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

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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 Ca²⁺-dependent manner, so we called it desmocalmin here. Desmocalmin also bound to the reconstituted keratin filaments in vitro in the presence of Mg²⁺, 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 polypeptide 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 Ca²⁺-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 desmosomal calmodulin-binding protein.
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 protease inhibitors. The desmosomes obtained from seven bovine muzzles were suspended in 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-l-naphthol solution.

For indirect immunofluorescence microscopy, the bovine muzzle epidermis was examined 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 ultracentrifugation at 100,000 g for 30 min. Immunoelectron microscopy was performed on 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-l-naphthol solution.
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 microscopic 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 Ca²⁺-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 Ca²⁺. When the column was eluted with the solution containing enough EGTA and EDTA to chelate Ca²⁺, the 240-kD protein was obtained in a high degree of purity (Fig. 2). By the calmodulin gel overlay technique, we detected Ca²⁺-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
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 isolectrically focused at an acidic pH of \(-\)5.5 (Fig. 4). When desmocalmin was co-electrophoresed 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.

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. 5). 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 nor desmoplakin II reacted with anti-desmocalmin and was retained at the origin without being focused.
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-shading 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, 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).
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. x 120,000. (b-g) Bar, 0.1 μm. x 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 Mg²⁺. 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.

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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 desmoplakin, 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 playa 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.

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