Desmoyokin/AHNAK Protein Localizes to the Non-Desmosomal Keratinocyte Cell Surface of Human Epidermis

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Desmoyokin, a high–molecular-weight protein of 680 kD with a 170-nm–long dumbbell shape, was originally thought to be localized to the desmosomal attachment plaque and to work as a kind of stabilizer of desmosomes. Recently, desmoyokin was shown to be widely detected in many types of cells that do not possess desmosomes. The purpose of the present study was to elucidate the precise localization and possible function of desmoyokin in human epidermis. In 0.2-μm ultrathin cryosections of human skin for immunofluorescence, anti-desmoyokin antibody showed a ladder-like staining pattern along the cell surface, whereas anti-desmocollin and anti-desmoplakin antibodies as controls showed a discontinuous dotted staining pattern, indicating their distinct localization. Post-embedding immunoelectron microscopy with cryofixation and cryosubstitution revealed that desmoyokin was localized mainly along the non-desmosomal and non-hemidesmosomal plasma membrane, but not to the desmosomes and hemidesmosomes themselves. This localization was further confirmed by double-labeling immunoelectron microscopy with antibodies against desmocollin, desmoplakin, or bullous pemphigoid antigen. Results indicate that desmoyokin was not localized to the desmosomes themselves as previously considered. Desmoyokin was localized to the non-desmosomal and non-hemidesmosomal epidermal keratinocyte cell surface as a plasma membrane-associated protein, and might play a role in cell adhesion that is not directly associated with desmosomes or hemidesmosomes. ***Key words: immunoelectron microscopy/cryofixation/cryosubstitution/desmosome. J Invest Dermatol 104:941–945, 1995***

Desmoyokin, a high–molecular-weight protein of 680 kD having a 170-nm–long dumbbell shape, was first identified and purified from desmosomes isolated from bovine muzzle epidermis and this molecule was found only in stratified epithelium [1]. The desmosome plays an important role in intercellular adhesion in stratified epithelium, and is composed of transmembrane glycoproteins, including Dsg1, 2 (desmoglein) [2–6], Dsg3 (pemphigus vulgaris antigen) [3,7–9], and Dsc (desmocollin) [2,3,10–13], and nonglycosylated attachment plaque proteins, including desmoplakin [2,14–16] and plakoglobin [17–19]. Desmoyokin was originally thought to be a desmosomal attachment plaque protein and to work as a kind of stabilizer of desmosomes [1].

AHNAK protein, which is expressed in many types of cells as an exceptionally large protein of 700 kD, was identified as one of the proteins that are downregulated in neuroblastoma [20]. As reported previously, desmoyokin and AHNAK protein are thought to be identical because of the extensive homology of the deduced amino acid sequence, the similarities in size and structure, and identical patterns on Southern blot analysis of genomic DNAs [21].

Manuscript received November 9, 1994; final revision received January 30, 1995; accepted for publication February 13, 1995.

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Desmoyokin/AHNAK protein is widely detected in many types of cells, which indicates that it may play important roles in fundamental cell functions [21,22]. Immunofluorescence showed an interesting distribution pattern of desmoyokin/AHNAK protein; i.e., it was associated closely with the plasma membrane in epidermal keratinocytes, but was distributed diffusely in the cytoplasm of cells other than epidermal keratinocytes [21]. Therefore, the precise localization of desmoyokin in epidermal keratinocytes would yield important information about its role.

Hieda *et al* reported that desmoyokin was localized exclusively to the periphery of the desmosomal attachment plaque of bovine muzzle epidermis [1]. However, we observed considerably different patterns of immunofluorescence staining between anti-desmoyokin and anti-desmoplakins I/II antibodies in normal human epidermis and cultured keratinocytes [21]. This finding suggests that localization of desmoyokin in human keratinocytes differs markedly from that of desmoplakin. The purpose of this study was to elucidate the precise ultrastructural localization of desmoyokin/AHNAK protein and its possible role in normal human epidermis.

MATERIALS AND METHODS

Antibodies Details of the production and characterization of rabbit polyclonal anti-desmoyokin antibody have been described previously [21]. Briefly, the fusion protein produced by the expression vector pGEX-3X inserted in-frame with the subcloned cDNA of desmoyokin, DY6, was used for immunizing rabbits. The DY6 cDNA clone corresponded to the central domain of desmoyokin, which showed a dumbbell shape. Production and

0022-202X/95/$09.50 • SSDI0022-202X(95)00068-V •

characterization of mouse monoclonal anti-desmoyokin antibodies, Yo-12086 (a generous gift from Dr. S. Tsukita, Kyoto University) [1] and 33A-3D [21], have been described previously. Mouse monoclonal antibodies against desmocollin and desmoplakin used as controls were kind gifts from Dr. D.R. Garrod, University of Manchester. Production and characterization of anti-desmocollin [10–13] and anti-desmoplakin [23] antibodies have been reported previously. Bullous pemphigoid (BP) serum that recognized the 180-kD antigen in the hemidesmosomes was used as control; its characterization was described previously [24]. Normal rabbit or mouse serum was also used as a control.

Immunoblot Analysis To analyze the specificity of the rabbit polyclonal anti-desmoyokin antibody used in this study, immunoblotting of dispase-treated normal human epidermal extract was performed, as described previously [25]. Two mouse monoclonal anti-desmoyokin antibodies [1,21] and mouse monoclonal anti-desmoplakin antibody [23] were used as controls. The specificity of serum from a patient with BP was also analyzed by immunoblotting.

Immunofluorescence on Ultrathin Cryosections Ultrathin cryosections were obtained essentially by the Tokuyasu method [26] after cryofixation. Briefly, small pieces of normal human skin were fixed in 95% ethanol at 4°C for 30 min followed by immersion in 2.3 M sucrose/phosphate-buffered saline (PBS) at 4°C for 20 min, and cryofixed by plunging them into liquid propane cooled to −190°C (KF-80, Reichert-Jung, Vienna, Austria). The 0.2-μm ultrathin cryosections were cut by Ultracut S with FCS cryo-attachment (Reichert-Jung) and collected with a droplet of 2.3 M sucrose/PBS on a slide glass. The sections were incubated with rabbit polyclonal anti-desmoyokin antibody diluted 1:40 or mouse monoclonal anti-desmocollin or anti-desmoplakin antibody at room temperature for 1 h, followed by incubation with fluorescein isothiocyanate–labeled secondary antibody (DAKO, Copenhagen, Denmark) at room temperature for 1 h. The sections were mounted with glycerol/PBS containing *p*-phenylenediamine as an antifading agent [27].

Post-Embedding Immunoelectron Microscopy Using Cryofixation and Cryosubstitution Without Chemical Fixative Post-embedding immunoelectron microscopy using cryofixed and cryosubstituted skin was carried out as described previously [28,29] with slight modification [2]. Briefly, small pieces of fresh normal human skin (from the thigh of a 35-year–old man) were cryoprotected with 15% glycerol/PBS at 4°C for 30 min and cryofixed by plunging them into liquid propane cooled to −190°C, followed by cryosubstitution (CS-auto, Reichert-Jung) with acetone at −80°C for 120 h; they were then embedded in Lowicryl K11M (Chemische Werke Lowi, Waldkraiburg, Germany) at −60°C. The specimens were polymerized by ultraviolet irradiation at −60°C for 72 h and at room temperature for another 72 h. Ultrathin sections were incubated overnight at 4°C with rabbit polyclonal anti-desmoyokin antibody diluted 1:100 or with mouse monoclonal anti-desmocollin or anti-desmoplakin antibody diluted 1:4. After being washed, each section was placed on a drop of 1-nm gold-labeled goat anti-rabbit or mouse IgG (Amersham International, Buckinghamshire, UK) diluted 1:40 at room temperature for 2 h, and was then washed with distilled water. For easier observation, the 1-nm gold particles were enlarged by incubation with immunogold silver-enhancement solution (Amersham International) at room temperature for 6 min (for observation at high magnification) or 10 min (for observation at low magnification) [29]. Instead of 1-nm gold-labeled goat anti-rabbit IgG combined with silver enhancement, 15-nm gold-labeled goat anti-rabbit IgG (Zymed Laboratories, San Francisco, CA, USA) was also used as a secondary antibody. The sections were counterstained with saturated uranyl acetate and lead citrate for 6 and 2 min, respectively.

Double-Labeling Post-Embedding Immunoelectron Microscopy The experimental procedure was the same as that for single-labeling immunoelectron microscopy, except for the omission of silver enhancement. A mixture of rabbit polyclonal anti-desmoyokin antibody and mouse monoclonal anti-desmocollin or anti-desmoplakin antibody or serum from a patient with BP was used as primary antibody. A mixture of 15-nm gold-labeled goat anti-rabbit IgG and 5-nm gold-labeled goat anti-mouse or human IgG (Amersham International) was used as secondary antibody.

RESULTS

Immunoblot Analysis of the Specificity of Antibodies In immunoblot analysis of the extract from normal human epidermis, rabbit polyclonal antibody against desmoyokin reacted specifically with a protein band estimated to be approximately 680 kD, corresponding to desmoyokin **(Fig 1)**. This was the same molecular weight as that of the protein band recognized by the two mouse monoclonal anti-desmoyokin antibodies, Yo-12086 and 33A-3D. A mouse monoclonal antibody against desmoplakin used as a control reacted with protein bands of desmoplakins I and II of approximately 250 and 210 kD, but not with desmoyokin.

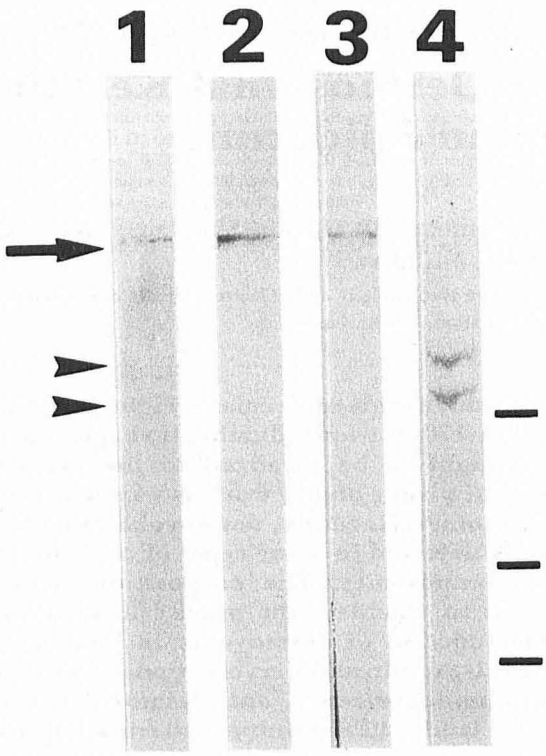

Figure 1. Immunoblot analysis of the specificity of rabbit polyclonal anti-desmoyokin antibody (*lane 1*), mouse monoclonal anti-desmoyokin antibodies Yo-12086 (*lane 2*) and 33A-3D (*lane 3*), and mouse monoclonal anti-desmoplakin antibody (*lane 4*). Dispase-treated normal human epidermal extract was used as antigen source. The rabbit polyclonal anti-desmoyokin antibody used in this study reacted specifically with a protein band of approximately 680 kD (*arrow*). The mouse monoclonal anti-desmoyokin antibodies reacted with a protein band of the same molecular weight. The anti-desmoplakin antibody reacted with protein bands of approximately 250 and 210 kD (*arrowheads*). Molecular weight markers for 205, 116.5, and 80 kD are indicated on the right.

The serum from a patient with BP used in this study recognized only 180-kD BP antigen (BPAG2) by immunoblot analysis as reported previously [24].

Desmoyokin Shows Different Distribution from Desmocollin and Desmoplakin by Immunofluorescence on 0.2-μm Ultrathin Cryosections Rabbit polyclonal anti-desmoyokin antibody showed a ladder-like staining pattern along the cell surface of normal human epidermis. No other specific labeling was observed in the epidermis **(Fig 2*A,B*)**. In contrast, immunofluorescence of anti-desmoplakin and anti-desmocollin antibodies showed a discontinuous dotted staining pattern along the cell surface through the entire epidermis **(Figs 2*C,D*)**. These results indicate that distribution of desmoyokin differed from that of desmoplakin and desmocollin.

Desmoyokin Is Localized to the Non-Desmosomal and Non-Hemidesmosomal Plasma Membrane by Post-Embedding Immunoelectron Microscopy Skin that was cryofixed and cryosubstituted without chemical fixatives showed a satisfactorily preserved ultrastructure, as described previously [2,24,28,29]. Desmosomes, hemidesmosomes, plasma membrane, and tonofilaments were clearly demonstrated.

When the 1-nm immunogold particles were silver-enhanced for 10 min at room temperature, labeling with rabbit polyclonal

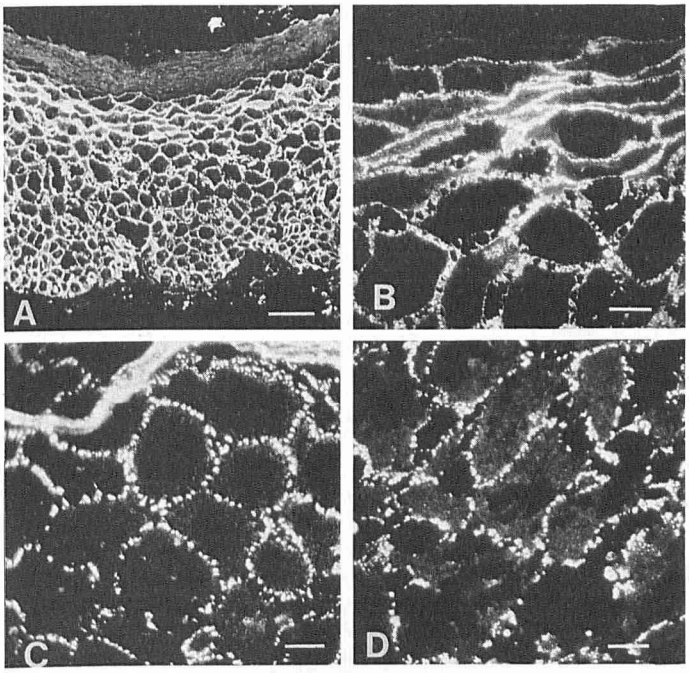

Figure 2. Immunostaining pattern of rabbit polyclonal anti-desmoyokin (*A,B*) is different from that of mouse monoclonal anti-desmoplakin (*C*) and anti-desmocollin (*D*) antibodies. A 0.2-μm ultrathin cryosection of normal human skin was used as substrate. Anti-desmoyokin antibody showed a ladder-like staining pattern along the cell surface of the keratinocyte. In contrast, anti-desmoplakin and anti-desmocollin antibodies showed a discontinuous dotted staining pattern on the cell surface in the entire epidermis. *Bars: A*, 50 μm; *B–D*, 10 μm.

anti-desmoyokin antibody was easily observed, even at very low magnification **(Fig 3*A*)**. The silver-enhanced immunogold labeling was localized to the periphery of keratinocytes in the entire epidermis, including the basal, spinous, and granular layers. Weak labeling was also localized to the keratinocyte cell surface along the basement membrane. There was no specific labeling within the nucleus. At this low magnification, the outline of the cell could be easily recognized. Each desmosomal structure could be clearly seen, unhindered by silver-enhanced gold particles **(Fig 3*B*)**.

More detailed localization could be observed at high magnification when silver enhancement time at room temperature was shortened to 6 min. Gold particles with rabbit polyclonal anti-desmoyokin antibody were localized along the non-desmosomal plasma membrane of the keratinocyte, with a major labeling of intracellular sites **(Fig 4*A,B*)**. No specific labeling was observed on the desmosomal structures themselves. The same result was obtained when 15-nm gold-labeled goat anti-rabbit IgG was used as secondary antibody without silver enhancement **(Figs 4*C,D*)**. In the basement membrane zone, immunolabeling was localized along the non-hemidesmosomal plasma membrane, with a major labeling of intracellular sites **(Fig 4*E*)**.

Desmoyokin Shows Different Localization from Desmocollin, Desmoplakin, and BP Antigen by Double-Labeling Immunoelectron Microscopy To further confirm the localization of desmoyokin, we carried out double-labeling immunoelectron microscopy with rabbit polyclonal anti-desmoyokin antibody and mouse monoclonal anti-desmocollin or anti-desmoplakin antibody or serum from a patient with BP **(Fig 5)**. Gold particles with desmocollin were localized on intra- and extracellular sites of desmosomes, and desmoplakin was immunolocalized to the cytoplasmic attachment plaques of desmosomes, as described previously [2]. The labeling of 180-kD BP antigen was localized along the plasma membrane of hemidesmosomes as shown previously [24].

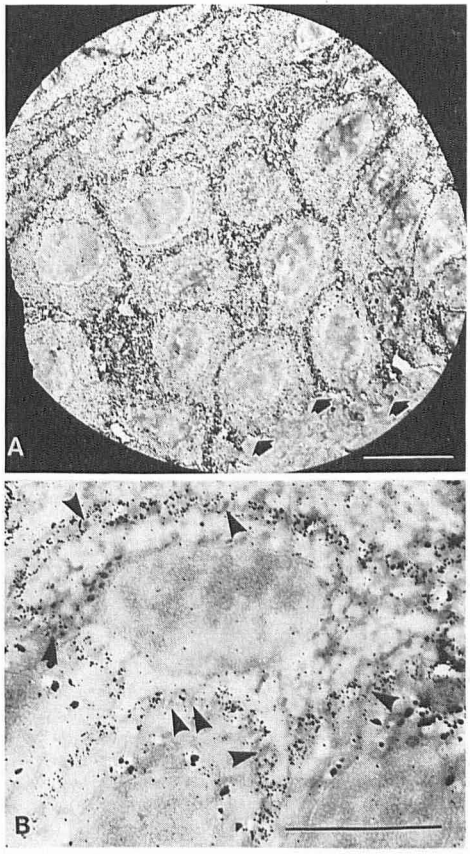

Figure 3. Low-magnification electron micrograph showing the localization of desmoyokin to the periphery of keratinocytes in normal human epidermis. Post-embedding immunoelectron microscopy was performed on the cryofixed and cryosubstituted normal human skin section. After immunostaining, 1-nm gold particles were silver enhanced for 10 min at room temperature. *A*) Labeling at the periphery of keratinocytes was observed clearly throughout the entire epidermis, including the basal, spinous, and granular layers, and the outline of the cell was easily recognized. Weak labeling was also observed along the basement membrane. *Arrows* indicate the basement membrane zone. *B*) Gold labeling was seen mainly on the non-desmosomal plasma membrane, but not on the desmosomes themselves (*arrowhead*). *Bars: A*, 10 μm; *B*, 5 μm.

The section that was double-labeled with anti-desmoyokin and anti-desmocollin antibodies showed that desmocollin (5-nm small gold particles) was localized on the intra- and extracellular sites of the desmosomes, whereas desmoyokin (15-nm large gold particles) was localized along the cell surface of non-desmosomal areas **(Figs 5*A,B*)**. The localization of each labeling was clearly distinguishable. Similarly, on the section stained with both anti-desmoyokin and anti-desmoplakin antibodies, the labeling of desmoyokin (15-nm large gold particles) on non-desmosomal areas was clearly distinguished from that of desmoplakin (5-nm small gold particles) on the cytoplasmic attachment plaques of desmosomes **(Figs 5*C,D*)**. On the basilar surface of basal cells, desmoyokin (15-nm large gold particles) was localized along the plasma membrane between hemidesmosomes, whereas the labeling of BP serum (5-nm small gold particles) was localized to the hemidesmosomal structures themselves **(Fig 5*E*)**. These results confirmed that desmoyokin was localized to the non-desmosomal and non-hemidesmosomal keratinocyte cell surface as a plasma membrane–associated protein in normal human skin, but not to desmosomes and hemidesmosomes themselves.

DISCUSSION

Our results showed that desmoyokin is localized along the non-desmosomal and non-hemidesmosomal keratinocyte cell surface of

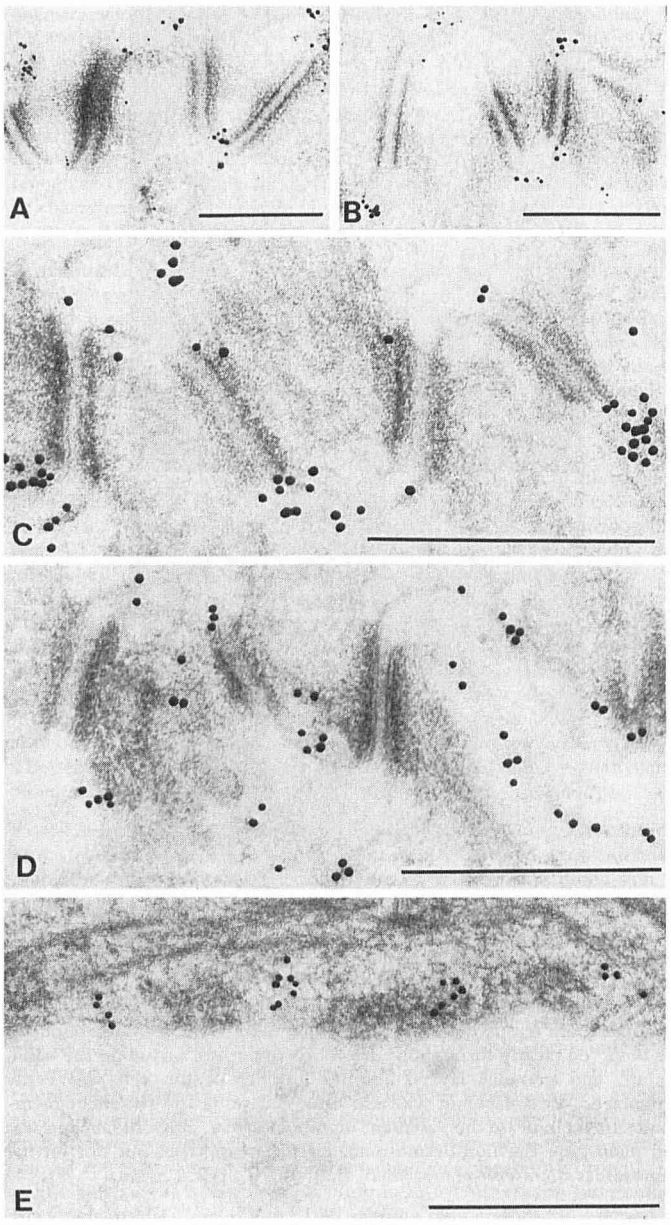

Figure 4. High-magnification electron micrograph showing the localization of desmoyokin to the non-desmosomal and non-hemidesmosomal plasma membrane. Experimental procedure was the same as described in **Fig 3** except for the use of 1-nm colloidal gold silver enhanced for 6 min at room temperature (*A,B*) or 15-nm colloidal gold (*C–E*) as the secondary antibody. *A–D*) Labeling was localized to the plasma membrane between desmosomes. There was no specific labeling on the desmosomes themselves. *E*) Immunogold particles were localized along the basilar surface of basal cells with major labeling of intracellular sites, but not to the hemidesmosomes themselves. *Bars*, 0.5 μm.

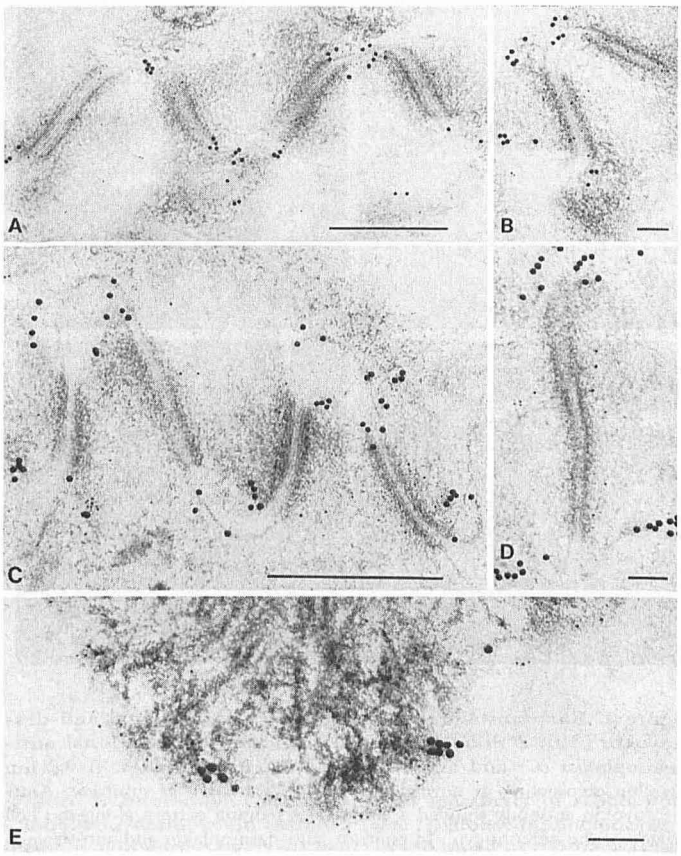

Figure 5. Double-labeling immunoelectron micrograph showing that the localization of desmoyokin is different from that of desmocollin (*A,B*), desmoplakin (*C,D*), and BP antigen (*E*). The cryofixed and cryosubstituted normal human skin section was used as substrate for post-embedding immunoelectron microscopy. *A,B*) Electron micrographs clearly showed the distinct localizations of desmoyokin (15-nm large gold particles) and desmocollin (5-nm small gold particles). Desmoyokin was localized to non-desmosomal plasma membrane, but not to the desmosomal structures themselves. *C,D*) Labeling with anti-desmoyokin (15-nm large gold particles) localized exclusively to non-desmosomal plasma membrane whereas labeling with anti-desmoplakin (5-nm small gold particles) localized to the cytoplasmic attachment plaque of the desmosome. *E*) Localization of desmoyokin (15-nm large gold particles) was clearly distinguishable from that of BP antigen (5-nm small gold particles), i.e., desmoyokin was localized along the plasma membrane between hemidesmosomes, whereas BP antigen was localized to the hemidesmosomes themselves. *Bars: A and C*, 0.5 μm; *B,D, and E*, 0.1 μm.

human epidermis, but not to desmosomes themselves where it was originally thought to be localized [1].

Desmoyokin was first shown to be localized at the periphery of the desmosomal attachment plaque in ethanol-fixed bovine muzzle epidermis by immunoelectron microscopic examination using ultrathin cryosections [1]. Cryoultramicrotomy is a method for preserving the antigenicity of a labile antigen such as desmoyokin. However, the disadvantage of using ultrathin cryosections for immunoelectron microscopy is a poorer ultrastructural preservation, especially that of the non-desmosomal cell surface. We therefore applied cryofixation and cryosubstitution techniques without using chemical fixatives for post-embedding immunoelectron microscopy, which has been proved to preserve both an adequate ultrastructure and antigenicity [2,24,28,29]. This technique enabled us to succeed in demonstrating the localization of desmoyokin to the non-desmosomal and non-hemidesmosomal plasma membrane, but not to desmosomes themselves.

Immunoelectron micrography using 6-min silver-enhanced 1-nm gold or 15-nm gold without silver enhancement provided a fine ultrastructural localization of desmoyokin. We used double-labeling immunoelectron microscopy to further confirm that desmoyokin is not localized to the desmosomal and hemidesmosomal structures themselves. These results clearly indicate that desmoyokin was not localized to the desmosomes and hemidesmosomes. The reason of the weaker labeling of desmoyokin at the basement membrane zone might be explained by the shorter non-hemidesmosomal region than the non-desmosomal region in the certain length of plasma membrane.

We used ultrathin cryosections of 0.2-μm thickness as the substrate for immunofluorescence to improve the resolution. In this method, it was unlikely that multiple desmosomes overlap in a

0.2-μm ultrathin cryosection. Desmocollin and desmoplakin were clearly demonstrated as a discontinuous dotted labeling pattern along the keratinocyte cell surface, which should correspond to the desmosomes. In contrast, desmoyokin showed a ladder-like staining pattern that apparently differed from the distribution pattern of desmocollin or desmoplakin, suggesting their distinct localization. The different localization of desmoyokin from that of desmoplakin shown by immunoelectron microscopy was further confirmed by immunofluorescence.

We found no specific labeling of desmoyokin in the nucleus of the keratinocyte although desmoyokin is identical to AHNAK protein [21], which was originally reported to be localized mainly in the nucleus of fibroblast [20,22]. At present, there is no definite explanation for this discrepancy.

In conclusion, light and electron microscopic immunolabeling studies of desmoyokin confirmed that this molecule is localized along the non-desmosomal and non-hemidesmosomal plasma membrane of normal human keratinocytes, but not to the desmosomes and hemidesmosomes themselves. The fact that desmoyokin was also localized to the basilar surface of basal cells supported the view that desmoyokin was not a desmosome-related protein as reported previously. Although Hieda *et al* thought that desmoyokin works as a kind of stabilizer of desmosomes [1], our results lead us to speculate that desmoyokin may not relate to the function of desmosomes, but play an important role in keratinocyte adhesion not directly associated with desmosomes.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (05404036, 01570575, and 02454280).

We thank Dr. S. Tsukita, Kyoto University, Japan, and Dr. D.R. Garrod, University of Manchester, UK, for providing us with specific monoclonal antibodies.

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