Plakoglobin, or an 83-kD Homologue Distinct from β-catenin, Interacts with E-cadherin and N-cadherin

Karen A. Knudsen* and Margaret J. Wheelock‡

*The Lankenau Medical Research Center, Wynnewood, Pennsylvania 19096; ‡Department of Biology, University of Toledo, Toledo, Ohio 43606

Abstract. E- and N-cadherin are members of a family of calcium-dependent, cell surface glycoproteins involved in cell–cell adhesion. Extracellularly, the transmembrane cadherins self-associate, while, intracellularly, they interact with the actin-based cytoskeleton. Several intracellular proteins, collectively termed catenins, have been noted to co-immunoprecipitate with E- and N-cadherin and are thought to be involved in linking the cadherins to the cytoskeleton. Two catenins have been identified recently: a 102-kD vinculin-like protein (α-catenin) and a 92-kD *Drosophila armadillo*/plakoglobin-like protein (β-catenin).

Here, we show that plakoglobin, or an 83-kD plakoglobin-like protein, co-immunoprecipitates and colocalizes with both E- and N-cadherin. The 83-kD protein is immunologically distinct from the 92-kD β-catenin and, because of its molecular mass, likely represents the cadherin-associated protein called γ-catenin. Thus, two different members of a plakoglobin family associate with N- and E-cadherin and, together with the 102-kD α-catenin, appear to participate in linking the cadherins to the actin-based cytoskeleton.

The cadherin family is comprised of a growing number of cell surface, calcium-dependent glycoproteins involved in cell–cell adhesion (reviewed by Takeichi, 1991; Magee and Buxton, 1991). Members of the cadherin supergene family are found in regions of cell–cell contact and in intermediate junctions, with E-cadherin in *zonula adherens* (Kemler et al., 1990), N-cadherin in *fascia adherens* (Volk and Geiger, 1984), and desmogleins and desmocollins in *macula adherens* (desmosomes) (Wheeler et al., 1991; Nilles et al., 1991; Koch et al., 1991, 1992; Parker et al., 1991). Optimal activity depends upon intracellular linkage of the transmembrane cadherins to the cytoskeleton, with E- and N-cadherin linking to f-actin microfilaments at *zonula* and *fascia adherens* junctions, respectively, and desmogleins and desmocollins interacting with intermediate filaments at desmosomes.

Several intracellular proteins have been postulated to participate in linking cadherins to the cytoskeleton. Such proteins have been identified in two ways: first, they colocalize with cadherins at cell–cell junctions and, second, they co-immunoprecipitate with solubilized cadherins extracted from cells or tissue. At least three proteins with molecular masses ranging from ~80–102 kD have been shown by co-immunoprecipitation to associate with E-, N-, and P-cadherin (Peyrieras et al., 1985; Ozawa et al., 1989; Nagafuchi and Takeichi, 1989; Wheelock, 1990; Wheelock and Knudsen, 1991*a*,*b*). These proteins, collectively termed catenins, appear to anchor the cytoplasmic domains of E- and N-cadherin to the microfilaments. Two catenins have been identified and characterized. One is a 102-kD, vinculin-like protein (CAP 102 or α-catenin) shown by Nagafuchi et al. (1991) to co-immunoprecipitate with E-cadherin and by Herrenknecht et al. (1991) to co-immunoprecipitate with N-cadherin and, in addition, to colocalize with both E- and N-cadherin in cells.

McCrea et al. (1991) showed that a second protein with a molecular mass of 92 kD, called β-catenin, co-immunoprecipitates with *Xenopus* E-cadherin. β-catenin has 70% predicted amino acid sequence identity to a protein encoded by the *Drosophila armadillo* gene (McCrea et al., 1991). *Armadillo* is a segment polarity gene required for normal pattern formation in the *Drosophila* embryo (Peifer and Wieschaus, 1990). Both the *armadillo* protein and the *Xenopus* 92-kD β-catenin share considerable amino acid sequence homology with human plakoglobin (Franke et al., 1989; McCrea et al., 1991; Peifer and Wieschaus; 1990).

Plakoglobin is an 83-kD intracellular protein that localizes to the cytoplasmic face of both desmosomes, which contain desmogleins and desmocollins, and *zonula adherens*, which contain E-cadherin (Cowin et al., 1986). Plakoglobin, as recognized by a mAb to bovine plakoglobin, has been reported to co-immunoprecipitate with desmogleins (Korman et al., 1989), suggesting a physical interaction between plakoglobin and desmogleins. McCrea et al. (1991) noted that a protein with a molecular mass of ~90 kD co-immunoprecipitated with E-cadherin from canine MDCK epithelial cells and was recognized by the same mAb to bovine plakoglobin. These authors concluded that the E-cadherin–associ-

ated protein recognized by antiplakoglobin was homologous to the 92-kD β-catenin they characterized in *Xenopus*.

In addition to the 102-kD vinculin-like α-catenin and the 92-kD *armadillo*/plakoglobin-like β-catenin, at least one additional protein with a molecular mass in the region of 80 kD, called γ-catenin, has been observed to co-immunoprecipitate with E-, P-, and N-cadherin (Peyrieras et al., 1985; Ozawa et al., 1989; Wheelock, 1990; Wheelock and Knudsen, 1991*a*,*b*). Although γ-catenin has not been identified, several proteins that have molecular masses of ~80 kD and, in addition, localize to the cytoplasmic face of the plasma membrane might be considered as candidates. They include plakoglobin (83 kD) (Cowin et al., 1986), which is found at regions of cell–cell contact, and zyxin (82 kD) (Crawford and Beckerle, 1991), which is found at both cell–cell and cell–matrix attachment sites. Other proteins include radixin (82 kD) (Tsukita et al., 1989; Funayama et al., 1991), moesin (77 kD) (Lankes and Furthmayr, 1991), and ezrin (77–81 kD) (Bretscher, 1983, 1989), all of which are related to each other and to the erythrocyte band 4.1 protein.

In this paper, we demonstrate that an 83-kD protein recognized by the mAb to bovine plakoglobin co-immunoprecipitates with both human E-cadherin and chicken N-cadherin. We further show that the 83-kD cadherin-associated protein is immunologically distinct from a 92-kD E- and N-cadherin–associated protein recognized by antibodies to the *Drosophila armadillo* protein, suggesting that two distinct members of a plakoglobin family interact with cadherins in vitro. As evidence that the 83-kD protein is associated with cadherins in cells, we show that protein recognized by antiplakoglobin colocalizes with the cadherins in fixed cells and physically redistributes with E- and N-cadherin in live cells. We propose that plakoglobin, or an 83-kD plakoglobin-like protein, is the cadherin-associated protein referred to as γ-catenin.

Materials and Methods

Antibodies and Other Reagents

A mouse mAb to bovine plakoglobin (PG5.1) (Cowin et al., 1986) was purchased from IBL Research Products Corp (Cambridge, MA). Mouse mAbs to desmoglein and desmoplakins I and II were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Affinity purified rabbit antiserum to CAP 102 (Nagafuchi et al., 1991) was a gift of Dr. Akira Nagafuchi (National Institute for Physiological Sciences, Okazaki, Japan). Affinity purified rabbit antiserum to the *Drosophila armadillo* protein (Piefer and Wieschaus, 1990) was a gift of Dr. Mark Peifer (University of North Carolina, Chapel Hill, NC). Rabbit antiserum to chicken zyxin (Crawford and Beckerle, 1991) was a gift of Dr. Mary Beckerle (University of Utah, Salt Lake City, UT). Rabbit antisera to chicken vinculin and chicken α-actinin were gifts from Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC). A mouse mAb to bovine moesin (38/87) (Lankes et al., 1988), a rabbit antiserum to human placental ezrin (90-3) (Saku and Furthmayr, 1989), and a rabbit antiserum to human placental moesin (90-7) (Saku and Furthmayr, 1989), the latter of which also recognizes ezrin and radixin, were gifts from Dr. Wolfgang Lankes (Stanford University School of Medicine, Stanford, CA). A rabbit antiserum to chicken brush border ezrin (Bretscher, 1983, 1989) was a gift of Dr. Anthony Bretscher (Cornell University, Ithaca, NY). A rabbit antiserum to bovine desmoplakins I and II (Angst et al., 1990) was a gift of Dr. Kathleen Green (Northwestern University Medical School, Chicago, IL). The hybridoma secreting a rat antibody to chicken N-cadherin, NCD-2 (Hatta and Takeichi, 1986), was a gift from Dr. Masatoshi Takeichi (Kyoto University, Kyoto, Japan). Rabbit anti-(chicken)N-cadherin is described by Wheelock and Knudsen (1991*a*). Rabbit polyclonal and rat monoclonal (E9) antibodies to human E-cadherin are described by Wheelock et al. (1987).

Cell Culture, Extraction, and Immunoprecipitation

Cardiac myocytes were isolated from 11-d chicken embryos and grown in DME with 10% FBS and 5% embryo extract as previously described (Wheelock and Knudsen, 1991*a*). The human choriocarcinoma epithelial cell line, JAR PR497 (JAR) was cultured as described (Wheelock et al., 1987).

For solubilization of N-cadherin, hearts from 11-d chick embryos were excised and extracted with 2% nonionic detergent as described (Wheelock and Knudsen, 1991*a*). Solubilization of E-cadherin from JAR cells with 0.5% NP-40 was performed as described (Wheelock et al., 1987).

The N-cadherin complex was affinity purified on immobilized anti-N-cadherin (NCD-2) (Wheelock and Knudsen, 1991*a*). The column was washed extensively with 10 mM Tris HCL, pH 8.0, containing 0.5% NP-40 and the complex eluted with 50 mM diethylamine, pH 11.0. The pH was adjusted to 8.0 and the complex was dialyzed against 10 mM Tris HCl, pH 8.0, and stored at −70°C. The E-cadherin complex was affinity purified by mixing JAR extract with anti-E-cadherin (E9) for 1 h at 4°C. Subsequently, the antigen-antibody complex was captured using immobilized anti-rat IgG. The E-cadherin complex was eluted as described above.

SDS Polyacrylamide Gel Electrophoresis and Western Immunoblot Analysis

SDS-PAGE of tissue and cell extracts was performed under reducing conditions as described by Laemmli (1970). Molecular mass markers (Sigma Chemical Co., St. Louis, MO) included myosin (205 kD), β-galactosidase (116 kD), phosphorylase b (97 kD), BSA (66 kD), ovalbumin (43 kD), and carbonic anhydrase (30 kD). SDS-PAGE–resolved proteins contained in cell and tissue extracts, or proteins in E- and N-cadherin immunoprecipitates, were transferred electrophoretically to nitrocellulose as described (Wheelock et al., 1987). The nitrocellulose was blocked with 3% BSA. The various proteins of interest were detected by specific polyclonal or monoclonal antibodies as indicated, followed by species-appropriate alkaline phosphatase-conjugated antibodies (Fisher Scientific Co., Pittsburgh, PA) and nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate substrates (NBT/BCIP) (Sigma Chemical Co.).

E- and N-cadherin "Patching"

To "patch" N-cadherin in embryonic chick cardiac cells, a specific polyclonal rabbit anti-N-cadherin serum (1:40) was added to live 48-h cultures for 1–2 h at 37°C. To patch E-cadherin in JAR human epithelial cells, a specific polyclonal anti-E-cadherin antiserum (1:50) was added to live cells for 2 h at 37°C. Subsequent to their exposure to anti-cadherin antisera, the cells were washed thoroughly with PBS, fixed with cold methanol, and processed for immunofluorescence light microscopy.

Immunofluorescence Light Microscopy

For immunofluorescence light microscopy, embryonic chick cardiac myocytes and JAR human epithelial cells were grown on glass coverslips. All cells were fixed with cold methanol for 10–15 min, blocked with 10% goat serum for 1–2 h, and exposed to first antibodies specific for the proteins of interest. First antibodies were detected by species-specific second antibodies tagged with either rhodamine or FITC (Fisher Scientific Co.). Fluorescence was detected with a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence and filters appropriate for visualizing rhodamine or fluorescein and photographed using Polaroid 3000 ASA Type 57 film (Polaroid Corp., Cambridge, MA) or Kodak T-MAX 3200 film (Eastman Kodak Co., Rochester, NY). All pictures were taken using either a 63× or 100× objective.

Results

Proteins Co-immunoprecipitating with N-cadherin and E-cadherin

We reported previously that a similar repertoire of noncadherin-related proteins with molecular masses in the region of 80–100 kD co-immunoprecipitated with both E-cadherin isolated from human epithelial cells and N-cadherin isolated from chicken muscle (Wheelock and Knudsen, 1991*a*). The

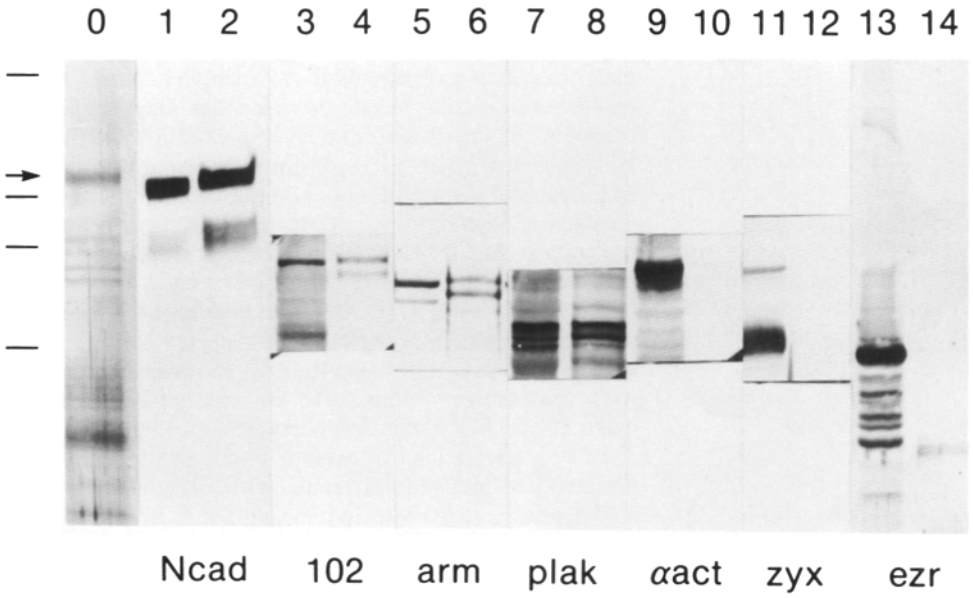

Figure 1. Immunoblot analysis of embryonic chicken heart extract and N-cadherin immunoprecipitate. Hearts of 11-d embryonic chicks were extracted with 2% NP-40. The same amount of heart extract was loaded in all odd-numbered lanes starting with lane *1*. N-cadherin was immunoprecipitated from the heart extract with immobilized NCD-2, a mAb to chicken N-cadherin. The identical amount of N-cadherin immunoprecipitate was loaded into lane *0* and all even-numbered lanes, starting with lane *2*. The proteins were resolved on a 7% SDS–polyacrylamide gel, transblotted to nitrocellulose, and immunoblotted with specific antibodies subsequently detected by species-specific, alkaline phosphatase-conjugated second antibodies and NBT/BCIP. Lane *0* shows the N-cadherin immunoprecipitate stained with India ink. The arrow marks N-cadherin at 135 kD. The following lanes were exposed to the following antibodies: lanes *1* and *2*, rat anti–N-cadherin (NCD-2) (conditioned supernate); lanes *3* and *4*, rabbit anti-CAP 102 antiserum (1:20); lanes *5* and *6*, anti-*armadillo* antiserum (1:100); lanes *7* and *8*, antiplakoglobin mAb (5 μg/ ml); lanes *9* and *10* anti–α-actinin antiserum (1:1,000); lanes *11* and *12*, antizyxin antiserum (1:500); lanes *13* and *14*, anti–(chicken)ezrin antiserum (1:500). The lines at the left mark molecular mass standards at 205, 116, 97, and 66 kD.

similarity in the number and molecular weights of the E- and N-cadherin–associated proteins led us to speculate that similar or homologous intracellular proteins interacted with the conserved cytoplasmic domains of E- and N-cadherin and served to link these cadherins to the actin cytoskeleton. As one approach to identifying the intracellular proteins coimmunoprecipitating with E-cadherin and N-cadherin, we determined whether or not they were immunologically related to previously identified proteins. We focused our attention on proteins known (*a*) to associate with E- and N-cadherin, (*b*) to localize at cell–cell junctions, or (*c*) to participate in anchoring components of the plasma membrane to the cytoskeleton. We therefore acquired antibodies to CAP 102 (α-catenin), the *Drosophila armadillo* protein, plakoglobin, vinculin, α-actinin, zyxin, ezrin, and moesin and used these antibodies to probe for the presence of immunocross-reactive proteins in chicken N-cadherin and human E-cadherin immunoprecipitates.

Fig. 1 shows that embryonic chicken heart extract contained proteins recognized by antibodies to N-cadherin (lane *1*), CAP 102 (lane *3*), the *armadillo* protein (lane *5*), plakoglobin (lane *7*), α-actinin (lane *9*), zyxin (lane *11*), and ezrin (lane *13*). Immunoreactive proteins were also detected in the heart extract by antibodies to vinculin and moesin (data not shown). The individual antibodies detected distinct bands with molecular weights similar, or identical, to those published for the individual proteins, demonstrating the presence of N-cadherin, α-catenin, β-catenin, α-actinin, vinculin, plakoglobin, zyxin, ezrin, and moesin (or closely related proteins) in the chick cardiac extract. It is to be noted that, although the *armadillo* protein and plakoglobin share considerable amino acid sequence homology (Peifer and Wieschaus, 1990), the anti-*armadillo* recognized a 92-kD doublet in the cardiac extract, but failed to recognize any band in the 83-kD molecular mass region of the same extract (Fig. 1, lane *5*). Conversely, the antibody to plakoglobin failed to recognize the 92-kD proteins recognized by anti-*armadillo*, but did recognize 2–3 bands in the molecular mass region of 83 kD (Fig. 1, lane *7*). The presence of plakoglobin in the heart extract was expected since cardiac myocytes have desmosomes.

Fig. 2 shows that similar results were observed when an extract of human epithelial cells was immunoblotted with anti-*armadillo* and antiplakoglobin. That is, anti-*armadillo* recognized a 92-kD protein (Fig. 2, lane *3*), whereas the antiplakoglobin recognized an 83-kD band (Fig. 2, lane *5*). The data indicate, therefore, that the antibodies to the *armadillo* protein and plakoglobin recognize distinctly different proteins in the extracts of both embryonic chicken heart and human epithelial cells.

Fig. 1 also depicts immunoblot analysis of the N-cadherin immunoprecipitate and demonstrates that three immunologically distinct proteins associate with N-cadherin. As expected, intact N-cadherin was readily detected at 135 kD (Fig. 1, lane *2*), while a broad band of proteolytic breakdown products was observed at a lower molecular mass region. It is to be noted that all lanes contain approximately equivalent amounts of N-cadherin, as determined by immunoblot analysis. To conserve antibodies that were costly or available only in limited supply, we excised the region surrounding the published molecular mass of the particular protein of interest and exposed this region to the antigen-specific antibody. The excised and immunoblotted region was then returned to its original position for further analysis, molecular mass determination, and photography.

The N-cadherin immunoprecipitate contained a band at about 100 kD recognized by antibodies to CAP 102 (Fig. 1, lane *4*). Presumably, this protein represents the vinculin-like

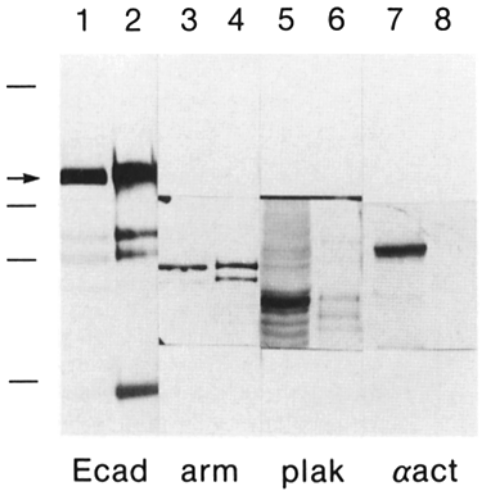

Figure 2. Immunoblot analysis of human epithelial cell extract and E-cadherin immunoprecipitate. JAR human epithelial cells were extracted with 0.5% NP-40. The odd-numbered lanes contain identical amounts of the whole extract. E-cadherin was immunoprecipitated from the epithelial cell extract using the E9 mAb to human E-cadherin. The even-numbered lanes contain identical amounts of E-cadherin immunoprecipitate. The proteins were resolved on a 7% SDS-polyacrylamide gel, transblotted to nitrocellulose, and probed with various specific antibodies, followed by species-specific, alkaline phosphatase-conjugated second antibodies and NBT/BCIP. The lanes were immunoblotted with the following antibodies: lanes *1* and *2*, E9 mAb (conditioned supernate); lanes *3* and *4*, anti-*armadillo* antiserum (1:100); lanes *5* and *6*, antiplakoglobm mAb (5 μg/ml); lanes *7* and *8*, anti-α-actinin antiserum (1:1,000). The arrow marks the location of E-cadherin at 120 kD. The lines at the left denote molecular mass standards of 205, 116, 97, and 66 kD.

α-catenin; this conclusion is consistent with the observation of Herrenknecht et al. (1991), who showed that α-catenin associates with chicken N-cadherin. Fig. 1 also shows that the polyclonal antiserum to the *Drosophila armadillo* protein recognized a doublet in the molecular mass region of 92 kD in the N-cadherin immunoprecipitate (Fig. 1, lane *6*). The molecular weight and the immunocross-reactivity with the *armadillo* protein suggest that this protein is likely the 92-kD β-catenin described by McCrea et al. (1991). The presence of multiple bands detected by both anti-CAP 102 and anti-*armadillo* is not understood at this time. Although the most likely explanation may be the presence of proteolytic breakdown products, the expression of modification variants of the *armadillo* protein has been suggested by Peifer and Wieschaus (1990).

Fig. 1 further shows that a mAb to plakoglobin (PG5.1) recognized two bands in the N-cadherin immunoprecipitate (Fig. 1, lane *8*). These proteins have molecular masses in the region of 83 kD, suggesting that plakoglobin or a plakoglobin-like protein(s) associates with N-cadherin. The presence of multiple bands detected by antiplakoglobin is not understood at this point. Multiple protein bands in the 83 kD region recognized by the same antiplakoglobin antibody used here have been detected by other labs (Cowin et al., 1986; Korman et al., 1989) and have been assumed to be breakdown products. However, the possibility that plakoglobin isoforms may exist cannot be ruled out, since at least two mRNAs of similar size have been detected in some cells (Cowin et al., 1986; Franke et al., 1989).

Fig. 2 shows that the E-cadherin immunoprecipitate also contains 2–3 bands recognized by the antiplakoglobin antibody. Fig. 2, lane *2* reveals E-cadherin at 120 kD (*arrow*) and lower molecular weight breakdown products in the immunoprecipitate. (In addition, the heavy chain of the E9 antibody is seen at 55 kD.) It is to be noted that similar amounts of E-cadherin were present in all lanes. The anti-*armadillo* detected a doublet at 92 kD in the E-cadherin immunoprecipitate (Fig. 2, lane *4*), indicating the presence of the E-cadherin-associated *armadillo*-like β-catenin described by McCrea et al. (1991). As in the case of the N-cadherin immunoprecipitate, no bands were detected by anti-*armadillo* in the region of 83 kD in the E-cadherin immunoprecipitate. Instead, the antibody to plakoglobin detected 2–3 bands at ~83 kD in the E-cadherin immunoprecipitate (Fig. 2, lane 6), suggesting that plakoglobin or a plakoglobin-like protein(s) co-immunoprecipitates with E-cadherin.

The presence of protein(s) recognized by the antiplakoglobin in the E- and N-cadherin immunoprecipitates was not because of a general contamination with intracellular, cytoskeletal proteins nor to nonspecific interaction of desmosomal proteins. Antibodies to α-actinin (Figs. 1 and 2) and vinculin (data not shown) reacted strongly with 110- and 130-kD bands, respectively, in both the chicken heart extract (Fig. 1, lane *9*) and the human epithelial cell extract (Fig. 2, lane *7*), but did not detect any bands in either the N-cadherin immunoprecipitate (Fig. 1, lane *10*) or the E-cadherin immunoprecipitate (Fig. 2, lane 8). In addition, while zyxin (Fig. 1, lanes *11* and *12*), ezrin (Fig. 1, lanes *13* and *14*), and moesin (data not shown) were all readily detected in the heart extract, none of these proteins were detected in the N-cadherin immunoprecipitate, except for trace amounts of ezrin. Lastly, the cadherin immunoprecipitate did not contain desmoplakin, an intracellular desmosomal protein thought to participate in linking the transmembrane desmosomal proteins to intermediate filaments (Kapprell et al., 1990). Fig. 3 shows that, as expected, desmoplakin is present in the heart extract (Fig. 3, lane *3*). In contrast, desmoplakin was not detected in the N-cadherin immunoprecipitate (Fig. 3, lane *4*).

Fig. 1, lane *0* shows the India ink-stained N-cadherin immunoprecipitate and indicates the complexity of this material. The major India ink-stained band clearly represents N-cadherin (*arrow*), whereas lower molecular mass bands appear to represent N-cadherin breakdown products and the multiple (at least six) bands recognized by antibodies to α-catenin, the *armadillo* protein, and plakoglobin. In addition, it is possible that as yet unidentified proteins are also present in the immunoprecipitate. In general, our observations on the composition of cadherin immunoprecipitates are consistent with those of others who have noted that the 92-kD β-catenin is the most tenaciously bound of the catenins (McCrea and Gumbiner, 1991; Ozawa and Kemler, 1992). More stringent washing of the immunoprecipitate results in loss of the associated proteins, with protein recognized by antiplakoglobin being lost most readily (data not shown), which is also a property shared by the cadherin-associated protein referred to as γ-catenin (Ozawa et al., 1989; McCrea and Gumbiner, 1991; Ozawa and Kemler, 1992).

In summary, our immunoprecipitation data indicate that at least three distinctly different proteins recognized by antibodies to CAP102 (α-catenin), the *armadillo* protein, and plakoglobin associate closely with E- and N-cadherin. In addition, our data indicate that α-actinin, vinculin, zyxin, ez-

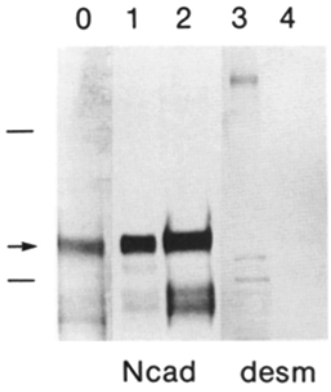

Figure 3. Immunoblot analysis of embryonic chick heart extract and N-cadherin immunoprecipitate using antidesmoplakin. Hearts from 11-d chick embryos were extracted with NP-40 and N-cadherin was immunoprecipitated from the heart extract with immobilized NCD-2 mAb to chicken N-cadherin. Identical amounts of the whole extract are present in lanes *1* and *3*, whereas identical amounts of N-cadherin immunoprecipitate are present in lanes *0*, *2*, and *4*. The proteins were resolved on a 5% SDS-polyacrylamide gel, transblotted to nitrocellulose, and probed with specific antibodies, followed by species-specific alkaline phosphatase-conjugated second antibody and NBT/BCIP. Lane *0* was stained with India ink. N-cadherin (135 kD) is marked with an arrow. Lanes *1* and *2* were exposed to rat NCD-2 anti-N-cadherin. Lanes *3* and *4* were exposed to rabbit antidesmoplakins I and II. The lines at the left denote the 205- and 116-kD molecular mass standards.

rin, moesin, and desmoplakin do not associate with the cadherins during immunoprecipitation.

Colocalization of Cadherins and Co-immunoprecipitating Proteins

To provide evidence that the proteins recognized by antibodies to the *armadillo* protein and plakoglobin associate with cadherins in cells, and not just in vitro, we colocalized the proteins in chicken cardiac cells and human epithelial cells. Since CAP 102 (α-catenin) has already been shown to colocalize with both E- and N-cadherin at areas of cell–cell contact (Herrenknecht et al., 1991), we did not focus our efforts on this cadherin-associated protein.

Fig. 4 indicates that N-cadherin and the 92-kD *armadillo*-like protein colocalize as assessed by immunofluorescence light microscopy. Fig. 4 *A* presents the phase-contrast image of embryonic chicken cardiac cells. These cells were exposed simultaneously to rat monoclonal anti-N-cadherin (NCD-2) and rabbit anti-*armadillo* protein. Fig. 4 *B* indicates the expression of N-cadherin detected by NCD-2 and rhodamine-labeled goat anti-rat IgG, whereas Fig. 4 *C* shows the expression of the *armadillo*-like protein detected by rabbit anti-*armadillo* and FITC-labeled goat anti-rabbit IgG. Note that the patterns of staining for the two proteins are virtually identical. In scrutinizing many different cells, we failed to detect one protein without detecting the other at the identical location.

Both E- and N-cadherin colocalized with proteins recognized by antiplakoglobin. The coincident anticadherin staining and the antiplakoglobin staining was continuous and linear in regions of cell–cell contact in chicken cardiac cells (Fig. 5, *A* and *B*) and in human epithelial cells (Fig. 6, *A* and *B*). Antidesmoplakin staining, denoting desmosomes, was also observed in the same regions of cell–cell contact; however, the antidesmoplakin staining was noted to be a discontinuous or dashed line, as opposed to being a continuous line. Our observation that antiplakoglobin staining appears to be both in and out of desmosomes is consistent with the published data of Cowin et al. (1986), who found antiplakoglobin staining

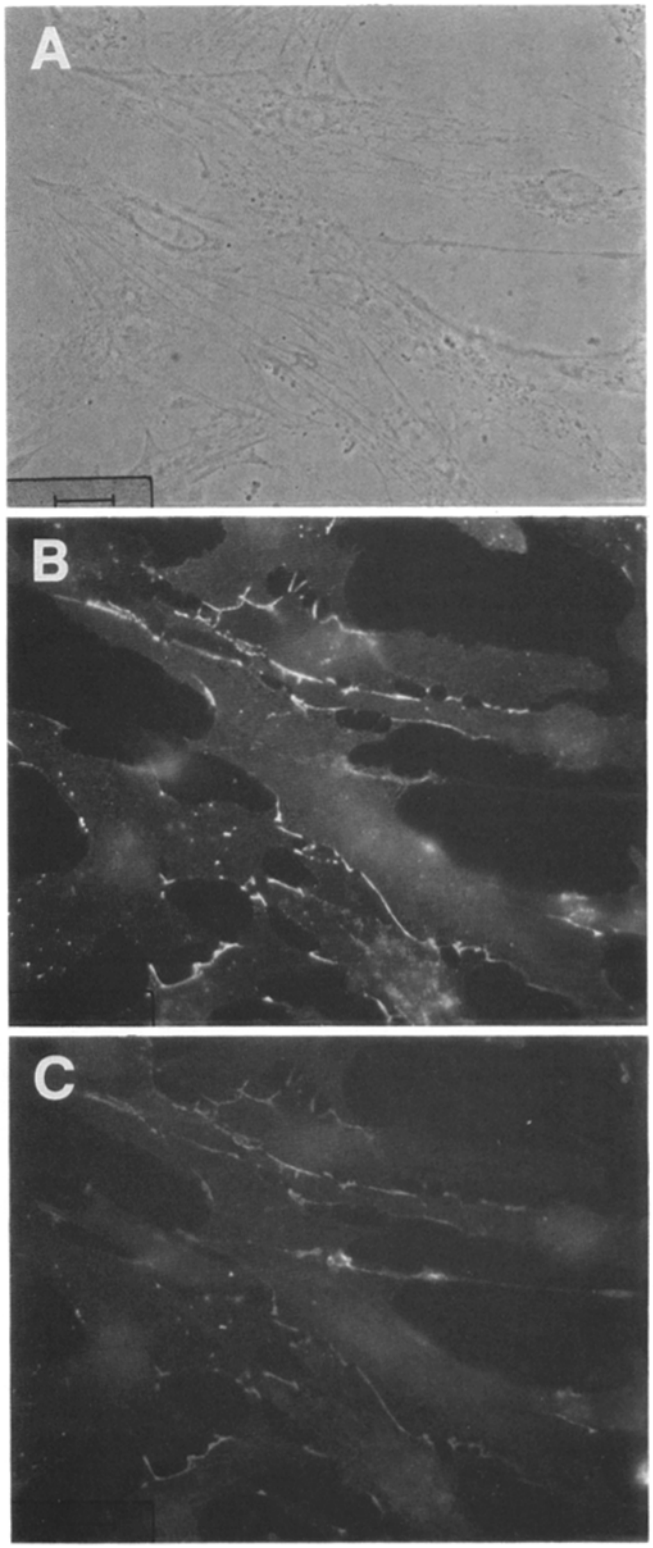

Figure 4. Immunofluorescence colocalization of N-cadherin and the *armadillo*-like protein. Cardiac myocytes were prepared from the hearts of 11-d chick embryos and grown for 48 h on glass coverslips. The cells were fixed with cold methanol, blocked with 10% goat serum, and exposed simultaneously to rat anti-N-cadherin (1:10 dilution of nude mouse ascites) and rabbit anti-*Drosophila armadillo* protein antiserum (1:10). The primary antibodies were detected by rhodamine-conjugated goat anti-rat IgG (1:50) and FITC-conjugated goat anti-rabbit IgG (1:100). *A*, phase-contrast; *B*, anti-N-cadherin; *C*, anti-*armadillo*. Bar, 15 μm.

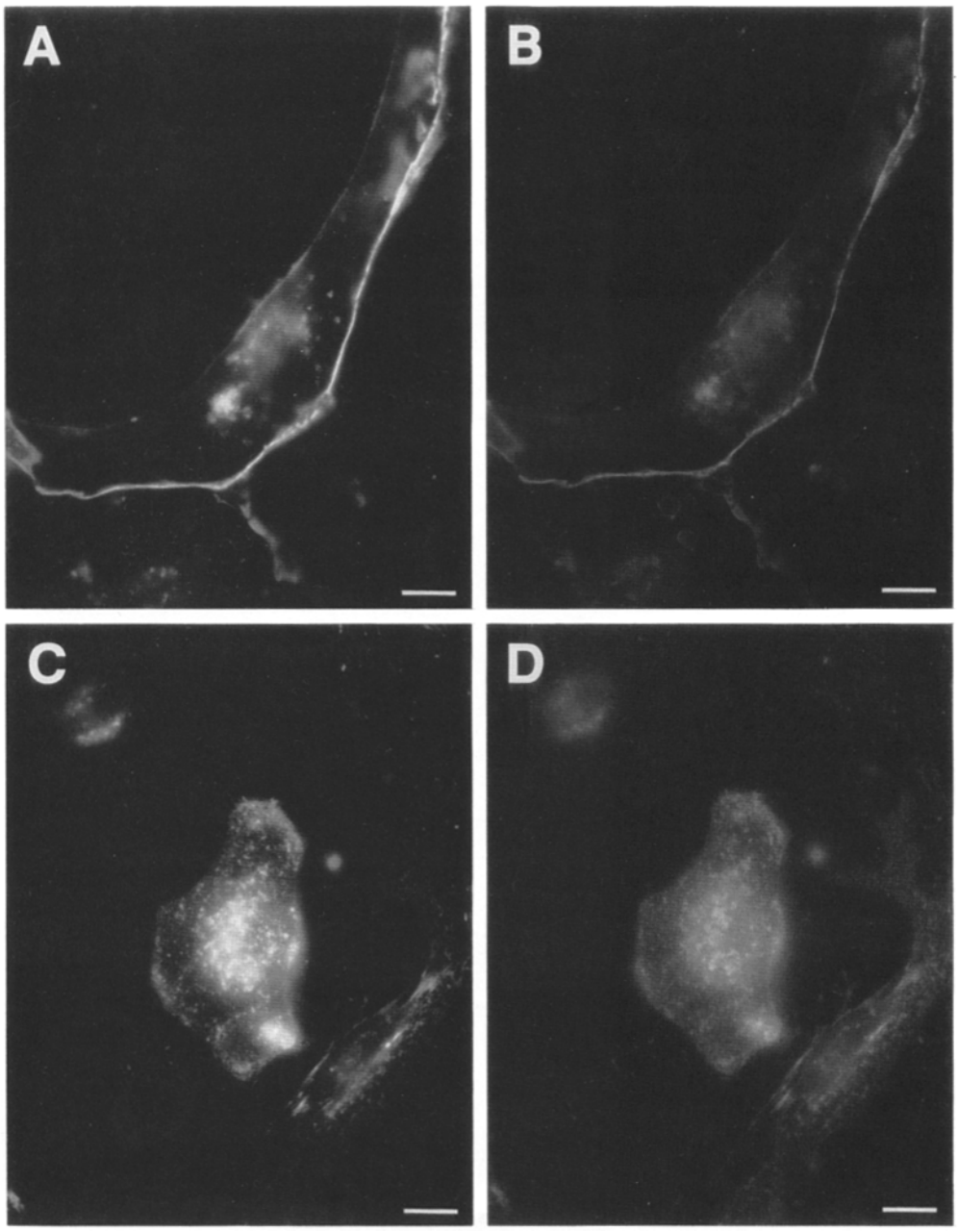

Figure 5. Immunofluorescence colocalization of anti-N-cadherin and antiplakoglobin before and after N-cadherin patching on live cardiac cells. Cardiac myocytes were prepared from the hearts of 11-d chick embryos and grown for 48 h on glass coverslips. Live cells were exposed either to preimmune serum (*A* and *B*) or to rabbit anti-N-cadherin (1:40) (*C* and *D*) for 2 h at 37°C to patch N-cadherin. The cells were then washed thoroughly with PBS, fixed with cold methanol, and simultaneously exposed to affinity purified anti-N-cadherin (estimated to be <1 μg/ml) plus a mouse mAb to plakoglobin (20 μg/ml). The primary antibodies were detected with rhodamine-conjugated goat anti-rabbit IgG (1:50) and FITC-conjugated goat anti-mouse IgG (1:100). (*A* and *B*) unpatched cells; (*C* and *D*) patched cells. (*A* and *C*) anti-N-cadherin; (*B* and *D*) antiplakoglobin. The plane of focus in *A* and *B* is on cell–cell contacts, whereas the plane of focus in *C* and *D* is on N-cadherin patches. Bar, 10 μm.

at both desmosomes and nondesmosomal regions of cell–cell contact.

To provide further evidence that N- and E-cadherin are physically linked to protein(s) recognized by antiplakoglobin, we experimentally redistributed N- and E-cadherin on the surface of cells and determined what effect this had on the pattern of antiplakoglobin staining. To redistribute cadherins we added specific anticadherin antibodies to live cells for 2 h. In this period of time no changes in cell morphology were noted. N-cadherin was patched by adding anti-N-cadherin to live cardiac cells (Fig. 5 *C*), whereas E-cadherin was patched by adding anti-E-cadherin to live epithelial cells (Fig. 6 *C*). The cells were then fixed for immunofluorescence microscopy. Protein recognized by antiplakoglobin was detected in the N-cadherin patches on cardiac cells (Fig. 5 *D*) and in E-cadherin patches on epithelial cells (Fig. 6 *D*). Antiplakoglobin staining was also observed at regions of cell–cell contact (Fig. 6, *E*), as was E- and N-cadherin (data not shown), indicating that not all the cadherin or protein recognized by antiplakoglobin was patched. The coincident redistribution of cadherin with protein(s) recognized by antiplakoglobin suggests a physical linkage between these proteins.

The anticadherin-induced redistribution of antiplakoglobin staining did not appear to result from a nonspecific movement of plasma membrane-associated proteins. For example, the pattern of expression of N-CAM in cardiac cells was not altered by anti-N-cadherin-induced patching of N-cadherin (data not shown). In addition, the pattern of expression of two desmosomal proteins, desmoplakin (an intracellular protein) and desmoglein (a transmembrane protein), was not altered by anti-E-cadherin (Fig. 6). Neither

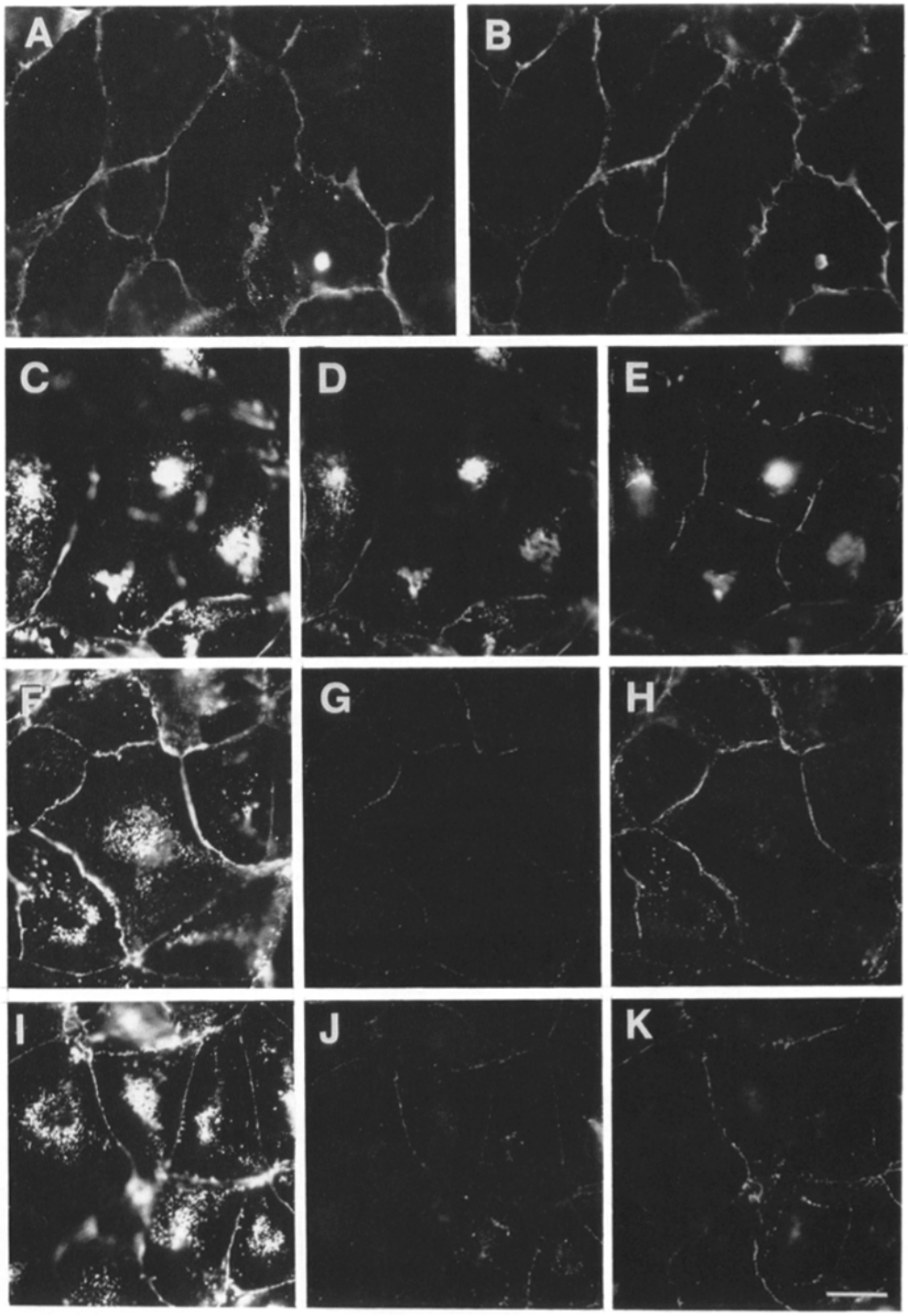

Figure 6. Immunofluorescence colocalization of anti-E-cadherin and antiplakoglobin before and after patching E-cadherin on live epithelial cells. Human epithelial JAR cells were grown on glass coverslips and exposed to normal rabbit serum (*A*,*B*) or rabbit anti-E-cadherin (1:50) (*C–K*) for 2 h at 37°C to patch E-cadherin. The cells in *A* and *B* (unpatched) were washed thoroughly, fixed with cold methanol, and simultaneously exposed to rat monoclonal anti-E-cadherin supernate (1:10) plus mouse antiplakoglobin (5 μg/ml). The cells in *C–K* (patched) were washed, fixed with cold methanol, and exposed to either mouse antiplakoglobin mAb (PG5.1) (5 μg/ml), mouse antidesmoplakin mAb (5 μg/ml), or mouse antidesmoglein mAb (5 μg/ml). Rat and rabbit anti-E-cadherin antibodies were subsequently detected by species-specific, rhodamine-conjugated goat anti-IgG, whereas the mouse antibodies were detected by FITC-conjugated goat anti-(mouse) IgG. (*A* and *B*) unpatched cells; (*C–K*) patched cells. (*A*, *C*, *F* and *I*) anti-E-cadherin; (*D* and *E*) antiplakoglobin; (*G* and *H*) antidesmoplakin; (*J* and *K*) antidesmoglein. The plane of focus in *A*, *B*, *E*, *H*, and *K* is on regions of cell–cell contact, whereas the plane of focus in *C*, *D*, *F*, *G*, *I*, and *J* is on E-cadherin patches. Bar, 12 μm.

desmoplakin (Fig. 6 *G*) nor desmoglein (Fig. 6 *J*) was detected in the E-cadherin patches. (What little fluorescence that was visible in the region of the E-cadherin patch in Fig. 6, *G*, *H*, *J*, and *K* was red and, therefore, due to rhodamine-labeled anti-E-cadherin staining). Rather, desmoplakin (Fig. 6 *H*) and desmoglein (Fig. 6 *K*) remained at sites of cell–cell contact. Thus, the antiplakoglobin staining in the cadherin patches does not occur because desmosomal proteins move nonspecifically into the cadherin patches. Rather, protein recognized by antiplakoglobin appears to move specifically with N- and E-cadherin in living cells, suggesting the E- and N-cadherin interact physically with plakoglobin or a plakoglobin-like protein.

Discussion

Cadherins have been shown to play important roles in the interaction of cells during the development and maintenance of multicellular organisms. Full activity of transmembrane cadherins involves their linkage to the cytoskeleton, with this linkage being essential for optimal adhesive activity and possibly for transmembrane signaling (Nagafuchi and Takeichi, 1988; Jaffe et al., 1990). Thus, a complete understanding of the roles cadherins play in multicellular organisms will include an understanding of the molecular mechanisms responsible for connecting cadherins to the cytoskeletons.

Two lines of evidence initially led to the suggestion that

cadherins, such as E- and N-cadherin, are part of a transmembrane complex that interacts with the actin cytoskeleton. First, the cadherins colocalized with known cytoskeleton-associated proteins, including vinculin and α-actinin, at sites where the membrane associates with microfilaments (Volk and Geiger, 1984; Hirano et al., 1987). Second, when solubilized and immunoprecipitated, E-, P-, and N-cadherin associated with at least three intracellular proteins with molecular masses in the regions of 100, 90, and 80 kD, referred to as α-, β-, and γ-catenin, respectively (Wheelock and Knudsen, 1991*b*; reviewed by Magee and Buxton, 1991). Co-immunoprecipitation and colocalization of intracellular proteins with transmembrane cadherins have been taken as evidence supporting a close physical interaction between cadherins and particular intracellular proteins.

Nagafuchi et al. (1991) and Herrenknecht et al. (1991) have presented evidence that a 102-kD protein (CAP 102) co-immunoprecipitates and colocalizes with both E- and N-cadherin and is the protein referred to as α-catenin. These data, along with CAP 102's amino acid sequence homology to the cytoskeleton-associated protein vinculin (Nagafuchi et al., 1991), provide strong evidence that this protein interacts with the conserved cytoplasmic domains of E- and N-cadherin and participates in the cadherin–cytoskeleton linkage at regions of cell–cell adhesion. In addition, the data from McCrea et al. (1991), together with those presented here, indicate that a 92-kD *armadillo*-like protein also co-immunoprecipitates and colocalizes with E- and N-cadherin. This protein, referred to as β-catenin, likely participates with α-catenin in linking cadherins to the cytoskeleton.

McCrea et al. (1991) suggested that plakoglobin was the homologue of the 92-kD *armadillo*-like β-catenin they characterized in *Xenopus*. However, our data indicate that plakoglobin is distinct from the 92-kD *armadillo*-like protein and, moreover, that two separate members of a plakoglobin family interact with E- and N-cadherin. We show that an 83-kD plakoglobin-like protein(s) co-immunoprecipitates with solubilized E- and N-cadherin and colocalizes with both cadherins in fixed cells. Furthermore, protein recognized by antiplakoglobin moves coincidently with the cadherins in living cells, providing additional evidence for a physical linkage between the two proteins. The 83-kD protein is immunologically distinct from a cadherin-associated 92-kD protein recognized by antibodies to the *Drosophila armadillo* protein, even though the *armadillo* protein shares amino acid sequence homology with plakoglobin. Because of its molecular weight, we suggest that the 83-kD protein represents γ-catenin.

The 83-kD cadherin-associated protein may be plakoglobin or it may be a plakoglobin homolog. Plakoglobin has been reported to localize to regions of cell–cell contact involving both desmosomes and cadherin-containing *zonula* and *fascia adherens* junctions and has been postulated to be involved in anchoring proteins of the plasma membrane to both microfilaments and intermediate filaments (Cowin et al., 1986). Using the same antiplakoglobin mAb described by Cowin et al. (1986), we show here that 83-kD immunocrossreactive protein(s) associates physically with E- and N-cadherin. The evidence that the cadherin-associated protein is actually plakoglobin includes both its molecular mass and its immunocross-reactivity with the antiplakoglobin mAb. In addition, we have determined that the pI of the denatured 83-kD catenin is 6.1 (data not shown), similar to the reported pI of denatured plakoglobin (Kapprell et al., 1990). More definitive proof that the 83-kD cadherin-associated protein is actually plakoglobin would require amino acid sequence information. Although we have attempted to obtain such information, the limited amount of protein that co-immunoprecipitates with the cadherins has precluded us from doing so. It is possible, of course, that the antiplakoglobin antibody detects either isoforms or highly related proteins. Both multiple antibody-binding proteins and more than one mRNA have been reported (Cowin et al., 1986; Franke et al., 1989).

Precisely how the three intracellular proteins (the vinculin-like α-catenin, the *armadillo*-like β-catenin, and plakoglobin or the plakoglobin-like γ-catenin) interact with E- and N-cadherin is not fully understood. Ozawa and Kemler (1992) have suggested that the E-cadherin complex is composed of one molecule of E-cadherin, one or two molecules of the β-catenin, and one molecule of α-catenin. Furthermore, their data indicate that CAP 102 can be released from E-cadherin immunoprecipitates under conditions where β-catenin remains associated with E-cadherin. This suggests that β-catenin interacts directly with E-cadherin, whereas α-catenin has either a weaker interaction with E-cadherin or, possibly, associates indirectly with E-cadherin via β-catenin. Since the *armadillo* protein and plakoglobin share considerable amino acid sequence homology, the two cadherin-associated members of the plakoglobin family may share a similar binding site on the intracellular domains of E- and N-cadherin.

If the 83-kD cadherin-associated protein is plakoglobin, its interaction with E- and N-cadherin may be similar or different in mechanism from its interaction with desmogleins. Desmogleins have a much larger cytoplasmic peptide domain than E- or N-cadherin (Nilles et al., 1991; Wheeler et al., 1991). They do, however, share amino acid sequence homology in the intracellular C1 subdomain (Nilles et al., 1991), suggesting a possible site for plakoglobin to interact with both desmogleins and cadherins. It is also possible that separate binding sites for plakoglobin are present on E/N-cadherin and desmogleins, since the association of plakoglobin with desmogleins has been suggested to involve disulfide bonding (Korman et al., 1989). The cytoplasmic domain of desmoglein I is rich in cysteine (Nilles et al., 1991), whereas the intracellular domains of E- and N-cadherin contain no cysteine.

A number of intracellular proteins known either to colocalize with E- or N-cadherin, to be present at cell–cell junctions, or to bridge the actin cytoskeleton and the plasma membrane are not detected in E- or N-cadherin immunoprecipitates, suggesting these proteins are not closely associated with E- or N-cadherin. They may not interact in any way with the cadherins. Alternatively, they may interact indirectly with the cadherins via CAP 102 (α-catenin), the *armadillo*-like protein (β-catenin), or the plakoglobin-like protein (γ-catenin). Such proteins include α-actinin, vinculin, zyxin, ezrin, and moesin. Our data initially suggested that ezrin might interact weakly with the N-cadherin complex, since a high titer antiserum to ezrin faintly detected a band of the appropriate molecular weight in the N-cadherin immunoprecipitate. However, two other antibodies failed to detect ezrin in the immunoprecipitate and, in addition, ezrin failed to colocalize with N-cadherin in cells.

In summary, our data show that plakoglobin, or a plakoglobin homolog, joins the vinculin-like α-catenin and the *armadillo*-like β-catenin as an E- and N-cadherin–associated protein. Together, the three intracellular proteins likely function to anchor E-cadherin, N-cadherin, and possibly other cadherins with homologous intracellular domains to the actin-based cytoskeleton. Exactly how the three catenins interact with E- and N-cadherin, and/or with each other, remains to be determined and is of future interest to our laboratories.

The authors wish to thank Karen Frutiger, Trudi St. Clair, Sheila McElwee, Lisa Young, and Linda Myers for technical assistance. We are grateful also to our many colleagues who so generously provided us with their specific antibodies.

This work was supported by grants CA 44464 and AR 37945 from the National Institutes of Health to M. J. Wheelock and K. A. Knudsen, respectively, and by grants from the National Science Foundation (DIR-8804006) and the Ohio Chapter of the American Heart Association to M. J. Wheelock and from The W. W. Smith Charitable Trust to K. A. Knudsen.

Received for publication 4 March 1992 and in revised form 22 May 1992.

Note Added in Proof. Since the submission of this paper we learned of similar results concerning the interaction of plakoglobin with E-cadherin (M. Peifer et al., 1992).

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