Journal of Cell Science 104, 751-762 (1993)
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Defining E-cadherin-associated protein complexes in epithelial cells: plakoglobin, β- and γ-catenin are distinct components

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SUMMARY

Ca^{2+}-dependent cell adhesion is mediated by a family of proteins termed cadherins, and is modulated by cytosolic proteins that include α-, β-, and γ-catenin and other cytoskeletal proteins that bind to the cytoplasmic domain of cadherins. Recent studies have suggested that either β- or γ-catenin may be identical to plakoglobin, a protein associated with *adherens* junctions. However, the relationship between these proteins, and their interaction with cadherins, are not well understood. In this study, we have further defined the relationship between plakoglobin and the catenins in complexes with E-cadherin in Madin-Darby canine kidney (MDCK) cells. Specific immunoprecipitations revealed that plakoglobin (86 kDa) and β-catenin (92 kDa) have different detergent extractabilities and apparent molecular weights in these cells; however, plakoglobin has an apparent molecular weight similar to that of γ-catenin (86 kDa). Immunoblotting of E-cadherin immunoprecipitates demonstrated that both plakoglobin and β-catenin co-immunoprecipitate with E-cadherin. Laser-scanning confocal microscopy demonstrated temporally and spatially co-ordinate redistribution of plakoglobin and E-cadherin following induction of cell-cell contact in MDCK cells. Although plakoglobin comigrated with γ-catenin on SDS-PAGE, quantitative analysis of E-cadherin and plakoglobin immunoprecipitates revealed that plakoglobin accounted for <50% of the γ-catenin signal. Two-dimensional gel electrophoresis resolved the γ-catenin protein band into two proteins. One protein was identified as plakoglobin, based upon apparent molecular weight, immunoreactivity and isoelectric point (pI ~6.1). The other protein comigrated with γ-catenin on SDS-PAGE, did not react with plakoglobin antibodies and had a pI of ~4.25; we refer to this protein as γ-catenin to distinguish it from plakoglobin. Two-dimensional gel electrophoresis further revealed that plakoglobin comprised multiple isoelectric variants, but that, within the newly synthesized pool of plakoglobin, only the most basic of these variants co-immunoprecipitated with E-cadherin; phosphorylation did not account for the plakoglobin isoelectric variants seen by two-dimensional gel electrophoresis. These results demonstrate directly that plakoglobin associates and co-localizes with the E-cadherin in MDCK epithelial cells in a complex that contains α-, β-, and γ-catenin. Although plakoglobin shares sequence similarity with β-catenin, and co-migrates with γ-catenin in SDS-PAGE, plakoglobin is distinct from the catenins. The asociation of plakoglobin with E-cadherin may be regulated by post-translational modifications of plakoglobin.

Key words: epithelial cells, cell adhesion, cadherin, catenin, plakoglobin

INTRODUCTION

Cell adhesion is vitally important during development and in the adult organism, being necessary in sorting of cells, induction of cellular morphogenesis and maintenance of tissue integrity (reviewed by Edelman and Crossin, 1991; Takeichi, 1991). The cadherins are a family of Ca^{2+}-dependent cell adhesion molecules that are involved in the formation and maintenance of cell-cell contacts (Kemler, 1992; Takeichi, 1991). Cadherin-mediated cell adhesion requires homotypic interactions between cadherin molecules on adjacent cells (Nagafuchi et al., 1987). There is increasing evidence that cadherin function is modulated by cytosolic proteins that bind to the C terminus of cadherins (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989, 1990). First, the amino acid sequence of the cytoplasmic domain of all cadherins is highly conserved (Hirano et al., 1987), and this domain associates with distinct cytosolic proteins, termed catenins (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989, 1990). Second, deletion of this catenin-binding domain generates cadherins that cannot mediate cell-cell adhesion (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990). Third, overexpression of the cadherin cytoplasmic domain acts as a dominant negative mutation that competes with endogenous cadherin for catenins and generates an adhesion-defective phenotype (Kintner, 1992).

Although the catenins are important for cadherin-mediated cell-cell adhesion, their functions are presently

unknown. Three catenins were originally identified that coimmunoprecipitated with E-cadherin (Ozawa et al., 1989): α-catenin (102-105 kDa), β-catenin (92-97 kDa), and γ-catenin (82-86 kDa). Catenins are co-immunoprecipitated with cadherins in all tissues and developmental stages examined, although the reports of their relative abundance and strength of interaction with E-cadherin have varied (Kemler, 1992). In their initial characterization, Ozawa et al. (1989) demonstrated that β- and γ-catenin could be preferentially removed from E-cadherin immunoprecipitates in buffers of pH 3.0. Recently, McCrea et al. (1991) reported that α- and γ-catenin could be selectively removed from E-cadherin immunoprecipitates in buffers containing 1 M NaCl. Taken together, these results indicate that α- and β-catenin bind to E-cadherin independently, while γ-catenin may bind through one of the other two catenins.

The recent cloning of α-catenin (Herrenknecht et al., 1991; Nagafuchi et al., 1991) and β-catenin (McCrea et al., 1991) has elucidated their identities and has suggested a function for α-catenin. Within its conserved domains, α-catenin shows 30% identity to vinculin, a protein found predominantly in focal cell-cell and cell-substratum adhesions and thought to be involved in linking membrane proteins to the cortical cytoskeleton (Burridge et al., 1988; Geiger, 1979; Geiger et al., 1980). By analogy, α-catenin may link cadherins to the cortical cytoskeleton, which is consistent with the previous findings that E-cadherin co-localizes with actin filaments (Hirano et al., 1987) and associates with components of the membrane-cytoskeleton, ankyrin and fodrin (Itoh et al., 1991; Nelson et al., 1990). β-catenin was cloned from *Xenopus* and shown to have ~60% amino acid identity to human plakoglobin (McCrea et al., 1991), a desmosomal component which has also been shown to co-localize with other *adherens* junctions (Cowin et al., 1986). β-catenin also exhibits ~65% identity to *Drosophila* armadillo protein (McCrea et al., 1991), product of a segment polarity gene (Peifer and Wiechaus, 1990). Based upon the amino acid sequence similarity and apparent co-migration in SDS-PAGE, McCrea et al. (1991) postulated that plakoglobin and β-catenin were the same protein. While our manuscript was in preparation, however, Butz et al. (1992), Peifer et al. (1992) and Knudson and Wheelock (1992) reported evidence that these two proteins are distinct. In those studies, plakoglobin and γ-catenin were found to have similar apparent molecular sizes, leading both Knudson and Wheelock (1992) and Peifer et al. (1992) to speculate that plakoglobin and γ-catenin are the same protein.

The importance of these cadherin-associated proteins in modulating cell-cell adhesion, and the poor understanding as to their identities relative to one another, led us to further define the protein composition of the E-cadherin complex. Here, we have used immunoprecipitation and immunoblotting with antibodies specific to plakoglobin, α-catenin, β-catenin and E-cadherin to demonstrate that plakoglobin and β-catenin are distinct proteins which co-immunoprecipitate and co-localize with E-cadherin in MDCK epithelial cells. Quantitation of sequential immunoprecipitates of protein complexes with E-cadherin and plakoglobin antisera demonstrates that although plakoglobin comigrates with γ-catenin in SDS-PAGE, plakoglobin alone cannot account for the γ-catenin protein band. We have confirmed this by performing two-dimensional gel electrophoresis of E-cadherin immunoprecipitates. This resolves several protein spots in the isoelectric focusing dimension that co-migrate with the γ-catenin protein band in the SDS-PAGE dimension. One set of proteins consists of plakoglobin isoelectric variants, based upon immunoreactivity and isoelectric point (pI 6.0-6.15). The other protein does not react with plakoglobin antibodies and has a pI of ~4.25; we refer to this protein as γ-catenin to distinguish it from plakoglobin. Phosphatase analysis demonstrates that the plakoglobin isoelectric variants are phosphorylated but that phosphorylation probably does not give rise to the heterogeneity in isoelectric point. These data demonstrate that plakoglobin and α-, β-, and γ-catenin are all distinct E-cadherin-associated proteins and that plakoglobin is a phosphoprotein which exists as multiple isoelectric variants within cells.

MATERIALS AND METHODS

Cell culture and metabolic labeling

Madin-Darby canine kidney cells were cultured and maintained as described previously (Nelson and Veshnock, 1986). For all experiments where cell-cell contact was synchronously induced, cells were rendered contact-naive. Briefly, confluent cultures of MDCK cells were subcultured by trypsinization with a solution containing 0.04% trypsin, 3 mM EDTA, and then replated at a density of ~1×10^5 cells/cm^2. 24 h later, cells were again trypsinized and replated at a density of ~1.5×10^4 cells/cm^2. After a further 24 h, cells were trypsinized and combined. Trypsin in the cell suspension was neutralized by adding 1/5 volume of low Ca^{2+} medium (LCM) containing DMEM supplemented with 5 μM Ca^{2+} and 10% FBS; FBS was extensively dialyzed against a solution of 10 mM Tris-HCl, pH 7.5, 120 mM NaCl. The cell suspensions were then centrifuged at 569 ***g*** for 5 min, resuspended in LCM to form a single-cell suspension, and replated at a density of ~3×10^5 cell/cm^2 onto collagen-coated, 35 mm plastic Petri dishes (Falcon). Rat tail collagen was prepared as previously described (Noda, 1960). After 4 h the cells adhered to the Petri dishes. LCM was aspirated and replaced with DMEM containing 1.8 mM Ca^{2+} and 10% undialyzed FBS to induce cell-cell contacts through the Ca^{2+}-dependent cell adhesion protein, E-cadherin. To metabolically label cells, LCM was replaced with DMEM lacking methionine and supplemented with 1.8 mM Ca^{2+} and 10% dialyzed FBS. Cells were preincubated for 30 min, and then incubated in fresh medium containing 100 μCi [^{35}S]methionine/cysteine (New England Nuclear; 8.81 μCi/μg).

Protein extraction

Unless otherwise indicated, both metabolically labeled and unlabeled cultures were processed 4 h after induction of cell-cell contacts Cell cultures were rinsed twice with ice cold PBS, and then 1 ml of ice cold CSK buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl, 0.5% (v/v) Triton X-100, 1.2 mM PMSF, 0.1 mg/ml DNAse I, 0.1 mg/ml RNAse) was added. The cultures were incubated for 10 min at 4˚C. Cells were vigorously scraped from the Petri dish with a rubber policeman, and the resulting suspension of buffer and insoluble cell debris was transferred to a 1.5 ml screw cap Eppendorf tube. The crude extracts were centrifuged at 48,000 ***g*** for 10 min. The supernatants

were transferred to separate Eppendorf tubes (CSK-soluble fraction). The pellets (CSK-insoluble fraction) were solubilized by adding 100 μl of SDS buffer (1% SDS, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), triturating the pellets and then heating the suspensions at 100˚C for 10 min. The SDS concentration of the resulting solutions was lowered to 0.1% by adding 900 μl of CSK buffer. Whole cell lysates were prepared by rinsing cell cultures twice with ice cold PBS, adding 100 μl of SDS buffer and immediately removing the cells from the Petri dishes as described above. The resulting whole cell extracts were transferred to Eppendorf tubes, triturated and boiled at 100˚C for 10 min to solubilize completely all proteins. The SDS concentration of the extracts was then lowered to 0.1% by adding 900 μl of CSK buffer. Both whole cell extracts and CSK buffer-solubilized fractions were flash frozen in liquid N_2 and stored at −70˚C.

Antibodies

The polyclonal antiserum directed against the purified extracellular domain of canine E-cadherin has been previously described (Shore and Nelson, 1991). The polyclonal antiserum directed against the E-cadherin cytoplasmic domain was provided by Dr James Marrs; the antibody was prepared against the cytoplasmic domain of canine E-cadherin expressed in *E. coli* as a fusion protein with glutathione-S-transferase (GST), and will be described in detail elsewhere (Marrs and Nelson, in preparation) The monoclonal anitibody 3G8 was generously provided by Dr Warren Gallin, University of Alberta, Canada. The polyclonal antiserum directed against bovine plakoglobin was raised in rabbits against SDS-denatured plakoglobin isolated from purified bovine desmosomes, and was a gift of Dr Manijeh Pasdar, University of Alberta, Canada. The antipeptide plakoglobin polyclonal antiserum was provided by Lindsay Hinck and Dr Jackie Papkof (Syntex Corp.) and was raised in rabbits against a KLH-conjugated peptide with the sequence CIDTYSDGLRPPYPTADH. The affinity-purified α- and β-catenin antibodies were provided by Drs Kurt Herrenknecht and Rolf Kemler and were raised in rabbits against the sequences HVDPVQALSEFK and PGDSNQLAWFDTDL, respectively.

Immunoprecipitation and immunoblot

All steps in the immunoprecipitation protocol were carried out either on ice or at 4˚C. Extracts used for immunoprecipitation were precleared by addition of 10 μl of nonimmune serum and 30 μl of fixed *Staphlococcus aureus*, followed by incubation for 1 h. Extracts were then centrifuged for 5 min at 14,900 ***g*** and the resulting supernatants were transferred to new tubes. Antiserum was added to the cleared extracts which were then mixed and incubated for 1 h; 30 μl of each of the polyclonal antisera, and 25 μl of each of the monoclonal hybridoma supernatant (ammonium sulfate-cut and concentrated 100 ×), were used per extract. 50 μl of a 1:1 slurry of Protein A-Sepharose 4B (Pharmacia) and high stringency buffer (HSB: 0.1% SDS, 1% deoxycholate, 0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM DTT) was added, and the extracts were incubated on a rocking platform for 1 h. The extracts were then centrifuged for 5 min at 14,900 ***g***. The resulting supernatants were aspirated and discarded. The pellets were resuspended in 800 μl of HSB; this was then underlayered with 130 μl of HSB containing 1 M sucrose. The immunoprecipitates were centrifuged for 5 min at 14,900 ***g***. The supernatants were aspirated and the pellets resuspended in 900 μl of HSB containing 1 M NaCl. The immunoprecipitates were centrifuged for 5 min at 14,900 ***g***. The supernatants were aspirated and the pellets resuspended in 900 μl of low salt wash buffer (2 mM EDTA, 10 mM Tris-HCl, pH 7.5, 0.5 mM DTT). The immunoprecipitates were once again centrifuged at 14,900 ***g***, and the supernatants were discarded.

Sequential immmunoprecipitations of E-cadherin and plakoglobin were conducted by first immunoprecipitating E-cadherin from a CSK-soluble fraction of metabolically labeled MDCK cells, as described above. The resulting E-cadherin immunoprecipitate was dissociated by resuspending the Protein A-Sepharose beads in 100 μl of SDS buffer and incubating on a rocking platform at room temperature for 1 h. The suspension was then centrifuged for 5 min at 14,900 ***g*** and the SDS buffer was transferred to a different tube. The SDS concentration was lowered to 0.1% by adding 900 μl of CSK buffer. Plakoglobin was immunoprecipitated from this buffer solution as described above and subjected to SDS-PAGE.

Immunoprecipitates were resuspended in 80 μl of SDS sample buffer (2% SDS, 40 mM Tris-HCl, pH 6.8, 7.5% glycerol, 50 mM DTT, 0.01% bromophenol blue) and heated at 100˚C for 10 min. Proteins were subjected to SDS-PAGE on 7.5% polyacrylamide gels, according to standard procedures (Laemmli, 1970). Fluorography was conducted by saturating gels with 20% diphenyloxazole in dimethyl sulfoxide, precipitating the diphenyloxazole with deionized water and then drying the gels. All flourograms were preflashed to an absorbance in the linear range and then analyzed using a Molecular Dynamics scanning densitometer.

For immunoblotting, proteins were electroblotted from SDS-polyacrylamide gels to nitrocellulose filters, as previously described (Nelson and Veshnock, 1986). Nitrocellulose filters were fixed in 50% isopropanol for 20 min, stained with 0.1% India ink for 1 h, and blocked overnight at room temperature in a solution containing 3% bovine serum albumin in gelatin wash buffer (150 mM Tris-HCl, pH 7.5, 1.3 M NaCl, 50 mM NaN_3, 10 mM EDTA, pH 7.5, 1% Tween-20, 0.1% gelatin). Nitrocellulose filters were then incubated with antisera diluted in gelatin wash buffer for 2 h at room temperature; polyclonal antisera to E-cadherin extracellular domain, E-cadherin cytoplasmic domain, purified bovine plakoglobin and plakoglobin peptide were all used at dilutions of 1:1000, while the α- and β-catenin antisera were used at dilutions of 1:37 and 1:22, respectively. Nitrocellulose filters were washed twice in gelatin wash buffer for 30 min at room temperature, and then incubated with 1 μCi of ^{125}I-Protein A in 10 ml of gelatin wash buffer for 1 h at room temperature. Finally, the nitrocellulose filters were washed twice in gelatin wash buffer for 30 min at room temperature, allowed to air dry and subjected to autoradiography.

Two-dimensional gel electrophoresis

Isoelectric focusing was performed as described previously (O'Farrell, 1975). Tube gels were prepared in 130 mm × 2.5 mm acid-washed glass tubes which had been sealed at one end with parafilm. A polyacrylamide gel mixture (9 M urea, 3.72% acrylamide, 0.22% bisacrylamide, 2% Nonidet P-40, 1.9% ampholines pH 5-7, 0.86% ampholines pH 4-6, 0.4% ampholines pH 3.5-10, 0.01% ammonium persulfate) was degassed, catalyzed with TEMED (7 μl for every 10 ml of solution), and added to the tubes to a height 13 mm from the top. After the isoelectric focusing gels had polymerized, they were prefocused. 2.5 l of 0.01 M H_3PO_4 were placed in the bottom reservoir of the isoelectric focusing apparatus (Hoeffer Scientific Instruments). The parafilm was removed from the bottom of the glass tubes and the isoelectric focusing gels were placed into the apparatus. 50 μl of lysis buffer (9.5 M urea, 2% Nonidet P-40, 1.9% ampholines pH 5-7, 0.86% ampholines pH 4-6, 0.4% ampholines pH 3.5-10, 50 mM DTT) was layered onto the top of each isoelectric focusing gel. The upper reservoir of the apparatus and the remaining space in each tube was filled with 0.02 M NaOH which had been extensively degassed. The isoelectric focusing gels were then prefocused at

200 V for 15 min, 300 V for 30 min, and then 400 V for 30 min. Immunoprecipitation samples were prepared as described above, with the exception that after the final wash in low salt wash buffer, the immunoprecipitates were resuspended in 50 μl of 0.05% SDS, 50 mM DTT and incubated at 40˚C for 1 h with occasional mixing. At the end of this incubation, 54 mg urea crystals were added to bring the final urea concentration to 9 M in a volume of 100 μl. The samples were incubated at 40˚C for another 15 min to dissolve the urea. The resulting supernatants were loaded onto the prefocused isoelectric focusing gels. Each sample was overlayered with 20 μl of buffer (9 M urea, 0.96% ampholines pH 5-7, 0.44% ampholines pH 4-6, 0.2% ampholines pH 3.5-10). The upper reservoir and remaining space in the tubes was filled with fresh 0.02 M NaOH which had been extensively degassed. The isoelectric focusing gels were run overnight at 400 V, and then at 500 V during the last 2 h to yield a total of 7000 - 8000 V·h. After isoelectric focusing had been completed, the tube gels were removed from their tubes and each was incubated in 10 ml of SDS sample buffer on a rocking platform for 30 min. The tube gels were then laid sideways on top of a 3 mm thick, 7.5% SDS-polyacrylamide gel and sealed in place with 1% agarose in SDS running buffer. Second dimension SDS-PAGE was conducted overnight at 10 mA/gel. The current was raised to 40 mA/gel the next day, and electrophoresis was continued until the bromophenol blue dye front reached the bottom of the second dimension gels. The pH gradient generated by isoelectric focusing was assayed by running a blank isoelectric focusing gel. After isoelectric focusing, the blank gel was cut up into 1 cm segments, and each segment was incubated in 1 ml of deionized water overnight. The pH of each solution was measured with a pH meter.

Phosphatase treatment

Phosphatase treatment was conducted as described by Cooper and King (1986) with minor modifications. Briefly, potato acid phosphatase (Sigma) was resuspended in storage buffer (10 mM HEPES, pH 7.4, 0.5 mM $MgCl_2$, 0.5 mM DTT, 50% glycerol) to a concentration of 1 mg/ml, divided into 20 μl samples and stored at −70˚C. Samples for phosphatase treatment were prepared by immunoprecipitation, as described above. After pelleting the Protein A-Sepharose and removing the supernatant, samples were resuspended in 50 μl of phosphatase buffer (40 mM PIPES, pH 6.0, 1 mM DTT, 2 mM PMSF, 0.1 mg/ml potato acid phosphatase) and incubated at 30˚C for 1 h with occasional mixing. After this incubation, the phosphatase was removed by stringently washing the phosphatase-treated immunoprecipitates through the series of buffers used for immunoprecipitations (see above). Control immunoprecipitates were either incubated under identical conditions in the presence of phosphatase and 100 mM NaF, 25 mM Na_3VO_4, or were processed immediately for two-dimensional gel electrophoresis. Activity of the phosphatase was determined by addition of *p*-nitrophenyl phosphate to a final concentration of 10 mM in 100 μl of the same phosphatase buffer used for phosphatase treatment. The resulting yellow color is diagnostic for the cleavage of the pseudosubstrate by the phosphatase.

Immunofluorescence

MDCK cells were plated onto collagen-coated glass coverslips and cell-cell contact was synchronously induced as described above. After 0, 1, 4, 8, 12 and 96 h, cells were rinsed twice with PBS, and extracted with 2 ml CSK buffer at room temperature for 10 min. At the end of the incubation, cells were rinsed twice with PBS and then fixed with 2 ml of 1.75% formaldehyde in PBS for 10 min at room temperature. Cells were rinsed twice with PBS, and then blocked for 30 min at room temperature with 1 ml of blocking solution (PBS containing 0.2% BSA, 50 mM NH_4Cl and a 1:32 dilution of goat serum). Cells were washed twice for 10 min each with PBS containing 0.2% BSA (PBS-BSA). 200 μl of the primary antibody solution (1:100 dilution of the 3G8 hybridoma supernatant and 1:200 dilution of the plakoglobin anti-peptide antiserum, in PBS-BSA) was then applied to each coverslip, and incubated for 1 h at room temperature. The cells were once again washed twice for 10 min each in PBS-BSA, and then incubated for 30 min with 200 μl of secondary antibody solution (1:100 dilution of anti-rabbit rhodamine and 1:100 dilution of anti-mouse fluorescein in PBS-BSA). The coverslips were rinsed twice for 10 min each in PBS-BSA and mounted with elvanol (PBS, pH 7.0, containing 33% glycerol and 16.7% mowiol (Calbiochem)) onto glass slides. The slides were viewed using a confocal microscope (Technical Instruments).

RESULTS

Plakoglobin and β-catenin are distinct proteins in MDCK epithelial cells

The previous work of McCrea et al. (1991) suggested that plakoglobin and β-catenin co-migrated in SDS-PAGE, but this was inconsistent with the published molecular weights of the proteins, 83 kDa and 94 kDa, respectively (Cowin et al., 1986; Ozawa et al., 1989). To resolve this issue, we analyzed the migration of [^{35}S]methionine/cysteine-labelled proteins by SDS-PAGE following protein extraction from MDCK cells in buffers containing Triton X-100 (CSK-buffer); for this analysis, proteins were metabolically labelled for 4 h following synchronous induction of cell-cell contact in confluent monolayers of contact-naive cells (Nelson and Veshnock, 1987). One half of each fraction extracted from cells was immunoprecipitated with a polyclonal antiserum directed against the E-cadherin extracellular domain; the other half was immunoprecipitated with an anti-peptide polyclonal antiserum raised against an amino acid sequence (CIDTYSDGLRPPYPTADH) that is specific for plakoglobin and not β-catenin. Immunoprecipitates were stringently washed by successive mixing and centrifugation through a sucrose cushion, a buffer containing 1 M NaCl and, finally, a 10 mM Tris buffer containing 2 mM EDTA.

E-cadherin (Fig. 1, lanes 1 and 2) partitions predominantly into the CSK-soluble fraction (88%), while plakoglobin (Fig. 1, lanes 3 and 4) partitions approximately equally into the CSK-soluble and -insoluble fractions. The E-cadherin immunoprecipitate from the CSK-soluble fraction contains three prominent protein bands in addition to E-cadherin. Based upon their association with E-cadherin and their apparent molecular weights, these proteins are defined as the catenins: α-catenin (102 kDa), β-catenin (97 kDa), and γ-catenin (86 kDa). Plakoglobin (86 kDa) comigrates with γ-catenin rather than β-catenin (compare Fig. 1, lanes 2 and 3). A protein band with apparent molecular size of 120 kDa is detected in the plakoglobin immunoprecipitate (Fig. 1, lane 4). Given that the intensity of this band is significantly above background and that the immunoprecipitates were stringently washed, it is most likely a plakoglobin-associated protein. It does not represent an immunoreacting protein, since a protein of 120 kDa is not seen in a corresponding plakoglobin immunoblot (see below).

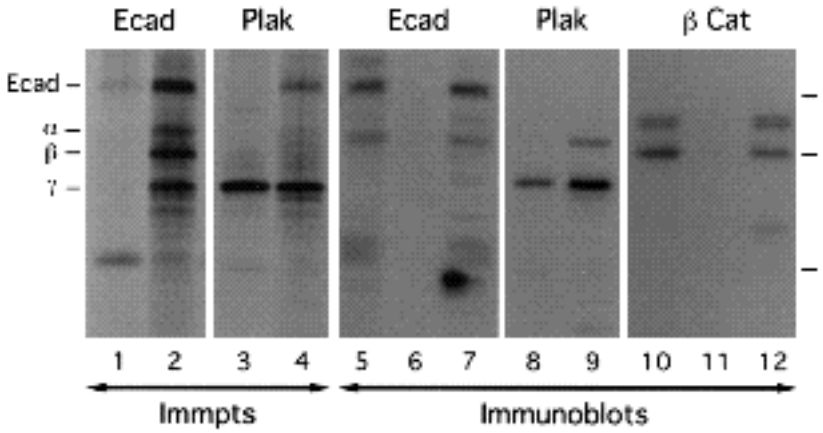

Fig. 1. Characterization of extractability and electrophoretic mobility in SDS-PAGE of E-cadherin, plakoglobin, and β-catenin. MDCK cells were directly extracted to prepare a whole cell extract, or extracted with CSK buffer as described (see Materials and Methods). CSK-soluble (lanes 2, 4, 7, 9 and 12) and -insoluble fractions (lanes 1, 3, 6, 8 and 11) and whole cell extracts (lanes 5 and 10) were subjected to immunoprecipitation, SDS-PAGE and fluorography (panels labeled 'immpts'), or to immunoblotting and autoradiography (panels labeled 'immunoblots'). The proteins analyzed by these techniques were as indicated: Ecad (E-cadherin, cytoplasmic domain antibody); Plak (plakoglobin, antipeptide antibody); β Cat (β-catenin polyclonal antibody). The tick marks to the right of the figure apply to all lanes and denote the positions of the 116, 97 and 68 kDa markers (from top to bottom, respectively).

The differences in apparent molecular size and extractability in buffers containing Triton X-100 of β-catenin and plakoglobin was demonstrated by direct immunoblotting of cell extracts. Proteins extracted with CSK buffer were separated by SDS-PAGE, transfered to nitrocellulose and probed with different antibodies. Plakoglobin was detected with the same antiserum as that used for the immunoprecipitations; E-cadherin was detected with a polyclonal antiserum directed against the cytoplasmic domain of E-cadherin; β-catenin was detected with a polyclonal antipeptide antibody directed against a sequence (PGDSNQLAWFDTDL) unique to β-catenin and not present in plakoglobin.

A 120 kDa protein is detected in the E-cadherin immunoblot in the CSK-soluble fraction (Fig. 1, lane 7) and in the whole cell lysate (Fig. 1, lane 5). E-cadherin is not detected in the CSK-insoluble fraction under these conditions. That E-cadherin immunoreactivity is detectable in whole cell lysates demonstates that the E-cadherin antibodies recognize SDS-denatured protein, and hence the failure to detect protein in the CSK-insoluble fraction is not due to SDS extraction and protein denaturation at the time of harvesting the cells. The faint lower molecular size bands seen in the E-cadherin immunoblot may represent degradation products generated during extraction.

The plakoglobin immunoblot reveals a protein of 86 kDa in both the CSK-soluble and -insoluble fractions; plakoglobin appears to be more abundant in the CSK-soluble fraction. A second protein of approximate molecular size 100 kDa is also detected in the CSK-soluble fraction of the plakoglobin immunoblot (Fig. 1, lane 9). This protein may represent a cross-reacting protein, since the intensity of that band is significantly above the background. Its absence from the plakoglobin immunoprecipitate of the CSK-soluble fraction (Fig. 1, lane 4) presumably reflects the preferential recognition of the SDS-denatured form of this protein by the antibody. It does not represent α-catenin or β-catenin since α-catenin, β-catenin and plakoglobin migrate differently in SDS-PAGE (see above).

The β-catenin immunoblot reveals three prominent proteins (97 kDa, and a doublet of 105 kDa) in the CSK-soluble fraction (Fig. 1, lane 12) and the whole cell lysate (Fig. 1, lane 10). The β-catenin antibody does not detect any proteins in the CSK-insoluble fraction at this time after induction of cell-cell contact (Fig. 1, lane 11). As in the case of the E-cadherin immunoblot detection of the same three bands in the whole cell lysate assures us that failure to detect protein in the CSK-insoluble fraction is not an artifact of our extraction conditions. The 97 kDa protein has the same electrophoretic mobility as β-catenin detected in E-cadherin immunoprecipitates (Fig. 1, lane 2). The upper doublet migrates at an apparent molecular size (105 kDa) similar to the predicted size of *Drosophila* armadillo protein. This may reflect the fact that the β-catenin antibody was raised against a peptide that shares ~80% amino acid sequence similarity with β-catenin and armadillo. This suggests that MDCK cells might contain one or more additional armadillo-like proteins.

Taken together, these results demonstrate that MDCK cells express immunologically distinct plakoglobin and β-catenin. Plakoglobin and β-catenin migrate with different apparent molecular sizes in SDS-PAGE, and exhibit different relative extractabilities in buffers containing Triton X-100. Clearly, plakoglobin and β-catenin are distinct proteins in MDCK cells.

Plakoglobin and β-catenin associate with the E-cadherin complex

The 120 kDa protein detected in plakoglobin immunoprecipitates (Fig.1) suggested to us that plakoglobin associates with E-cadherin. We sought to test this possibility directly by probing E-cadherin immunoprecipitates with antibodies specific for either plakoglobin or β-catenin. MDCK cells were extracted with CSK buffer and the soluble fractions were immunoprecipitated with the antibody to the E-cadherin extracellular domain. The E-cadherin immunoprecipitates were then subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies specific for the extracellular domain of E-cadherin (Fig. 2, lane 1), plakoglobin (Fig. 2, lane 2), α-catenin (Fig. 2, lane 3) or β-catenin (Fig. 2, lane 4). All antibodies reacted with specific proteins of the appropriate molecular size in the E-cadherin immunoprecipitates: E-cadherin, 120 kDa; plakoglobin, 86 kDa; α-catenin, 105 kDa; and β-

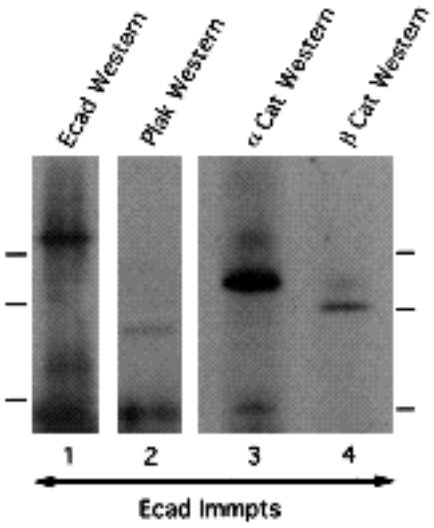

Fig. 2. α-Catenin, β-catenin and plakoglobin are distinct proteins that co-immunoprecipitate in a complex with E-cadherin. MDCK cells were extracted with CSK buffer. The CSK buffer-soluble fractions were subjected to immunoprecipitation with antibody raised against E-cadherin extracellular domain (see Materials and methods). The immunoprecipitates were subjected to SDS-PAGE, and then transfered to nitrocellulose filters and immunoblotted with antibodies to E-cadherin (lane 1, Ecad Western), plakoglobin (lane 2, Plak Western), α-catenin (lane 3, α Cat Western) or β-catenin (lane 4, β Cat Western). The dark areas seen at the bottom of lanes 1, 2 and 3 represent the IgG heavy chain detected with ^{125}I-Protein A. The tick marks to either side of the figure denote the positions of the 116, 97 and 68 kDa markers (from top to bottom, respectively); the tick marks on the left apply to lanes 1 and 2, and those on the right apply to lanes 3 and 4.

catenin, 97 kDa. The lower molecular weight band observed in the E-cadherin immunoblot (Fig. 2, lane 1) presumably represents a partial degradation product of E-cadherin migrating with an apparent molecular size of 76 kDa, distinct from that of plakoglobin and α-, β- and γ-catenin. The reactivity of the 97 kDa protein in the E-cadherin immunoprecipitates with the β-catenin antiserum demonstrates that this protein is the same as the one revealed in the β-catenin immunobots of CSK-soluble fractions of whole cell extracts (Fig. 1). The presence of α-catenin in the E-cadherin immunoprecipitates confirms that the E-cadherin immunoprecipitates from MDCK cells are composed of proteins similar to those reported for cadherins in other cell types. The presence of plakoglobin immunoreactivity in the E-cadherin immunoprecipitates demonstrates directly that plakoglobin comprises one of the E-cadherin-associated proteins.

Temporally and spatially co-ordinated accumulation of plakoglobin and E-cadherin at lateral borders following induction of cell-cell adhesion

We sought to determine whether plakoglobin and E-cadherin are co-ordinately localized to cell-cell contacts following induction of synchronous cell-cell adhesion in MDCK cells by double immunofluorescence and confocal microscopy. At each time point, the cells were extracted with CSK buffer, fixed in formaldehyde, and then double labelled with a monoclonal antibody directed against the extracellular domain of E-cadherin and the plakoglobin anti-peptide polyclonal antibody. Fig. 3 shows that plakoglobin (panels labeled Plak) and E-cadherin (panels labeled Ecad) co-accumulate at the lateral cell borders in a CSK-insoluble form with the same time course. The CSK-insoluble E-cadherin staining presumably corresponds to the small population of CSK-insoluble E-cadherin seen by immunoprecipitation (see Fig. 1, lane 1). The staining pattern at cell-cell contacts is initially punctate (1 h time point), but becomes progressive less punctate over the time course of analysis. By 96 h after contact induction, the cells have formed a tightly packed monolayer in which they have assumed a polygonal shape and each cell covers a smaller surface area of the plate relative to cells seen at the earlier time points. The staining for both plakoglobin and E-cadherin is continuous along the lateral membranes from the apex to the base of the cells. These results suggest that despite having a different extractability in buffers containing Triton X-100, a fraction of the plakoglobin participates in the same contact-induced process(es) as E-cadherin.

γ-Catenin is comprised of two proteins, one of which is plakoglobin

The results shown in Fig. 2 indicate that γ-catenin and plakoglobin co-migrate in SDS-PAGE. However, comparison of the relative amounts of plakoglobin and γ-catenin in E-cadherin immunoprecipitates suggests that the situation is more complex. MDCK cells were metabolically labeled with [^{35}S]methionine/cysteine and then extracted with CSK buffer. One of the CSK-soluble fractions was immunoprecipitated with polyclonal E-cadherin antibody directed against the extracellular domain. The resulting immunoprecipitate was dissociated in buffer containing 1% SDS. The SDS concentration was lowered to 0.1%, and the resulting solution was immunoprecipitated with a polyclonal antiserum directed against purified bovine plakoglobin (for details, see Materials and Methods). The plakoglobin immunoprecipitate was subjected to SDS-PAGE (Fig. 4, lane 3). A single 86 kDa protein representing plakoglobin is detected. Plakoglobin (Fig. 4, lane 2) and E-cadherin (Fig. 4, lane 1) were immunoprecipitated from other CSK-soluble fractions without subsequent dissociation and re-immunoprecipitation. E-cadherin (120 kDa) and α-, β- and γ-catenin (105, 97, and 86 kDa, respectively) are detected in the E-cadherin immunoprecipitate. The specificity of the procedure was checked by immunoblotting a sample identical to that in lane 3 with the independent antipeptide plakoglobin antiserum (Fig. 4, lane 4). Quantitation of the intensity of the 86 kDa protein detected in the fluorograms shown in Fig. 4 (lanes 1 and 3) by scanning densitometry indicates that plakoglobin can account for no more than 50% of the γ-catenin signal (data were normalized to take into account loss of protein antigenicity during dissociation protocol - controls not shown). These results confirm the association of plakoglobin with E-cadherin and

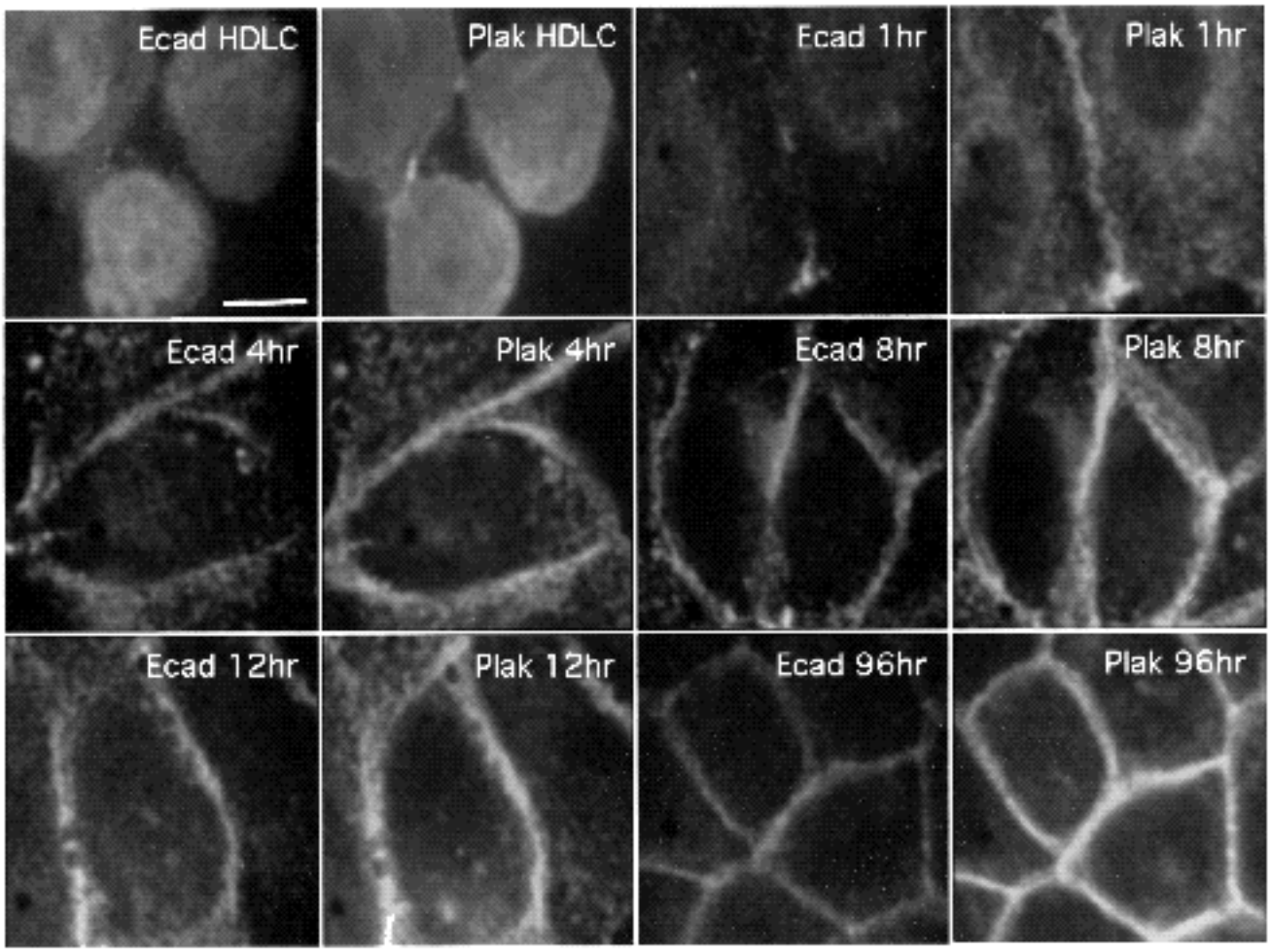

Fig. 3. Co-localization of E-cadherin and plakoglobin following induction of cell-cell contact. MDCK cell cultures were established on collagen-coated glass coverslips, as described in Materials and methods. At various times after induction of cell-cell contact, two cultures were removed and processed for double immunofluorescence confocal microscopy, as described in Materials and methods. E-cadherin was detected with the monoclonal antibody 3G8 and a flourescein-conjugated, anti-mouse secondary antibody (Ecad). Plakoglobin was detected with antipeptide plakoglobin antibody and a rhodamine-conjugated, anti-rabbit secondary antibody (Plak). The cultures were processed either at the time of induction of cell-cell contact (HDLC), or at 1, 4, 8, 12 or 96 hours after cell-cell contact. Bar, 5 μm.

suggest that the E-cadherin-associated protein band refered to as γ-catenin following SDS-PAGE actually consists of more than one protein.

Taken together, the results described above indicate that in MDCK cells, E-cadherin complexes with α-catenin, β-catenin, plakoglobin and possibly one or more other proteins which comigrate with γ-catenin. To investigate whether γ- catenin, as defined by migration on SDS-PAGE, contains more than one protein, and to gain further insight into the composition of the E-cadherin complex in MDCK cells, we performed two-dimensional gel electrophoresis (Fig. 5). E-cadherin was immunoprecipitated from [^{35}S]methionine/cysteine-labeled CSK-soluble fraction, prepared as described above. The E-cadherin immunoprecipitate was first subjected to equilibrium isoelectric focusing, and then subjected to SDS-PAGE in the second dimension.

A total of six proteins were resolved (Fig. 5, top panel; Ecad Immpt). For comparison, SDS-PAGE of an E-cadherin immunoprecipitate not subjected to isoelectric focusing is shown on the right of the panel. Spot 1 represents E-cadherin based upon its apparent molecular size in the SDS-PAGE dimension (120 kDa), and apparent isoelectric point of pI 4.2. Protein spot 4 is poorly resolved and appears to represent one of the minor background bands seen in the adjacent SDS-PAGE E-cadherin immunoprecipitate. Protein spot 3 is non-specific, and is detected in control immunoprecipitates in which the primary antibody is omitted (data not shown). α- and β-catenin are not resolved on this gel system, but the results presented above (Figs 1 and 2) clearly distinguish them from both plakoglobin and γ-catenin.

Protein spots 2, c and d are specific for the E-cadherin immunoprecipitate and represent the components of γ-catenin, as defined by their co-migration with γ- catenin in the corresponding SDS-PAGE. The very small difference in isoelectric point between protein spots c and d (pI 6.1 and pI 6.15, respectively) suggests that they may represent different isoelectric variants of the same protein (see below). On the other hand, the large difference in isoelec-

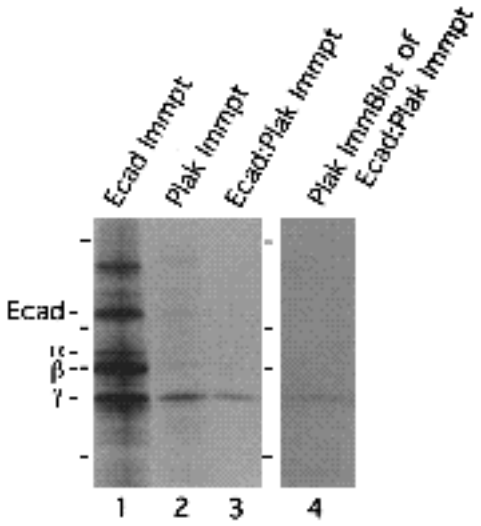

Fig. 4. Direct evidence that E-cadherin complex contains plakoglobin by sequential immunoprecipitation of E-cadherin and plakoglobin. E-cadherin and plakoglobin were sequentially immunoprecipitated from a metabolically labeled CSK-soluble fraction as described in Materials and Methods (lane 3, Ecad:Plak Immpt). For comparison, E-cadherin and plakoglobin were immunoprecipitated separately from parallel metabolically labeled fractions without subsequent dissociation and re-immunoprecipitation (lane 1, Ecad Immpt; lane 2, Plak Immpt). To confirm the specificity of the procedure, E-cadherin and plakoglobin were sequentially immunoprecipitated, and the final immunoprecipitate was immunoblotted with plakoglobin antipeptide antiserum (lane 4, Plak Immblot of Ecad:Plak Immpt). The tick marks to the left of lane 1 and to the right of lane 3 apply to all lanes and denote the positions of the 116, 97 and 68 kDa markers (from top to bottom, respectively).

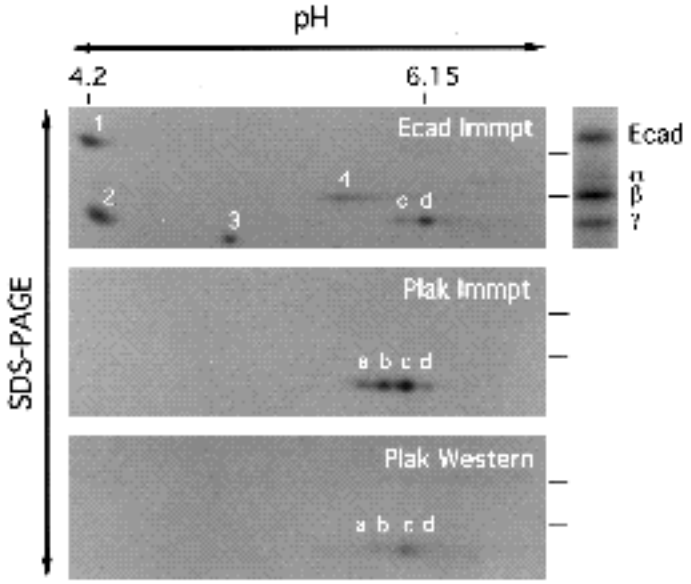

Fig. 5. Distinguishing plakoglobin and γ-catenin by two-dimensional gel electrophoresis. E-cadherin was immunoprecipitated from a metabolically labeled CSK buffer-soluble fraction using the 3G8 monoclonal hybridoma supernatant (ammonium sulfate cut and concentrated 100 ×). The immunoprecipitated proteins were subjected to two-dimensional gel electrophoresis as described in Materials and Methods (Ecad Immpt). Tick marks to the right of the panels indicate the position of the 97 kDa and 116 kDa molecular size markers in the SDS-PAGE dimension. An identical E-cadherin immunoprecipitate was subjected to SDS-PAGE without prior isoelectric focusing and is shown to the right of the top panel for comparison of the electrophoretic mobilities of E-cadherin and the catenins in the SDS-PAGE dimension. The middle panel shows plakoglobin immunoprecipitated from a metabolically labeled whole cell extract and subjected to two-dimensional gel electrophoresis (Plak Immpt). The bottom panel shows an E-cadherin immunoprecipitate from an unlabeled CSK-soluble fraction that was subjected to two-dimensional gel electrophoresis, and then immunoblotted with plakoglobin antiserum (Plak Western). All gels are aligned with their pH gradients in register. The numbers and letters refer to individual protein spots resolved on these two-dimensional gels; the letters a-d refer to the same isoelectric variants in all three panels (for details, see text).

tric point between protein spots c/d and 2 (pI 6.15 and pI 4.25, respectively) suggests that they represent different proteins.

We sought to determine whether plakoglobin represented any or all of the protein spots in the E-cadherin immunoprecipitate that comigrated with γ-catenin. First, a [^{35}S]methionine/cysteine-labelled whole cell lysate was immunoprecipitated with anti-plakoglobin antiserum. The resulting immunoprecipitate was subjected to two-dimensional gel electrophoresis as above (Fig. 5, middle panel; Plak Immpt). The 2-dimensional gels are aligned with their pH gradients in register. The letters a-d refer to the same isoelectric variant in all three panels. Four discrete plakoglobin spots are detected with an apparent molecular size of 86 kDa (spots a-d; pI 6.0-6.15). The isoelectric points of the proteins in spots c and d are similar to those previously reported for plakoglobin translated in vitro (Cowin et al., 1986). No protein is detected at pI 4.25 in the plakoglobin immunoprecipitate, demonstrating that protein spot 2, and protein spots c and d in the E-cadherin immunoprecipitate (Fig. 5, top panel), represent different proteins. Two of the plakoglobin isoforms, c and d (middle panel), are also detected in the E-cadherin immunoprecipitate (top panel). Plakoglobin isoelectric variant d is relatively more intense in the E-cadherin immunoprecipitate, whereas variant c is relatively more intense in the total plakoglobin immunoprecipitate. Note that isoelectric variant d is a relatively minor component of newly synthesized plakoglobin, but it is the major newly synthesized variant co-immunoprecipitated with E-cadherin. These results have also been obtained using the anti-peptide plakoglobin antiserum (data not shown) and suggest that the E-cadherin associates with a distinct subset of the plakoglobin isoelectric variants.

As a second means of confirming that protein spots c and d in E-cadherin immunoprecipitates (Fig. 5, top panel) were different isoelectric variants of plakoglobin, an E-cadherin immunoprecipitate was subjected to 2-dimensional gel electrophoresis and then immunoblotted with the anti-peptide plakoglobin antiserum (Fig. 5, bottom panel; Plak West-

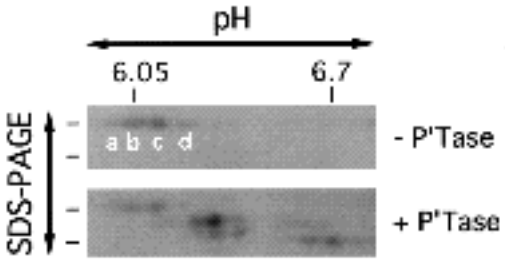

Fig. 6. Phosphatase analysis of plakoglobin. Plakoglobin was immunoprecipitated with the antipeptide plakoglobin antiserum from two-metabolically labeled whole cell extracts. One of the plakoglobin immunoprecipitates was subjected to two-dimensional gel electrophoresis without phosphatase treatment (top panel: −P'Tase). The other immunoprecipitate was treated with phosphatase as described in Materials and methods, and then subjected to two-dimensional gel electrophoresis (bottom panel: +P'Tase). The pH gradient of the isoelectric focusing dimension is indicated above the top panel and applies to both panels. The tick marks to the left of the panels indicate the size of the different protein spots: 86 kDa and 77 kDa, top to bottom. The letters a-d refer to the different plakoglobin isoelectric variants observed in the non-phosphatase-treated sample.

ern). The plakoglobin isoelectric variants b and c are clearly detected in the E-cadherin immunoprecipitate. These correspond to the two most abundant isoelectric variants detected in the plakoglobin immunoprecipitate shown (Fig. 5, middle panel). Variants a and d are also present and are evident upon longer exposure of the autoradiogram. Note that no protein is detected by the plakoglobin antibody at pI 4.25. It is interesting that the composition of plakoglobin isoelectric variants associated with E-cadherin detected by immunoblot and co-immunoprecipitation are different. The most basic isoelectric variant of newly-synthesized plakoglobin is preferentially associated with E-cadherin.

The above data rule out the possibility that protein spot 2 is plakoglobin. The possible identity of protein spot 2 as an E-cadherin degradation product can also be eliminated. While one of the degradation products representing the E-cadherin extracellular domain is similar in size to plakoglobin, previous studies have shown that γ-catenin co-immunoprecipitated with E-cadherin does not share identity with E-cadherin based upon peptide mapping (Ozawa et al., 1989; Peyrieras et al., 1985). Furthermore, we do not detect any immunoreactivity at the apparent molecular weight of γ-catenin and plakoglobin in our E-cadherin immunoprecipitates using an antiserum directed against the extracellular domain of E-cadherin (Fig. 2, lane 1). Based upon the relative abundance of E-cadherin and protein spot 2 seen in Fig. 5, one would expect to detect a protein band at 86 kDa if protein spot 2 were an E-cadherin degradation product. That this is not the case clearly indicates that protein spot 2 represents an independent protein.

Plakoglobin is a phosphoprotein

We sought to determine if phosphorylation was responsible for the presence of isoelectric variants of plakoglobin. Two whole cell lysates were prepared from parallel cultures of MDCK cells labelled with [^{35}S]methionine/cysteine, as described above. The lysates were subjected to immunoprecipitation with antipeptide plakoglobin antiserum. One immunoprecipitate was washed and immediately prepared for isoelectric focusing (Fig. 6; −P'Tase). The same results are obtained when the control immunoprecipitate is treated with phosphatase and the specific phosphatase inhibitors NaF and Na_3VO_4 and incubated in parallel with the phosphatase-treated immunoprecipitate (data not shown). The other immunoprecipitate was incubated with potato acid phosphatase in phosphatase buffer for 1 h at 30°C, and then prepared for isoelectric focusing (Fig. 6; +P'Tase); the pH gradients of the resulting fluorograms are aligned in register. Following phosphatase treatment, four plakoglobin isoelectric variants (a-d) are still evident compared to the control (−P'Tase). In addition to these four isoelectric variants, various other spots are present, which fall into three groups. The first group consists of three protein spots (pI ~6.3.) with an increased electrophoretic mobility in the SDS-PAGE dimension, representing a shift in apparent molecular size from 86 kDa to 82 kDa. The second group consists of two proteins with pI values similar to the two most basic isoelectric variants of the first group; however, this group has a decreased apparent molecular size of 80 kDa. The third group consists of three protein spots (pI ~6.7) with an apparent molecular size of 77 kDa. These results demonstrate that plakoglobin is a phosphorylated protein but that phosphorylation probably does not account for the different plakoglobin isoelectric variants.

DISCUSSION

Cell-cell adhesion plays a critical role in the development and maintenance of the structural and functional organization of diverse cells (Edelman and Crossin, 1991; Takeichi, 1991). Despite extensive analysis of the proteins involved, it remains unclear how extracellular contact between cells is transduced into alterations in the internal structure and function of cells. Recent studies have shown that cytosolic proteins that bind to the cytoplasmic domain of cadherins modulate cadherin function, and, therefore, are good candidates for mediating transduction of cell-cell contact to the cell interior (Kemler, 1992; Nagafuchi, 1988; Ozawa et al., 1989, 1990). At this stage it is important that the identities of these cytosolic proteins are defined.

In this study, we have clarified the relationships between plakoglobin and the catenins. Human plakoglobin and *Xenopus* β-catenin share a high degree of amino acid sequence similarity (60%). A previous report by McCrea et al. (1991) left unclear the question of whether or not these two proteins are species-specific variants of the same protein; in addition, evidence was presented which can be interpreted to indicate that plakoglobin and β-catenin are the same protein in MDCK cells. Our data, however, clearly demonstrate that plakoglobin and β-catenin are distinct proteins, both of which associate with E-cadherin in MDCK cells. Our immunoblot analysis of E-cadherin immunoprecipitates (Fig. 2) demonstrates that plakoglobin is bound to E-cadherin, in addition to α- and β-catenin in MDCK cells. The association of E-cadherin and plakoglobin was confirmed by sequential immunoprecipitations and immunoblotting (Fig. 4). These results confirm conclusions

made in two recent studies published during the preparation of this manuscript (Knudsen and Wheelock, 1992; Peifer et al., 1992). However, our results show that the association of both of these proteins with the cadherin complex is relatively strong and is resistant to extraction with buffers containing 1 M NaCl.

Our results demonstrate that plakoglobin and β-catenin have different extractability properties in buffers containing Triton X-100. While β-catenin was readily extracted from cells under these buffer conditions, like E-cadherin, we detected a significant proportion of plakoglobin that was resistant to extraction. This Triton X-100-insoluble pool of plakoglobin may be associated with desmosomes. This is supported by the fact that plakoglobin co-localizes with desmosomes in epithelial cells (Cowin et al., 1986), and our previous studies have shown that components of desmosomes rapidly become insoluble in CSK buffer following induction of cell-cell contact in MDCK cells (Pasdar and Nelson, 1988a,b, 1989; see also Penn et al., 1987). If true, our immunofluorescence results (Fig. 3) would suggest that there is concurrent contact-induced regulation of different *adherens* junctions.

The finding that plakoglobin and β-catenin are distinct proteins in MDCK cells is especially intriguing given the similarity of both proteins to the *Drosophila* segment polarity protein armadillo (McCrea et al., 1991). Armadillo is hypothesized to be involved in intercellular signalling in the *Drosophila* embryo. A protein doublet of approximate molecular size 105 kDa is detected in MDCK cells with our β-catenin antibody; note that the antibody was raised against a peptide shared by both *Xenopus* β-catenin and *Drosophila* armadillo. The protein doublet detected with this antibody is similar to the predicted size of the *Drosophila* armadillo protein (Peifer and Wiechaus, 1990) and may indicate the presence of one or more additional armadillo-related proteins in MDCK cells.

Careful analysis of the migration of E-cadherin-associated proteins in SDS-PAGE showed that plakoglobin comigrated with γ-catenin (Fig.1, lanes 2 and 3), indicating that they might be the same protein, as suggested in recent studies (Knudsen and Wheelock, 1992; Peifer et al., 1992). The results of our sequential immunoprecipitation experiments, however, indicate that the situation is more complex (Fig. 4). This was confirmed by performing two-dimensional gel electrophoresis of E-cadherin immunoprecipitates. Based upon differences in pI and immunological reactivity with a plakoglobin antiserum, at least two different proteins comprise the protein band referred to as γ-catenin as detected by SDS-PAGE (Fig. 5). One of these proteins is plakoglobin. The other is a previously uncharacterized acidic protein with a pI of 4.25. This protein spot does not represent an E-cadherin degradation product, as evidenced by its lack of immunoreactivity with an E-cadherin-specific antiserum despite its apparent relative abundance with respect to E-cadherin (Fig. 2, lane 1 and Fig. 5). We refer to this protein as γ-catenin to distinguish it from plakoglobin. Its identity is currently unknown, but since it associates with E-cadherin it presumably plays some role in the process of cadherin-mediated cell adhesion. These questions are currently under investigation.

It was of particular interest to us to note that there are multiple isoelectric variants of plakoglobin present in these cell extracts. Furthermore, within the pool of plakoglobin synthesized during the first four hours after contact induction, only a subset of these isoelectric variants associated with E-cadherin (Fig. 4). Specifically, we detected the most basic isoelectric variant of plakoglobin associated preferentially with E-cadherin. This variant is likely the least processed, since its pI is nearly identical to that reported for plakoglobin translated in vitro (Cowin et al., 1986); the pI values of the more acidic forms diverge increasingly from this value. These data suggest that the mechanism which gives rise to the different isoelectric variants of plakoglobin is also responsible for regulating the association of plakoglobin with E-cadherin.

However, we also note that plakoglobin immunoblots of E-cadherin immunoprecipitates resolved on two-dimensional gels revealed the presence of all the same isoelectric variants as those seen in total plakoglobin immunoprecipitates. The different results obtained following plakoglobin immunoprecipitation or plakoglobin immunoblotting of E-cadherin immunoprecipitates cannot be due to preferential binding of the plakoglobin antibodies to certain of the isoelectric variants; both the material from the whole cell extract used for immunoprecipitation and the material present on the immunoblot have been SDS-denatured, and both procedures have been performed with the same anti-peptide plakoglobin antibody (see Results). This indicates that if there is regulation of association of plakoglobin with E-cadherin, it only occurs within the newly synthesized pool of proteins. Formally, this raises two possible interpretations for the relationship of E-cadherin with the newly synthesized and steady state pools of plakoglobin. First, the most basic plakoglobin isoelectric variant associates with E-cadherin and is then chased into the more acidic plakoglobin variants while associated with E-cadherin. Second, the most basic isoelectric variant of plakoglobin associates more rapidly with E-cadherin but does not chase into the other isoelectric variants once associated. While mechanistically different, both scenarios could provide the means for controlling plakoglobin/E-cadherin association. This may be important in regulating plakoglobin association with E-cadherin and desmoglein I (Korman et al., 1989), a component of the desmosome, to coordinate assembly of different types of *adherens* junctions.

Previous studies have indicated that phosphorylation of components of cell-cell and cell-substratum junctions is important in regulating protein assembly and function (Geiger and Ginsberg, 1991; Shattil and Brugge, 1991; Takeichi, 1991; Tsukita et al., 1991). The existence of multiple plakoglobin isoelectric variants and their possible role in regulating plakoglobin function prompted us to examine whether phosphorylation played a role in generating these isoelectric variants. We tested this idea by treating plakoglobin immunoprecipitates with potato acid phosphatase, which cleaves both serine/threonine and tyrosine phosphates; thus, our results cannot be taken as evidence of a particular type of phosphorylation. The results show a clear shift in isoelectric point of plakoglobin variants from pI ~6.1 to pI ~6.7, nearly identical to the isoelectric point of plakoglobin translated in vitro.

The shift of plakoglobin isoelectric variants to a more

basic pI and decrease in apparent molecular size (86 kDa to 77 kDa) upon phosphatase treatment clearly demonstrates that plakoglobin is phosphorylated in MDCK cells. The decrease in apparent molecular size cannot be accounted for by the mass of potential phosphates removed and is not fully understood. However, such effects have been reported by others upon the removal of phosphate groups (I. Näthke, personal communication). That the plakoglobin isoelectric variants do not collapse into a single spot upon dephosphorylation suggests that modifications other than phosphorylation give rise to plakoglobin isoelectric variants. Formally, it is possible that the phosphatase is unable to remove all phosphate groups which give rise to the isoelectric variants. We consider this unlikely because the entire group of variants is shifted to a pI which is nearly identical to that of in vitro-translated plakoglobin (Cowin et al., 1986). The nature of the different plakoglobin isoelectric variants remains in question. The alternatives include proteolytic processing followed by N-terminal myristylation, differential RNA splicing or tyrosine sulfation. These possibilities are currently under investigation.

In summary, the data presented in this report demonstrate that plakoglobin, β-catenin and γ-catenin are distinct proteins that are associated with the cadherin complex. At least plakoglobin and β-catenin appear to possess similar but subtly different biochemical and cell biological properties. Although plakoglobin and γ-catenin co-migrate on SDS-PAGE, our analysis reveals that the protein band designated as γ-catenin in E-cadherin immunoprecipitates contains two proteins that are immunologically distinct and have substantially different isoelectric points; one of these proteins is plakoglobin, the other protein is unknown. The elucidation of the relationships between these proteins lays a framework for investigating the function of different E-cadherin complexes during cell-cell adhesion and subsequent cellular remodelling.

We wish to thank Drs Kurt Herrenknecht and Rolf Kemler (Max Planck Institut für Immunbiologie; Freiburg, Federal Republic of Germany) for providing the α-catenin and β-catenin antisera, Lindsay Hinck and Dr Jackie Papkoff (Syntex Corporation, Palo Alto) for providing the antipeptide plakoglobin antiserum and Dr James Marrs for providing the E-cadherin cytoplasmic domain antiserum. We would like to thank Technical Instruments (San Hose, CA) for providing the confocal microscope and technical advice. This work was supported in part by grants to W. J. Nelson from the National Institutes of Health (GM 35527) and from the American Cancer Society (BE-144). Peter Piepenhagen is a recipient of a predoctoral fellowship from the National Science Foundation. W. James Nelson is a recipient of an Established Investigator Award from the American Heart Association.

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(Received 21 October 1992 - Accepted 2 December 1992)