Desmocalmin: A Calmodulin-binding High Molecular Weight Protein Isolated from Desmosomes

SACHIKO TSUKITA and SHOICHIRO TSUKITA
Department of Anatomy, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT A unique high molecular weight protein (240,000 mol wt) has been purified from isolated desmosomes of bovine muzzle epidermis, using low-salt extraction at pH 9.5–10.5 and gel-filtration followed by calmodulin-affinity column chromatography. This protein was shown to bind to calmodulin in a Ca^{2+}-dependent manner, so we called it desmocalmin here. Desmocalmin also bound to the reconstituted keratin filaments in vitro in the presence of Mg^{2+}, but not to actin filaments. By use of the antibody raised against the purified desmocalmin, desmocalmin was shown by both immunoelectron and immunofluorescence microscopy to be localized at the desmosomal plaque just beneath the plasma membrane. Judging from its isoelectric point and antigenicity, desmocalmin was clearly distinct from desmoplakins I and II, which were identified in the desmosomal plaque by Mueller and Franke (1983, *J. Mol. Biol.*, 163:647–671). In the low-angle, rotary-shadowing electron microscope, the desmocalmin molecules looked like flexible rods ~100-nm long consisting of two polypeptide chains lying side by side. The similar rodlike structures were clearly identified in the freeze-etch replica images of desmosomes. Taken together, these findings indicate that desmocalmin could function as a key protein responsible for the formation of desmosomes in a calmodulin-dependent manner (Trinkaus-Randall, V., and I. K. Gipson, 1984, *J. Cell Biol.*, 98:1565–1571).

Association of the cytoskeleton with cell membranes is a universal organizational feature in cells (for reviews, see references 1 and 2). Through this association, various functions of the cell membranes are controlled by the cytoskeleton (3–5). In this connection, the physiological roles of the cytoskeleton in the cell adhesion mechanism have been paid special attention by many investigators. It has been shown that, together with vinculin and talin, the actin filaments play a crucial role in cell-to-cell or cell-to-substrate adhesion (for reviews, see references 1 and 6). The other specialized junction is the desmosome (for reviews, see references 7 and 8). In the desmosome, the plasma membranes of neighboring cells are closely associated through intercellular matrix, and 10-nm filaments are associated with the plasma membranes, extending deep into the cytoplasm (9–13). Thus, the desmosome is suitable for the study of how 10-nm filaments are involved in the cell-to-cell adhesion.

Desmosomes are particularly accumulated in the epithelial cells. Skerrow and Matoltsy have succeeded in isolating desmosomes from cow muzzle epidermis, showing ~24 polypeptides bands upon SDS PAGE (14–16). Gorbsky and Steinberg (17) have prepared a desmosomal core fraction that consists primarily of highly glycosylated proteins including desmocollins I and II (18, 19). Recently, antigens of two high molecular weight polypeptides have been detected in desmosomal plaque structures and have been designated as desmoplakin I and II by Mueller and Franke (20) and Franke et al. (21). Most recently, desmoplakin III (81 kD) has been immunolocalized to the desmosomal plaque (22). However, since desmoplakins have not yet been isolated in their native forms, the association between 10-nm filaments and plasma membranes in desmosomes remains to be elucidated at the molecular level.

Recent studies have shown that the desmosome is formed in a Ca^{2+}-dependent manner (11, 23–26) and that calmodulin may be involved in its formation (25). Therefore, we have attempted to purify calmodulin-binding proteins from desmosomes without denaturing reagents. In the present paper, we describe the purification procedure and some biochemical, morphological, and immunological aspects of a new calmodulin-, keratin filament–binding protein isolated from desmosomes of cow muzzle epidermis. We have designated it desmocalmin for the desmosome calmodulin-binding protein.

THE JOURNAL OF CELL BIOLOGY · VOLUME 101 DECEMBER 1985 2070-2080

Desmocalmin is shown to be distinct from other desmosomal plaque components, especially from desmoplakins in its isoelectric point and antigenicity.

MATERIALS AND METHODS

Isolation of Desmosomes: Bovine muzzle was obtained fresh at the slaughterhouse. After the outermost layer of mainly stratum corneum of seven muzzles was removed with razor blades, the underlying layer (stratum spinosum) was collected and soaked in 0.1 M citric acid–sodium citrate buffer (pH 2.6) containing 0.05% Nonidet P-40 and 5 μg/ml each of leupeptin and pepstatin A. The desmosome-enriched fraction was isolated basically according to the method of Gorbsky and Steinberg (17). The slices of the stratum spinosum were finely minced with razor blades and then stirred vigorously in the above buffer for 3 h at 4°C. After the homogenate was passed through 50-μm nylon mesh, the filtrate was centrifuged at 13,000 *g* for 20 min at 4°C. The pellet was then resuspended in 0.1 M citric acid–sodium citrate buffer (pH 2.6) containing 0.01% Nonidet P-40 and protease inhibitors, and centrifuged at 750 *g* for 20 min at 4°C. After the supernatant was spun at 12,000 *g* for 20 min at 4°C, the upper layer of the pellet was resuspended and centrifuged again at 12,000 *g* for 20 min at 4°C. This process was repeated twice more. The obtained desmosome fraction (see Fig. 1) was used for purification of desmocalmin.

Purification of Desmocalmin: All procedures were performed at 4°C. The desmosomes obtained from seven bovine muzzles were suspended in 100 ml of low-salt extraction buffer consisting of 0.1 mM EDTA, leupeptin (10 μg/ml, pH 7.2), and its pH was adjusted to 9.5–10.5 by the addition of NaOH. After 30 min with gentle stirring, the suspension was centrifuged at 100,000 *g* for 60 min. Ammonium sulfate was added to the supernatant to 80% saturation and stirred for 30 min. The resultant precipitate was collected by centrifugation at 220,000 *g* for 20 min and dissolved in 10 ml buffer A. Buffer A was composed of 100 mM KCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, leupeptin (1 μg/ml), pepstatin A (1 μg/ml), 2 mM phenylmethylsulfonyl fluoride. After dialysis against 1 liter buffer A, the solution was clarified by centrifugation at a force of 220,000 *g* for 20 min and subjected to gel filtration on a column (3.5 × 50 cm) of Sepharose CL-4B equilibrated with buffer A. The fractions enriched in high molecular weight proteins that retarded from the void volume fractions were combined. The addition of ammonium sulfate to 80% saturation was followed by gentle stirring for 30 min. After the solution was centrifuged at 220,000 *g* for 20 min, the precipitate was dissolved in 4 ml buffer A containing 0.3 mM $CaCl_2$ and dialyzed against the buffer A, 0.3 mM $CaCl_2$. To remove insoluble material the solution was spun at 220,000 *g* for 20 min, and the supernatant was applied to the calmodulin-affinity column (Affigel-calmodulin, purchased from Bio-Rad Laboratories, Richmond, CA) equilibrated with buffer A containing 0.3 mM $CaCl_2$. Desmocalmin was eluted from the column with buffer A containing 1 mM EGTA and 3 mM EDTA.

Calmodulin-binding Assay: The calmodulin gel overlay technique was used, basically according to the method of Glenney and Weber (27), using *N*-(7-dimethylamino-4-methylcoumarinyl)-maleimide (DACM)[1]-labeled calmodulin. DACM-calmodulin was prepared by the method of Hamaguchi and Iwasa (28).

F-Actin-binding Assay: G-actin (5 mg/ml in 2 mM Tris-HCl, 0.2 mM ATP, pH 7.5) obtained from rabbit skeletal muscle (29) was mixed with an equal volume of buffer A and incubated at room temperature for 1 h for polymerization. The F-actin thus obtained was added to 8 vol desmocalmin solution (30 μg/ml), which had been clarified by centrifugation at 100,000 *g* for 1 h in buffer A, 1 mM EGTA, 3 mM EDTA, with or without 6 mM $MgSO_4$. After F-actin was incubated with desmocalmin on ice for 1 h, the mixture was spun at 220,000 *g* for 15 min at 4°C, and then the supernatant and pellet were analyzed by SDS PAGE.

Keratin Filament–binding Assay: Keratins were extracted from bovine epidermis in the posterior region of the hoof with a solution of 8 M urea, 50 mM Tris-HCl (pH 9.0), 25 mM 2-mercaptoethanol according to the method developed by Steinert and Idler (30). Keratin filaments were reconstituted by dialysis of the extracted polypeptides (1–2 mg/ml) against 1,000 vol 5 mM Tris-HCl (pH 7.6) in the presence of 25 mM 2-mercaptoethanol. We used two-cycle reconstituted keratin filaments for binding assay with desmocalmin after they were dialyzed against buffer A. 100 μl of the filament solution (1 mg/ml) was mixed with 3 ml of desmocalmin solution (35 μg/ml in buffer A, 1 mM EGTA, 3 mM EDTA) in the presence or absence of 10 mM $MgCl_2$. Before incubation, desmocalmin solution was clarified by centrifugation at 220,000 *g* for 20 min at 4°C. The incubation was continued on ice for 2 h and followed by centrifugation at 9,000 *g* for 15 min. For control experiments, 100 μl buffer A was used instead of keratin filaments. The supernatant and pellet were examined by SDS PAGE.

Immunological Methods: Antisera to desmocalmin were raised in rabbits. 5 ml desmocalmin solution (35 μg/ml in buffer A, 1 mM EGTA, 3 mM EDTA) was concentrated to 0.5 ml by ultrafiltration in the presence of 0.1% Tween 20 on ice and thoroughly emulsified with 0.5 ml of Freund's complete adjuvant. After the preimmune serum was collected, the rabbits were injected subcutaneously with the emulsified antigen. 3 wk later, the rabbits were boosted with the antigen in Freund's complete adjuvant. Blood was taken from the hearts of the anesthetized rabbits. After the blood was clotted at room temperature for 3 h and placed at 4°C overnight to facilitate clot retraction, the serum was collected by centrifugation at 100,000 *g* for 30 min. Immune sera were assayed by a solid-phase enzyme-linked immunosorbent assay. IgG was purified from the serum using ammonium sulfate precipitation and DEAE-cellulose column chromatography.

Immunoblotting was performed by one- or two-dimensional gel electrophoresis, followed by electrophoretic transfer to nitrocellulose sheets as described by Vaessen et al. (31). Nitrocellulose sheets were treated with the anti-desmocalmin antibody and then with horseradish peroxidase–labeled goat anti-rabbit IgG, and the localization of peroxidase was detected by the reaction using chloro-1-naphthol solution.

For indirect immunofluorescence microscopy, the bovine muzzle epidermis

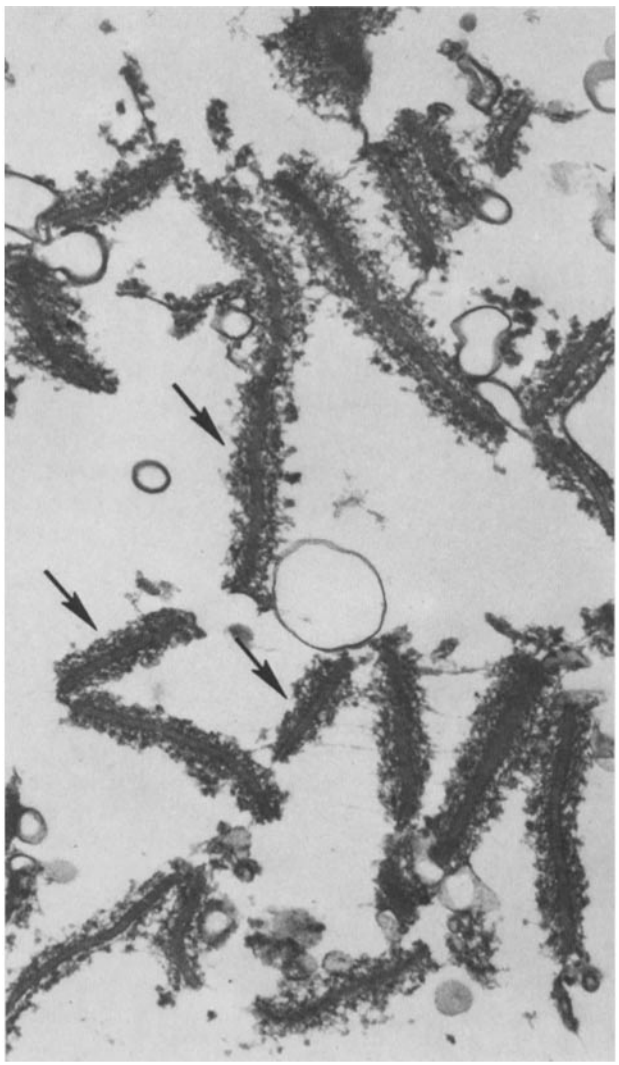

FIGURE 1 Electron micrograph of the desmosomes isolated from bovine muzzle epidermis. Each desmosome (arrows) is seen to be composed of two plasma membranes that are closely associated by the intercellular matrix. Almost all keratin filaments are dissolved, leaving short filamentous structure on the cytoplasmic surface of the desmosomes. × 23,000.

[1] *Abbreviation used in this paper:* DACM, *N*-(7-dimethylamino-4-methylcoumarinyl)-maleimide.

and the bovine esophagus epithelium were frozen using liquid nitrogen, and frozen sections were immunofluorescently stained as described by Levine and Willard (32). For electron microscopical localization of desmocalmin in the bovine muzzle epidermis, the immunoperoxidase procedure was applied to the frozen sections according to the method of Jarasch et al. (33) and Franke et al. (34).

Gel Electrophoresis: One-dimensional SDS PAGE was based on the method of Laemmli (35), and the stain Coomassie Brilliant Blue R-250 was used. For two-dimensional gel electrophoresis, the isoelectric focusing was followed by SDS PAGE, according to the method of O'Farrell (36), with the slight modification that the sample was applied on the acidic sides in the isoelectric focusing. The silver staining of the two-dimensional gels employed the method of Oakley et al. (37).

Thin-Section Electron Microscopy: Samples were processed as previously described, using 0.5% tannic acid, 2.5% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.4) as a fixative (38, 39).

Low-Angle Rotary-Shadowing Electron Microscopy: The molecular shape of the purified desmocalmin was examined by means of low-angle rotary-shadowing electron microscopy according to the method described by Tyler and Branton (40). To detect the binding sites of the keratin filaments in the desmocalmin molecules, a mixture of desmocalmin and the keratin filaments was also examined.

Rapid-Freeze Deep-Etch Replica Electron Microscopy: The stratum spinosum of fresh bovine muzzle was cut into small pieces and soaked in buffer A containing 0.05% Nonidet P-40 at 4°C for 10 h. The samples were washed with buffer A several times at 4°C for 2 h and then soaked in buffer A diluted 10-fold with distilled water. They were immediately placed in the freezing apparatus (RF-10, Eiko Engineering Co., Ltd., Mito, Japan) and rapidly frozen by being touched against a pure copper block cooled to 4°K. Then the samples were processed for deep-etching and subsequent replication, as described by Tsukita et al. (5, 41).

RESULTS

Calmodulin-binding Protein in the Desmosome

Taking advantage of the application of the calmodulin gel overlay technique to the isolated desmosome fraction, we screened the calmodulin-binding proteins in desmosomes. For this purpose, we used the desmosome fraction from bovine muzzles, as shown in Fig. 1. As a result, only one polypeptide of ~240 kD was shown to bind to calmodulin in a Ca^{2+}-dependent manner. Thus, we have attempted to purify this high molecular weight polypeptide without denaturing agents. On one-dimensional gel electrophoresis of the bovine muzzle epidermis, stratum spinosum, the keratin polypeptides ranging from 48 to 68 kD were prominent (see reference 13), and some minor polypeptides were also present in the high molecular weight region (Fig. 2). These high molecular weight polypeptides were strikingly enriched in the desmosome fractions and effectively solubilized with a low-salt solution at pH 9.5–10.5. Subsequent ammonium sulfate fractionation and gel filtration on Sepharose CL-4B yielded a fraction enriched in the four to five major polypeptides of 180–240 kD. The final steps in purification of desmocalmin were ammonium sulfate fractionation and Affigel-calmodulin column chromatography. Among the high molecular weight polypeptides, only the 240-kD protein remained exclusively in the calmodulin-affinity column in the presence of Ca^{2+}. When the column was eluted with the solution containing enough EGTA and EDTA to chelate Ca^{2+}, the 240-kD protein was obtained in a high degree of purity (Fig. 2). By the calmodulin gel overlay technique, we detected Ca^{2+}-dependent binding of DACM-labeled calmodulin to this purified 240-kD protein (Fig. 2). Hence, we here call this protein desmocalmin, for desmosome calmodulin-binding protein. As far as we determined, desmocalmin resisted extraction in buffers of a broad range of salt concentrations (1 mM–0.6 M) at pH 4–8. Only by the low-salt alkaline solution treatment was desmocalmin

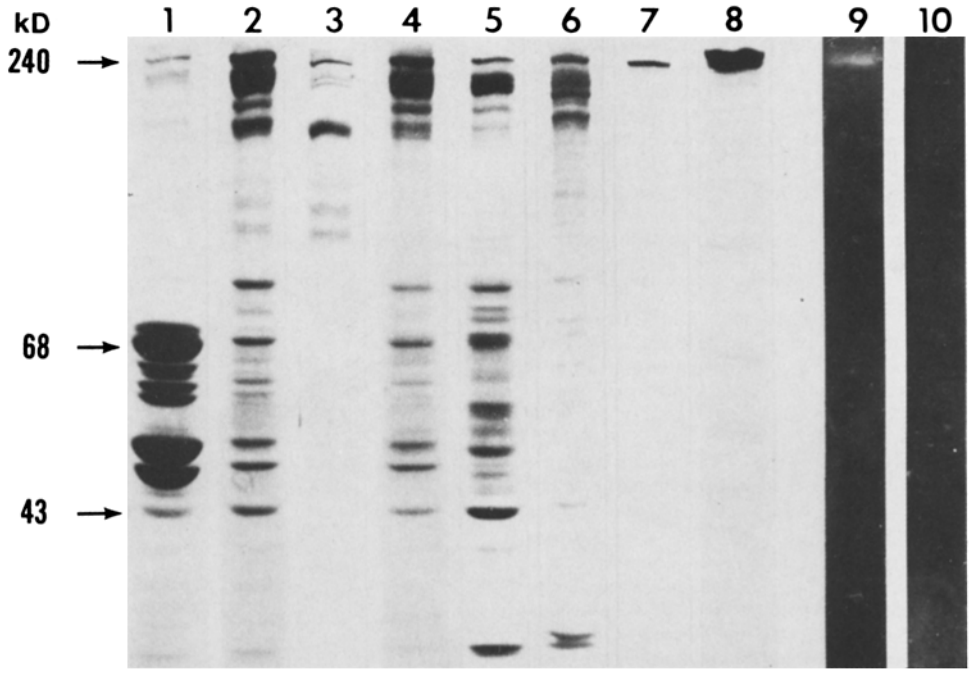

FIGURE 2 SDS polyacrylamide gels showing the protein profiles during the purification of desmocalmin, and calmodulin-binding assay of purified desmocalmin. Lane *1*, bovine muzzle epidermis (stratum spinosum). Lane *2*, isolated desmosome fraction. Lanes *3* and *4*, pellet and supernatant after centrifugation of the low-salt extract of desmosomes, respectively. Lane *5*, the proteins applied on a Sepharose CL-4B column after ammonium sulfate precipitation. Lane *6*, the gel-filtrated fractions that retarded from the void volume fractions. The fractions enriched in the high molecular weight proteins were combined. Lanes *7* and *8*, purified desmocalmin eluted from calmodulin affinity column. In lane *8*, the desmocalmin was heavily loaded on the gel for confirming the purity. Lanes *9* and *10*, DACM-calmodulin binding on nitrocellulose sheets in the presence or absence of Ca^{2+}, respectively, after electrophoretic transfer from SDS polyacrylamide gels of the purified desmocalmin.

solubilized from desmosomes, and, once extracted, desmocalmin was soluble even in the near-physiological buffer.

To determine the apparent molecular weight of desmocalmin, we performed co-electrophoresis with human erythrocyte spectrin (240 and 220 kD) and bovine brain spectrin (calspectin or fodrin, 240 and 235 kD). The 240-kD polypeptide of the erythrocyte and brain spectrins co-migrated with desmocalmin, indicating that the molecular weight of desmocalmin was 240-kD on the SDS gel (Fig. 3).

Two-Dimensional Gel Electrophoresis

The pattern of two-dimensional gel electrophoresis of isolated desmosomes has already been described by Mueller and Franke (20). We re-examined this pattern and focused our attention on the high molecular weight region. As already mentioned, desmoplakins I and II were isoelectric at approximately neutral pH. In contrast, purified desmocalmin was isoelectrically focused at an acidic pH of ~5.5 (Fig. 4). When desmocalmin was co-electrophoresced with desmosome fractions on the two-dimensional gel, it became clear that the isoelectric points of desmocalmin and desmoplakins differed (Fig. 4). On the two-dimensional gel of isolated desmosomes, the clear spot was barely identified in the region where desmocalmin should be focused (Fig. 4). Furthermore, in co-electrophoresis of desmocalmin with isolated desmosome fractions, the longer the incubation before co-electrophoresis, the less intense was the desmocalmin spot. These results indicated that in the desmosome fraction desmocalmin was closely associated with some components, so it was barely solubilized for the isoelectric focusing. To examine this interpretation, the immunological method was used.

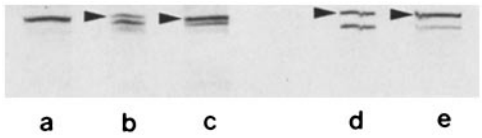

FIGURE 3 Co-electrophoresis of purified desmocalmin with brain and erythrocyte spectrins on SDS polyacrylamide gels. Lane *a*, purified desmocalmin. Lane *b*, bovine brain spectrin. Lane *c*, mixture of desmocalmin and bovine brain spectrin. Lane *d*, human erythrocyte spectrin. Lane e, mixture of desmocalmin and human erythrocyte spectrin. The 240-kD polypeptide of the spectrins (arrowheads) co-migrates with desmocalmin.

Immunoblotting with Anti-Desmocalmin

Desmocalmin was characterized immunologically by the immunoblotting procedure using peroxidase. On one-dimensional gels of isolated desmosome fractions, anti-desmocalmin stained only the 240-kD polypeptide, showing no cross-reactivity with any other polypeptides (Fig. 5). When purified desmocalmin was subjected to two-dimensional gel electrophoresis and then to immunoblotting, anti-desmocalmin was associated with the spot of desmocalmin (Fig. 4). When the isolated desmosome fraction was analyzed by two-dimensional electrophoresis and then by immunoblotting, anti-desmocalmin stained exclusively one spot of 240 kD in the origin of the isoelectric gel (Fig. 4). Neither desmoplakin I

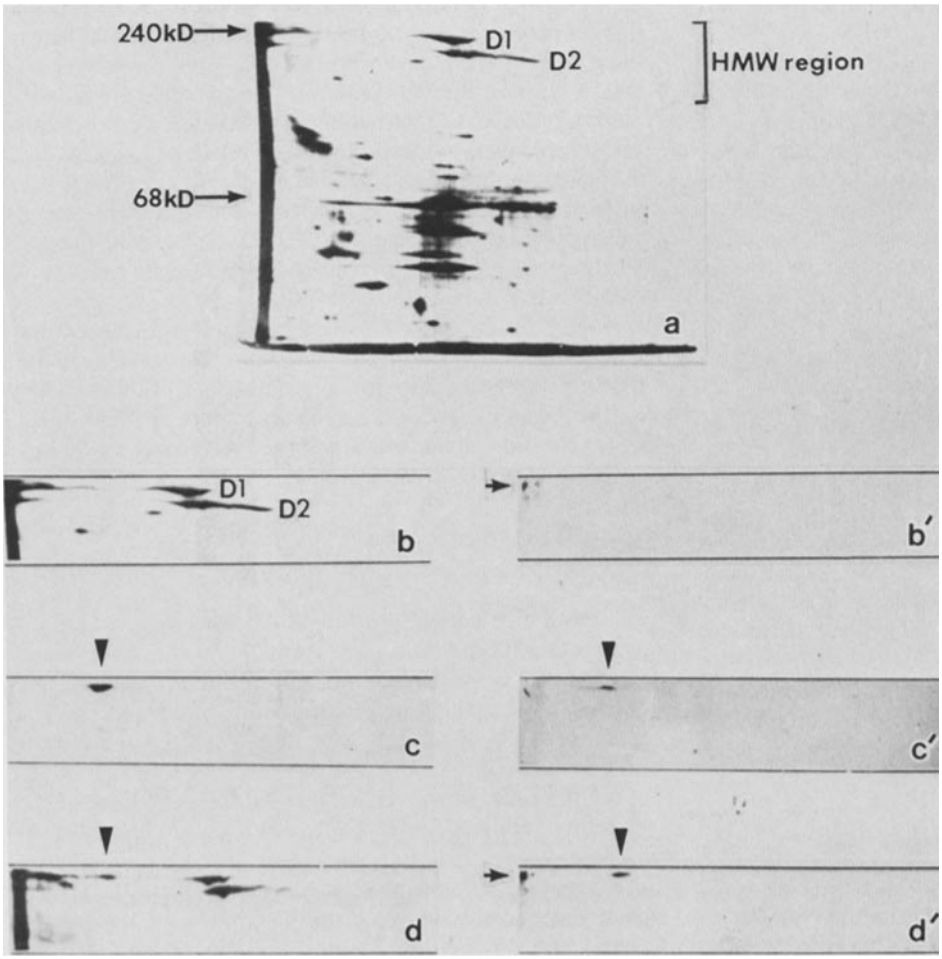

FIGURE 4 (*a–d*) Comparison between desmocalmin and desmoplakins I and II by two-dimensional gel electrophoresis. The samples were applied on the acidic sides in the isoelectric focusing. The gels were stained by the silver staining method (37). In *a*, the whole gel was provided, whereas in *b–d*, only the high molecular weight (*HMW*) regions were shown. In the two-dimensional gel pattern of desmosome fractions (*a*, *b*), desmoplakins I and II (*D1*, *D2*) were easily identified based on their molecular weights and isoelectric points (see reference 20). Purified desmocalmin (*c*) showed a different isoelectric point (arrowheads). This difference was further confirmed when purified desmocalmin and desmosome fractions were co-electrophoresed (*d*). (*b'–d'*) Immunoblots of *b–d* with anti-desmocalmin. These immunoblots showed that neither desmoplakin I nor II reacted with anti-desmocalmin and that in desmosome fractions (arrows) desmocalmin was retained at the origin without being focused.

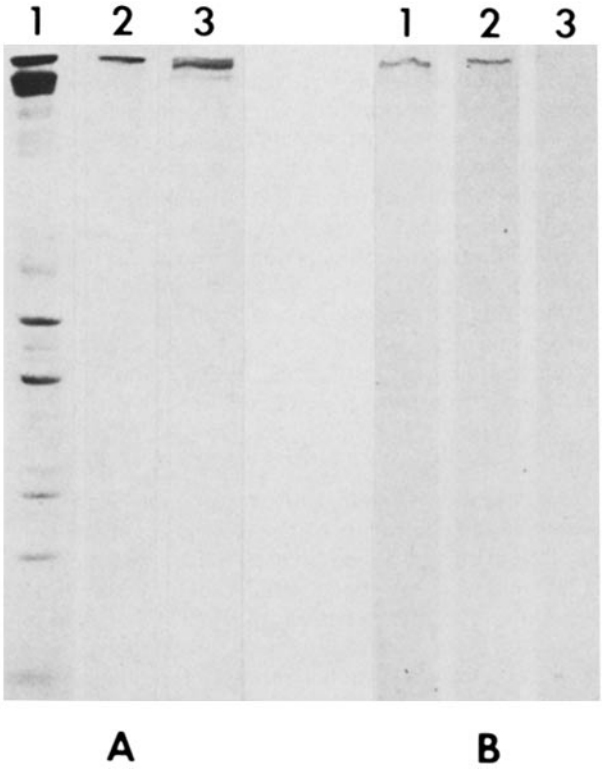

FIGURE 5 One-dimensional gel electrophoresis (*A*) and accompanying immunoblot with anti-desmocalmin. Lane *1*, desmosomes. Lane *2*, purified desmocalmin. Lane *3*, bovine brain spectrin. Anti-desmocalmin reacted with a single band (240 kD) in desmosome fractions, but not with brain spectrin.

nor II reacted with anti-desmocalmin. On the two-dimensional gel of the mixture of purified desmocalmin and the isolated desmosome fractions, anti-desmocalmin stained both of the spots of desmocalmin and the origin of 240 kD (Fig. 4). These results led us to conclude that, in the isoelectric focusing of the isolated desmosome fractions, desmocalmin was retained at the origin without being focused and that the purified desmocalmin was different from desmoplakins in its isoelectric point and antigenicity.

Molecular Shape of Desmocalmin

Desmocalmin molecules looked like elongated rods under low-angle rotary-shadowing electron microscopy (Fig. 6). The rods appeared to consist of two polypeptide chains lying side by side. The overall contour length was 100 ± 10 nm, mean ± SD. Considering that this morphology was similar to that of the dimeric forms of erythrocyte and brain spectrin molecules, the isolated desmocalmin molecules could be side-by-side dimers of the major species. We failed to detect tetrameric forms of desmocalmin in various conditions used for spectrin-like proteins. Actually, when the purified desmocalmin molecules were passed through our Sepharose CL-4B column together with human erythrocyte spectrin dimers and tetramers, they were eluted together with the spectrin dimers.

Co-sedimentation of Desmocalmin with Keratin Filaments

We examined the interactions of desmocalmin with 10-nm filaments of keratin by co-sedimentation experiments. For this purpose, keratin filaments were reconstituted from the bovine epidermis of the posterior hoof region (Figs. 7 and 8). The hoof keratin filament preparations did not contain any high molecular weight proteins, an advantageous condition for an experiment on binding of desmocalmin with keratin filaments. In the presence of Mg^{2+} desmocalmin co-precipitated with keratin filaments, whereas in the absence of Mg^{2+} it showed no ability to bind keratin filaments (Fig. 7).

The mixture of desmocalmin and keratin filaments was examined in the presence of Mg^{2+} by low-angle rotary-shadowing electron microscopy. Desmocalmin molecules seemed to attach to the keratin filaments at their ends in an end-on fashion (Fig. 8). Some molecules appeared to cross-link 10-nm filaments, although the cross-linking ability failed to be biochemically detected.

For comparison, similar co-sedimentation of desmocalmin was done using actin filaments; these experiments showed no binding affinity between desmocalmin and actin filaments (Fig. 9).

Localization of Desmocalmin in Muzzle Epidermis and Esophagus Epithelium

Using anti-desmocalmin, we tested whether desmocalmin was localized only in desmosomes in the bovine muzzle epidermis. By indirect immunofluorescence microscopy, desmocalmin specific fluorescence was highly concentrated at the periphery of epidermal cells other than those in the stratum corneum (Fig. 10). When the *en face* view of the cell periphery was revealed in obliquely cut sections, the spots of fluorescence were seen to be scattered (Fig. 10). To identify these spots at the electron microscopic level, the frozen sections of bovine muzzle epidermis were incubated with anti-desmocalmin and then treated with peroxidase-labeled anti-rabbit IgG, and the localization of anti-rabbit IgG was detected using diaminobenzidine (Fig. 10). As a result, the cytoplasmic surfaces of the desmosomes appeared to be intensely stained, which suggests that desmocalmin was localized in the desmosomal plaque, especially enriched just beneath the plasma membrane of the desmosome.

Epithelial cells of the bovine esophagus also showed desmocalmin-specific fluorescence at the cell periphery in the stratum basale and stratum spinosum (Fig. 11). Depending on the plane of sections, the distinct spots of fluorescence were resolved. In the stratum corneum, almost no fluorescence was detected.

Rapid-Freeze Deep-Etch Replica Image of Desmosomes

The question naturally arose whether the elongated rodlike structures ~100-nm long were identified in the desmosomal plaque at the electron microscopic level. For this purpose, the rapid-freeze deep-etch replica method is highly potent. The epidermal cells of stratum spinosum of the bovine muzzle were treated with Nonidet P-40 to extract the soluble proteins inside the cells. In the replica images, the cytoplasm of the epidermal cells was densely packed with keratin filaments. At the desmosomes in the periphery of the cells, keratin filaments were seen to graze along the cytoplasmic surface of the plasma membranes and were associated laterally with the plasma membranes through thin strands (Fig. 12). Careful observa-

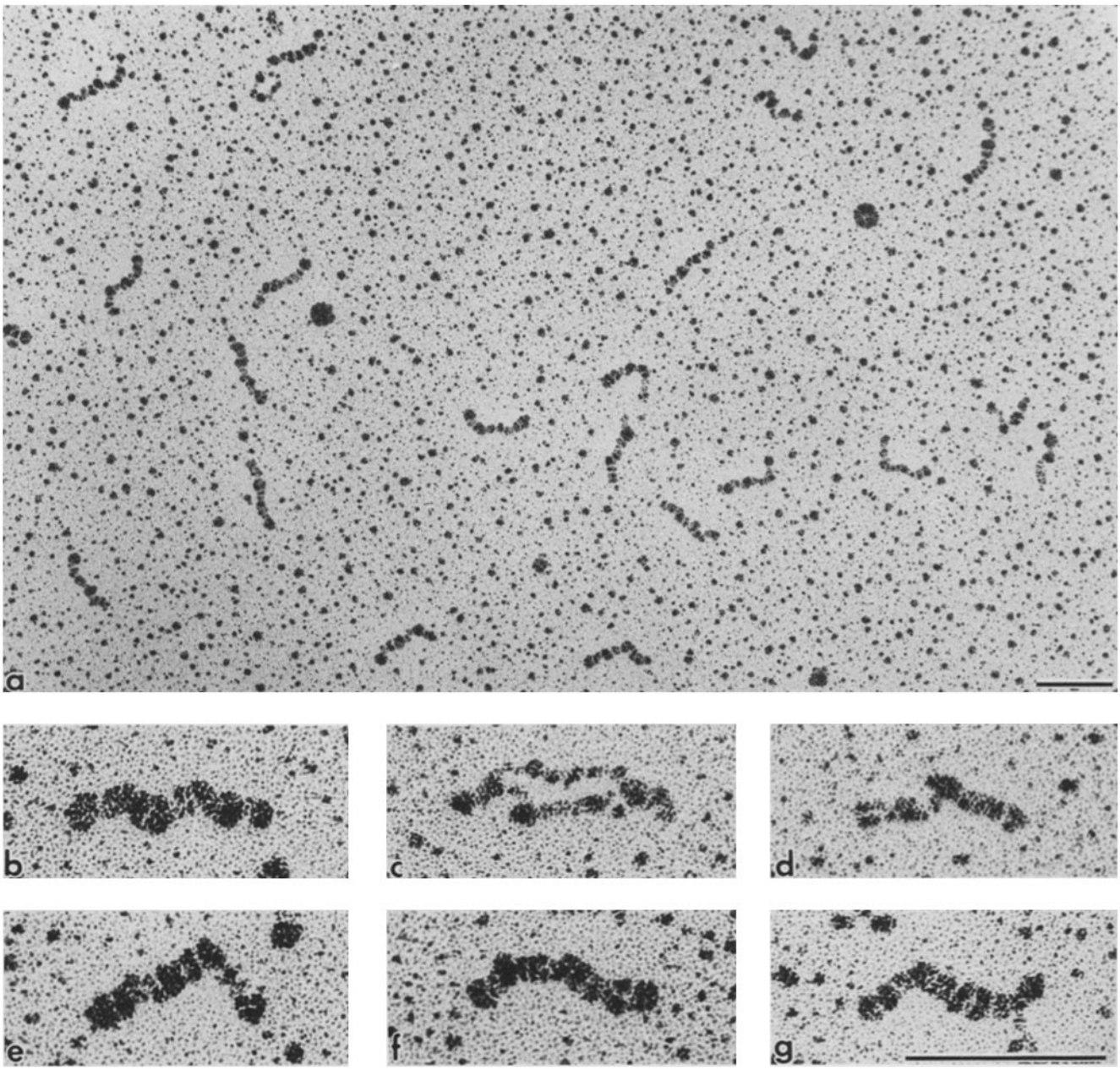

FIGURE 6 Morphology of desmocalmin molecules in rotary-shadowed preparations. Desmocalmin molecules resemble elongated rods consisting of two polypeptide chains lying side by side. In *c*, note the separation of two twisted strands in the middle portion. (*a*) Bar, 0.1 μm. × 120,000. (*b–g*) Bar, 0.1 μm. × 320,000.

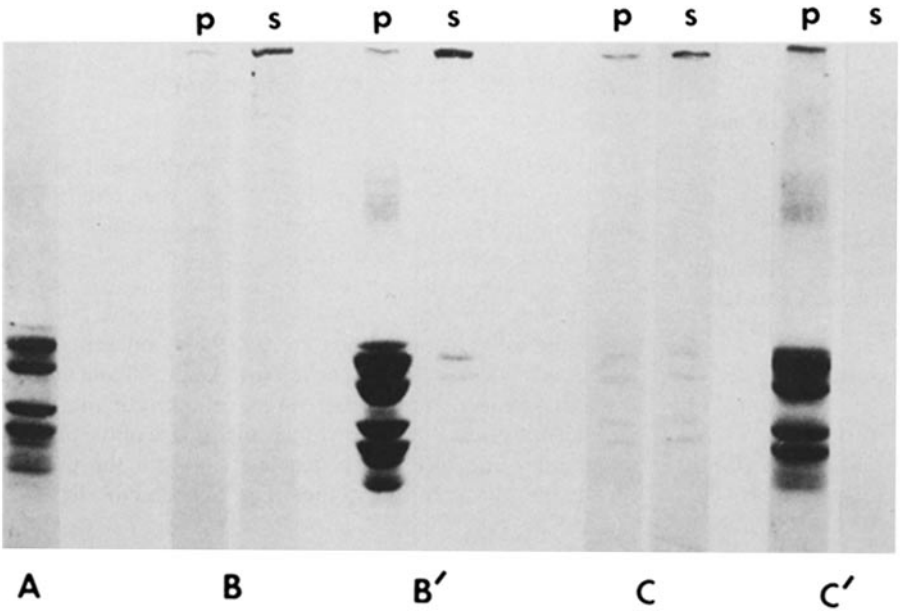

FIGURE 7 Co-sedimentation of desmocalmin with keratin filaments. (*A*) Keratin filaments reconstituted from bovine epidermis of hoof. Note that no high molecular weight polypeptides are contained in this fraction. (*B* and *B′*) Co-sedimentation experiments in the absence of Mg^{2+}. With (*B′*) or without (*B*) keratin filaments, almost all desmocalmin was recovered in the supernatant (*s*). (*C* and *C′*) Co-sedimentation in the presence of Mg^{2+}. Without keratin filaments (*C*) most desmocalmin was recovered in the supernatant, whereas with keratin filaments (*C′*) desmocalmin was largely pelleted (*p*).

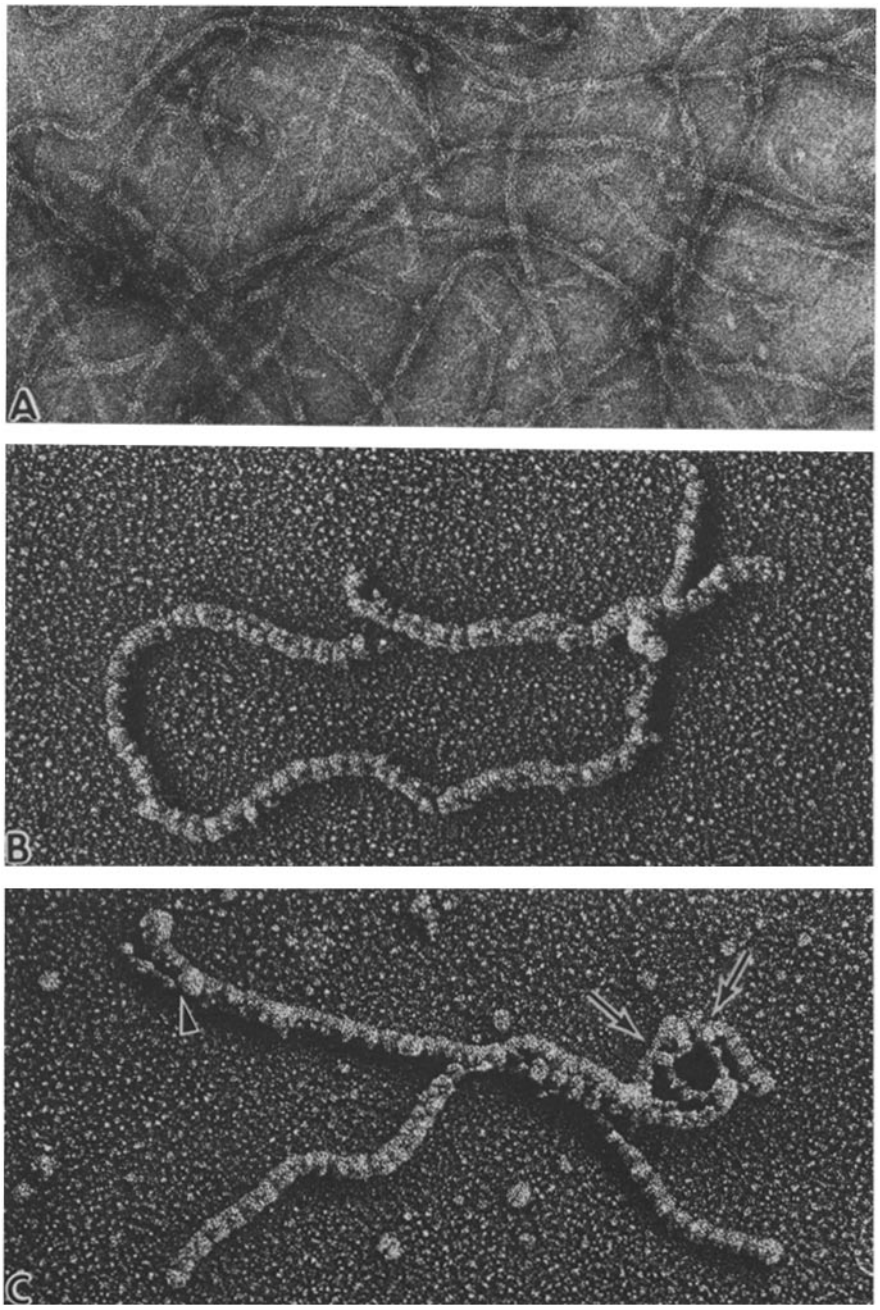

FIGURE 8 Electron micrographs of keratin reconstituted filaments (*A*, *B*) and a mixture of keratin filaments and purified desmocalmin (*C*). (*A*) Negative stained preparations. The typical 10-nm filaments are reconstituted. × 150,000. (*B*) Rotary-shadowed preparations. × 120,000. Keratin filaments are seen to be thickened mainly due to platinum deposition. No protofilamentous thin structures are observed. (*C*) Rotary-shadowed preparations. × 120,000. Desmocalmin molecules seem to attach to keratin filaments at one (arrowhead) or both (arrows) ends.

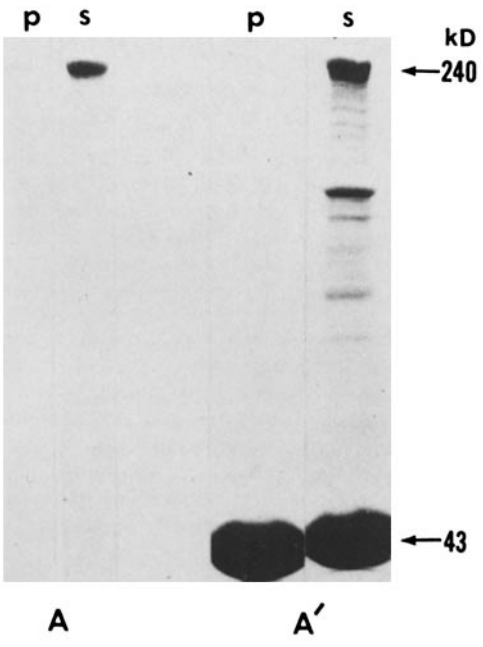

FIGURE 9 Co-sedimentation of desmocalmin with actin filaments in the presence of Mg^{2+}. Desmocalmin was incubated with (*A′*) or without (*A*) actin filaments and so showed no binding affinity between desmocalmin and actin filaments. *p*, pellet. *s*, supernatant.

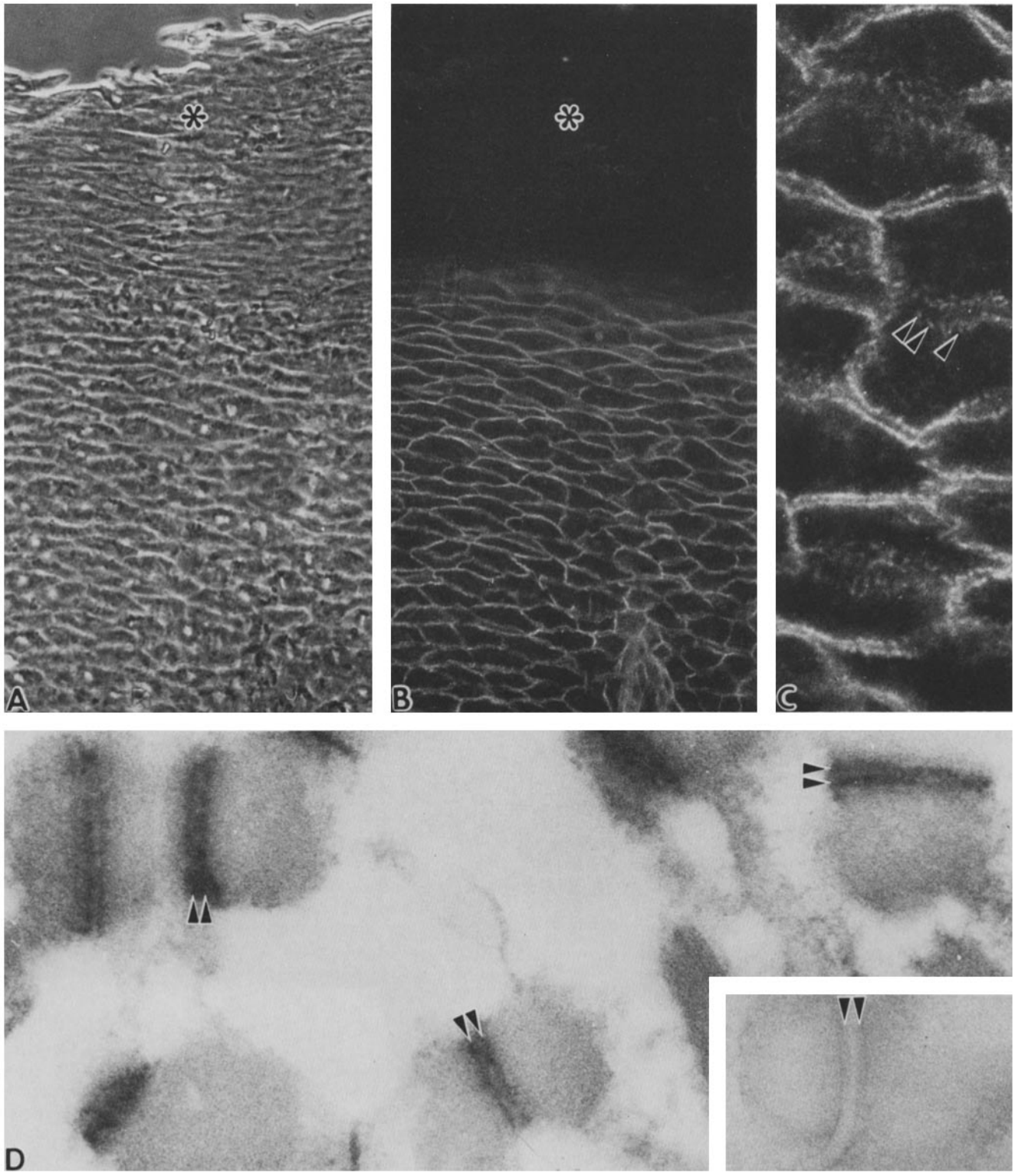

FIGURE 10 Localization of desmocalmin in muzzle epidermis. (*A*) Phase-contrast micrograph of bovine epidermis. Note the stratum spinosum and stratum corneum (asterisk). (*B*) Indirect immunofluorescence micrographs of the same section with anti-desmocalmin. Intense fluorescence appears at the periphery of each epidermal cell in stratum spinosum but not in stratum corneum (asterisk). When the preimmune serum was used instead of anti-desmocalmin, no fluorescence was detected. × 230. (*C*) Higher magnification of immunofluorescence micrographs. The spots (arrowheads) of fluorescence are clearly identifiable. × 1,200. (*D*) Immunoelectron microscopy. The frozen sections of the bovine muzzle epidermis were incubated with anti-desmocalmin and then treated with peroxidase-labeled anti-rabbit IgG, which was localized by the use of diaminobenzidine. The cytoplasmic surfaces of desmosomes appeared to be intensely stained (arrowheads). *Inset*: control experiments. The frozen sections were incubated with preimmune serum. × 100,000.

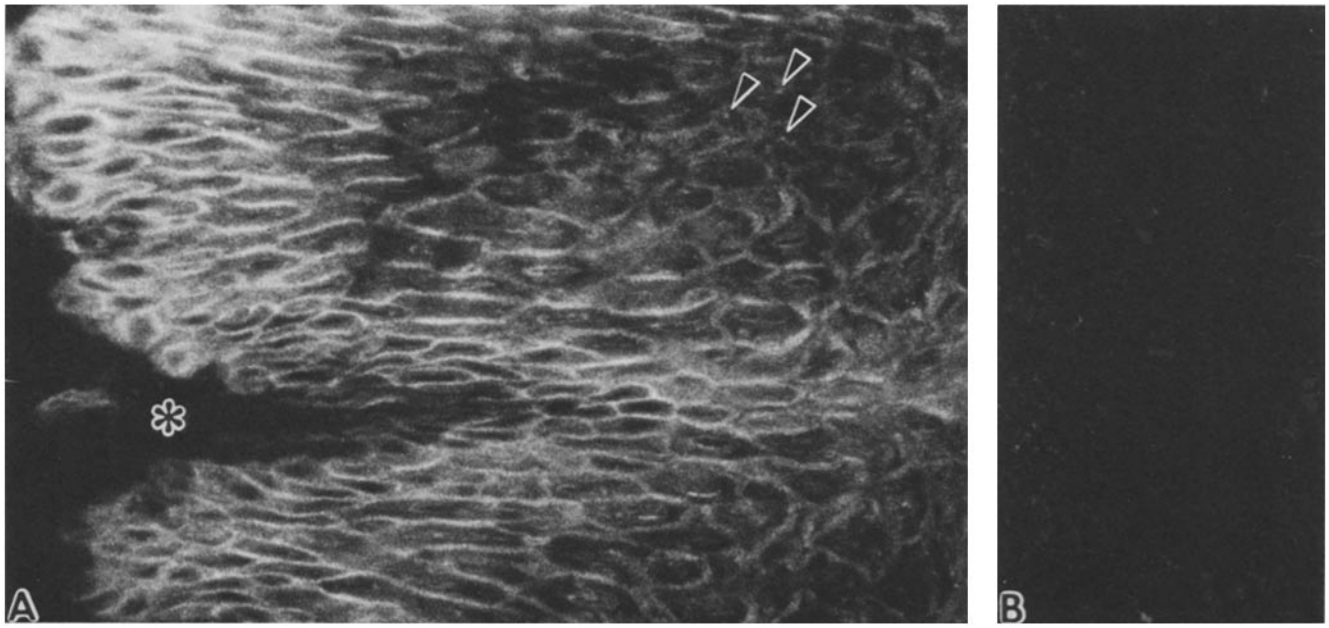

FIGURE 11 Localization of desmocalmin in esophagus epithelium by indirect immunofluorescence microscopy. (*A*) In the stratum basale and stratum spinosum, fluorescence appears at the cell periphery. The spots (arrowheads) of fluorescence are resolved in the obliquely cut region. No fluorescence is detected in the connective tissue (asterisk). (*B*) Almost no fluorescence is observed in the stratum corneum. × 540.

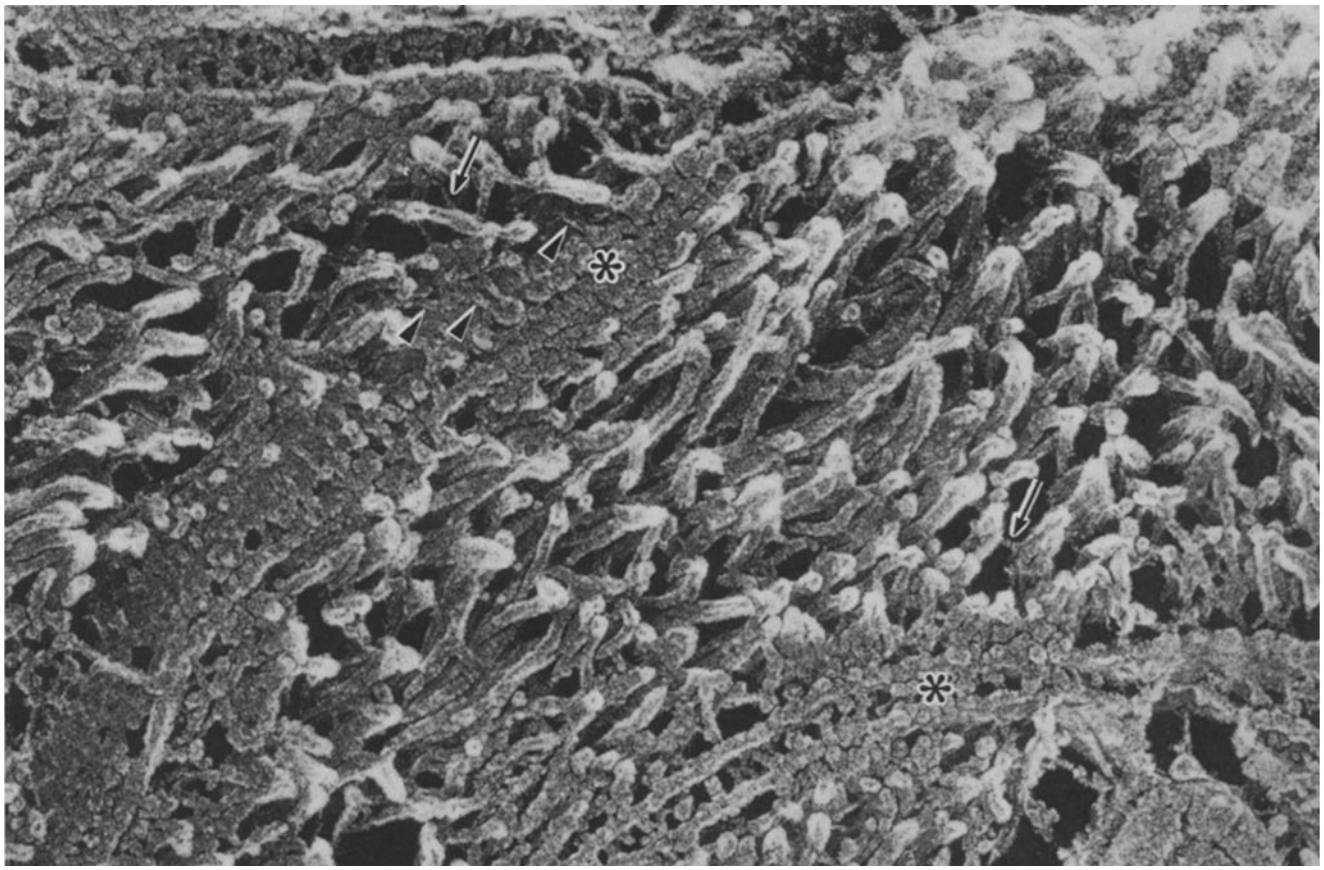

FIGURE 12 Rapid-freeze deep-etch replica electron micrograph of muzzle epidermal cells of stratum spinosum. After the epidermal cells were treated with Nonidet P-40 to wash out the soluble proteins, they were rapidly frozen using liquid helium and processed for the deep-etch replica electron microscopy. At the desmosomes (asterisks), keratin filaments are seen to attach to the cytoplasmic surfaces through the rodlike strands (arrowheads). The cytoplasm is densely packed by keratin filaments, which seem to be cross-bridged each other by thin strands (arrows). × 180,000.

tions revealed that the keratin filaments appeared to be cross-bridged by similar thin strands in the desmosomal plaque.

DISCUSSION

In this study, we have successfully purified a unique 240-kD protein designated here as desmocalmin, from isolated desmosomes in bovine muzzle epidermal cells. This protein could bind to calmodulin and keratin filaments and was localized in the desmosomal plaque, especially just beneath the desmosomal plasma membrane. Its calmodulin-binding ability showed a calcium-dependent character. Recently, two high molecular weight polypeptides, desmoplakin I (240 kD) and II (210 kD), have been immunologically shown to be localized in the desmosomal plaque, although these polypeptides failed to be purified in their native forms (20, 21). Most recently desmoplakin III, an 81-kD desmosomal protein, has been immunolocalized to the plaque (22). Our present study clearly revealed that the isoelectric point and antigenicity of desmocalmin differed from those of desmoplakins, leading us to conclude that desmocalmin and desmoplakins were distinct proteins.

In the desmosomes, it has been believed that the desmosomal plaque may play an important role in connecting 10-nm filaments to the plasma membrane (7, 8, 10). At present, our knowledge of the molecular organization of the desmosomal plaque is still fragmentary, mainly due to the difficulty of solubilizing its constituents in their native form. In addition to desmoplakins and desmocalmin, another high molecular weight polypeptide (300 kD), called plectin, has been detected by immunological methods at the desmosomal plaque (42). Plectin has been reported to occur in many locations other than desmosomes and is thought to be distinct from desmoplakins and desmocalmin, based on its antigenicity and its molecular weight.

Among these types of polypeptides in the desmosomal plaque, only desmocalmin was successfully purified without denaturing reagents. The molecular shape of the purified desmocalmin molecules resembled flexible rods. In the freeze-etch replica images, similar rodlike structures were observed to cross-bridge a keratin filament to the neighboring keratin filament or to the plasma membrane. It is possible that some of these rodlike structures are the morphological counterparts of desmocalmin in the desmosomes, but it is still premature to discuss further the molecular organization of the desmosomal plaque. Detailed biochemical and morphological analyses of desmoplakins and plectin in their native forms will provide us with a clear picture of how 10-nm filaments are associated with plasma membranes in the desmosomes.

The major physiological roles of desmosomes are in cell-to-cell recognition and adhesion (7–12). For a better understanding of the molecular mechanism of these physiological roles, it is necessary first to analyze the regulation mechanism of the formation and destruction of desmosomes in situ. Recently, it has been stressed that extracellular Ca^{2+} is a crucial factor for desmosome formation in the culture system of epidermal cells. Furthermore, in the epithelial-basal lamina culture system, it has been demonstrated that hemidesmosome formation is inhibited reversibly by the calmodulin antagonist (24), which suggests that the formation of desmosomes may be regulated by the intracellular calmodulin. Our results have shown that desmocalmin is a major calmodulin-binding protein in the desmosome. Therefore, it is natural to speculate that desmocalmin may be a key protein in the regulation mechanism of the desmosome formation. To clarify further this point we must answer the following questions in future: What type of desmocalmin function is affected by calmodulin in vitro? In what manner is desmocalmin localized in the epidermal cell during desmosome formation?

The molecular weight of 240,000 and the calmodulin-binding ability of desmocalmin have persuaded us to consider the relation of desmocalmin and spectrin-like proteins. Spectrin-like proteins have been reported to be composed of two distinct high molecular weight polypeptides, one of which, the 240-kD polypeptide, occurs in all types of spectrin-like proteins and shows calmodulin-binding ability (32, 43–48). Furthermore, both desmocalmin and spectrin-like protein molecules resembled flexible rods ~100-nm long in dimeric forms (46). However, desmocalmin should not be included in the family of spectrin-like proteins, mainly for the following reasons: (*a*) The electrophoretic banding patterns of the spectrin-like proteins isolated so far are characterized by two distinct bands. (*b*) Our anti-desmocalmin antibody has not cross-reacted with bovine brain spectrin (Fig. 5). (*c*) Desmocalmin does not bind to actin filaments, although one of the major characteristics of the spectrin-like proteins is an actin-binding property. (*d*) There is no indication that the spectrin-like proteins can be immunologically detected in desmosomes (45–49).

Spectrin-like proteins have been shown to occur in the cell periphery of various cell types and play an important role in the association of actin filaments with the plasma membranes. In a similar sense, it is possible to speculate that the desmocalmin-like proteins may be widely distributed in many cells and contribute to the 10-nm filament-plasma membrane association. At present, some important but unsettled questions remain to be elucidated about the immunological identification of desmocalmin in various types of tissues and the interaction of desmocalmin with 10-nm filaments other than keratin filaments. Studies along these lines are being conducted in our laboratory.

We are particularly grateful to Dr. Takaaki Kobayashi (Jikei University) for his collaboration in raising the anti-desmocalmin antibody in rabbits, to Dr. Kentaro Kuramochi and Dr. Issei Mabuchi (University of Tokyo) for their collaboration in calmodulin gel overlay technique using DACM-labeled calmodulin, and to Prof. Masanori Kurokawa (University of Tokyo) for the use of the biochemical facilities. Our thanks are also due to Dr. Masafumi Yano (University of Tokyo) for generously providing G-actin and to Dr. Yumiko Sugioka (Gunma University) for her technical advice on the two-dimensional electrophoretic analysis. Also, we would like to express our sincere appreciation to them and Prof. Harunori Ishikawa (Gunma University) for their helpful discussions and encouragement throughout this study.

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

Received for publication 23 April 1985, and in revised form 22 July 1985.

REFERENCES

1. Mangeat, P., and K. Burridge. 1984. Actin-membrane interaction in fibroblasts: What proteins are involved in this association? *J. Cell Biol.* 99(No. 1, pt. 2):95s–103s.
2. Ishikawa, H. 1979. Identification and distribution of intracellular filaments. *In* Cell Motility: Molecules and Organization. S. Hatano, H. Ishikawa, and H. Sato, editors. University of Tokyo Press, Tokyo. 417–444.
3. Nicolson, G. L. 1976. Transmembrane control of the receptors on normal and tumor

cells. I. Cytoplasmic influence over cell surface components. *Biochim. Biophys. Acta.* 457:57–108.
4. Singer, S. J. 1974. The molecular organization of membranes. *Annu. Rev. Biochem.* 43:805–831.
5. Tsukita, S., S. Tsukita, and H. Ishikawa. 1983. Association of actin and 10 nm filaments with the dense body in smooth muscle cells of the chicken gizzard. *Cell Tissue Res.* 229:233–242.
6. Geiger, B., Z. Adnur, G. Rinnerthaler, H. Hinssen, and V. J. Small. 1984. Microfilament-organizing centers in areas of cell contact: cytoskeletal interactions during cell attachment and locomotion. *J. Cell Biol.* 99(1, Pt. 2):83s–91s.
7. Weinstein, R. S., F. B. Merk, and J. Alroy. 1976. The structure and function of intercellular junctions in cancer. *Adv. Cancer Res.* 23:23–89.
8. Staehelin, A. L. 1974. Intercellular junctions. *Int. Rev. Cytol.* 39:191–283.
9. Overton, J. 1977. Formation of junctions and cell sorting in aggregates of chick and mouse cells. *Dev. Biol.* 55:103–116.
10. Wiseman, L., and J. Strickler. 1981. Desmosome frequency: experimental alteration may correlate with differential cell adhesion. *J. Cell Sci.* 49:217–223.
11. Jones, J. C. R., A. E. Goldman, P. M. Steinert, S. Yuspa, and R. D. Goldman. 1982. Dynamic aspects of the supramolecular organization of intermediate filament networks in cultured epidermal cells. *Cell Motil.* 2:197–213.
12. Dembitzer, H. M., F. Herz, A. Schermer, R. C. Wolley, and L. G. Koss. 1980. Desmosome development in an in vitro model. *J. Cell Biol.* 85:695–702.
13. Drochmans, P., C. Freudenstein, J. C. Wanson, L. Laurent, T. W. Keenan, J. Stadler, R. Leloup, and W. W. Franke. 1978. Structure and biochemical composition of desmosomes and tonofilaments isolated from calf muzzle epidermis. *J. Cell Biol.* 79:427–443.
14. Skerrow, C. J., and A. G. Matoltsy. 1974. Isolation of epidermal desmosomes. *J. Cell Biol.* 63:515–523.
15. Skerrow, C. J., and A. G. Matoltsy. 1974. Chemical characterization of isolated epidermal desmosomes. *J. Cell Biol.* 63:524–530.
16. Skerrow, C. J. 1979. Selective extraction of desmosomal proteins by low ionic strength media. *Biochim. Biophys. Acta.* 342:367–371.
17. Gorbsky, G., and M. S. Steinberg. 1981. Isolation of the intercellular glycoproteins of desmosomes. *J. Cell Biol.* 90:243–248.
18. Cohen, S. M., G. Gorbsky, and M. S. Steinberg. 1983. Immunochemical characterization of related families of glycoproteins in desmosomes. *J. Biol. Chem.* 258:2621–2627.
19. Cowin, P., D. Mattey, and D. Carrod. 1984. Identification of desmosomal surface components (desmocollins) and inhibition of desmosome formation by specific Fab'. *J. Cell Sci.* 70:41–60.
20. 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.
21. Franke, W. W., R. Moll, D. L. Schiller, E. Schmid, J. Kartenbeck, and H. Mueller. 1982. Desmoplakins of epithelial and myocardial desmosomes are immunologically and biochemically related. *Differentiation* 23:115–127.
22. Gorbsky, G., S. M. Cohen, H. Shida, G. J. Giudice, 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.
23. Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S. H. Yuspa. 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell.* 19:245–254.
24. Hennings, H., and K. A. Holbrook. 1983. Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. An ultrastructural study. *Exp. Cell Res.* 143:127–142.
25. Trinkaus-Randall, V., and I. K. Gipson. 1984. Role of calcium and calmodulin in hemidesmosome formation in vitro. *J. Cell Biol.* 98:1565–1571.
26. Watt, F. M., D. L. Mattey, and D. R. Garrod. 1984. Calcium-induced reorganization of desmosomal components in cultured human keratinocytes. *J. Cell Biol.* 99:2211–2215.
27. Glenney, J. R., Jr., and K. Weber. 1980. Calmodulin-binding proteins of the microfilaments present in isolated brush borders and microvilli of intestinal epithelial cells. *J. Biol. Chem.* 25:10551–10554.
28. Hamaguchi, Y., and F. Iwasa. 1980. Localization of fluorescently labeled calmodulin in living sea urchin eggs during early development. *Biomed. Res.* 1:502–509.
29. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
30. Steinert, P. M., and W. W. Idler. 1976. Self-assembly of bovine epidermal keratin filaments in vitro. *J. Mol. Biol.* 108:547–567.
31. Vaessen, R. T. M. J., J. Kreike, and G. S. P. Groot. 1981. Protein transfer to nitrocellulose filters. A simple method for quantitation of single proteins in complex mixtures. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 124:193–196.
32. Levine, J., and M. Willard. 1981. Fodrin: axonally transported polypeptides associated with the internal periphery of many cells. *J. Cell Biol.* 90:631–643.
33. Jarasch, E. D., C. Grund, G. Bruder, H. W. Heid, T. W. Keenen, and W. W. Franke. 1981. Localization of xanthine oxidase in mammary gland epithelium and capillary endothelium. *Cell.* 25:67–82.
34. Franke, W. W., H. W. Heid, C. Grund, S. Winter, C. Freundenstein, E. Schmid, E. D. Jarasch, and T. W. Keenan. 1981. Antibodies to the major unsoluble milk fat globule membrane-associated protein: specific localization in apical regions of lactating epithelial cells. *J. Cell Biol.* 89:485–494.
35. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
36. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
37. Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified, ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105:361–363.
38. Tsukita, S., S. Tsukita, and H. Ishikawa. 1980. Cytoskeletal network underlying the human erythrocyte membrane. Thin-section electron microscopy. *J. Cell Biol.* 85:567–576.
39. Tsukita, S., S. Tsukita, H. Ishikawa, S. Sato, and M. Nakao. 1981. Electron microscopic study of reassociation of spectrin and actin with the human erythrocyte membrane. *J. Cell Biol.* 90:70–77.
40. Tyler, J. M., and D. Branton. 1981. Rotary shadowing of extended molecules from glycerol. *J. Ultrastruct. Res.* 71:95–102.
41. Tsukita, S., S. Tsukita, J. Usukura, and H. Ishikawa. 1983. ATP-dependent structural changes of the outer dynein arm in tetrahymena cilia: a freeze-etch replica study. *J. Cell Biol.* 96:1480–1485.
42. 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.
43. Kakiuchi, S., K. Sobue, and M. Fujita. 1981. Purification of a 240,000 Mr calmodulin-binding protein from a microsomal fraction of brain. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 132:144–148.
44. Glenney, J. R., Jr., P. Glenney, M. Osborn, and K. Weber. 1982. An F-actin- and calmodulin-binding protein from isolated intestinal brush borders has a morphology related to spectrin. *Cell.* 28:843–854.
45. Repasky, E. A., B. L. Granger, and E. Lazarides. 1982. Widespread occurrence of spectrin in nonerythroid cells. *Cell.* 29:821–833.
46. Kakiuchi, S., K. Sobue, K. Kanda, K. Morimoto, S. Tsukita, S. Tsukita, and H. Ishikawa. 1982. Correlative biochemical and morphological studies of calspectin: a spectrin-like calmodulin-binding protein. *Biomed. Res.* 3:400–410.
47. Shimo-oka, T., and Y. Watanabe. 1981. Stimulation of actomyosin Mg^{2+}-ATP activated by a brain microtubule-associated protein fraction. High-molecular-weight protein is the stimulating factor. *J. Biochem.* 90:1297–1307.
48. Tsukita, S., S. Tsukita, H. Ishikawa, M. Kurokawa, K. Morimoto, K. Sobue, and S. Kakiuchi. 1983. Binding sites of calmodulin and actin on the brain spectrin, calspectin. *J. Cell Biol.* 97:574–578.
49. Lazarides, E., and W. J. Nelson. 1982. Expression of spectrin in nonerythroid cells. *Cell.* 31:505–508.