Association of a Basic 25K Protein with Membrane Coating Granules of Human Epidermis

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Abstract. Keratinocytes of the upper granular layers contain unique round-to-oval granules, 100–500 nm in diameter, in their peripheral cytoplasm. These granules (known as membrane coating granules [MCG], or lamellar granules) fuse with the apical cell surface of uppermost granular cells and discharge their contents into the intercellular space, where they are believed to play a role in establishing the permeability barrier of the epidermis and possibly in regulating the orderly desquamation of terminally differentiated keratinocytes. Using two monoclonal antibodies originally prepared against hair follicle antigens, we have identified a 25K epidermal protein in association with both MCG-like granules in the peripheral cytoplasm of granular cells as well as MCG-derived intercellular material. This protein is relatively basic (pI > 8), largely aqueous soluble, methionine deficient, and is relatively abundant in epidermis (comprising up to ~0.1% of soluble proteins). Its distribution is restricted to the granular layer of keratinized (cornified) stratified squamous epithelia. The identification of this protein component opens new avenues for studying the molecular mechanisms underlying the establishment of permeability barrier and/or regulation of desquamation.

The epidermis consists of regenerating basal keratinocytes which give rise to suprabasally located, terminally differentiating cells dedicated to the synthesis and assembly of various components that eventually form the nonliving but functional layers of the stratum corneum (for review see Matoltsy, 1986). There are two major functions performed by the stratum corneum of all land animals. First, it provides physical protection which is contributed mainly by keratin filaments and a cross-linked cell envelope (Green, 1980). Second, and most critical in terms of terrestrial existence, it provides a highly efficient permeability barrier which prevents the rapid loss of water and water-soluble components to the external environment. Central in performing this second function are a class of cytoplasmic granules called membrane coating granules (MCG); also known as lamellar granules, Odland bodies, cementosomes, or keratinosomes; see Odland and Holbrook, 1981). With a diameter of 100–500 nm, these oval to rod shaped structures are present in the peripheral cytoplasm of granular cells where they later fuse with the apical cell surface and discharge their contents into the intercellular space beneath the stratum corneum. The lipid components thus discharged are believed to be ultimately responsible for the hydrophobic nature of the permeability barrier of the skin (Matoltsy and Parakkal, 1965; Elias and Friend, 1975; Elias et al., 1977a, b; Wertz and Downing, 1982). Protein components are also thought to be discharged and are involved in regulating the postsecretory modifications of these lipids or in mediating the process of desquamation in which the terminally differentiated keratinocytes are sloughed off (Elias, 1983). Evidence for this includes the histochemical demonstration of several classes of hydrolytic enzyme activities including acidic hydrolases and phospholipases in MCGs and their discharges (Eisen et al., 1964; Elias et al., 1988; Frienkel and Traczyk, 1983; Gonzalez et al., 1976; Weinstock and Wigram, 1970; Wolff-Shreiner, 1977). There is also little doubt that some proteins exist in MCGs that play a structural role in maintaining membrane structure and/or modulating the organization of intercellular lamellae. However, since no one has been able to isolate large amounts of MCGs with sufficient purity for biochemical analysis, not a single protein has yet been identified unambiguously in this special class of cytoplasmic granules (Frienkel and Traczyk, 1983; Grayson et al., 1985).

As part of a continuing effort directed toward the identification and characterization of protein molecules involved in various stages of keratinocyte differentiation, we have recently generated a panel of monoclonal antibodies against some hair-follicle antigens (O’Guin, W. M., M. Manabe, and T.-T. Sun, manuscript submitted for publication). Two of these antibodies showed an interesting cross-reactivity with some granules located in the peripheral cytoplasm of upper granular cells of the epidermis. Double staining experiments showed that these granules are clearly distinguishable from keratohyalin granules. Ultrastructural localization studies further established that the staining was due to cytoplasmic granules having the physical dimension of MCGs, as well as some membranous, intercellular material known

1. Abbreviation used in this paper: MCG, membrane coating granules.
to represent MCG discharges. The protein responsible for this staining was identified by immunoblotting as a novel, 25K epidermal protein that is reasonably abundant in vivo (comprising up to \( \sim 0.1\% \) of soluble proteins) but greatly diminished in cultured keratinocytes. This molecule provides a valuable marker for studying the biochemical and molecular mechanisms underlying the final stage of epidermal differentiation.

**Materials and Methods**

Monoclonal antibodies AE17 and AE18 were originally prepared against hair antigens; they recognize a 220K protein of the trichohyalin granule which is absent in the epidermis or any other nonhair, soft epithelial tissues (O’Guin et al., 1989). Polyclonal rabbit antiserum to human filaggrin was the generous gift of Dr. Beverly Dale (University of Washington, Seattle). Polyclonal rabbit antiserum to human involucrin was the generous gift of Dr. Robert Rice (Harvard University). Monkey (M. nemestrina) skin was obtained from the Regional Primate Center at the University of Washington (Seattle). Fresh human skin was obtained from surgical procedures or from autopsy specimens. For immunolocalization studies, the skin was embedded in OCT medium, frozen in isopentane cooled in a liquid nitrogen bath. Unfixed cryostat sections (4–6 \( \mu m \)) of tissue were used for both indirect immunofluorescence and immunoelectron microscopy.

For indirect immunoelectron microscopy, frozen sections were incubated in primary antibody (AE17 or AE18), washed, and incubated with peroxidase-conjugated goat anti-mouse IgG. Peroxidase activity was localized using diaminobenzidine. A nonspecific myeloma supernatant (P3) was used as a negative primary antibody. The stained sections were subsequently fixed, dehydrated, and embedded in Epon. Thin sections were viewed at 80KV in a JEOL 100S transmission electron microscope.

For protein analyses, fresh skin was frozen on a block of dry ice and the epidermis was removed by scraping with a scalpel. The frozen epidermal shavings were then transferred to 9.5 M urea containing 25 mM Tris-HCl (pH 7.4) and protease inhibitors (Eichner et al., 1984) or transferred to SDS sample buffer. SDS-PAGE was performed on 10% gels as previously described (Laemmli, 1970). Two-dimensional NEPHGE was according to O’Farrell et al. (1977). CNBr cleavage was performed as described by Lonsdale-Eccles et al. (1980). For immunoblot analyses, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (Towbin et al. 1979) and subsequently stained using either the peroxidase-antiperoxidase method for AE17 and AE18 or peroxidase-conjugated goat anti-rabbit IgG for polyclonal antifilaggrin. In both cases, peroxidase activity was detected using diaminobenzidine.

**Results**

In the course of characterizing a panel of monoclonal antibodies to hair antigens, we found that two of these antibodies, AE17 and AE18, showed strong reactivity with some granules in the granular cells of normal human epidermis (Fig. 1). Although this staining pattern resembles that seen for filaggrin, a keratohyalin granule–associated protein (Dale et al., 1985), careful double-staining experiments revealed some differences in their detailed histological distribution (Fig. 2, a and b). Such a difference was even more obvious when we double-stained the thick human sole epidermis which possesses four to five layers of granular cells (Fig. 2, d and e) instead of just one or two layers as in normal trunk epidermis (Fig. 2, a–c). This analysis clearly established that, unlike keratohyalin granules which are distributed rather evenly in the cytoplasm of all granular cells, the AE17-positive granules tend to be localized toward the cell periphery, a phenomenon becoming increasingly more conspicuous in upper cells located immediately underneath the first layer of cornified cells (Fig. 2 d). There is also clear indication that both filaggrin and involucrin (another marker for an

*Figure 1. Indirect immunofluorescence of unfixed, frozen sections of human abdominal epidermis using monoclonal antibody (a) AE17 and (b) AE18. Note that the granular staining of the antibody is restricted to the uppermost granular layer (G) of the epidermis and is not seen in the basal (B), spinous (S), or cornified (C) layers. (c) The corresponding phase contrast image of b. Arrows indicate the epidermal–dermal junction; asterisks (+) identify a corresponding cell in b and c. Bar, 5 \( \mu m \).*
advanced stage of epidermal differentiation) make their first appearance in lower cell layers than AE17 antigen (Fig. 3). This makes the AE17 antigen one of the last differentiation markers known to appear during terminal stages of epidermal differentiation.

The peripheral location of these AE17-reactive granules in upper granular cells strongly suggests that they are related to MCGs. To exploit this possibility, we performed localization studies of human epidermis on an ultrastructural level. Since the immunoreactivity of both antibodies diminished upon tissue fixation, frozen sections were used in these experiments. Although under these conditions relatively few MCGs were preserved, a number of cytoplasmic granules with physical dimensions of MCGs were indeed strongly stained by AE17 (short arrows in Fig. 4 d). In addition, striking staining was observed along and between the lamellar,
membranous material deposited in the intercellular space between the upper granular and lower cornified cells (Fig. 4, c-f). Virtually identical AE17 localization data were obtained in monkey epidermis (Fig. 5); this antibody does not cross-react with the epidermis from any other species tested including rabbit, rat, and cow. AE18 produced a similar staining of cytoplasmic granules (Fig. 4 b) in human epidermis; this antibody can be distinguished from AE17 in that its staining is somewhat stronger and "coarser" and that this antibody does not react with monkey epidermis. Since it has been well-established that the intercellular, lamellar material is MCG derived (Elias and Friend, 1975), these results strongly support the notion that these immunoreactive cytoplasmic granules and intercellularly located antigen(s) are MCG related.

The antigen responsible for these staining reactions was identified using the immunoblotting technique (Towbin et al., 1979). Total proteins from human epidermis were solubilized in 9.5 M urea, separated by SDS-PAGE, transferred to nitrocellulose, and stained immunochemically with AE17 and AE18 antibodies. A 25K epidermal protein was identified by both antibodies (Fig. 6). This protein is extractable in aqueous buffer, although roughly equal amounts of additional antigen can be extracted from the aqueous-insoluble pellet using urea or SDS (Fig. 7). The relative amount of this protein in extracts from different skin samples was variable with, for example, trunk skin consistently yielding more of the 25K component than breast skin. Since reducing agent has no apparent effect in facilitating the solubilization, intermolecular disulfide bonds are apparently not involved in regulating the solubility of this protein.

Two-dimensional gel electrophoretic analysis showed that the 25K band seen in one-dimensional SDS gels (arrow in Fig. 6) can be resolved into a group of AE17-positive spots (bracketed in Fig. 8) which are more basic than the most basic 67K keratin, K1, and a 25K "contaminant" (circled in Fig. 8), which is much more acidic and does not react with the antibody. The immunoreactive protein was resolved into a major and two or three minor, more acidic charge isomers. The relative intensity of antibody staining is such that the minor spots actually reacted more strongly than the major one (Fig. 8). Similar results were obtained with AE18 antibody (not shown). Since AE17 and AE18 are indistinguishable by immunoblot analysis it is clear that the slightly different immunolocalization patterns (Fig. 4) are due to the fact that these two antibodies recognize different epitopes of the same protein. These immunoblot data indicate that AE17 and AE18 reactivity could involve some posttranslational modification(s) of the 25K protein; the same modification may also be present on the 220K hair antigen (O'Guin, W. M., M. Manabe, and T.-T. Sun, manuscript submitted for publication) thus accounting for their immunocrossreactivity.

An unusual property of this 25K protein is that it is cyanogen bromide resistant, suggesting that it lacks methionine. This is demonstrated by using a diagonal gel system (Lonsdale-Eccles et al., 1981) in which proteins resolved during the first-dimensional SDS-PAGE were treated in situ with either formic acid alone (as a control) or CNBr in formic acid.

Figure 4. Immunoelectron microscopic localization of AE17 and AE18 antigens in human abdominal epidermis. (a) A control section stained with a nonrelevant P3 antibody illustrating background staining. C indicates the first layer of the stratum corneum and G is part of the uppermost granular cell. Note the absence of staining in the intercellular spaces marked by Xs. (b) The staining pattern obtained with AE18 is generally granular throughout the upper granular cell cytoplasm. c-f show the immunoreactivity of AE17 is primarily located in the intercellular space (x) where the reaction product is clearly associated with lipid leaflets (large arrows). Some cytoplasmic granules are also seen in the upper granular cells (d, small arrows). D, desmosome. Bar, 1 μm.
Second-dimensional separation was achieved using an identical SDS-PAGE system. In a control experiment all unmodified epidermal proteins remained, as expected, on the diagonal (Fig. 9 a), while CNBr treatment resulted in the cleavage of most proteins including keratins, with their degradation products migrating below the diagonal (Fig. 9 b). The only two major epidermal proteins that are CNBr resistant and thus remain on the diagonal are filaggrin, a keratohyalin granule component (Fig. 9 c; Lynley and Dale, 1983), and the 25K protein (Fig. 9 d).

**Discussion**

Using two monoclonal antibodies, AE17 and AE18, we have...
Two-dimensional immunoblot analyses of human abdominal epidermal protein using monoclonal antibody AE17. An urea extract of human epidermis was first separated on NEPHGE and subsequently separated on standard SDS polyacrylamide gels. These proteins were then transferred to nitrocellulose and stained with fast green giving the profile shown in a. b is the immunoblot of a using monoclonal antibody AE17 and indicates immunoreactivity with three relatively basic isoelectric variants of the 25K protein. Brackets identify the major isoforms of the protein recognized by the monoclonal antibody. The circle denotes an acidic protein which co-migrates with the immunoreactive proteins in one-dimensional gels but is not actually recognized by the antibodies. K1, K5, K10, and K14 identify the major epidermal keratins as classified by Moll et al. (1982).

identified a new differentiation-dependent protein characteristic of the granular layer of human epidermis. Indirect immunofluorescence and immunoelectron microscopy localization studies demonstrate that it is present only in granular inclusions present in the cytoplasm of upper granular cells and in the intercellular space between the stratum granulosum and stratum corneum. This antibody staining appears to be epidermis specific, as we have not detected it in several other epithelia including esophageal and corneal epithelia (see below).

The epidermal antigen responsible for this staining reaction was identified as a novel 25K protein. This protein is clearly distinguished from all of the previously characterized proteins known to be expressed in the granular layer of the epidermis. Most significantly, no previously described epidermal-specific protein has been localized in the intercellular space. There are additional differences. Filaggrin, a major protein of keratohyalin granules (Steinert et al., 1981; for review see Dale et al., 1985), showed no immunological cross-reactivity with the 25K protein (Fig. 9). Moreover, the staining of sole epithelium revealed a complete segregation of staining by antibodies to these two proteins (Fig. 2, d and e). The possibility that the 25K antigen is located in a subclass of filaggrin-negative keratohyalin granules is eliminated by our immunoelectron microscopy data demonstrating that AE17 and AE18 antibodies did not react with keratohyalin granules of any kind (Fig. 4 and data not shown). We therefore conclude that the 25K antigen is not related to either filaggrin or any other keratohyalin components.

Several cornified envelope proteins are known to be present in the granular layer but they can be differentiated from this 25K protein by both their molecular weights and their histological distribution. These include involucrin (140K; Simon and Green, 1984; Rice and Green, 1977; Rice et al., 1984; see Fig. 3), keratinin (6K; Zettergren et al., 1984), and a number of conditionally defined components ranging from 70 to 330K (Simon and Green, 1984). While relatively little information is currently available about some of these recently described molecules, they seem to be clearly different from the 25K protein in tissue distribution. Transglutaminase is an enzyme involved in covalently cross-linking cornified envelope precursors and has a tissue distribution similar to that of involucrin. While membrane-bound transglutaminase has a somewhat granular distribution, its subunit molecular weight of 92K easily distinguishes it from the 25K protein (Thatcher and Rice, 1985). Finally, our fine structural studies of the 25K protein are inconsistent with its involvement in the formation of cornified envelopes (see Warhol et al., 1982; Rice et al., 1984).

The fact that the 25K protein is found only in the upper granular layer and at the interface between the living granular cell and the stratum corneum in trunk epidermis is consistent with known localization of MCG and their secreted contents (Matoltsy and Parakkal, 1965). Since the only mechanism known to be involved in discharging or transporting components from the cytoplasm of upper granular cells into the intercellular space is through the membrane coating granules (Lavker, 1976; Landmann, 1986), the finding that AE17 and AE18 antibodies react with the intercellularly located material strongly support the no-
tion that their 25K antigen is a component of membrane coating granules. Moreover, the fact that this 25K protein cannot be detected by our antibodies in esophageal or any other non-cornified epithelia that are also known to possess MCGs suggests that MCGs are structurally heterogeneous and that the 25K protein is specialized for MCGs of the epidermis and potentially other cornified (orthokeratotic) epithelia.

Although the function of this 25K MCG-related protein is presently unknown, it is possible that it represents one of the several known MCG-associated enzymatic activities, including acid hydrolases and phospholipases, and several widely expressed lysosomal enzymes that have been histochemically localized to the intercellular space beneath the stratum corneum (Eisen et al., 1964; Elias et al., 1988; Frienkel and Traczyk, 1983; Gonzalez et al., 1976; Menon et al., 1986; Weinstock and Wilgram, 1970; Wolff-Schreiner, 1977). These MCG-associated enzymes are thought to be involved in a number of processes including the postsecretory modification of MCG lipids converting them into a functional permeability barrier, and/or the degradation of intercellular constituents such as desmosomal components resulting in desquamation. If so, the 25K protein would represent a novel epidermal-specific enzyme differentially expressed by the granular cells. Alternatively, this relatively abundant protein may play a structural role in the organization and stabilization of permeability barrier components. This possibility is supported by our EM localization data showing that this protein is localized along both the short disks of the initially secreted MCG lamellae as well as the broad lipid sheets of the mature permeability barrier derived from the smaller lamellae. Finally, this protein may be involved in strengthening the adherence of cornified cells to the underlying living cell layers. Ongoing molecular cloning and expression experiments should provide clues to the detailed structure and function of this protein which provides an excellent molecular marker of an advanced stage of epidermal differentiation.

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Figure 9. Cyanogen bromide resistance of the 25K protein. (a) Control: total proteins of human epidermis were separated by SDS-PAGE, treated in situ with 70% formic acid alone, separated on the second dimension by SDS-PAGE, transferred to nitrocellulose, and stained with Fast Green. Note that all proteins including keratins (K) remain intact and stay on the diagonal. (b) A CNBr-treated sample, Fast Green stained. A similar sample was treated with CNBr after the first dimensional but before the second dimensional separation. Note the complete removal of keratins (K) from the diagonal indicating their susceptibility to CNBr. Also note the presence of a 37K filaggrin and the 25K protein on the diagonal indicating their resistance to CNBr. (c) A CNBr-treated sample stained with a polyclonal antiserum to filaggrin. (d) Another CNBr-treated sample stained with AE17.
