Detection of Several Families of Deiminated Proteins Derived from Filaggrin and Keratins in Guinea Pig Skin

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Detection of Several Families of Deiminated Proteins Derived from Filaggrin and Keratins in Guinea Pig Skin

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ABSTRACT—Structural proteins in the mammalian epidermis contain citrulline residues generated by enzymatic deimination of arginine residues. We analyzed deiminated proteins solubilized from sequentially stripped layers of guinea pig epidermis. Deiminated proteins were localized in the granular and cornified layers. Those in the inner layer enriched with granular cells were resolved into numerous components by two-dimensional gel electrophoresis. An arc-shaped high-molecular-weight smear and two series of charged isomers among them coincided with filaggrin immunoreactivity. Several groups of filaggrin-negative spots appeared to be generated by further deimination and proteolysis of these filaggrins. Deiminated protein spots co-migrating with type II and type I keratins were also detected. Deiminated filaggrins and their further processed derivatives disappeared in the outer layer, while deiminated keratins persisted. These data suggested that filaggrin as well as profilaggrin were deiminated during the posttranslational processing in guinea pig skin, and that some keratins were deiminated preferentially during the cornification of epidermis. Possible biological significance of protein deimination in guinea pig skin was discussed in comparison with our recent finding on deiminated proteins in rat skin.

INTRODUCTION

The process of normal epidermal differentiation is characterized by a series of morphologic and biochemical changes as keratinocytes progress from the germinative basal layer through the spinous and granular layers to the outer cornified layer. Aggregation of keratin filaments mediated by filaggrin is one of the key events underlying the process (Lynley and Dale, 1983; Manabe et al., 1991; Steinert et al., 1981). Filaggrin is generated from its high-molecular-weight precursor (profilaggrin), which does not aggregate keratin filaments (Haydock and Dale, 1986; Meek et al., 1983; Rothnagel et al., 1987; Scott and Harding, 1981) and is deposited in keratohyalin granules in highly phosphorylated forms (Lonsdale-Eccles et al., 1980, 1982; Resing et al., 1985; Scott and Harding, 1981). Profilaggrin is dephosphorylated during processing to filaggrin (Lonsdale-Eccles et al., 1982; Resing et al., 1984, 1985; Scott and Harding, 1981), and filaggrin is further degraded in the cornified layer to amino acids, which are thought to be important for retaining moisture in the cornified cells (Scott et al., 1982; Scott and Harding, 1986). Therefore, both phosphorylation/dephosphorylation and proteolysis are involved in the functional role of filaggrin. A relatively unexplored posttranslational modification, which filaggrin seems to be subjected to, is “deimination”. Deimination is catalyzed by “peptidylarginine deiminases (EC 3.5.3.15)” which deiminate arginine residues generating citrulline residues (Fujisaki and Sugawara, 1981; Kubilus et al., 1980; Rogers et al., 1977; Rothnagel and Rogers, 1984; Terakawa et al., 1991; Watanabe et al., 1988). Harding and Scott (1983) suggested that the deimination of arginine residues of filaggrin modulates its interaction with keratins and facilitates its breakdown to amino acids. However, information concerning biological significance of protein deimination is limited, partly because sensitive enough methods to detect deiminated proteins specifically were not available.

We recently showed that not only filaggrin but also keratins were deiminated in rat skin (Senshu et al., 1995). These deiminated proteins were detected specifically by means of chemically modifying citrulline residues then probing them with a monospecific antibody (Senshu et al., 1992). We also showed that deiminated proteins were localized in the granular and cornified layers, and suggested a possible role for protein deimination during the terminal stage of epidermal differentiation (Senshu et al., 1995). However, in rat skin, not only the number of arginine residues modified in a given filaggrin molecule but also the amount of deiminated filaggrin in the total filaggrin pools appeared to be too small to argue for the functional significance of filaggrin deimination. We therefore extended our studies using guinea pig skin, in which multiple charged isomers of filaggrin were detected as...
Animals and tissue samples

Five-week-old female albino guinea pigs (Hartley strain) weighing 300-350 g were obtained from Japan SLC Co. Ltd. (Shizuoka, Japan). All operations were performed under pentobarbital anesthesia or after decapitation under the anesthesia. Back skin was clipped and deplilated using depilatory cream (Epilat; Kanebo, Japan). Epidermal layers were stripped using cyanacrylate glue. The glue was spread as a thin film over a delineated area, and then removed with the attached cell layers. This operation was repeated three times. Histological examination showed relative enrichment of the upper cornified cells in the first layer, that of the lower cornified cells in the second layer, and that of the granular cells in the third layer.

Materials and Methods

Extraction of proteins

The stripped layers were minced in 9.5 M urea (Ultra Pure, ICN Biochemicals, Inc., Ohio), 2% Nonidet P-40, 2% Ampholines (pH 3.5-10, Pharmacia LKB), and 5% 2-mercaptoethanol, and insoluble materials were removed by brief centrifugal filtration through a 200-mesh nylon screen. Protein concentrations in the extracts were estimated according to Lowry et al. (1951) after precipitation with 20% trichloroacetic acid. Bovine serum albumin was used as the standard.

SDS-PAGE and immunoblotting

The extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional gel electrophoresis using a nonequilibrium pH gradient gel electrophoresis (pH 3.5-10) in the first dimension as described (Senshu et al., 1995). Samples for SDS-PAGE were diluted at least 5-fold to reduce the concentration of Nonidet P-40. They were not heated to avoid artificial carbamylation of proteins. Resolved proteins were Western blotted to a BA-S85 membrane (S & S, Dassel, Germany). The stripped layers were incubated in modification medium free of diacetyl monoxime, incubated in modification medium at 37°C for 3 hr (Asaga and Senshu, 1993; Senshu et al., 1995). Control sections were incubated in medium free of diacetyl monoxide, antipyrine, and acetic acid. The resulting sections were incubated with anti-modified citrulline IgG (0.125 µg/ml). Sections for filaggrin immunostaining were incubated with the affinity purified antibody to rat filaggrin (13 ng/ml). Bound antibodies were stained by means of a VECTASTAIN ABC Elite kit (Vector Laboratories), using 3,3'-diaminobenzidine as the chromogenic substrate. Stained sections were counterstained with hematoxylin.

Results

Detection of deiminated proteins in stripped epidermal layers

We first compared SDS-PAGE profiles of proteins extracted from the upper three layers of stripped epidermis. Three major bands (195, 61, and 56 kDa) were detected in the total protein profile (Fig. 1a). These bands were detected with the mixture of monoclonal antibodies AE1 and AE3, although the 61-kDa band was under-represented probably due to its lower affinity to the monoclonal antibodies (Fig. 1b). Two minor bands (66 and 46 kDa) were detectable in the total protein profiles of the inner two layers. The former but not the latter was detected with the monoclonal antibody mixture.

Immunochemical detection was confirmed by probing equivalent blots migrated with those detected by the AE1/AE3 mixture, although the 61-kDa band was under-represented probably due to its lower affinity to the monoclonal antibodies (Fig. 1c). The band intensity increased in the inner layers along with a leading smear. These signals probably represented profilaggrin and its high-molecular-weight processing intermediates. Two additional bands co-migrating with the 66- and 46-kDa bands found in the total protein profile were detected in the second layer, and their signal intensity increased further in the third layer. Detection of deiminated proteins on equivalent blots showed four major bands (206, 165, 61, and 56 kDa) in the first layer (Fig. 1d). The signal intensity of the two smaller bands, which co-migrated with those detected by the AE1/AE3 mixture, decreased in the inner layers, whereas two additional bands (66 and 46 kDa) co-migrating with those detected by anti-filaggrin became detectable. In addition, broad bands (about 34, 30, and 23 kDa) were discernible above the background smear. The signal intensity of the 46-, 34-, 30-, and 23-kDa bands appeared to be stronger in the second layer than in the third layer, while that of the 66-kDa band remained about equal.

This and the relative abundance of 66- and 46-kDa filaggrins
in these layers suggested that these filaggrins in the second layer were deiminated at larger numbers of arginine residues than those in the third layer.

Identification of deiminated proteins on two-dimensional blots

The above one-dimensional analyses allowed quantitative comparison of the band intensity between different samples. We next performed two-dimensional Western blotting of the first and the third layer fractions to identify deiminated proteins. Keratins, filaggrin, and deiminated proteins were detected on equivalent blots and results then correlated with the stained total protein profiles. It should be mentioned that each immunoblot was displayed at an appropriate intensity to show its protein composition. This does not necessarily mean that the data are suitable for quantitative comparison between different samples. The first layer showed two diffuse major spots of deiminated proteins (Fig. 2d). Overlapping spots were visible in the total protein (Fig. 2a). The less anodic spots co-migrated with a diffuse type II keratin spot corresponding to the 61-kDa band in the one-dimensional analysis (Fig. 2b). The other spot co-migrated with a 56-kDa type I keratin spot. These keratins are probably the products of various posttranslational modifications as measured from the rather diffuse appearance of the spots. A few curved smears of deiminated proteins were found as minor signals near the upper anodic corner. Similar signals were detected in infant rat skin (Senshu et al., 1995), which were thought to be derived from trichohyalin (Rogers et al., 1977). These deiminated proteins did not coincide with weak signals detected by anti-

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The total protein profile of the third layer showed an arc-shaped smear in the upper cathodic region, multiple spots of keratins in the neutral to acidic region, and two series of charged isomers (at about 66 and 46 kDa) scattered in the cathodic region (Fig. 2e). The mobility of these charged isomers in the second dimension appeared to decrease slowly with the decreasing first dimensional mobility. An additional series of charged isomers (about 34 kDa) was also discernible in the neutral region. Immunoblotting for filaggrin showed a major signal co-migrating with the arc-shaped smear representing high-molecular-weight processing intermediates, and minor signals co-migrating with basic components of the 66- and 46-kDa charged isomers (Fig. 2g). Detection of deiminated proteins on equivalent blots visualized a prominent signal co-migrating with the arc-shaped smear. This suggests deimination of high-molecular-weight processing intermediates of profilaggrin (Fig. 2h). Two series of charged isomers (66 and 44 kDa) co-migrating with those in the total protein profile were also detected. The arrays of these charged isomers extended further towards the neutral region than those detected with anti-filaggrin, suggesting that less basic components were deiminated at larger numbers of arginine residues. In addition, weak signals of 34-kDa charged isomers were detected in the neutral region. Series of smaller charged isomers could be detected when the data were displayed at increased sensitivity. These isomers appeared to be vertically or near vertically linked with trailing smears, giving the overall impression of a flowing stream. This layer also contained a series of intense signals that co-migrated with a series of 61-kDa type II keratins and minor signals migrating in the 56-kDa type I keratin region (Fig. 2f). These spots of deiminated proteins accounted for all the bands found in the one-dimensional analysis. It should be noted that only limited numbers of keratin spots were deiminated.

Immunolocalization of deiminated proteins

Figure 3 shows micrographs of immunohistochemically stained sections in comparison with that stained with hematoxylin and eosin (Fig. 3a). Filaggrin immunoreactivity was localized in the granular and lower cornified layers (Fig. 3b). Deiminated proteins were localized in the whole cornified layer as well as in the granular layer (Fig. 3c). Control sections incubated in modification medium without diacetyl monoxime, antipyrine, and acetic acid were not stained (Fig. 3d).

DISCUSSION

Filaggrin is the product of the dephosphorylation and proteolytic processing of the precursor profilaggrin (Haydock and Dale, 1986; Lonsdale-Eccles et al., 1980, 1982; Meek et al., 1983, Resing et al., 1984, 1985; Rothnagel et al., 1987; Scott and Harding, 1981). Filaggrin can be separated by two-
Fig. 2. Characterization of the deiminated proteins by two-dimensional gel electrophoresis. Samples were resolved by nonequilibrium pH gradient gel electrophoresis and SDS-PAGE in the first and second dimensions, respectively. Total proteins (a, e), keratins (b, f), filaggrin (c, g), and deiminated proteins (d, h) were visualized as described. Panels a-d, proteins extracted from the first layer; Panels e-h, proteins extracted from the third layer. The amount loaded was about 30 µg. Acidic type I and neutral-basic type II keratins were distinguished by separately probing equivalent blots with AE1 and AE3. Reference points are shown by numbered arrowheads.
dimensional gel electrophoresis into families differing in molecular weight and/or charge, depending on the species (Harding and Scott, 1983). Guinea pig filaggrin has been shown to be highly complex, including high-molecular-weight variants (200 kDa or above) (Harding and Scott, 1983; Scott, 1986). We used an affinity purified polyclonal antibody to rat filaggrin for immunoblotting. The two-dimensional immunoblot reported here resembled those of guinea pig filaggrin reported by other investigators (Harding and Scott, 1983; Scott, 1986). In addition, the antibody specifically stained the lower cornified and granular layers. These results indicated the acceptable cross-reactivity and specificity of our antibody for probing guinea pig filaggrin.

Harding and Scott (1983) suggested the deimination of guinea pig filaggrin based on amino acid analysis and on the detection of $[^3H]$citrulline in filaggrin derived from guinea pig skin pre-labeled with $[^3H]$arginine. They further proposed that deimination progressively lowers the affinity of filaggrin for keratins based on their observation that less basic variants of filaggrins interacted poorly with keratins in vitro. However, direct identification of deiminated filaggrins on the two-dimensional map was not conducted by these authors. Here we used a highly sensitive and specific method for the detection of deiminated proteins on the two-dimensional Western blot, and detected several families of deiminated filaggrins at various stages of processing, ranging from the arc-shaped smear in the high-molecular-weight region to multiple charged isomers in the low-molecular-weight region. Furthermore, it should be noted that the present method enabled us to detect multiply modified or extensively processed components that were not detectable with anti-filaggrin. The enrichment of deiminated filaggrins in the second layer and their almost complete deprivation in the first layer probably reflected increased deimination of filaggrins in the lower cornified layer followed by their degradation in the upper cornified layer. This is in accord with the proposal of Harding and Scott (1983) suggesting that the deimination of filaggrin facilitates its breakdown to amino acids. However, the biological significance of filaggrin deimination or even filaggrin itself in guinea pig skin is still unclear. We do not know which are functional filaggrin units to aggregate keratins among various filaggrin-positive components detected on the Western blots. The 66-kDa component may be regarded to be functional from its relative abundance. However, if so, why was the high-molecular-weight smear already deiminated? If they were deiminated prior to binding to keratins, did it reflect the regulated production of non-deiminated, functional filaggrin and deiminated, non-functional filaggrin? Furthermore, it is puzzling that deimination of filaggrin appeared to occur at only few arginine residues in a minor fraction of the total filaggrin in rat skin (Senshu et al., 1995). Nevertheless, filaggrin was thought to be efficiently degraded as measured from its deprivation in the upper cornified layer. These data suggest that deimination of filaggrin may not be an obligatory step for its breakdown in rat skin. In addition, deimination of high-molecular-weight processing intermediates were not detected.

Fig. 3. Micrographs of stained skin sections. Cryosections were processed as described in the text. (a) Stained with hematoxylin and eosin; (b) anti-filaggrin; (c) post-fixed, incubated in the modification medium, and stained with anti-modified citrulline IgG; (d) treated similarly to b except that diacetyl monoxime, antipyrine, and acetic acid were omitted from the modification medium. (C) Stratum corneum; (G) stratum granulosum; (S) stratum spinosum. Dashed line, epidermal-dermal junction. Bar, 50 µm.
in rat skin. Therefore, the biological significance of filaggrin deimination might differ between different species.

It is well-known that a basic type II keratin K5 and its acidic type I keratin partner K14 are expressed in the basal layer of epidermis forming fine cytoskeletal networks (Steinert and Freedberg, 1991). Another basic type II keratin K1 and its acidic type I keratin partner K10 are expressed in the suprabasal cells and incorporated into the pre-existing networks of K5 and K14 (Steinert and Freedberg, 1991). The resulting keratin networks are converted to a densely packed filamentous structure mediated by filaggrin during the transition of the granular cells to the cornified cells (Lynley and Dale, 1983; Manabe et al., 1991; Steinert et al., 1981). The presence of deiminated keratins and filaggrins in these upper epidermal layers is consistent with our proposal that the protein deimination was playing a possible role during the terminal stages of epidermal differentiation (Senshu et al., 1995). It should be noted that only a few of keratin spots were deiminated, as has been observed in rat skin. This suggests a preferential action of peptidylarginine deiminases towards these keratins. The major deiminated products are type II keratins. The deimination of specific arginine residues of such keratins might play a role in modulating interactions with their direct partners, other keratins in the cytoskeletal network, or keratin-asssociated protein filaggrin. Detailed identification of deiminated keratins was not attempted because antibodies specific enough to identify guinea pig keratins were not available. We are in the process of identifying the deiminated residues in defined keratins.

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