A New High Molecular Mass Protein Showing Unique Localization in Desmosomal Plaque

Yohki Hieda,*‡ Sachiko Tsukita,* and Shoichiro Tsukita*

*Department of Ultrastructural Research, The Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan; ‡Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464, Japan

Abstract. A high molecular mass protein of 680 kD was identified and purified from the isolated desmosomes in bovine muzzle epidermal cells. This protein, called "desmoyokin" (from the English, yoke) here, showed no binding ability with keratin filaments in vitro, and its molecule had a characteristic dumbbell shape ~170 nm in length. We have succeeded in obtaining one monoclonal antibody specific to desmoyokin. By the use of this monoclonal antibody and antidesmoplakin monoclonal antibody, desmoyokin was shown to be colocalized with desmoplakin at the immunofluorescence microscopic level; desmoyokin occurred only in the stratified epithelium, not in the simple epithelium nor in the other tissues. At the electron microscopic level, these two proteins were clearly seen to be sorted out in the plaque of desmosomes with desmoyokin at the periphery and desmoplakin at the center of the disk-shaped desmosomal plaque, suggesting that these two plaque proteins play distinct roles in forming and maintaining the desmosomes in stratified epithelium.

Among the intercellular junctions are two categories showing prominent interaction with cytoskeletons: adherens junctions and desmosomes (6, 8, 14, 24). Actin-containing microfilaments and intermediate-sized filaments attach to adherens junctions and desmosomes, respectively, through their cytoplasmic dense plaques (undercoats). In the case of adherens junctions, no biochemical enrichment procedure has been available so far, and only quite recently has this type of junction been successfully isolated from rat liver (29). In sharp contrast, through the use of bovine muzzle epidermis, an isolation procedure for desmosomes was established over the past decade, allowing us to systematically analyze the molecular organization of this type of junction in detail (5, 9, 22). Desmosomal proteins identified so far are categorized into two groups: glycosylated and nonglycosylated. The glycosylated proteins, called desmogleins, include three polypeptides (DGI, -2, and -3, with molecular masses of 150, 110, and 22 kD, respectively), some of which are thought to be integral adhesion molecules in desmosomes (1, 4, 9, 12, 23, 25).

The nonglycosylated proteins are constituents of desmosomal plaque and possibly link intermediate-sized filaments to the plasma membrane. Major desmosomal plaque proteins are desmoplakins I and II with molecular masses of 240 and 215 kD, respectively, which are biochemically very similar but have some distinct antigenic determinants (2, 11, 19). Immunoelectron microscopic analyses have revealed that desmoplakins are really localized at desmosomal plaques with oriented arrays (7). Another high molecular mass constituent of the desmosomal plaque is desmocalmin with a molecular mass of 240 kD, which is a calmodulin- and keratin filament-binding protein distinct from desmoplakin I biochemically and immunologically (28). Two other proteins have been reported to be localized in the desmosomal plaque: band 5 protein (plakoglobin) (3) and band 6 protein (13). Interestingly, plakoglobin occurs not only in desmosomes but also in adherens junctions (3).

So far little attention has been paid to desmosomal plaque proteins in the molecular mass range of >300 kD. However, in the SDS–polyacrylamide gel banding patterns of the isolated desmosomes, some unknown polypeptides with molecular masses >300 kD can be reproducibly identified. Considering that titin (connectin) (18, 32) and nebulin (31), both with molecular masses >300 kD, are thought to play crucial roles in maintaining the structural integrity of myofibrils in skeletal muscle cells, it appears to be of great value to analyze the high molecular mass proteins of >300 kD in the desmosomal plaque. Here, we describe a new desmosomal plaque protein with a molecular mass of ~680 kD. Biochemical and immunohistochemical analyses have been performed, leading us to conclude that the single most characteristic feature of this protein is its distribution pattern in the desmosomal plaque. By virtue of its characteristic localization in the desmosomal plaque, this protein is tentatively called "desmoyokin" (for the English, yoke) in this study.

Materials and Methods

Isolation of Desmosomes

Bovine muzzles were obtained fresh at a slaughterhouse. Desmosomes were isolated mainly according to the method developed by Gorbsky and Stein-

berg (9). All procedures were performed at 4°C. After the removal of stratum corneum from seven pieces of bovine muzzle with razor blades, stratum spinosum specimens were collected and soaked in citric acid/sodium citrate (CASC) buffer containing 0.05% Nonidet P-40. CASC buffer was composed of 0.1 M sodium citrate buffer (pH 2.6), 5 μg/ml leupeptin, 5 μg/ml pepstatin A. The slices of stratum spinosum were finely minced and then vigorously stirred in the above solution for 3 h. After the filtration of homogenate with nylon mesh, the filtrate was centrifuged at 20,000 *g* for 15 min. The pellet was suspended in CASC buffer containing 0.01% Nonidet P-40 and centrifuged at 500 *g* for 15 min. The centrifugation of the supernatant at 20,000 *g* for 15 min resulted in a pellet enriched in desmosomes, which was used for partial purification of desmoyokin.

Partial Purification of Desmoyokin

All procedures were performed at 4°C. Desmosomal plaque proteins were extracted without denaturing agents from isolated desmosomes according to the method developed by Tsukita and Tsukita (28), except for the replacement of 0.1 mM EDTA by 1 mM CHES (2-[cyclohexylamino] ethanesulfonic acid) (pH 10.0). After saturated ammonium sulfate was added to the extract up to 90% followed by stirring for 30 min, the extract was centrifuged at 20,000 *g* for 30 min. The pellet was resuspended and dialyzed against buffer 1 for 24 h. Buffer 1 was made up of 20 mM Hepes (pH 8.0), 5 mM EDTA, 200 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. After centrifugation at 100,000 *g* for 1 h, the supernatant was subjected to gel filtration on a column of Sepharose CL-2B(Pharmacia Fine Chemicals, Uppsala, Sweden), which had been equilibrated with buffer 1. Each fraction was analyzed by SDS-PAGE, and the fractions enriched in desmoyokin were pooled.

Low-angle, Rotary-shadowing Electron Microscopy

The molecular shape of desmoyokin was analyzed by low-angle, rotary-shadowing electron microscopy, mainly according to the method developed by Tyler and Branton (30). The solution containing partially purified desmoyokin was sprayed onto freshly cleaved mica. The droplets on the mica were dried at room temperature in a vacuum (1×10^{-6} torr) in JEOL freeze-etch equipment (JFD 7,000) for 10 min. Platinum was then rotary-shadowed at an angle of 5°, followed by coating from above with carbon. The replica was floated off on distilled water and picked up on formvar-filmed grids. The samples were examined in a JEOL 1200 EX electron microscope.

Gel Electrophoresis and Molecular Mass Estimation of Desmoyokin

One-dimensional SDS-PAGE was based on the method of Laemmli (17); the stain Coomassie brilliant blue R-250 or silver staining (Wako silver-staining kit; Wako Pure Chemicals, Tokyo, Japan) was used.

For molecular mass estimation of desmoyokin, the SDS-PAGE system developed by Weber and Osborn (33) was used, and the relative mobility of desmoyokin was compared to that of cross-linked phosphorylase *b* (Sigma Chemical Co., St. Louis, MO).

High molecular mass, microtubule-associated proteins were obtained from bovine brain mainly according to the method of Shelanski et al. (21). The cytoskeleton fractions containing "plectin" were prepared from C6 glioma cells according to the method of Pytela and Wiche (20). C6 glioma cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

Monoclonal Antibody Production

After electrophoresis of the extract from isolated desmosomes obtained from 12 bovine muzzles, the bands of desmoyokin were removed with a razor blade. Such bands were pooled, and desmoyokin was eluted electrophoretically. The eluted protein was precipitated by adding acetone, and the pellet was resuspended in 1 ml of distilled water. This solution was used as immunogen in vitro.

Monoclonal antibodies were obtained essentially according to the procedure of Köhler and Milstein (16), except that the immunization was performed in vitro. The spleen was removed from a 7-wk-old BALB/c mouse, and the splenocytes were incubated with the above immunogen in the presence of 140 μg/ml *N*-acetylmuramyl-L-alanyl-D-isoglutamine for 4 d. The splenocytes were then fused with mouse P3 myeloma cells. 50% polyethylene glycol (PEG 4000; Merck and Co., Rahway, NJ) 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 assay. The crude extract from isolated desmosomes was used for ELISA assay.

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 immunoblotting analyses. The clones that produced antibodies specific to desmoyokin were expanded and cloned again. The antibody-rich supernatant or ascites was used for immunoblotting and immunohistochemistry.

Immunofluorescence Microscopy

For indirect immunofluorescence microscopy of frozen sections, samples were frozen using liquid nitrogen, and sections ~10-μm thick 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 the first antibody (antidesmoyokin Yo-12086 IgM and/or antidesmoplakin IgG). Antidesmoplakin monoclonal antibody was purchased from Boehringer Mannheim Biochemica (Mannheim, FRG). They were then washed three times with PBS containing 1% BSA and 0.1% Triton X-100, followed by incubation with the FITC-conjugated goat anti-mouse IgM and/or the rhodamine-conjugated goat anti-mouse IgG for 30 min. After being washed in PBS again, they were examined with an Olympus fluorescence microscope (Vanox-S).

For indirect immunofluorescence microscopy of cultured cells, rat keratinocytes (FRSK cells obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan) were cultured on cover glasses and fixed with methanol at −20°C for 10 min. After being washed with PBS, the cells were treated with 0.5% Triton X-100 in PBS for 15 min and washed three times with PBS. The samples were soaked in PBS containing 1% BSA for 10 min, and treated with the first antibodies (see above) for 1 h in a moist chamber. The samples were then washed with PBS containing 1% BSA three times, followed by incubation with the second antibodies (see above) for 30 min. After incubation, the sample was washed with PBS three times and examined with an Olympus Vanox-S microscope.

Immunoblotting

Immunoblotting was performed by one-dimensional electrophoresis, followed by electrophoretic transfer to nitrocellulose sheets. Nitrocellulose sheets were treated with antibody followed by horseradish peroxidase-labeled goat anti-mouse IgG (heavy and light chains) (Bio-Rad Laboratories, Richmond, CA), and the localization of peroxidase was detected by the reaction using diaminobenzidine in the presence of Ni and Co ions.

Immunoelectron Microscopy

Immunoelectron microscopy using ultrathin cryosections was performed essentially according to the method developed by Tokuyasu (27) and modified by Keller et al. (15). Small pieces of bovine muzzle epidermis were fixed in 95% ethanol at 4°C for 30 min. The fixed specimen blocks were infused with 2.3 M sucrose, rapidly frozen, and ultrathin sectioned in the frozen state using glass knives with a DuPont Co., Sorvall Instruments Div. (Newton, CT) ultramicrotome MT-2 equipped with the LTC-2 cryoattachment. The first antibodies used were antidesmoyokin mouse monoclonal antibody (Yo-12086, IgM) and/or antidesmoplakin mouse monoclonal antibody (IgG). As second antibodies (purchased from Janssen Pharmaceutica, Beerse, Belgium), goat anti-mouse IgM coupled to 10-nm gold (GAMIgM G10) and/or goat anti-mouse IgG coupled to 15-nm gold (GAMIgG G15) were used. Finally, the samples were infused and embedded in LR White acrylic resin (London Resins Co., London, UK).

Results

Proteins in High Molecular Mass Range of Desmosomes

So far, it has been reported that desmoplakin I and desmocalmin (240 kD) are the desmosomal plaque proteins with the highest molecular masses (19, 28). However, close inspection of the electrophoretic banding pattern of the iso-

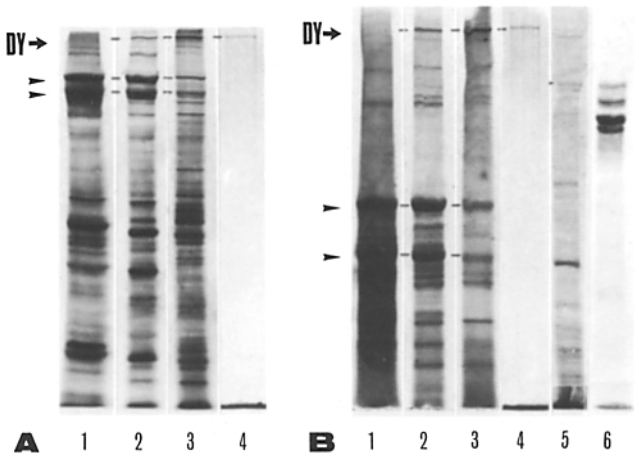

Figure 1. Identification and partial purification of desmoyokin in 7.5% gel (*A*) and 4% gel (*B*). From four to seven bands can be identified in the molecular mass range greater than desmoplakin I and II (*large arrowheads*) in the desmosome fraction (lane *1*). Desmoyokin (*DY*) can be extracted (lane *2*) and enriched by addition of ammonium sulfate (lane *3*). Desmoyokin was partially purified by gel filtration (lane *4*). Compare the molecular mass of desmoyokin to that of plectin (*small arrowhead*, lane *5*) in the cytoskeleton fraction from C6 glioma cells (lane *5*) and that of the high molecular mass, microtubule-associated proteins (lane *6*).

lated desmosomes, and especially of the crude extract of desmosomal plaque, revealed that four to seven minor but clear bands occur in the molecular mass range higher than desmoplakin I and desmocalmin (Fig. 1 *A*, lanes *1* and *2*). The addition of ammonium sulfate into the extract up to 90% saturation has mostly precipitated these bands, leaving the highest molecular mass polypeptide (Fig. 1 *A*, lane *3*). Careful analyses by the use of 4% polyacrylamide gels have confirmed that this highest molecular mass polypeptide can be reproducibly identified as a single band with a constant mobility (Fig. 1 *B*, lanes *1–3*). In this study, we have attempted to analyze the in vitro properties and in vivo distribution of this high molecular mass protein (desmoyokin) showing a single sharp band in the SDS-polyacrylamide gel.

Partial Purification of Desmoyokin

To investigate the in vitro characteristics of desmoyokin, we have partially purified this protein. By the treatment with low ionic strength and alkaline solution, desmoyokin was effectively extracted from desmosomes together with other desmosomal plaque proteins (Fig. 1, *A* and *B*, lanes *2*). After ammonium sulfate precipitation, the sample was applied to the gel filtration column, so that the fraction rich in desmoyokin was recovered in the void volume (Fig. 1, *A* and *B*, lanes *4*). The major contaminant was the polypeptide with

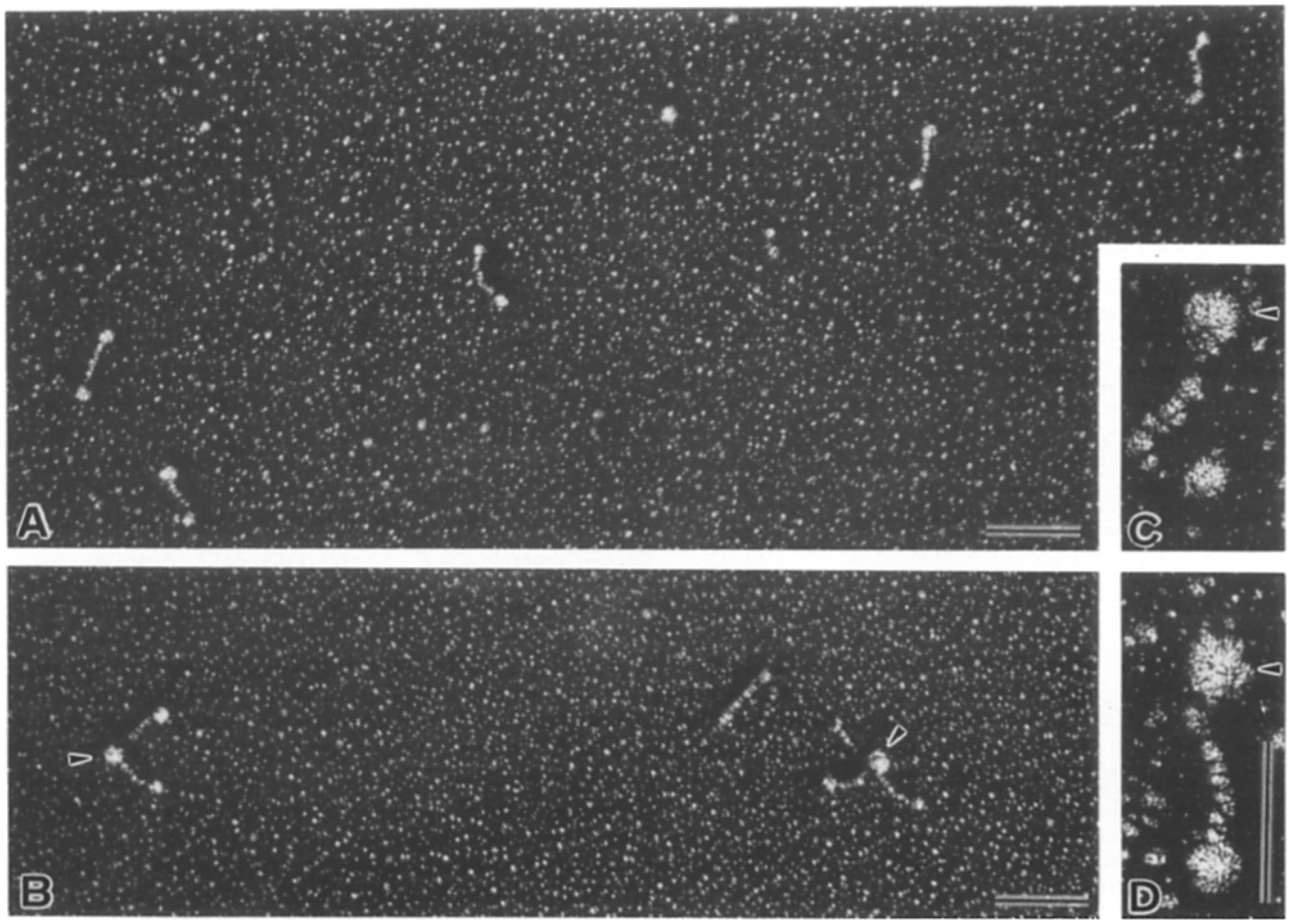

Figure 2. Molecular shape of desmoyokin. The molecule looks like a dumbbell ~170 nm in length (*A*, *C*, and *D*); one head (*arrowheads*, *C* and *D*) appears to be larger in diameter than the other. These molecules occasionally aggregate with each other through end-to-end association (*arrowheads*, *B*). Bars: (*A* and *B*) 200 nm; (*C* and *D*) 100 nm.

the molecular mass of 240 kD, which was recognized neither by antidesmocalmin nor by antidesmoplakin I antibodies.

At first, using this partially purified desmoyokin, its binding ability with keratin filaments was examined by cosedimentation experiments mainly according to the method used previously (28); the partially purified desmoyokin was mixed with the two-cycled keratin filaments (26) and incubated together followed by centrifugation. As a result, the cosedimentation of desmoyokin with keratin filaments was hardly detected irrespective of the presence of divalent cations.

Next, by the use of the low-angle, rotary-shadowing technique, the molecular shape of desmoyokin was analyzed (Fig. 2). Desmoyokin molecules had a characteristic dumbbell structure ~170 nm long; the globular structures at both ends appeared to be distinct in diameter from each other. These dumbbell-shaped molecules occasionally appeared to aggregate with each other through end-to-end association.

Molecular Mass Estimation

When the molecular mass of desmoyokin was compared to that of plectin (34, 35) and the high molecular mass, microtubule-associated proteins in SDS-PAGE, the molecular mass of desmoyokin was much larger than that of these proteins (Fig. 1 *B*, lanes *5* and *6*). Using cross-linked phosphorylase *b* as molecular mass marker, we have estimated the molecular mass of desmoyokin in the SDS–polyacrylamide gels. Since, in the gel system used here, each cross-linked phosphorylase *b* showed a semilogarithmic linear relationship between its relative mobility and molecular mass, the molecular mass of desmoyokin was estimated to be ~680 kD (Fig. 3).

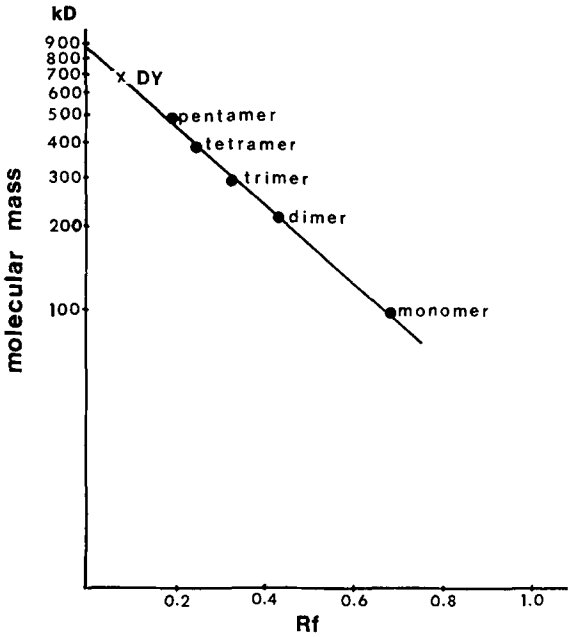

Figure 3. Molecular mass estimation of desmoyokin. From the calibration curve obtained with cross-linked phosphorylase *b* (from *monomer* to *pentamer*), the molecular mass of desmoyokin (*DY*) was extrapolated to be ~680 kD.

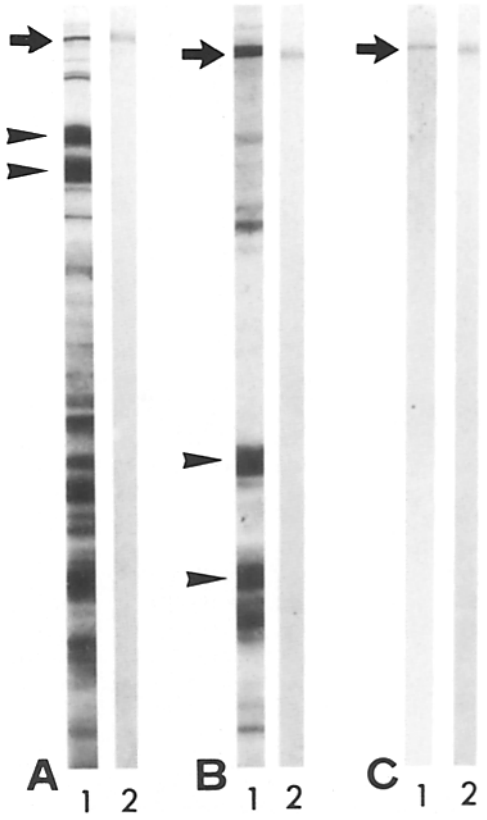

Figure 4. One-dimensional electrophoresis (lanes *1*; Coomassie brilliant blue staining) of desmosome extract (*A* and *B*) and partially purified desmoyokin (*C*), and accompanying immunoblot (lanes *2*) with antidesmoyokin monoclonal antibody. (*A*) 7.5% gel; (*B* and *C*) 4% gel. In all cases, antidesmoyokin specifically reacted with a single band of desmoyokin (*arrows*). *Arrowheads*, desmoplakin I and II. The transfer of a 680-kD band from gels to nitrocellulose membranes requires such a long blotting time that almost all low molecular mass bands would have been lost from the blot. To eliminate this possibility, blots with a shorter blotting time were examined, resulting that this antibody did not cross-react with any of the low molecular mass proteins.

Monoclonal Antibody Production

To obtain a monoclonal antibody specific to desmoyokin, the spleen cells from four BALB/c mice were immunized in vitro with the electrophoretically purified desmoyokin. After the first cloning, in the ELISA assay using the crude low salt extract of desmosomes, supernatants from eight clones were positive. Among them, only one supernatant, Yo-12086, was shown to be specific to desmoyokin in the immunoblot assay. This hybridoma was cloned twice by limiting dilution and grown as ascitic tumors in mice. Although BALB/c mice were also immunized in vivo with desmoyokin, no monoclonal antibody with specific reactivity to desmoyokin was obtained, suggesting that the antigenicity of desmoyokin is not so strong. Therefore, in this study, we have not attempted to search further for hybridomas specific to desmoyokin; the in vivo distribution of desmoyokin was analyzed by the use of Yo-12086.

In immunoblot analysis, this Yo-12086 monoclonal antibody specifically and sharply reacted with the band of desmoyokin in the crude extract of desmosomal plaque proteins

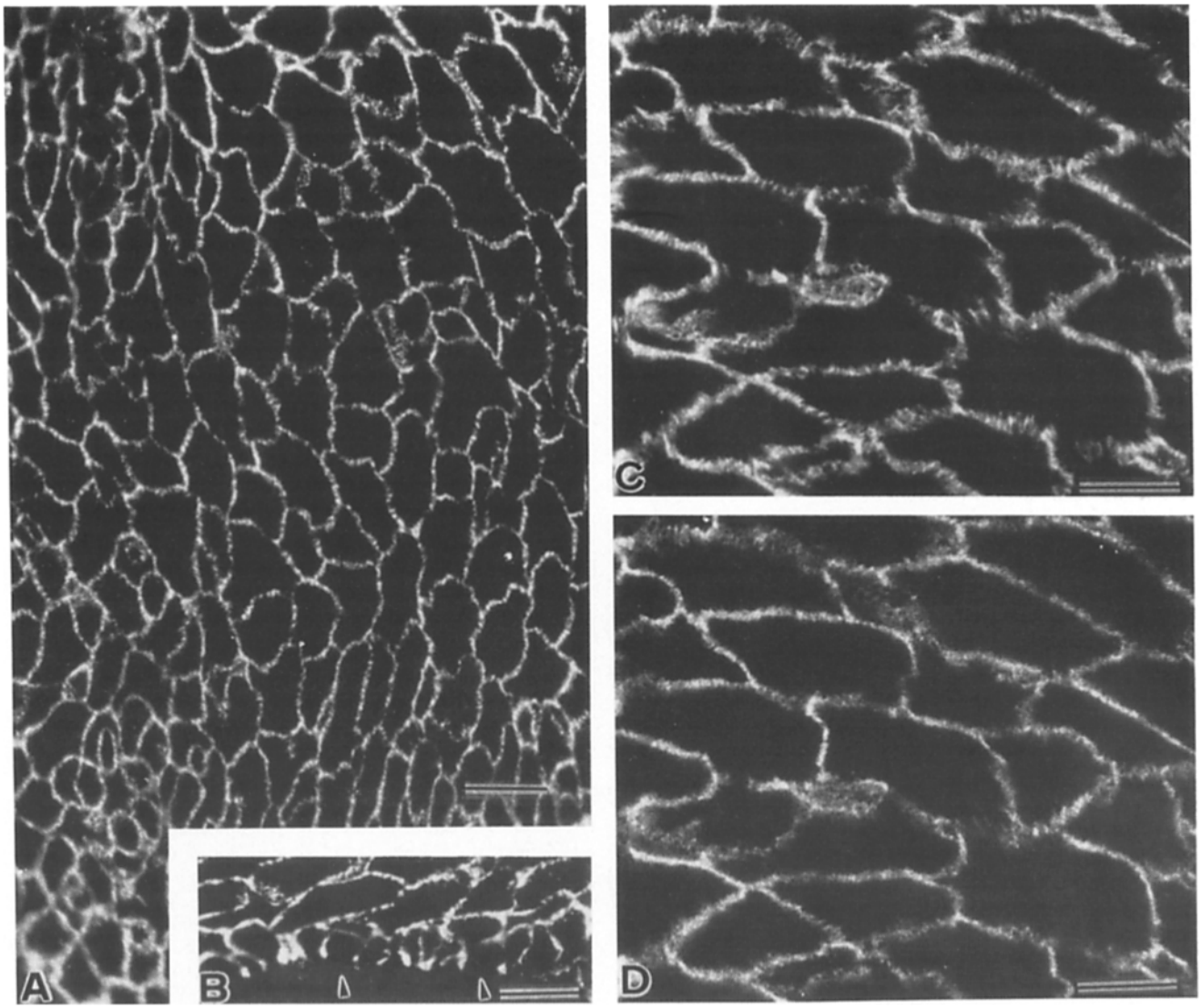

Figure 5. Immunofluorescent microscopic localization of desmoyokin (*A–C*) and desmoplakin (*D*) in frozen sections of bovine muzzle epidermis (*A* and *B*) and bovine tongue epidermis (*C* and *D*). Antidesmoyokin showed a short linear or patchy staining pattern in cell boundaries (*A*), but not in the cell-to-substrate boundaries (*arrowheads*, *B*). Double staining analysis revealed colocalization of desmoyokin (*C*) and desmoplakins (*D*). Bars, 20 μm.

(Fig. 4, *A* and *B*). Furthermore, when the partially purified desmoyokin was transferred to nitrocellulose, only the band of desmoyokin was detected; the band with the molecular mass of 240 kD, the major contaminant, was not recognized (Fig. 4 *C*).

Distribution of Desmoyokin at Light Microscopic Level

Using Yo-12086 monoclonal antibody, the frozen sections of some bovine tissues have been examined by immunofluorescence to study the distribution of the antigen. In the muzzle and tongue epidermis, intense labeling was detected at the boundaries of epidermal cells (Fig. 5). Very occasionally the staining patterns were either short and linear or patchy, which might correspond to the cellular distribution of desmosomes. The boundaries between the basal layer of epidermal cells and connective tissues were not fluorescently stained, indicating that the desmoyokin did not occur at hemidesmosomes (Fig. 5 *B*). When the tongue epidermis was doubly stained by the Yo-12086 and antidesmoplakin antibody, these two proteins were clearly shown to colocalize with each other (Fig. 5, *C* and *D*). In sharp contrast to epidermal cells, neither liver nor small intestine epithelial cells have been specifically stained by Yo-12086 (data not shown). Taking these findings together, it appears that desmoyokin is a desmosomal protein specific for stratified epithelial cells. Desmoyokin was detected neither in the cardiac muscle cells nor in the other junction-bearing but desmosome-negative tissues such as lens and aorta.

Next, using rat keratinocyte cell lines (FRSK cells), we further compared the distribution of desmoyokin with that of desmoplakin (Fig. 6). This type of cell showed a large number of desmosomes between cells, forming compact colonies at the appropriate cell density. At the central part of each colony, the staining pattern of desmoyokin was almost the

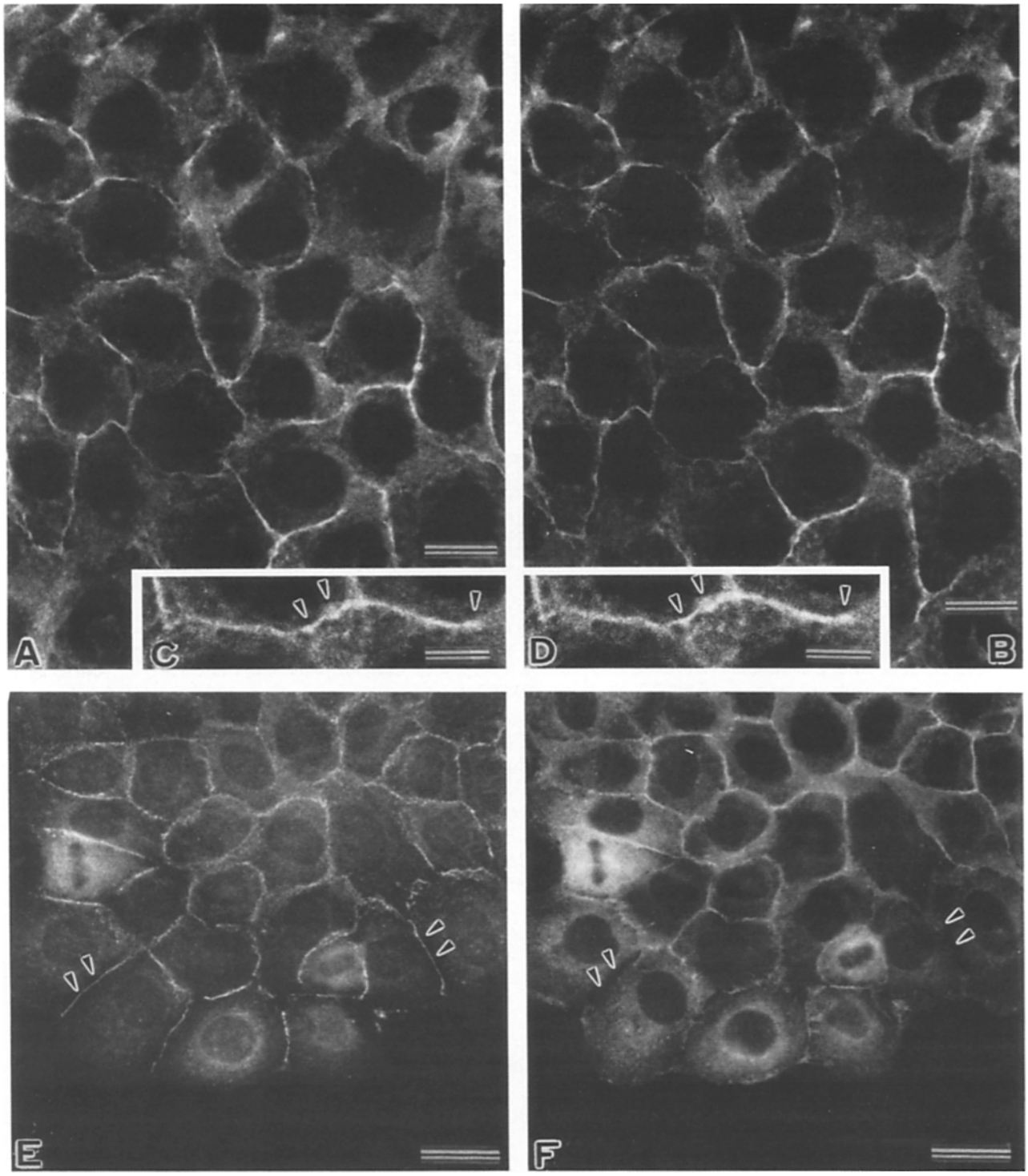

Figure 6. Localization of desmoyokin in cultured rat keratinocytes (FRSK cells). Double staining with antidesmoplakin (*A*, *C*, and *E*) and antidesmoyokin (*B*, *D*, and *F*). At the central part of each colony, the staining pattern of desmoyokin (*B* and *D*) was almost the same as that of desmoplakins (*A* and *C*). At the periphery, in the cell boundaries, desmoyokin was occasionally negative (*arrowheads*, *F*), while desmoplakins were positive (*arrowheads*, *E*). Bars: (*A* and *B*) 20 μm; (*C* and *D*) 10 μm; and (*E* and *F*) 30 μm.

same as that of desmoplakin (Fig. 6, compare *A* and *C* with *B* and *D*, respectively), while at the periphery the cell boundaries showing the desmoplakin-specific fluorescence occasionally lacked the desmoyokin staining (Fig. 6, *E* and *F*).

Distribution of Desmoyokin at Electron Microscopic Level

The ultrathin cryosections of bovine muzzle epidermis were subjected to immunoelectron microscopic observations, in

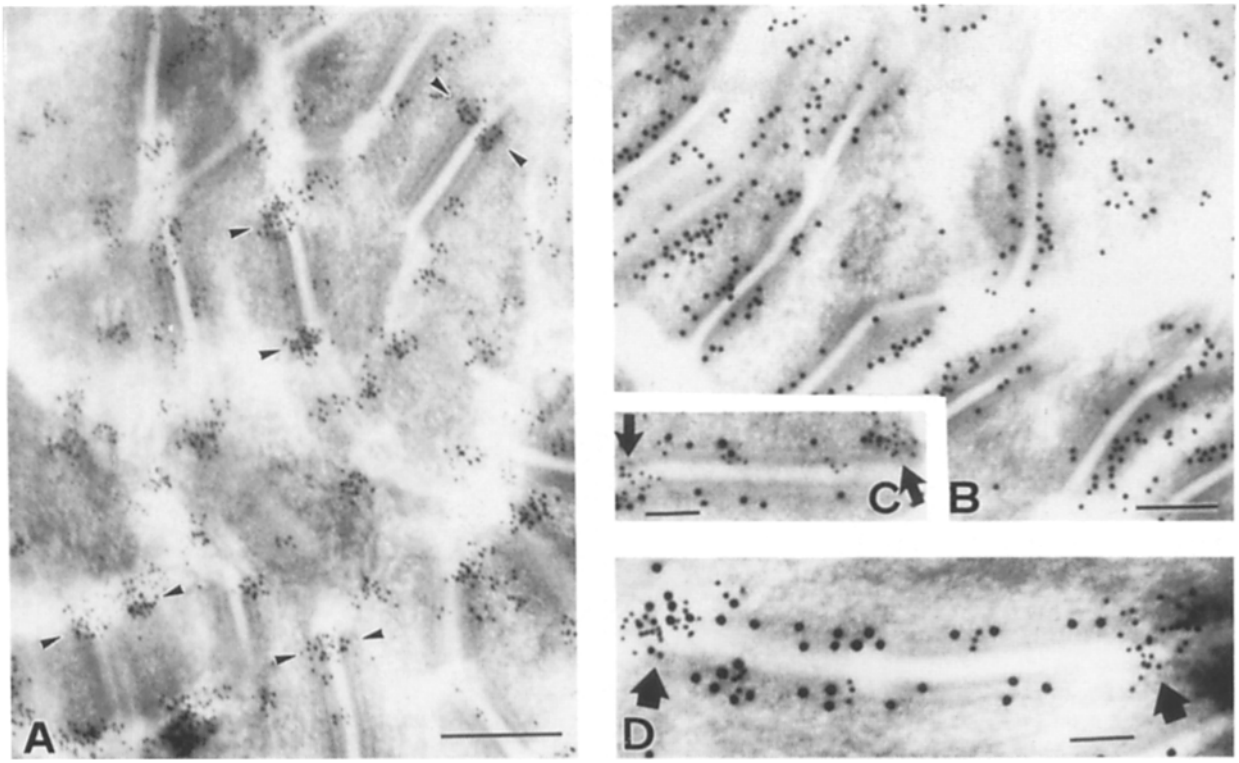

Figure 7. Localization of desmoyokin in the desmosomal plaque of bovine muzzle epidermis at electron microscopic level. (*A*) Localization of desmoyokin. Gold particles are distributed only at the periphery of each desmosomal plaque (*arrowheads*). (*B*) Localization of desmoplakin. Each desmosomal plaque is evenly labeled. (*C* and *D*) Double labeling with antidesmoyokin and antidesmoplakin. Desmoplakins (*large gold particles*) are seen to be sandwiched by desmoyokins (*small gold particles, arrows*). Bars: (*A*) 500 nm; (*B*) 250 nm; and (*C* and *D*) 100 nm.

combination with colloidal gold particle–conjugated secondary antibodies. As the first antibody, the monoclonal antibody to desmoplakins (IgG) and the Yo-12086 (IgM) was used (Fig. 7). At low magnification, both desmoyokin and desmoplakins were reproducibly detected in the desmosomal plaques. Close inspection, however, revealed that with the Yo-12086 the gold particles were distributed unevenly in each desmosomal plaque (Fig. 7 *A*), while with the antidesmoplakin antibody they were evenly distributed (Fig. 7 *B*). Each plaque was observed as a thick rod, and both ends were heavily labeled by the Yo-12086. These observations strongly suggested that desmoyokin (the epitope site for Yo-12086) was exclusively localized at the periphery of the disk-shaped desmosomal plaque, not at the central portion.

This interpretation was confirmed by the double-labeling experiment (Fig. 7, *C* and *D*). The antidesmoplakin antibody was located by the 15-nm gold particle–conjugated, anti-mouse IgG antibody; the Yo-12086, by the 10-nm gold particle–conjugated anti-mouse IgM antibody. When the same ultrathin cryosection was doubly stained, desmoplakins were observed to be sandwiched by desmoyokins in each plaque. The distribution of each protein partially overlapped.

Discussion

In this study, we have partially purified a high molecular mass protein (desmoyokin, 680 kD in SDS-PAGE) from isolated desmosomes in bovine muzzle epidermal cells. This protein did not bind to keratin filaments in vitro, and its molecule looked like a dumbbell ~170 nm long. Furthermore, we have succeeded in obtaining one monoclonal antibody specific to this protein. By the use of this monoclonal antibody and antidesmoplakin monoclonal antibody, desmoyokin appeared to be colocalized with desmoplakin at immunofluorescence microscopic level. However, at the electron microscopic level, these two proteins seemed to be sorted out in the plaque of desmosomes: desmoyokin at the periphery and desmoplakin at the center of disk-shaped desmosomal plaque.

Intensive studies during the past decade have clarified that the plaque of desmosomes possesses a rather complicated molecular organization consisting of several distinct proteins: desmoplakin I, desmoplakin II, desmocalmin, desmoplakin III (plakoglobin or band 5 protein), band 6 protein, etc. (3, 7, 10, 13, 19, 28). Among them, the orientation of desmoplakin molecules in the plaque was well analyzed electron microscopically by the use of several distinct monoclonal antibodies. Although all monoclonal antibodies appeared to label the plaque of desmosomes evenly, the distance of the labels from the membrane differed from monoclonal antibody to antibody (7). This result indicated that the rod-shaped desmoplakin molecules might be oriented vertical to the membrane in the plaque. In sharp contrast to this orientation of the desmoplakin molecule, the monoclonal antibody (Yo-12086) obtained in this study specifically labeled only the periphery of the plaque of desmosomes. Since the antibody labeling was performed after thin sectioning, the possibility was not likely that the lack of antibody

staining at the center of the plaque by Yo-12086 might be due to the antigen masking. The orientation of this molecule will be further clarified when the monoclonal antibodies specific to different epitopes in the desmoyokin molecule are obtained. The difference in the distribution of desmoplakin and desmoyokin seems to imply the distinct roles of these proteins in forming and maintaining desmosomes.

Recently, the polyclonal antibody specific to band 6 protein was obtained, and its distribution was clarified (13); this protein occurred only in the desmosomes of stratified epithelium, not in those of the simple epithelium nor in other tissues. Interestingly, this tissue specificity is almost the same as that of desmoyokin, although at present it is not clear why band 6 protein and desmoyokin are expressed only in the stratified epithelium. In this respect, the difference between the staining patterns of antidesmoplakin and antidesmoyokin in the cultured keratinocytes (FRSK cells) is interesting. At the periphery of each colony, the cell boundaries are intensively stained by antidesmoplakin, but not by antidesmoyokin. Considering that the desmosomes at the periphery appear to be in a dynamic state compared with those at the center of each colony, it is fascinating to speculate that desmoyokin may work as a kind of stabilizer for desmosomes. This speculation seems to be consistent with the distribution of desmoyokin in the plaque of desmosomes; desmoyokin molecules are observed to yoke the desmosomal plaque.

Desmoyokin described in this paper is the first example of a constituent of desmosomal plaques which shows uneven distribution inside the plaque. Further studies of the unidentified constituents of the desmosomal plaque will lead us to better understanding of the molecular organization and functions of desmosomes.

We thank Dr. H. Sawada (University of Tokyo) for technical advice on immunoelectron microscopy and Dr. H. Hosoya (The Tokyo Metropolitan Institute of Medical Science) for kindly giving us the microtubule-associated proteins fraction and for helpful discussions. Our thanks are also due to Drs. E. Muto, S. Yonemura, and Mr. T. Yagi, (The Tokyo Metropolitan Institute of Medical Science) for their useful discussions and encouragements through this study. We wish to acknowledge Miss N. Funayama for her excellent photographic work and Mrs. C. Torii for technical assistance. Y. Hieda thanks Dr. K. Owaribe (Nagoya University) for giving him a chance to work in the Department of Ultrastructural Research in the Tokyo Metropolitan Institute of Medical Science for two years and for his encouragement far from the German Cancer Research Center in West Germany.

This study was supported by research grants from the Ministry of Education, Science, and Culture of Japan.

Received for publication 31 March 1989 and in revised form 7 June 1989.

References

1. Blashuk, O. W., R. L. Manteuffel, and M. S. Steinberg. 1986. Purification of desmoglein II: a method for the preparation and fractionation of desmosomal components. *Biochim. Biophys. Acta.* 883:426–431.
2. Cowin, P., H.-P. Kapprell, and W. W. Franke. 1985. The complement of desmosomal plaque proteins in different cell types. *J. Cell Biol.* 101:1442–1454.
3. Cowin, P., H.-P. Kapprell, W. W. Franke, J. Tamkun, and R. O. Hynes. 1986. Plakoglobin: a protein common to different kinds of intercellular adhering junctions. *Cell.* 46:1063–1073.
4. Cowin, P., D. Mattey, and D. Garrod. 1984. Identification of desmosomal surface components (desmocollins) and inhibition of desmosome formation by specific Fab'. *J. Cell Sci.* 70:41–60.
5. Drochmans, P., C. Freudenstein, J.-C. Wanson, L. Laurent, T. W. Keenan, J. Stadler, R. Leloup, and W. W. Franke. 1978. Struture and biochemical composition of desmosomes and tonofilaments isolated from calf muzzle epidermis. *J. Cell Biol.* 79:427–443.
6. Farquhar, M. G., and G. E. Palade. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* 17:375–412.
7. Franke, W. W., P. Cowin, M. Schmelz, and H.-P. Kapprell. 1987. The desmosomal plaque and the cytoskeleton. Junctional complexes of epithelial cells. *Ciba Found. Symp.* 125:26–48.
8. Geiger, B., A. H. Dutton, K. T. Tokuyasu, and S. J. Singer. 1981. Immunoelectron microscope studies of membrane-microfilament interactions: distribution of α-actinin, tropomyosin, and vinculin in intestinal epithelia brush border and in chicken gizzard smooth muscle cells. *J. Cell Biol.* 91:614–628.
9. Gorbsky, G., and M. S. Steinberg. 1981. Isolation of the intercellular glycoproteins of desmosomes. *J. Cell Biol.* 90:243–248.
10. Gorbsky, G., S. M. Cohen, M. Shida, G. J. Guidice, and M. S. Steinberg. 1985. Isolation of the non-glycosylated proteins of desmosomes and immunolocalization of a third plaque protein: desmoplakin III. *Proc. Natl. Acad. Sci. USA.* 82:810–814.
11. Green, K. J., R. D. Goldman, and R. L. Chisholm. 1988. Isolation of cDNAs encoding desmosomal plaque proteins: evidence that bovine desmoplakins I and II are derived from two mRNAs and a single gene. *Proc. Natl. Acad. Sci. USA.* 85:2613–2617.
12. Jones, J. C. R., K. M. Yokoo, and R. D. Goldman. 1986. A cell surface desmosome-associated component: identification of a tissue-specific cell adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 83:7282–7286.
13. Kapprell, H.-P., K. Owaribe, and W. W. Franke. 1988. Identification of a basic protein of M_r 75,000 as an accessory desmosomal plaque protein in stratified and complex epithelia. *J. Cell Biol.* 106:1679–1691.
14. Kartenbeck, J., E. Schmid, W. W. Franke, and B. Geiger. 1982. Different modes of internalization of proteins associated with adherens junctions and desmosomes: experimental separation of lateral contacts induces endocytosis of desmosomal plaque material. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:725–732.
15. Keller, G. -A., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1984. An improved procedure for immunoelectron microscopy: ultrathin plastic embedding of immunolabeled ultrathin frozen sections. *Proc. Natl. Acad. Sci. USA.* 81:5744–5747.
16. Köhler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and cell lines by cell fusion. *Eur. J. Immunol.* 6:511–519.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature (Lond.).* 227:680–685.
18. Maruyama, K., S. Matsubara, R. Natori, Y. Nonomura, S. Kimura, K. Ohashi, F. Murakami, S. Handa, and G. Eguchi. 1977. Connectin, an elastic protein of muscle: characterization and function. *J. Biochem. (Tokyo).* 82:317–337.
19. Mueller, H., and W. W. Franke. 1983. Biochemical and immunological characterization of desmoplakins I and II, the major polypeptides of the desmosomal plaque. *J. Mol. Biol.* 163:647–671.
20. Pytela, R., and G. Wiche. 1980. High molecular weight polypeptides (270,000–340,000) from cultured cells are related to hog brain microtubule-associated proteins but copurify with intermediate filaments. *Proc. Natl. Acad. Sci. USA.* 77:4808–4812.
21. Shelanski, M. L., E. Gaskin, and C. R. Cantor. 1973. Microtubules assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA.* 70:765–768.
22. Skerrow, C. J., and A. G. Matoltsy. 1974. Isolation of epidermal desmosomes. *J. Cell Biol.* 63:515–524.
23. Sherrow, C. J., I. Hunter, and D. Skerrow. 1987. Dissection of the bovine epidermal desmosome into cytoplasmic protein and membrane glycoprotein domains. *J. Cell Sci.* 87:411–421.
24. Staehelin, A. L. 1974. Structure and function of intercellular junctions. *Int. Rev. Cytol.* 39:191–283.
25. Steinberg, M. S., H. Shida, G. J. Giudice, M. Shida, N. H. Patel, and O. W. Blaschuk. 1987. On the molecular organization, diversity and functions of desmosomal proteins. Junctional complexes of epithelial cells. *Ciba Found. Symp.* 125:3–25.
26. Steinert, P. M., and W. W. Idler. 1976. Self-assembly of bovine epidermal keratin filaments in vitro. *J. Mol. Biol.* 108:547–567.
27. Tokuyasu, K. T. 1980. Immunochemistry on ultrathin frozen sections. *Histochem. J.* 12:381–403.
28. Tsukita, S., and S. Tsukita. 1985. Desmocalmin: a calmodulin-binding high molecular weight protein isolated from desmosomes. *J. Cell Biol.* 101:2070–2080.
29. Tsukita, S., and S. Tsukita. 1989. Isolation of cell-to-cell adherens junctions from rat liver. *J. Cell Biol.* 108:31–42.
30. Tyler, J. M., and D. Branton. 1981. Rotary shadowing of extended molecule from glycerol. *J. Ultrastruct. Res.* 71:95–102.
31. Wang, K., and C. L. Williamson. 1980. Identification of an N2 line protein of striated muscle. *Proc. Natl. Acad. Sci. USA.* 77:3254–3258.
32. Wang, K., J. McClure, and A. Tu. 1979. Titin: major myofibrillar component of striated muscle. *Proc. Natl. Acad. Sci. USA.* 76:3698–3702.
33. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.
34. Wiche, G., H. Herrmann, F. Leichtfried, and R. Pytela. 1982. Plectin: a high-molecular weight cytoskeletal polypeptide component that copurifies with intermediate filaments of the vimentin-type. *Cold Spring Harbor Symp. Quant. Biol.* 46:475–482.
35. Wiche, G., R. Krepler, U. Artlieb, R. Pytela, and H. Denk. 1983. Occurrence and immunolocalization of plectin in tissues. *J. Cell Biol.* 97:887–901.