cell maintains cell contacts, or whether it loses cell contacts and divides.

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