

A 220-kD Undercoat-constitutive Protein: Its Specific Localization at Cadherin-based Cell-Cell Adhesion Sites

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Abstract. Recently we developed an isolation procedure for the cell-to-cell adherens junctions (AJ; cadherin-based junctions) from rat liver (Tsukita, Sh. and Sa. Tsukita. 1989. *J. Cell Biol.* 108:31-41). In this study, using the isolated AJ, we have obtained two mAbs specific to the 220-kD undercoat-constitutive protein. Immunofluorescence and immunoelectron microscopy with these mAbs showed that this 220-kD protein was highly concentrated at the undercoat of cell-to-cell AJ in various types of tissues and that this protein was located in the immediate vicinity of the plasma membrane in the undercoat of AJ. In the cells lacking typical cell-to-cell AJ, such as fibroblasts, the 220-kD protein was immunofluorescently shown to be coconcentrated with cadherin molecules at cell-cell adhesion sites. These localization analyses appeared to indicate the possible direct or indirect association of the 220-kD protein with cadherin molecules. Further-

more, it was revealed that the 220-kD protein and α -spectrin were coimmunoprecipitated with the above mAbs in both the isolated AJ and the brain. The affinity-purified 220-kD protein molecule looked like a spherical particle, and its binding site on the spectrin molecule was shown to be in the position ~ 10 - 20 nm from the midpoint of spectrin tetramer by low-angle rotary-shadowing electron microscopy. Taking all these results together with biochemical and immunological comparisons, we are persuaded to speculate that the 220-kD protein is a novel member of the ankyrin family. However, the possibility cannot be excluded that the 220-kD protein is an isoform of β -spectrin. The possible roles of this 220-kD protein in the association of cadherin molecules with the spectrin-based membrane skeletons at the cadherin-based cell-cell adhesion sites are discussed.

THE adherens junction (AJ)¹ is a site of cell contact which may play an important role in cell differentiation, growth, and transformation (Geiger, 1983; Burridge et al., 1988; Tsukita et al., 1990). AJ can be categorized into two types: cell-to-cell and cell-to-substrate (Geiger et al., 1985), in which the respective adhesion molecules are cadherin (E-cadherin/uvomorulin, P-cadherin/A-cell adhesion molecule [CAM], P-cadherin, and L-CAM) (Boller et al., 1985; Volk and Geiger, 1986; Gallin et al., 1987; Nagafuchi et al., 1987; Nose et al., 1987; Ringwald et al., 1987; Hatta et al., 1988; Takeichi, 1988, 1990) and integrin (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). In both types of AJ, actin filaments are associated with the plasma membrane through its well-developed undercoat (Farquhar and Palade, 1963; Staehelin, 1974; Geiger et al., 1984; Geiger, 1989). Recently, the molecular architecture of the undercoat of AJ has attracted increasing interest. Some unique proteins have been demonstrated to be concentrated in the

undercoat of AJ, mainly by immunohistochemical analyses: vinculin (Geiger, 1979), α -actinin (Lazarides and Burridge, 1975), filamin (Langanger et al., 1984), and 82-kD proteins (Beckerle, 1986) in both types of AJ, plakoglobin (Cowin et al., 1986) in the cell-to-cell type, and talin (Burridge and Connell, 1983), paxillin (Turner et al., 1990), and fimbrin (Bretscher and Weber, 1980) in the cell-to-substrate type. Recently some regulatory enzymes such as calcium-dependent protease (type II) (Beckerle et al., 1987), C-kinase (type 3) (Jaken et al., 1989), and *c-yes* and *c-src* kinases (Tsukita et al., 1991) have been reported to be concentrated at the undercoat of AJ; these are thought to be important for signal transduction. However, our knowledge is still limited as to how the undercoat regulates cell-to-cell and cell-to-substrate adhesions and how it participates in the signal transduction from the adhesion molecules to the nucleus. To answer these questions, it is prerequisite to clarify the whole picture of the molecular architecture of the undercoat of AJ, i.e., to clarify the molecular linkage from the adhesion molecule (cadherins or integrins) to the actin filaments in the undercoat of AJ.

Recently, we have developed a method to isolate cell-to-

1. *Abbreviations used in this paper:* AJ, adherens junction; CAP, cadherin-associated protein; MDBK, Madin-Darby bovine kidney; pAb, polyclonal antibody.

cell AJ from liver cells and introduced a way to systematically analyze its molecular organization (Tsukita and Tsukita, 1989). When the isolated AJ was dialyzed against a low-salt alkaline solution, most of the undercoat-constitutive proteins were effectively extracted, leaving the "AJ membrane" fraction. The "AJ extract" was mainly composed of 10 polypeptides including vinculin, α -actinin, and actin. Using AJ extract, two novel AJ undercoat-constitutive proteins, radixin (82 kD) and tenuin (400 kD), have been identified and characterized (Tsukita et al., 1989*a,b*; Sato et al., 1991). Most recently, we have found that some types of undercoat-constitutive proteins such as CAP102 (102-kD cadherin-associated protein) are rather resistant to the low-salt alkaline treatment, so that they are left on the AJ membrane as major constituents (Nagafuchi et al., 1991). Considering that CAP102 is tightly bound to the cytoplasmic domain of cadherin molecules, it is tempting to speculate that these undercoat-constitutive proteins that are resistant to low-salt extraction include some key proteins important for the cadherin-actin association. Thus, we have attempted to analyze these low-salt extraction-resistant undercoat-constitutive proteins on the AJ membranes in detail. For this purpose, we generated many mAbs using the AJ membrane as an antigen.

In this study, we have focused on two mAbs specific for the 220-kD undercoat-constitutive protein, one component of the AJ membrane fraction. This 220-kD protein was highly concentrated not only at the undercoat of the cell-to-cell AJ in various types of AJ-bearing cells, but also at the cadherin-based cell-cell adhesion sites in the cells lacking the typical cell-to-cell AJ such as fibroblasts. Immunoelectron microscopy with ultrathin cryosections has revealed that this protein is located in the immediate vicinity of the cytoplasmic surface of the membrane in the undercoat of AJ. Furthermore, we have demonstrated that the purified 220-kD protein is tightly associated with spectrin molecules at the position \sim 10–20 nm from the midpoint of the spectrin tetramer. These data suggest that the 220-kD protein may be a novel member of the ankyrin family, but the possibility cannot be excluded that it is an isoform of β -spectrin. The possible roles of this unique molecule in the association between cadherin molecules and actin-based cytoskeletons are discussed.

Materials and Methods

Cells and Antibodies

Rat 3Y1 cells were a generous gift from Dr. Chikako Sato (Aichi Cancer Center Research Institute, Aichi, Japan), and Madin-Darby bovine kidney (MDBK) cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Rat epidermal keratinocytes were primarily cultured from newborn rats according to the method described previously (Green et al., 1987; O'Keefe et al., 1987). All these cells were cultured in DME supplemented with 10% FCS.

Antisera against mouse E-cadherin and P-cadherin were generous gifts from Dr. Masatoshi Takeichi (Kyoto University, Kyoto, Japan). Anti-mouse vinculin polyclonal antibody (pAb) was obtained as described previously, and anti-chicken vinculin mAb was purchased from Sigma Chemical Co. (St. Louis, MO). Antiserum against the α -chain of chicken erythrocyte spectrin, which can specifically recognize rat nonerythrocyte α -spectrin, was purchased from Chemicon International Inc. (Temecula, CA). Antiserum against human erythrocyte ankyrin, which can recognize some types of rat brain ankyrin, was generously provided by Dr. V. Bennett (Duke University, Durham, NC).

Isolated AJ, Low-Salt Extract of AJ, and AJ Membrane Fraction

The fraction rich in AJ was obtained from the liver of 7–9-w-old rats through crude membrane and bile canaliculus fractions, as described previously (Tsukita and Tsukita, 1989). To extract most of the undercoat-constitutive proteins from the isolated AJ, the isolated AJ was dialyzed against a low-salt extraction solution (1 mM EGTA, 0.5 mM PMSF, 1 μ g/ml leupeptin, and 2 mM Tris-HCl [pH 9.2]), followed by centrifugation at 100,000 *g* for 1 h. After repeating the low-salt extraction three times, the AJ membrane fraction was obtained as the remnant precipitate. This AJ membrane fraction is composed of the tightly bound AJ undercoat-constitutive proteins and the membrane proper, including the integral membrane proteins; the tightly bound AJ undercoat-constitutive proteins can be selectively extracted by treating the AJ membrane fraction with 1 M acetic acid (pH 2.3) as described previously (Nagafuchi et al., 1991).

To confirm that a protein in the isolated AJ is not an integral membrane protein but an undercoat-constitutive protein, we usually check whether the protein can be extracted from the bile canaliculus fraction by treatment with 1 M acetic acid (pH 2.3) (without nonionic detergent). Since AJ was isolated from the bile canaliculus fraction using 0.1% NP-40, the AJ fraction itself is not appropriate for this kind of assessment.

mAb Production

mAbs were obtained essentially according to the procedure of Köhler et al. (1980). Eight BALB/c mice were immunized with the AJ membrane fraction as follows: day 1, intraperitoneally in complete Freund's adjuvant; day 14, intraperitoneally in complete Freund's adjuvant; day 28, intraperitoneally without adjuvant. 3 d after final injection, the spleen was removed and the splenocytes were fused with mouse P3 myeloma cells. 50% polyethylene glycol (PEG4000; E. Merck, Darmstadt, Germany) in RPMI-1640 was used as the fusogen. The initial fusion products were plated in four 24-well plates in hypoxanthine/aminopterin/thymidine medium. 6 d after fusion, fusion plates were screened for antibody production on an ELISA. The AJ membrane fraction was used for the ELISA.

The wells that showed positive activity were immediately expanded and then plated out at clonal density (1.2 cell/well) in a 96-well dish together with feeder cells (thymus lymphocytes). 1 wk after cloning, wells with a single clone were tested for antibody with a double screening of the ELISA and the immunoblotting analysis using the AJ membrane fraction. The clones that produced antibodies positive in both the ELISA and the immunoblotting assay were expanded, frozen, and stored in liquid nitrogen.

Nonionic Detergent Treatment of Cultured 3Y1 Cells

To extract the "soluble" form of cadherins from 3Y1 cells, the cells cultured on a 10-cm dish or on cover glasses were incubated with Hepes-buffered Mg^{2+} -free solution (150 mM NaCl, 1 mM $CaCl_2$, and 10 mM Hepes [pH 7.5]) containing 0.5% NP-40 at room temperature for 30 min (Nagafuchi and Takeichi, 1988). For immunoblotting analyses, the extracted cells on a 10-cm dish were collected into a microtube (Eppendorf Inc., Fremont, CA) by scraping with a rubber policeman, together with the extract containing NP-40, and then centrifuged at 100,000 rpm for 30 min. To the supernatant, 2 \times SDS sample buffer was added to make the total volume 250 μ l; this sample was then used as the detergent-soluble fraction. On the other hand, the pellet fraction was dissolved in 250 μ l of 1 \times SDS sample buffer, and this sample was then used as the detergent-insoluble fraction. For immunofluorescence microscopy, the extracted cells on cover glasses were washed with HMF three times and fixed with 1% formaldehyde in PBS for 15 min, and then processed for indirect immunofluorescence microscopy.

Gel Electrophoresis and Immunoblotting

Proteins were separated by the one-dimensional SDS-PAGE or the two-dimensional NEPHGE method. SDS-PAGE was based on the discontinuous Tris-glycine system of Laemmli (1970). For NEPHGE, slab gel electrophoresis was used (O'Farrell, 1975). The stain Coomassie brilliant blue R-250 or silver staining (silver staining kit; Wako, Osaka, Japan) was used.

Immunoblotting was performed by one- or two-dimensional electrophoresis, followed by electrophoretic transfer to nitrocellulose sheets. Nitrocellulose sheets were treated with the first antibody followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA), and the localization of peroxidase was detected by enhanced chemiluminescence system (Amersham International PLC, Amersham, UK).

Immunofluorescence Microscopy

For indirect immunofluorescence microscopy of frozen sections, samples were frozen using liquid nitrogen and the frozen sections ($\sim 5 \mu\text{m}$) were cut in a cryostat, mounted on glass slides, air-dried, and fixed in 95% ethanol at 4°C for 30 min and in 100% acetone at room temperature for 1 min. After being rinsed in PBS (150 mM NaCl, 10 mM phosphate buffer [pH 7.5]) containing 1% BSA for 15 min, the sections were incubated with a mixture of anti-220-kD protein mAb (T8-754) and anti-E-cadherin pAb or with a mixture of mAb T8-754 and anti-vinculin pAb. They were then washed three times with PBS containing 1% BSA and 0.1% Triton X-100, followed by incubation with the mixture of rhodamine-conjugated sheep anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG in PBS/1% BSA/0.1% Triton X-100 for 30 min. After being washed with PBS, they were embedded in 95% glycerol-PBS and examined with a fluorescence microscope, a Zeiss Axiophot photomicroscope (Carl Zeiss, Inc., Thornwood, NY).

For indirect immunofluorescence microscopy of cultured cells, cells were cultured on cover glasses and fixed with 1% formaldehyde in PBS for 15 min. The fixed cells were treated with 0.2% Triton X-100 in PBS for 15 min and washed three times with PBS. After being soaked in PBS containing 1% BSA, the samples were treated with the first antibodies (see above) for 1 h in a moist chamber. The samples were then washed three times with PBS containing 1% BSA, followed by incubation with the second antibodies (see above) for 30 min. After incubation, the samples were washed with PBS three times, embedded in 95% glycerol-PBS, and examined with a Zeiss Axiophot photomicroscope. When the cells extracted with NP-40 before fixation were immunofluorescently stained, the Triton X-100 treatment was omitted.

Immunoelectron Microscopy

Immunoelectron microscopy using ultrathin cryosections was performed essentially according to the method developed by Tokuyasu (1980, 1989) and modified by Keller et al. (1984). Small pieces of rat cardiac muscle were fixed in 4% formaldehyde in 0.1 M Hepes (pH 7.5) for 1 h at room temperature. The fixed samples were infused with 2.0 M sucrose containing 10% polyvinylpyrrolidone at room temperature for 2 h, rapidly frozen using liquid nitrogen, and ultrathin-sectioned in the frozen state using glass knives with an FC-4E low temperature sectioning system (Reichert-Jung). The sectioned samples were collected on formvar-film grids, washed three times with PBS containing 30 mM glycine (PBS-glycine), and incubated with PBS-glycine containing 2% goat serum for 3 min. Then the samples were incubated with anti-220-kD protein mAb (T4-192, IgM) or with a mixture of mAb T4-192 and anti-vinculin mAb (IgG) for 15 min. After being washed with PBS-glycine three times, the samples were blocked with PBS-glycine containing 2% goat serum for 3 min and then incubated with goat anti-mouse IgM coupled to 10 nm gold (GAMIgM G10) or with a mixture of GAMIgM G10 and goat anti-rabbit IgG coupled to 15 nm gold (GAMIgG G15; Janssen Life Science Products, Piscataway, NJ). After being washed

with PBS and distilled water, the samples were incubated with distilled water containing 2% uranyl acetate for 10 min and then with distilled water containing 2% polyvinylalcohol and 0.2% uranyl acetate for 10 min, air-dried, and examined in an electron microscope (1200 EX; JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV.

Immunoprecipitation of AJ Extract with Anti-220-kD Protein mAb and Anti- α -Spectrin pAb

Immunoprecipitation of the low-salt extract of isolated AJ with anti-220-kD protein mAb (T8-754) was performed by the use of Sepharose-bound mAb T8-754. IgG was purified from ascites of mAb T8-754 using a MAPS kit (Bio-Rad Laboratories) and coupled to a CNBr-Sepharose 4B column. The low-salt extract of isolated AJ was applied onto this column and washed extensively with 1 M KCl in DG solution (0.1 mM DTT/1 mM EGTA/10 mM Hepes [pH 7.5]/0.5 mM PMSF/1 $\mu\text{g}/\text{ml}$ leupeptin) and then with DG solution. The bound proteins including the 220-kD protein were eluted with 3 M MgCl_2 in DG solution, and the eluates were subjected to SDS-PAGE. For a control experiment, instead of the mAb T8-754 IgG, nonimmune mouse IgG was coupled to a CNBr-Sepharose 4B column.

The low-salt extract of isolated AJ was mixed and incubated with anti- α -spectrin pAb or with nonimmune rabbit IgG for 2 h at 4°C . Then the protein G-bound Sepharose beads (Pharmacia-LKB, Uppsala, Sweden) were added, followed by incubation for 1 h at 4°C . The beads were collected by centrifugation at 15,000 rpm for 5 min and washed five times with DG solution containing 1 M KCl and once with distilled water. The immunoprecipitants were released from the beads with 1 M acetic acid (pH 2.3), lyophilized, and separated by SDS-PAGE.

Purification of the 220-kD Protein and Spectrin from Rat Brain

The 220-kD protein was purified from rat brain by affinity chromatography on Sepharose-bound mAb T8-754. Rat brain was homogenized with a Waring blender (Waring, New Hartford, CT) in a hypotonic solution (1 mM NaHCO_3 /1 $\mu\text{g}/\text{ml}$ leupeptin [pH 8.0]) followed by centrifugation at 100,000 g for 60 min at 4°C . The pellet was resuspended and incubated in a high-salt solution (1 M KCl/1 mM EGTA/0.1 mM DTT/1 $\mu\text{g}/\text{ml}$ leupeptin/0.5 mM PMSF/10 mM Hepes [pH 7.5]) for 1 h at 4°C , and then centrifuged at 100,000 g for 90 min. The supernatant was applied onto the affinity column and washed extensively with 1 M KCl in DG solution (0.1 mM DTT/1 mM EGTA/10 mM Hepes [pH 7.5]/0.5 mM PMSF/1 $\mu\text{g}/\text{ml}$ leupeptin), followed by washing with DG solution. Then the bound proteins were eluted with 2.5 M MgCl_2 , followed by 3 M MgCl_2 . The 2.5 M MgCl_2 eluate contained both brain spectrin and the 220-kD protein, while the 3 M MgCl_2 eluate contained only the 220-kD protein. The purified 220-kD protein was dialyzed against the DGKM solution (DG solution plus 150 mM KCl and 1 mM MgCl_2).

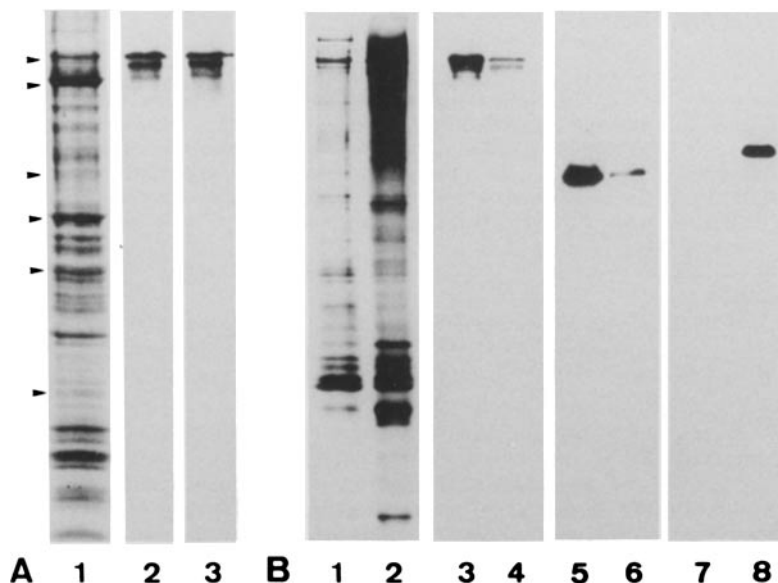


Figure 1. Identification and characterization of the 220-kD protein by immunoblotting. (A) Lane 1, Coomassie blue-stained gel (10%) of AJ membrane; lanes 2 and 3, accompanying immunoblots by the use of mAb T4-192 (IgM) and mAb T8-754 (IgG), respectively. Arrowheads indicate molecular sizes as 240, 200, 130, 95, 70, and 43 kD from the top. Both mAbs recognize the 220-kD band and several lower molecular weight bands, but the increase of these lower molecular weight bands with time suggests that they are proteolytic degradation products. (B) Acetic acid extraction experiments using the bile canalculus fraction. Lanes 1, 3, 5, and 7, acetic acid extract; lanes 2, 4, 6, and 8, extracted bile canalculus membranes. Lanes 1 and 2, Coomassie blue-stained gels (10%); lanes 3–8, accompanying immunoblots with mAb T8-754 (lanes 3 and 4), anti-vinculin mAb (lanes 5 and 6), and anti-E-cadherin pAb (lanes 7 and 8). Note that in sharp contrast to E-cadherin both the 220-kD protein and vinculin are effectively extracted from the bile canalculus fraction.

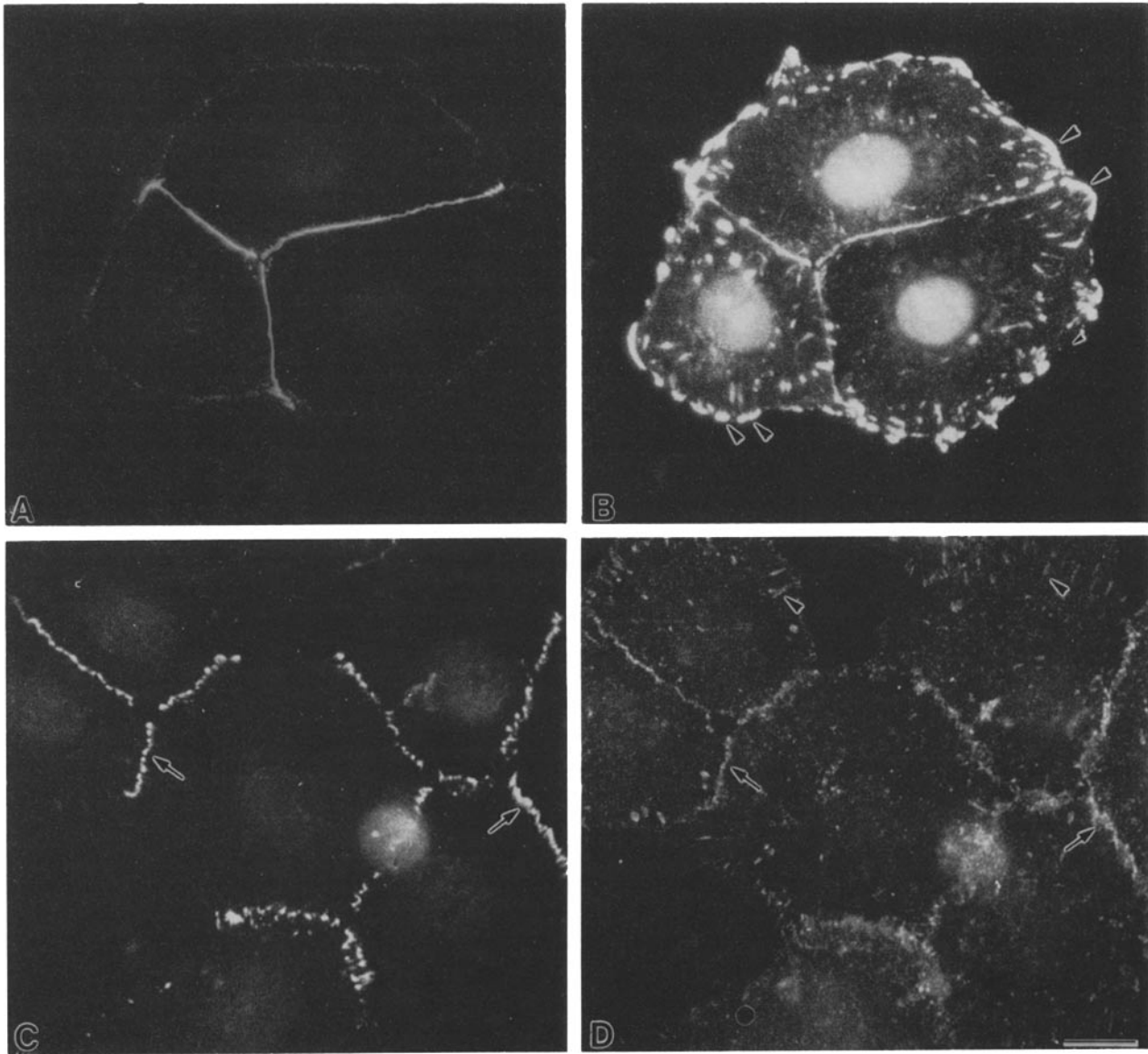
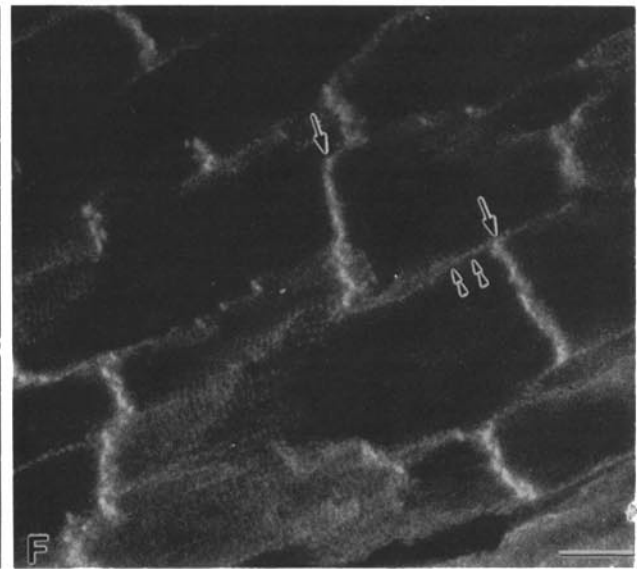
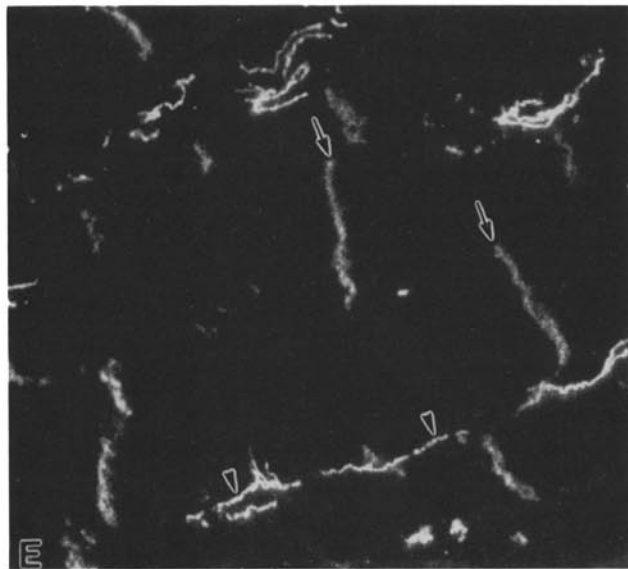
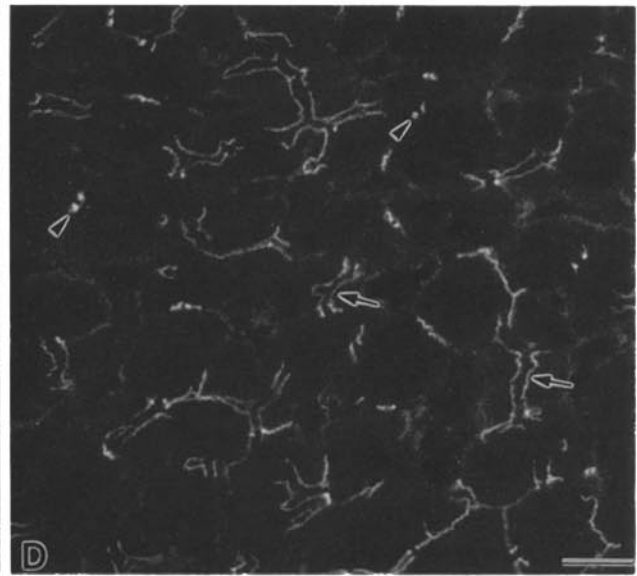
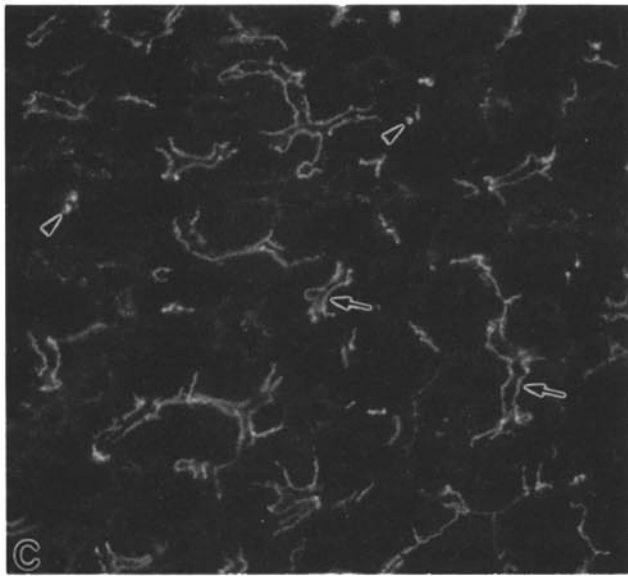
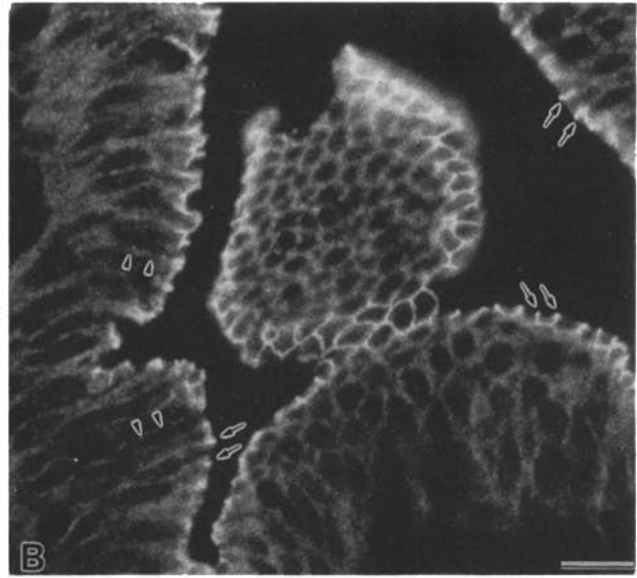
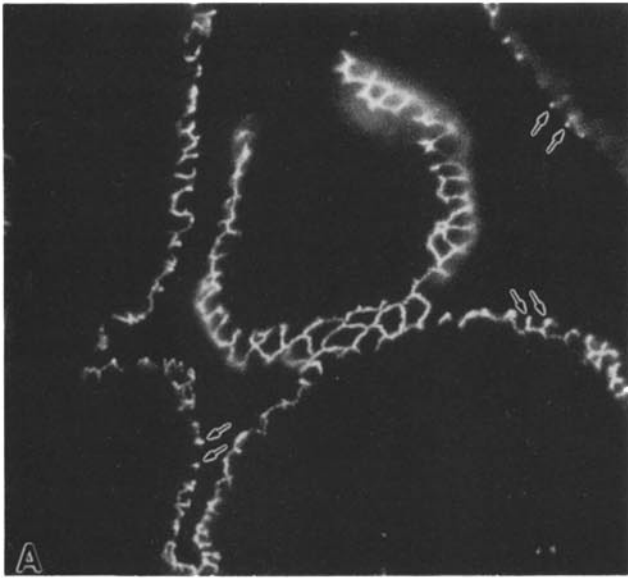


Figure 2. Double staining of MDBK cells (*A* and *B*) and rat keratinocytes (*C* and *D*) with mAb T8-754 (*A* and *C*) and anti-vinculin pAb (*B* and *D*). In both types of cells, the 220-kD protein is highly concentrated at the vinculin-positive cell-to-cell AJ, which is beltlike and spotlike (arrows) in MDBK cells and keratinocytes, respectively. Note that the 220-kD protein staining is not associated with the vinculin-positive cell-to-substrate AJ (arrowheads). Bar, 10 μ m.

Brain spectrin was purified from rat brain mainly according to the method developed by Glenney et al. (1982). Rat brain was homogenized with the solution containing 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM DTT, and 1 mM PMSF, and centrifuged at 100,000 g for 90 min. The pellet was resuspended and stirred in solution A (0.6 M KCl/1 mM DTT/1 mM Tris-HCl [pH 8.0]) for 30 min at 4°C, followed by centrifugation at 100,000 g

for 90 min. Ammonium sulfate was added to the supernatant at a concentration of 0.258 g/ml. It was then stirred for 30 min at 4°C and centrifuged at 100,000 g for 60 min. The pellet was resuspended in solution A and dialyzed against solution A overnight. The sample was centrifuged at 100,000 g for 60 min, and the supernatant obtained was applied onto the Sepharose CL-4B column. The fractions rich in spectrin molecules were combined

Figure 3. Localization of the 220-kD protein, E-cadherin, and vinculin in rat AJ-bearing tissues. (*A* and *B*) Double staining of the frozen section of intestinal epithelium with mAb T8-754 (*A*) and anti-E-cadherin pAb (*B*). Both the 220-kD protein and E-cadherin are concentrated at the cell-to-cell AJ (arrows), but the degree of concentration of the 220-kD protein is much higher than that of E-cadherin; the lateral membrane (arrowheads) of the epithelial cells is clearly stained with anti-E-cadherin pAb but not with mAb T8-754. (*C* and *D*) Double staining of the frozen section of liver with mAb T8-754 (*C*) and anti-vinculin pAb (*D*). In liver, the 220-kD protein and vinculin are colocalized at the beltlike cell-to-cell AJ along the bile canaliculi (arrows, longitudinal-section view; arrowheads, cross-section view). (*E* and *F*) Double staining of the frozen section of heart with mAb T8-754 (*E*) and anti-vinculin pAb (*F*). In heart, the intercalated discs (arrows) are intensely stained with both antibodies. Note that the 220-kD protein staining is also associated with the blood vessels (single arrowheads) and that the patchy labeling of vinculin is detected along the lateral borders of cells (double arrowheads). Bars: (*A–D*) 10 μ m; (*E* and *F*) 5 μ m.



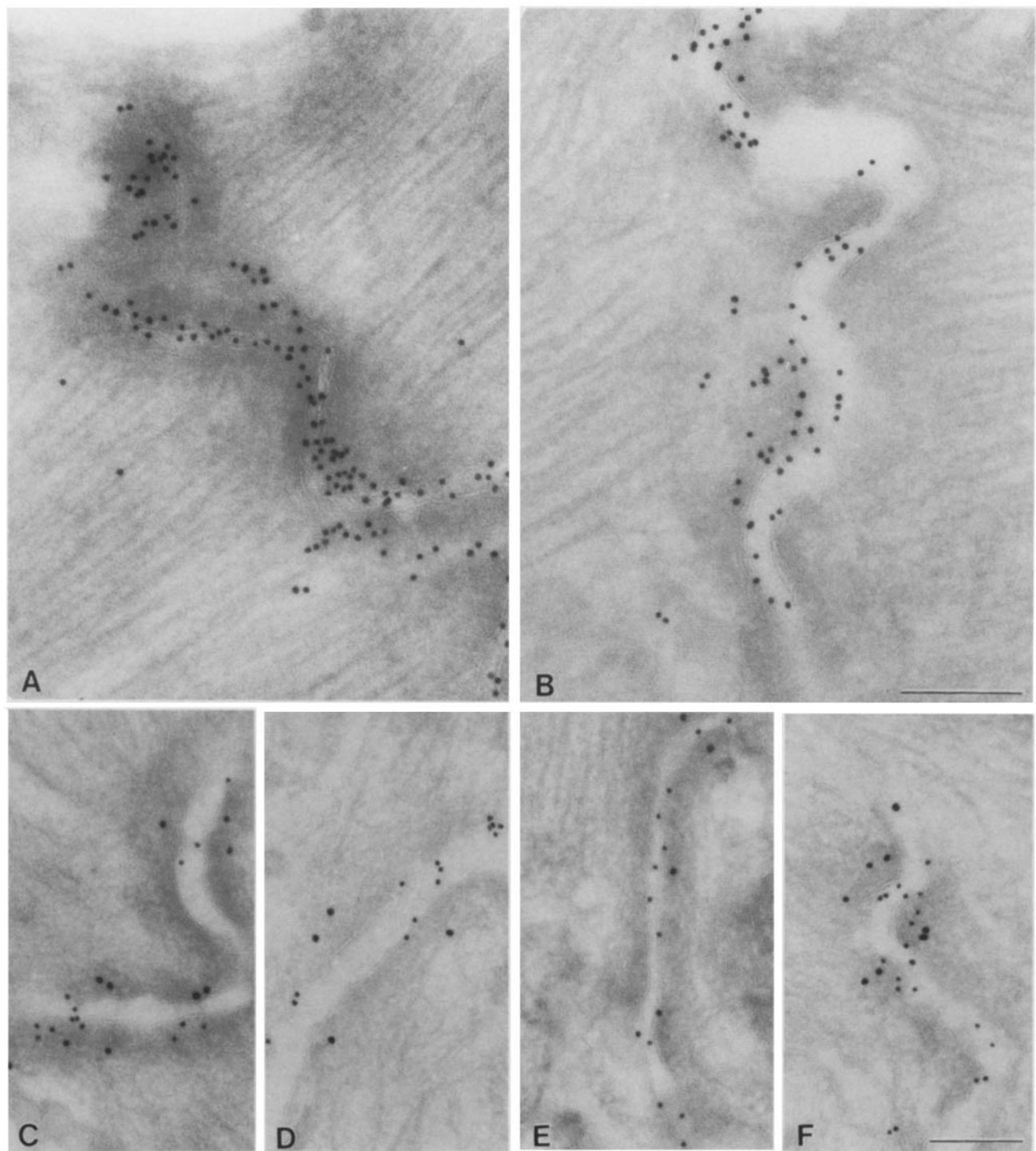


Figure 4. Ultrastructural localization of the 220-kD protein in rat heart. Longitudinal ultrathin cryosections of cardiac muscle cells are labeled singly with mAb T4-192 (*A* and *B*) or doubly with mAb T4-192 (10 nm gold) and anti-vinculin mAb (15 nm gold) (*C-F*). Gold particles accumulate at the cell-to-cell AJ in the intercalated discs. In dual-labeling images, it is clear that the 220-kD protein is concentrated much closer to the membrane than is vinculin (see Fig. 5). Bars, 0.2 μm .

and dialyzed against solution B (60 mM KCl/1 mM MgCl_2 /0.1 mM CaCl_2 /0.1 mM DTT/10 mM Hepes [pH 7.5]) overnight, followed by centrifugation at 100,000 g for 30 min. Brain spectrin molecules were recovered in the supernatant.

Low-Angle Rotary-shadowing Electron Microscopy

The molecular shape of the purified 220-kD protein and its manner of binding with brain spectrin were analyzed by low-angle rotary-shadowing electron microscopy, mainly according to the method developed by Tyler and

Branton (1981). The samples observed in this experiment were as follows: (*a*) purified 220-kD protein ($\sim 10 \mu\text{g/ml}$ in DGKM solution), (*b*) purified brain spectrin ($\sim 100 \mu\text{g/ml}$ in the DGKM solution), and (*c*) 2:1 (vol/vol) mixture of *a* and *b* (incubated at room temperature for 30 min). After the equivolume of glycerol was added to these samples, the samples were sprayed onto freshly cleaved mica. The droplets on the mica were dried at room temperature in a vacuum (1×10^{-5} torr) in freeze-etch equipment (Balzers 301) for 10 min. Platinum was then rotary-shadowed at an angle of 1° , followed by coating from above with carbon. The replica was floated off on distilled water and picked up on a formvar-film grid. The samples

were examined in an electron microscope (1200 EX; JEOL) at an accelerating voltage of 100 kV.

Results

Production of mAbs Specific for a 220-kD Undercoat-constitutive Protein of the Isolated AJ

As previously reported, when the isolated AJ was dialyzed against the low-salt alkaline solution, most of its undercoat-constitutive proteins were extracted, leaving the AJ membrane fraction (Tsukita and Tsukita, 1989). This fraction is composed of lipids, the membrane integral proteins, and the undercoat-constitutive peripheral proteins which are tightly associated with the membrane proper. Using this AJ membrane fraction as an antigen in eight mice, we have attempted to raise mAbs that can recognize each constituent of this fraction. As a result, 1,000 clones were obtained and frozen. In this study we have picked up two clones (T4-192 and T8-754), both of which recognize the same antigen; the affinity-purified antigen using mAb T8-754 (see Fig. 9) was specifically recognized by mAb T4-192 (data not shown). Using these mAbs, we have analyzed the *in vivo* distribution and the *in vitro* properties of this antigen. mAbs T4-192 and T8-754 were found to be IgM and IgG classes, respectively.

As shown in Fig. 1 *A*, these mAbs specifically recognized a high molecular weight protein in the AJ membrane fraction by immunoblotting. They also blotted several lower molecular weight bands rather weakly, but the increase of these weakly blotted bands with time suggested that they were proteolytic degradation products. As far as we investigated, no protease inhibitors were effective to inhibit this degradation, suggesting that this protein might be highly susceptible to proteolytic degradation. Although it is difficult to precisely determine the molecular mass of this antigen by SDS-PAGE, we designate it the 220-kD protein in this paper, mainly because of its identical mobility to ankyrin in 4% polyacrylamide gel (see Fig. 11).

First, to check whether this 220-kD protein is a membrane-integral protein or an undercoat-constitutive peripheral protein, we have examined the extractability of this protein from the membrane fractions (the bile canaliculi fraction). Since the 220-kD protein was rather resistant to the low-salt alkaline extraction, the bile canaliculi fraction was treated with 1 M acetic acid (pH 2.3) at 4°C for 30 min (Fig. 1 *B*). With this acid treatment, the 220-kD protein was effectively extracted together with other membrane peripheral proteins such as vinculin, while E-cadherins were by no means extracted. Thus, we have concluded that the 220-kD protein is not a membrane-integral protein but a membrane-peripheral protein. Neither of the mAbs specific for the 220-kD protein immunofluorescently stained the nonpermeabilized cultured cells, indicating that the 220-kD protein is not an extracellular but an intracellular protein (data not shown).

Immunofluorescence and Immunoelectron Microscopic Localization of the 220-kD Protein in Various Types of Cells Bearing Cell-to-Cell AJ

Using mAb T8-754 we have examined the distribution of the 220-kD protein in various types of cells bearing cell-to-cell

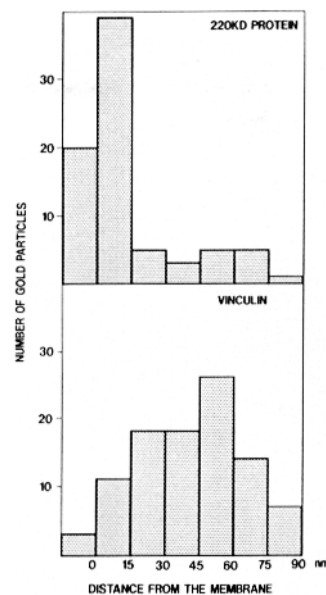


Figure 5. Immunogold label density of the 220-kD protein and vinculin as a function of distance from the plasma membrane of cell-to-cell AJ in heart. The numbers of gold particles are counted in 15-nm-wide zones starting at the center of the bilayer. The 220-kD protein labeling is mostly confined to a narrow area 0–15 nm away from the membrane, while the vinculin labeling exhibits a broad peak at distances of 30–75 nm from the membrane.

AJ by immunofluorescence microscopy. In cultured MDBK cells the beltlike cell-to-cell AJ was exclusively stained by mAb T8-754, while anti-vinculin pAb stained not only cell-to-cell AJ but also cell-to-substrate AJ (focal contacts) (Fig. 2, *A* and *B*). Primary-cultured rat keratinocytes are known to form spotlike cell-to-cell AJ. Also in these cells, the 220-kD protein staining was localized specifically at the cell-to-cell AJ, while it was not associated with the vinculin-positive focal contacts (Fig. 2, *C* and *D*). When the frozen sections of tissues bearing cell-to-cell AJ such as intestine, liver, and heart were doubly stained with mAb T8-754/anti-E-cadherin pAb or with mAb T8-754/anti-vinculin pAb, the 220-kD protein staining was reproducibly and exclusively found in vinculin- and cadherin-positive cell-to-cell AJ in all tissues examined (Fig. 3). In intestinal epithelia the degree of concentration of the 220-kD protein at cell-to-cell AJ was much higher than that of E-cadherin (Fig. 3, *A* and *B*), and in cardiac muscle cells the patchy labeling was detected periodically along the lateral borders of cells with anti-vinculin pAb (“costamere” pattern) but not with mAb T8-754 (Fig. 3, *E* and *F*). Taking these findings together, we are led to conclude that the 220-kD protein is highly concentrated at the cell-to-cell AJ in various types of cells, but is not detected in the cell-to-substrate AJ.

As shown in Fig. 3 *E*, the 220-kD protein was immunofluorescently shown to be localized at the intercalated discs of cardiac muscle cells. Corresponding labeling of ultrathin cryosections using the immunogold technique verified that the staining for this protein was specifically associated with the undercoat of cell-to-cell AJ, through which actin filaments are densely bound to the plasma membrane (Fig. 4, *A* and *B*). The characteristic feature of the immunogold labeling in Fig. 4, *A* and *B*, was that the gold particles located in the extracellular space were not so small in number. Taking the intrinsic resolution of immunogold labeling (20–25 nm) into consideration, this labeling pattern appeared to indicate that the 220-kD protein might be located immediately next to the cytoplasmic surface of the plasmalemma. To confirm this interpretation, we doubly labeled the ultrathin cryosections of cardiac muscle cells with mAb T4-192 (IgM) and anti-vinculin mAb (IgG) (Fig. 4,

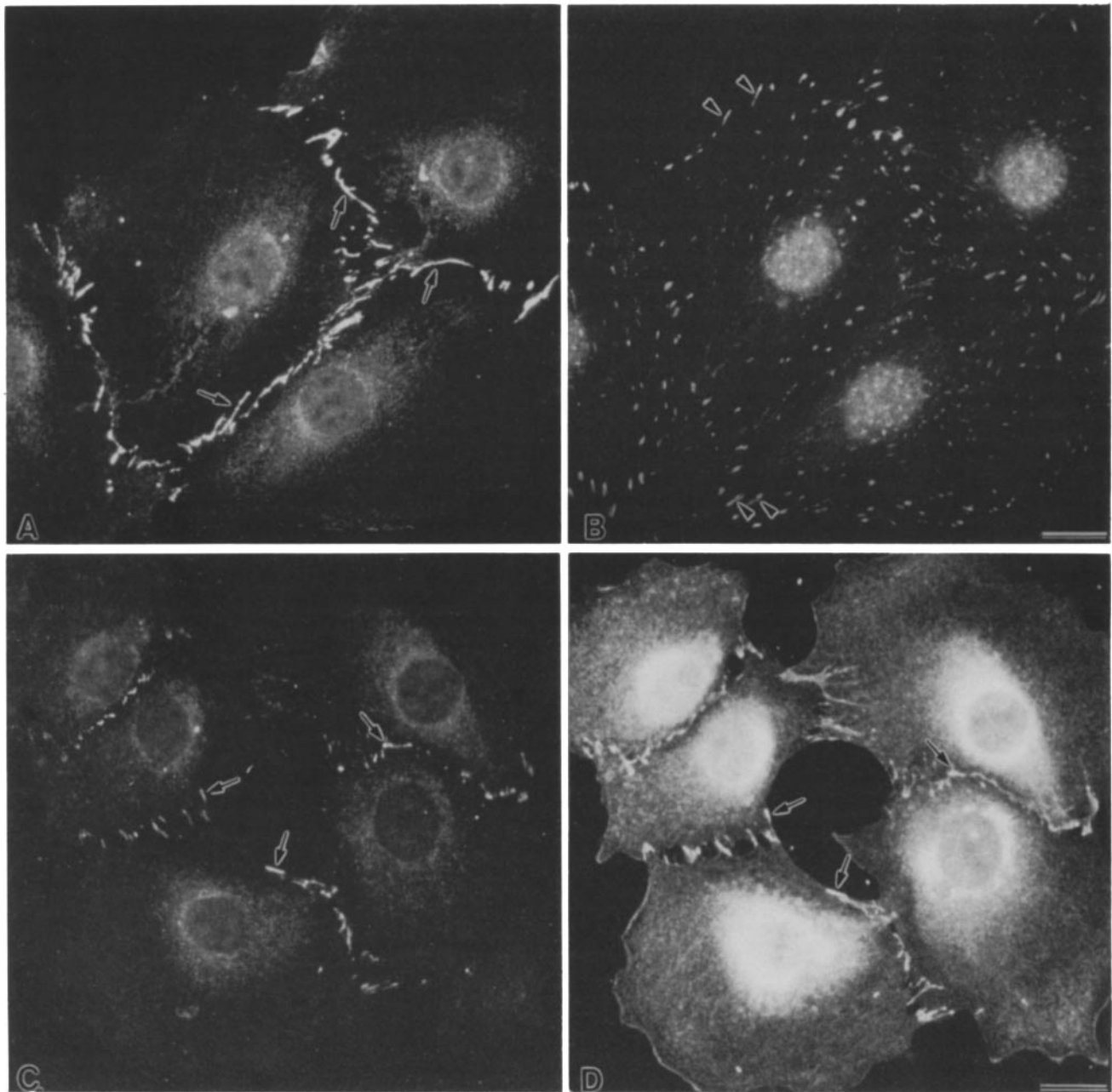


Figure 6. Localization of the 220-kD protein, vinculin, and P-cadherin in rat fibroblasts (3Y1 cells). 3Y1 cells are doubly stained with mAb T8-754 (A) and anti-vinculin pAb (B) or with mAb T8-754 (C) and anti-P-cadherin pAb (D). The 220-kD protein is exclusively concentrated at the cadherin-based cell-cell adhesion sites (arrows), where no accumulation of vinculin is detected. Arrowheads, vinculin-positive cell-to-substrate AJ. Note that cadherin is also diffusely distributed at the ventral cell surface (D). Bars: (A and B) 50 μm ; (C and D) 35 μm .

C-F); high-power electron micrographs, in which the section angle was nearly perpendicular to the plane of the membrane, were used to quantitate the labeling density as a function of distance from the membrane (Fig. 5). The results clearly indicated that vinculin was widely distributed through the undercoat of cell-to-cell AJ, exhibiting a broad peak at distances of 30–75 nm from the membrane. In sharp contrast, the 220-kD protein labeling was mostly confined to a narrower area 0–15 nm away from the membrane. Considering that both vinculin and the 220-kD protein molecules are spherical and that vinculin is smaller in diameter than the

220-kD protein (see Fig. 10), it is safe to say that the distribution difference shown by mAbs is significant. Therefore, we can conclude that the 220-kD protein is highly concentrated in the immediate vicinity of the membrane in the undercoat of cell-to-cell AJ.

Distribution of the 220-kD Protein and Cadherin in Fibroblasts

Fibroblasts such as rat 3Y1 cells and mouse 3T3 cells express a large number of cadherin molecules on their cell surface

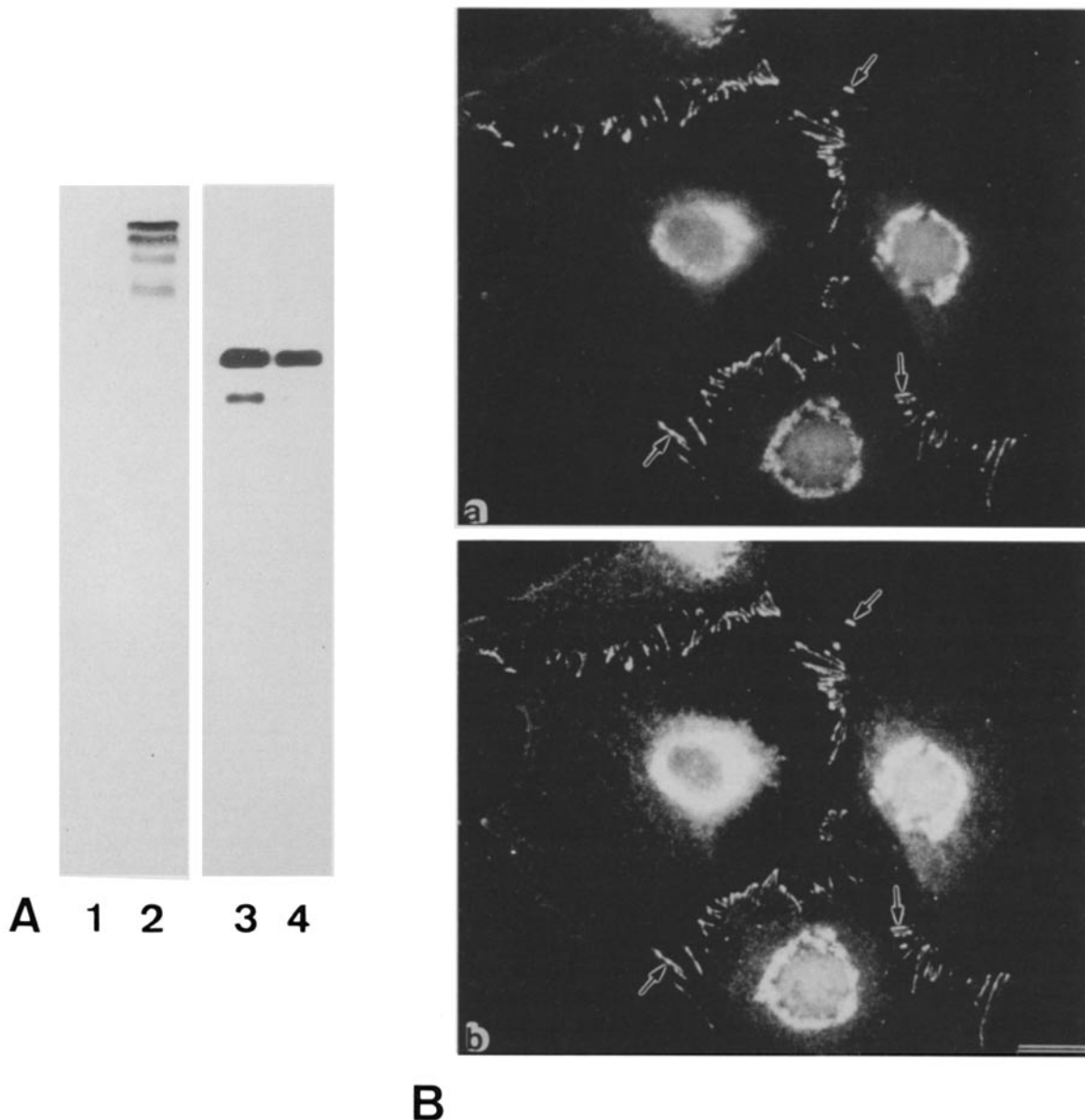


Figure 7. Extractability of the 220-kD protein and P-cadherin from 3Y1 cells with NP-40. Cells are treated with 0.5% NP-40 and separated into the extract and the extracted cells, followed by immunoblot and immunofluorescence analyses. (A) Immunoblot analyses with mAb T8-754 (lanes 1 and 2) and anti-P-cadherin pAb (lanes 3 and 4). Lanes 1 and 3, the extract from one dish; lanes 2 and 4, the extracted cells from one dish. Under this extraction condition, almost half of the cadherin molecules are extracted, while the 220-kD protein is hardly extracted. (B) Double-staining immunofluorescence analyses of the extracted cells with mAb T8-754 (a) and anti-P-cadherin pAb (b). Note that the distribution pattern of the insoluble cadherin molecules completely coincides with that of the 220-kD protein (arrows). In these samples, there is significant perinuclear and nuclear staining with antibodies to both the 220-kD protein and P-cadherin, but the significance of this staining is not clear. Bar, 50 μ m.

and exhibit a typical Ca^{2+} -dependent cell-cell adhesion. When two 3Y1 cells expressing P-cadherin come in contact with each other, P-cadherin molecules are rapidly concentrated at the adhesion sites (Fig. 6 D). However, vinculin is not so much concentrated in these adhesion sites, indicating that in these cells the typical cell-to-cell AJ is not formed (Fig. 6 B). Neither α -actinin, radixin, nor tenuin was accumulated in these sites (data not shown). Interestingly, the 220-kD protein staining was exclusively associated with these cadherin-based adhesion sites (Fig. 6, A and C). Close inspection of the 3Y1 cells doubly stained with mAb T8-754

and anti-P-cadherin pAb revealed that P-cadherin molecules were not only concentrated at the cell adhesion sites but also diffusely distributed on the ventral cell surface, while the 220-kD protein was exclusively localized at the cadherin-based cell-cell adhesion sites.

When semi-confluent 3Y1 cells were treated with 0.5% NP-40 for 30 min, immunoblotting analyses revealed that almost half of the P-cadherin molecules were extracted, while the 220-kD protein was hardly extracted (Fig. 7 A). The double-staining immunofluorescence microscopy of the NP-40-extracted cells has led us to the following conclusions

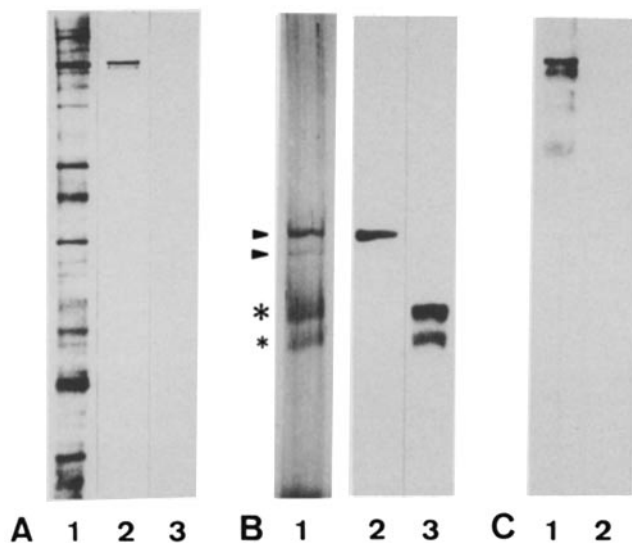


Figure 8. Association of the 220-kD protein with spectrin in the isolated AJ. (A) Immunoprecipitation from the low-salt extract of AJ with mAb T8-754. Lane 1, silver-stained gel (10%) of the low-salt extract of AJ which is applied onto the affinity column; lane 2, silver-stained gel of 3 M MgCl₂ eluate from the mAb T8-754-coupled Sepharose column; lane 3, silver-stained gel of 3 M MgCl₂ eluate from nonimmune mouse IgG-coupled Sepharose column. (B) Immunoblot analyses of the 3 M MgCl₂ eluate from mAb T8-754-coupled Sepharose column (same as that in A, lane 2). When this eluate is separated in 4% gel, four bands (arrowheads and asterisks) are detected in silver staining (lane 1). The largest band is specifically recognized by anti- α -spectrin pAb (lane 2). The lower two bands are recognized by mAb T8-754 (lane 3). The increase of the lowest bands (small asterisk) with time suggests that it is a proteolytic degradation product of the 220-kD band (large asterisk). (C) The immunoprecipitants from the low-salt extract of AJ with anti- α -spectrin pAb (lane 1) and with nonimmune rabbit IgG (lane 2) are analyzed by immunoblotting with mAb T8-754. Note that the 220-kD protein is coimmunoprecipitated with α -spectrin from the AJ extract.

(Fig. 7 B): (a) P-cadherin molecules on the ventral surface were mostly extracted, while those concentrated at the adhesion sites were resistant to the NP-40 treatment. (b) The distribution pattern of these "insoluble" cadherin molecules completely coincided with that of the 220-kD protein. Therefore, it is likely that the 220-kD protein plays a crucial role in making cadherin molecules insoluble by directly or indirectly linking them to cytoskeletons.

Binding of the 220-kD Protein with α -Spectrin in Cell-to-Cell AJ

As described above, the 220-kD protein was resistant to low-salt alkaline extraction, but some 220-kD protein molecules were included in the low-salt extract as a native form. To further characterize the 220-kD protein, we have attempted to immunoprecipitate this native 220-kD protein from the low-salt extract of isolated AJ by affinity chromatography on Sepharose-bound mAb T8-754. The low-salt extract was applied onto the affinity column followed by extensive washing, and it was found that the 220-kD protein was effectively eluted from the column by 3 M MgCl₂. In 10% polyacrylamide gel, this eluate was shown to contain only high molec-

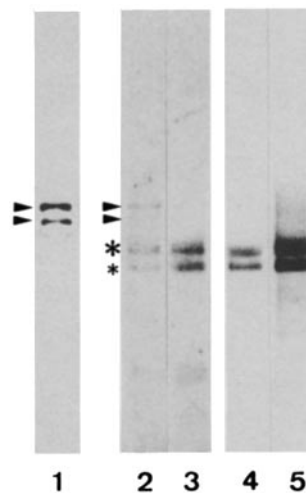


Figure 9. Purification of spectrin and the 220-kD protein from rat brain. (Lane 1) Silver-stained gel (4%) of purified brain spectrin. Brain spectrin was purified from rat brain according to the method developed by Glenney et al. (1982). (Lanes 2-5) Purification of the 220-kD protein. The crude extract from rat brain was applied onto the mAb T8-754-bound Sepharose column followed by extensive washing. In silver-stained gel (4%), 2.5 M MgCl₂ eluate (lane 2) contains both the 240-kD/235-kD bands (arrowheads) and the 220-kD protein (asterisk), while only the 220-kD protein is detected in 3 M MgCl₂ eluate (lane 3). (Lanes 4 and 5) Accompanying immunoblots of lanes 2 and 3 with mAb T8-754, respectively.

ular weight polypeptides (Fig. 8 A), which were separated into four bands in 4% gel (Fig. 8 B, lane 1). In immunoblotting, only the lower two of these bands (the lowest one may be the degradation product) were recognized by mAb T8-754 (Fig. 8 B, lane 3), suggesting that the upper two polypeptides were tightly associated with the 220-kD protein. The molecular masses (240 kD/235 kD) of these upper two bands persuaded us to speculate that they were α - and β -chains of liver spectrin. This speculation was partly verified by immunoblotting analysis with anti- α -spectrin pAb (Fig. 8 B, lane 2), although it was difficult to identify the 235-kD band as β -spectrin. To further confirm the binding of the 220-kD protein with spectrin molecules (at least with α -spectrin) in cell-to-cell AJ, the low-salt extract of the isolated AJ was immunoprecipitated with anti- α -spectrin pAb. The results clearly indicated that the 220-kD protein and spectrin molecules were coimmunoprecipitated with anti- α -spectrin pAb (Fig. 8 C). Taking all these findings together, we can conclude that in the undercoat of cell-to-cell AJ the 220-kD protein was tightly and specifically associated with α -spectrin, directly or indirectly.

Molecular Shape of the 220-kD Protein Molecule and Its Binding Site on a Spectrin Molecule

Next, we have attempted to purify the 220-kD protein. Through preliminary experiments, we have found that rat brain is a good source for the purification of the 220-kD protein. Therefore, as described in Materials and Methods, the 220-kD protein was enriched from the brain extract and applied onto the column of mAb T8-754-bound Sepharose. Also in the case of the brain, the 220-kD protein was released from the column together with spectrins (at least with α -spectrin) by 3 M MgCl₂ (Fig. 9). However, we have found that after washing with 2.5 M MgCl₂, the 240-kD/235-kD bands were preferentially released from the column, leaving only the 220-kD protein on the Sepharose. Thus, the purified 220-kD protein was obtained by washing this Sepharose with 3 M MgCl₂ (Fig. 9).

The molecular shape of this purified 220-kD protein was

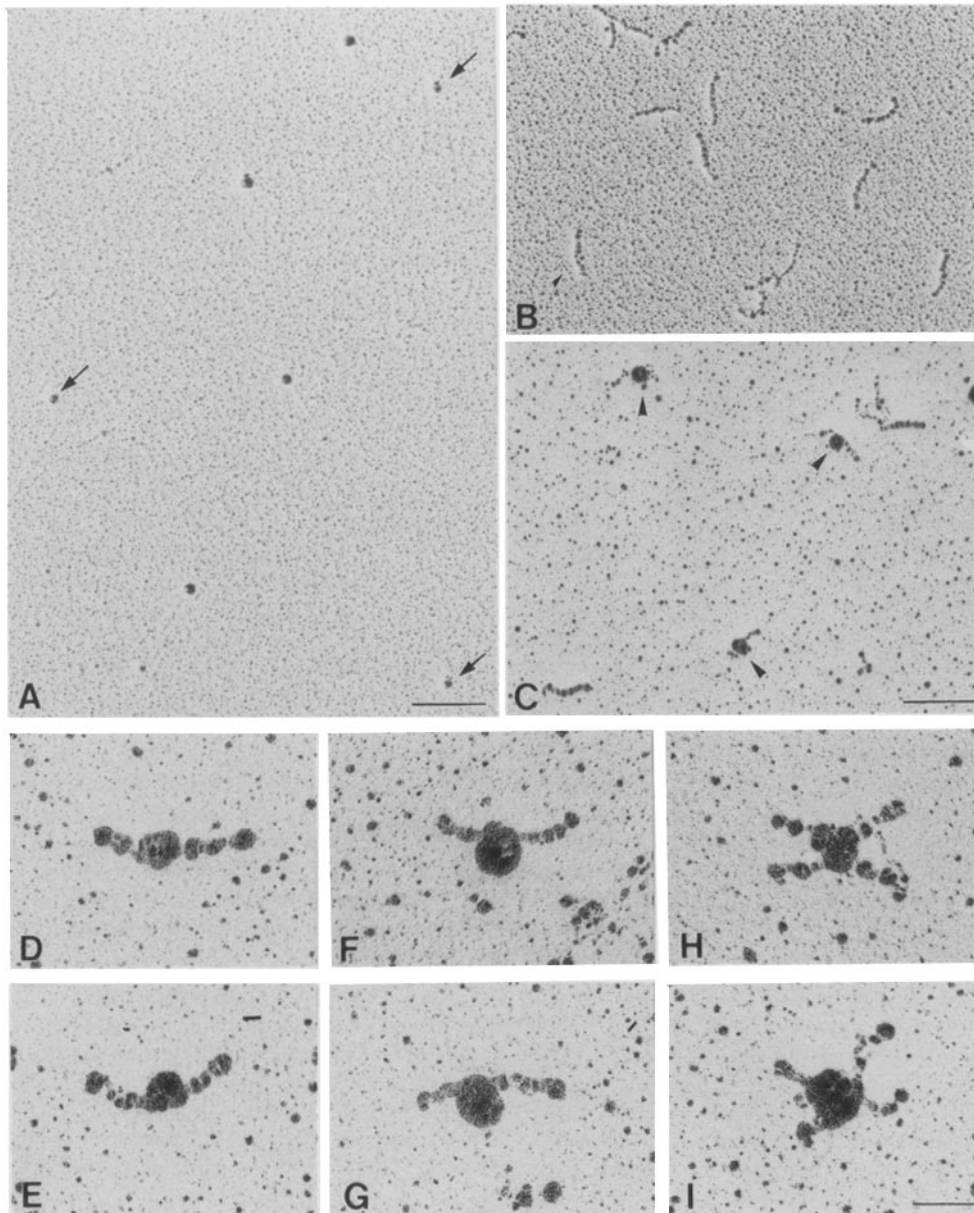


Figure 10. Molecular shape of the 220-kD protein molecule and its binding site on a spectrin molecule revealed by low-angle rotary-shadowing electron microscopy. (A) The 220-kD protein molecule purified from rat brain (see Fig. 9, lane 3). It looks like a spherical particle, but its diameter does not appear to be constant. The diameter mostly distributes from 25 nm (arrows) to 50 nm. Occasionally particles >50 nm in diameter are found. (B) Spectrin tetramers 200 nm in length purified from rat brain (see Fig. 9, lane 1). (C-I) Mixture of the purified 220-kD protein and the purified brain spectrin. Most of the 220-kD proteins are seen to be bound to spectrin molecules (C). The binding site is the position ~ 10 –20 nm from the midpoint of the length of the spectrin tetramer (D–G). Large particles >100 nm in diameter (aggregates of the 220-kD protein) are occasionally seen to crosslink two spectrin tetramers laterally (H and I). Bars: (A) 0.6 μm ; (B and C) 0.3 μm ; (D–I) 0.1 μm .

examined by low-angle rotary-shadowing electron microscopy. As shown in Fig. 10 A, the purified 220-kD protein molecule looked like a spherical particle, although its diameter did not appear to be constant; it was mostly distributed from 25 to 50 nm. Larger particles >50 nm in diameter were frequently observed, suggesting that the 220-kD protein molecules were aggregated to a certain extent, probably due to the treatment with 3 M MgCl_2 . To determine the binding site of this 220-kD protein molecule on a spectrin molecule, the purified 220-kD protein was mixed and incubated with the purified brain spectrin (tetramers) under physiological conditions, and then the mixture was examined by low-angle rotary-shadowing electron microscopy (Fig. 10, B and C). Most of the 220-kD protein molecules appeared to be bound to the spectrin tetramers; the binding site is not the midpoint of the length of the tetramer, but the position ~ 10 –20 nm from the midpoint (Fig. 10, D–G). Interestingly, the large particles, which might be aggregates of the

220-kD protein, were occasionally seen to crosslink two spectrin tetramers laterally (Fig. 10, H and I).

These images strongly suggest that the 220-kD protein may belong to the ankyrin family (Bennett and Stenbuck, 1979; Davis and Bennett, 1984a,b). The structure and functions of ankyrin were first analyzed in detail using human erythrocytes. Since pAb raised against the human erythrocyte ankyrin was reported to recognize the rat brain ankyrins, we have tested whether this pAb can recognize the 220-kD protein purified from rat brain. As shown in Fig. 11 A, the rat brain 220-kD protein was not specifically recognized by this pAb, indicating that the 220-kD protein is not identical to any of the brain ankyrins reported so far. Then, after the crude extract of rat brain was electrophoresed in 4% polyacrylamide gel and transferred to the nitrocellulose sheet, the molecular masses of these proteins were compared by immunoblotting. The electrophoretic mobility of the 220-kD protein was almost equal to that of the brain

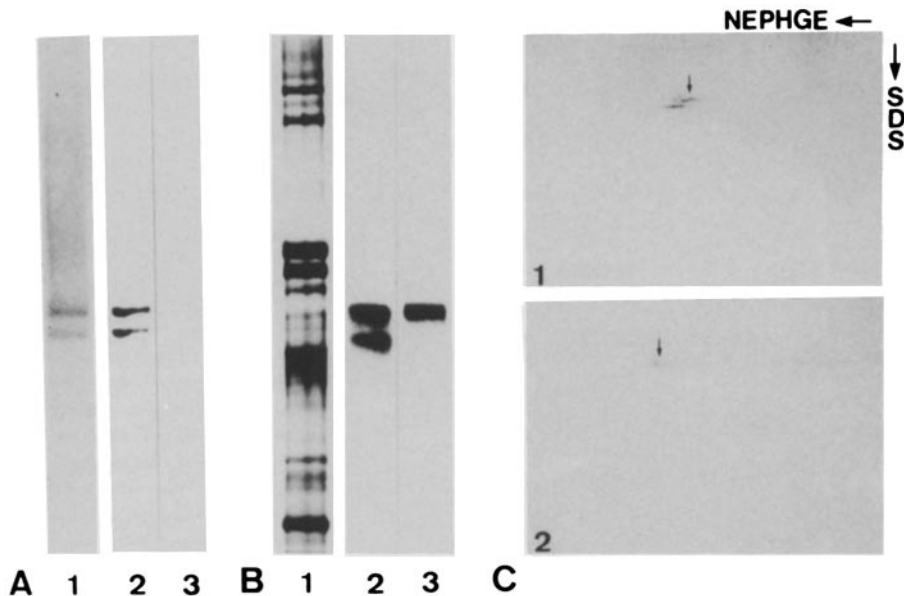


Figure 11. Comparison of the 220-kD protein with ankyrin. (A) Immunoblot analysis of the 220-kD protein purified from rat brain with anti-human erythrocyte ankyrin pAb. Lane 1, silver-stained gel (4%) of the purified 220-kD protein (see Fig. 9, lane 3); lane 2, accompanying immunoblot with mAb T8-754; lane 3, accompanying immunoblot with anti-ankyrin pAb. (B) Immunoblot analysis of the rat brain crude extract after one-dimensional electrophoresis. Lane 1, silver-stained gel (4%) of the crude extract; lane 2, accompanying immunoblot with mAb T8-754; lane 3, accompanying immunoblot with anti-ankyrin pAb. (C) Immunoblot analysis of the rat brain crude extract with mAb T8-754 (1) and anti-ankyrin pAb (2) after two-dimensional electrophoresis.

ankyrin (Fig. 11 B). The isoelectric focusing points of these proteins were also similar (Fig. 11 C), but clearly differed when the nitrocellulose sheet was blotted doubly with mAb T8-754 and anti-ankyrin pAb (data not shown). Taking all these results together, we are led to speculate that the 220-kD protein is a novel member of the ankyrin family.

Discussion

In this study, we have raised many types of mAbs against the "AJ membrane" obtained from the isolated AJ of rat liver; using two of these mAbs, the 220-kD undercoat-constitutive protein of cell-to-cell AJ was identified. So far many undercoat-constitutive proteins of cell-to-cell AJ were identified and characterized: tenuin (400 kD), filamin (260 kD), vinculin (130 kD), α -actinin (100 kD), placoglobin (83 kD), radixin (82 kD), etc. (Larazides and Burridge, 1975; Geiger, 1979; Langanger et al., 1984; Cowin et al., 1986; Tsukita et al., 1989a,b). In sharp contrast to those proteins so far reported, this 220-kD protein appeared to be unique in some aspects. First, this protein is highly concentrated at the cadherin-based cell-cell adhesion sites in fibroblastic cells as well as at the undercoat of cell-to-cell AJ in various types of tissues. As shown in Fig. 6, when cadherin molecules on a fibroblastic cell collide with those on a neighboring cell, they accumulate at the cell boundary and become resistant to the extraction treatment with the nonionic detergent. In the fibroblastic cells, the accumulation of cadherin molecules does not proceed to form a typical cell-to-cell AJ. The cell-cell adhesion sites of fibroblastic cells where the insoluble cadherins are concentrated are not associated with the concentration of vinculin, α -actinin, tenuin, or radixin, while the distribution of the 220-kD protein precisely coincides with that of these insoluble cadherins. This strongly suggests that the direct or indirect association of cadherin molecules with the 220-kD protein may occur at a very early stage of the AJ formation process in the AJ-bearing cells. Second, as shown by immunogold labeling of ultrathin cryosections, the 220-kD protein is located in the immediate vicinity of the membrane in the undercoat of cell-to-cell AJ; its labeling

was mostly confined to a very narrow area 0–15 nm away from the membrane. Considering that the cell-to-cell AJ is the very site where the insoluble cadherins work as adhesion molecules, these immunolabeling data again indicate the very close spatial relationship of the 220-kD protein with the insoluble cadherin molecules in vivo. Of course, whether these molecules are associated with each other directly or indirectly remains to be clarified, but it is not possible to analyze this association by immunoprecipitation experiments, because the 220-kD protein is not extracted by nonionic detergents.

It is clear that this 220-kD protein is not associated with the soluble cadherin molecules, because it is hardly coimmunoprecipitated with the soluble cadherins by anti-cadherin antibodies (data not shown). By the immunoprecipitation experiments, the soluble cadherin (uvomorulin) was reported to be associated with some cytoplasmic proteins, such as catenin α (102 kD), β (88–94 kD), and γ (80 kD) (Vestweber and Kemler, 1984; Peyrieras et al., 1985; Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; McCrea and Gumbiner, 1991). Therefore, it is tempting to speculate that the 220-kD protein may play a pivotal role in making the cadherin-catenin complex insoluble (i.e., in binding the complex to underlying cytoskeletons). The present data showing the binding of the 220-kD protein with spectrin molecules favor this speculation. The molecular shape of the 220-kD protein and its binding site on spectrin molecules persuade us to consider the relation of this protein and ankyrins (Branton et al., 1981; Bennett, 1985, 1989). Ankyrin was first reported in human erythrocytes to directly bind a membrane integral protein, band 3 protein, to the underlying spectrin molecules (Bennett and Stenbuck, 1979; Luna et al., 1979; Yu and Goodman, 1979). Now ankyrin is believed to be associated with the plasma membrane in many tissues, where it is also likely to interconnect various types of integral proteins such as Na^+/K^+ ATPase and voltage-dependent Na channel with the spectrin-based membrane skeletons (Bennett, 1979; Davis and Bennett, 1984a,b; Nelson and Veshnock, 1987; Srinivasan et al., 1988; Morrow et al., 1989). The 220-kD protein resembles ankyrin in the fol-

lowing points: (a) Its molecular mass and isoelectric point are similar to those of ankyrin (see Fig. 11). (b) Both of them specifically and tightly bind to spectrin molecules. (c) Its binding site on spectrin molecules is almost the same as that of ankyrin (see Fig. 10). (d) Both of them locate very near the cytoplasmic surface of the plasma membrane (compare Fig. 4 in this article with Fig. 7 in Kordeli et al., 1990). However, as far as has been examined immunochemically, the 220-kD protein is not identical to any isoform of ankyrins reported up to now; the isoforms of brain ankyrins so far identified were all recognized by anti-human erythrocyte ankyrin pAb, while the 220-kD protein purified from brain was not (Kordeli et al., 1990). The idea that a specific isoform of ankyrin so far unidentified occurs at cell-to-cell AJ is not so peculiar, because one specific isoform of ankyrin is known to be exclusively localized at the node of Ranvier in the brain (Kordeli et al., 1990).

The possibility cannot be excluded that the 220-kD protein is an isoform of β -spectrin. In the affinity-purified state, many nonerythroid spectrin subunits behave poorly and are prone to aggregation, thus showing a spherical molecular shape in electron microscopy. It is not peculiar that the aggregated β -spectrin binds to α -spectrin in the spectrin tetramer at specific sites. Recently, a protein with a molecular mass of 220 kD that is immunologically related to β -spectrin was reported to associate with motor endplates in skeletal muscle (Block and Morrow, 1989). Therefore, it appears to be possible that a specific isoform of β -spectrin is responsible for the formation of the cell-to-cell AJ (Bennett, 1990). The relationship of the 220-kD protein with ankyrins and β -spectrin will be determined more conclusively once the primary sequences of the 220-kD protein and each isoform of ankyrin and β -spectrin are determined. In this connection, we should refer here to a recent study suggesting that uvomorulin (E-cadherin) is associated with ankyrin and spectrin in MDCK cells; a small fraction of uvomorulin/E-cadherin was reported to cofractionate with ankyrin and spectrin (Nelson et al., 1990). It remains to be clarified whether this cofractionated ankyrin or β -spectrin is identical to the 220-kD protein characterized in this study.

Recently cloning and sequencing of cDNA encoding radixin, one of the major undercoat-constitutive proteins of cell-to-cell AJ, revealed that radixin is a novel member of the band 4.1 protein family (Funayama et al., 1991). The band 4.1 protein is one of the major constituents of the undercoat of erythrocyte membranes (Branton et al., 1981). As previously reported, α -spectrin is not concentrated at the cell-to-cell AJ (Bennett, 1985; Tsukita et al., 1991), but it is one of the major components of the low-salt extract of the isolated AJ. Considering that the 220-kD protein resembles ankyrin (or β -spectrin), it appears likely that the molecular architecture of the undercoat of cell-to-cell AJ may be somewhat similar to that of the erythrocyte membrane (Tsukita et al., 1980, 1981; Branton et al., 1981; Byers and Branton, 1985): Cadherin molecules may directly or indirectly bind to the 220-kD protein, which is associated with the underlying spectrin-actin network. In the network, spectrin molecules may form a complex together with radixin and actin filaments. On the other hand, the recent sequence analysis of cDNA encoding another cell-to-cell AJ undercoat-constitutive protein, CAP102, revealed that this protein has a similarity to vinculin and suggested the possible binding

between CAP102 and vinculin (Nagafuchi et al., 1991). Vinculin was reported to directly bind to α -actinin in vitro (Belkin and Kotliansky, 1987; Wachsstock et al., 1987), which can crosslink actin filaments (Burrige and Feramisco, 1982). Therefore, at present, two types of molecular linkage from cadherin molecules to actin filaments can be supposed. How are all these undercoat-constitutive proteins integrated into the densely packed structure of cell-to-cell AJ undercoat? How are these undercoat-constitutive proteins of AJ segregated from those of other membrane domains? Further efforts to identify and characterize the other constituents of the undercoat of the isolated AJ and to isolate cDNA encoding these proteins will lead us to a complete understanding of the complicated molecular architecture of the undercoat of cell-to-cell AJ and the molecular mechanism of the AJ formation. We believe this understanding is required to know how the undercoat of AJ regulates the cell adhesion and how it participates in the signal transduction from the adhesion molecule to the nucleus.

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