Evidence that filaggrin is a component of cornified cell envelopes in human plantar epidermis

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Cornified cell envelope (CE) is generated during the late stages of epidermal differentiation and is made up of proteins covalently linked together by transglutaminases. To determine whether filaggrin is a component of this structure in humans, we analysed highly purified CE from plantar stratum corneum. An immunoelectron microscopy analysis showed specific binding of four different anti-(pro)filaggrin monoclonal antibodies to the surface of the CE, proved previously to be free of non-covalently linked proteins. Moreover, the anti-filaggrin activity of one of the antibodies was absorbed by preincubation with the plantar CE, as determined by ELISA. Convincingly, fragments of CE produced by proteolytic digestion of the structures were stained by this antibody on immunoblots. These data provide direct evidence that filaggrin is a component of CE purified from human plantar stratum corneum. Cross-linking between CE and the filaggrin-containing fibrous matrix may enhance the structural cohesion of the corneocytes and thus the resistance of the stratum corneum.

INTRODUCTION

During the late stages of epidermal differentiation, a highly insoluble structure termed the cornified cell envelope (CE) is formed beneath the keratinocyte plasma membrane. This rigid scaffolding is thought to be critical for the barrier function and the cohesion of the stratum corneum, the cornified upper layer of epidermis. CE is formed by covalent cross-linking between glutamine and lysine residues of both cytosolic and particulate proteins, in a reaction catalysed by epidermal transglutaminases [1,2]. Defects in these enzymes or in the protein components of the CE may result in the lamellar ichthyosis phenotype [3]. Several proteins have been proposed as potential CE precursors, but the definite composition of the structure is unknown because of the insoluble character of the cross-linked components. Direct evidence for such a precursor role has nevertheless been reported for a number of proteins, including involucrin, loricrin, cystatin α/keratolinin, and small proline-rich proteins [1,2,4–8].

Profilaggrin, the filaggrin precursor, is synthesized in the granular cells of the epidermis and accumulates in the keratohyalin granules. It is highly phosphorylated, and consists of 10–12 filaggrin repeats separated by linker peptides. During the transition from the granular to the cornified cell, profilaggrin is dephosphorylated, and basic filaggrin units are produced. The basic filaggrins interact with and aggregate the cytokeratin filaments, facilitating formation of intermolecular disulphide bonds to yield the intracellular fibrous matrix of the cornified cells [9–16]. Further conversion, by a peptidylarginine deiminase, of arginine residues of filaggrin into citrullines results in a lower affinity of the molecule for cytokeratins [11,17]. We recently described a neutral/acidic isoform of filaggrin. This protein shows an identical peptide mapping and the same amino acid composition as the basic filaggrin, but a more acidic pI. Moreover, it is recognized by eight monoclonal antibodies (MoAbs) directed against different epitopes of basic filaggrin. Its origin and function are not clearly known [18]. Finally, filaggrin is proteolysed into free amino acids in the upper stratum corneum [19]. It has been shown that rat filaggrin might be cross-linked to the CE. Rat filaggrin is a substrate of epidermal transglutaminase *in vitro*, and the anti-filaggrin activity of a rabbit antiserum is absorbed on partially purified envelopes from newborn rat epidermis [20]. However, since recent data indicate that rigorous purification of the CE is critical for an accurate identification of their cross-linked components [4], the latter result could be questioned. Mathematical modelling of the amino acid composition of human foreskin CE [21] and an immunocytochemical analysis of human plantar CE [22] suggested that human filaggrin might also be a CE constituent. Identification of filaggrin and loricrin amino acid sequences joined by isodipeptide cross-links, in peptides recovered after limited proteolysis of purified human foreskin CE, recently demonstrated that human filaggrin is indeed covalently linked to the non-palmoplantar envelopes [8].

In the present study, we provide immunomorphological and immunochemical evidence that filaggrin is a true component of human plantar CE.

MATERIALS AND METHODS

Filaggrin extraction and purification

The neutral/acidic isoform of human filaggrin was extracted from breast skin (obtained from patients undergoing plastic surgery) and affinity-purified, as reported previously [23].

Preparation of CE

Human CE were purified from plantar stratum corneum and from breast epidermis, as described previously [4,24], with some modifications. All plasticware was siliconized. The samples were homogenized, and extracted by boiling with vigorous agitation for 10 min in a solution containing 2% (w/v) SDS, 25 mM dithiothreitol, 50 mM Tris/HCl, pH 7.4, and 5 mM EDTA (TESD buffer). The extract was centrifuged for 5 min at 4000 ***g***.

Abbreviations used: CE, cornified cell envelope; MoAb, monoclonal antibody; IIF, indirect immunofluorescence.

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Pelleted envelopes were resuspended in fresh TESD buffer, boiled for 10 min, and spun down. This procedure was repeated three times. CE were then extracted at 37 °C for 72 h in a solution containing 8 M urea, 25 mM dithiothreitol, 50 mM Tris/HCl, pH 7.4, 5 mM EDTA and 4 μg/ml of aprotinin. The urea-extracted envelopes were pelleted, resuspended in 0.1 % SDS, 192 mM glycine and 125 mM Tris, and electrodialysed against the same buffer, at 50 V for 72 h in a Biotrap apparatus (Schleicher and Schuell, Inc., Dassel, Germany). The purified CE were collected by centrifugation, then washed three times with distilled water and counted.

MoAbs

AKH1, a MoAb specific for human (pro)filaggrin, and a rabbit antiserum specific for involucrin, were purchased from Biomedical Technologies, Inc. (Stoughton, MA, U.S.A.). AHF1, AHF2 and AHF3, three MoAbs specific for human (pro)filaggrin, AHF7, a MoAb specific for human filaggrin, and G36-19, a MoAb directed against corneodesmosin, were produced and characterized in our laboratory [24,25]. BL6 MoAb (Immunotech, Marseille, France), specific for a CD1a antigen expressed by epidermal Langerhans cells [26], was used as a negative control in immunoelectron microscopy. MOPC, a control MoAb, was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

CE analysis

Indirect immunofluorescence (IIF) was performed on cyto-centrifuged CE, as described previously [24].

Plantar CE were analysed with post-embedding indirect immunogold labelling, as reported earlier [22,25].

ELISA was done, as described previously [25], in polystyrene microtitration plates (Nunc, Copenhagen, Denmark) coated overnight at 37 °C with the affinity-purified neutral/acidic filaggrin, solubilized in PBS (2 μg/ml). In the absorption experiments, anti-(pro)filaggrin MoAbs were pre-incubated for 2 h at room temperature with various concentrations of the purified filaggrin (1–10 μg/ml) or with various amounts of plantar CE (10^5–10^6 CE/ml, corresponding to roughly 0.1–1 mg/ml of protein). The mixture was centrifuged, and the supernatant was applied to the coated plates, instead of the antibody alone. In absorption experiment controls, a peroxidase-conjugated sheep antibody raised to mouse IgG (Zymed, San Francisco, CA, U.S.A.) was assayed, using a similar protocol, on the IgG fraction of a non-immune mouse serum, coated in plates. The antibody was pre-incubated as described, with various concentrations of mouse IgG or with various amounts of CE.

For protease digestion, 10^5 CE were resuspended in 90 μl of 0.5 % SDS/10 % (v/v) glycerol/125 mM Tris/HCl, pH 6.8, containing 0.1 mg/ml of *Staphylococcus aureus* V8 protease, and incubated at 37 °C for 72 h. The reaction was stopped by boiling in sample buffer. After centrifugation, proteins in the supernatant were separated by gel electrophoresis.

Protein analysis

Proteins were separated by SDS/PAGE on 10 % (w/v) acrylamide gels and electrotransferred to nitrocellulose membranes. The membranes were stained with Protogold (British BioCell International, Cardiff, U.K.) and probed, as reported previously [23], with MoAbs diluted to 0.2 μg/ml, and with the anti-involucrin serum diluted to 1/20. The immunoreactive proteins were visualized with peroxidase-conjugated sheep antibodies to mouse IgG or rabbit IgG (Zymed) and a chemiluminescent substrate (ECL Western Blotting kit, Amersham International, Aylesbury, Bucks., U.K.), according to the manufacturer's protocol. Protein markers from Bio-Rad Laboratories (Richmond, CA, U.S.A.) were used as molecular-mass references.

Transglutaminase cross-linking

Human breast epidermis was cleaved from dermis and homogenized in 0.2 ml/cm^2 of an ice-cold solution containing 40 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5 % Nonidet P-40, 0.1 % sodium azide and 0.1 mM PMSF. The homogenate was then clarified by centrifugation at 15000 ***g*** for 10 min. After addition of dithiothreitol to 5 mM and $CaCl_2$ to 20 mM, the homogenate was incubated at 37 °C for 0–6 h. Samples incubated without $CaCl_2$ and dithiothreitol served as controls. Reactions were stopped by the addition of sample buffer. After centrifugation, proteins were separated by SDS/PAGE and analysed by immunoblotting.

RESULTS AND DISCUSSION

CE were purified from human plantar stratum corneum by repeated boiling in a buffer containing SDS and a reducing agent, an extensive extraction in 8 M urea, and an extensive electrodialysis in the presence of SDS. The resulting highly purified envelopes were free of soluble and non-covalently bound proteins as judged by SDS/PAGE and staining of the gel with Coomassie Blue or silver reagent (results not shown), or by transfer to nitrocellulose membrane and staining of the membrane with Protogold (see Figure 3A, lane 6). Omission of the urea extraction step did not affect the CE purity, as checked by SDS/PAGE. In contrast to cultured keratinocyte CE [4], a sonication step was not necessary to get pure skin envelopes. This may indicate structural differences between epidermal and cultured keratinocyte CE. More probably, the electrodialysis step in the preparation of the envelopes is an alternative to the sonication step to extract soluble trapped proteins.

The plantar envelopes were analysed for the presence of filaggrin by IIF with four AHF MoAbs specific for (pro)filaggrin. They were weakly and diffusely labelled by the MoAbs. The labelling contrasted with the completely negative controls obtained with IgG of a non-immune mouse, or when the primary antibody was omitted (results not shown). This result suggested that human filaggrin is a minor component of plantar envelopes. Because of the faintness of the labelling, more sensitive methods were then used.

Post-embedding immunoelectron microscopy confirmed the presence of filaggrin in the CE purified from plantar stratum corneum (Figure 1). All of the four anti-(pro)filaggrin MoAbs weakly but clearly labelled the structures (Figures 1A–1C). The intensity of the labelling varied between individual CE samples (some fragments showing no reactivity), but apparently did not vary from one MoAb to another. No background labelling was noted on the plastic around the CE, and no labelling at all was observed when an irrelevant primary antibody (BL6 MoAb) was used, indicating the high specificity of the immunocytochemical binding. The considerably stronger labelling produced by an anti-involucrin serum (Figure 1D) suggests that filaggrin is hardly accessible to the AHF MoAbs when incorporated in the envelopes, or, more probably, that filaggrin is a minor component of plantar CE. Indeed, it was recently published that filaggrin makes up a very small proportion of foreskin CE [8].

The anti-filaggrin activity of AHF3, analysed by ELISA, was progressively absorbed by pre-incubation with increasing

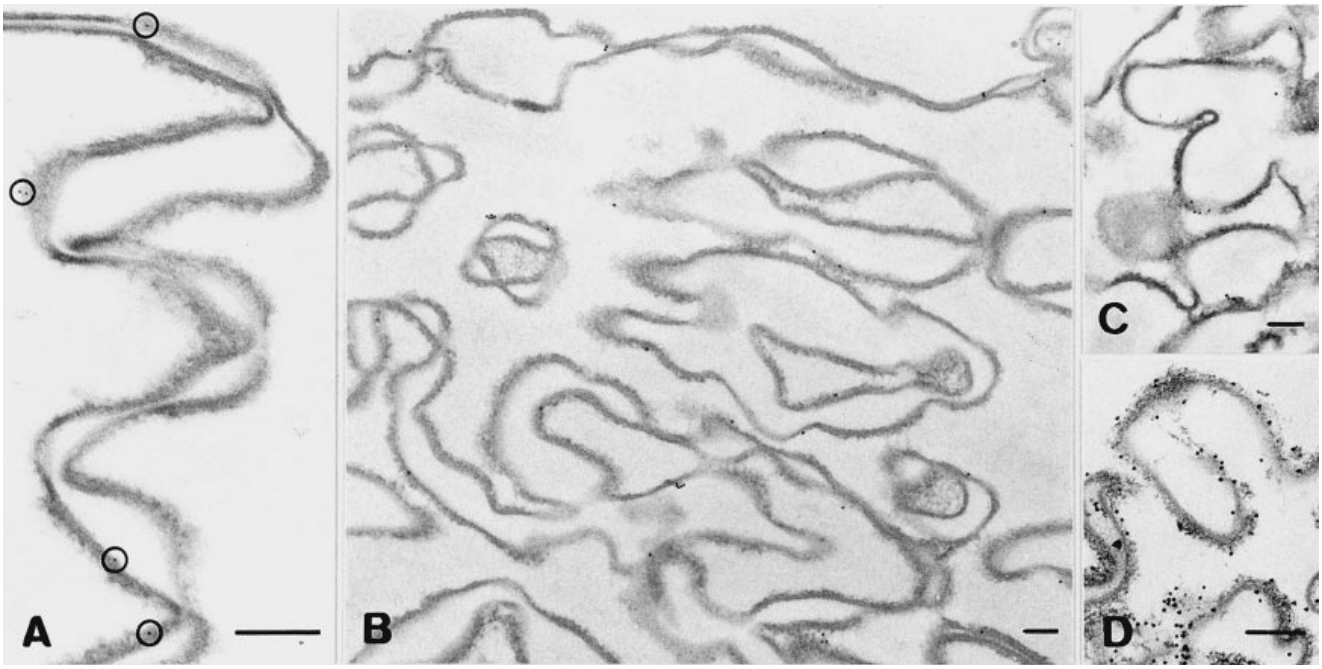

Figure 1 Anti-(pro)filaggrin MoAb-labelled plantar CE: immunogold labelling

CE were extracted from plantar stratum corneum and highly purified, as described in the Materials and methods section, except for the urea extraction step. Ultrathin sections of Lowicryl K4M-embedded material were used for indirect immunogold 'on grid' labelling. (**A**, **B**) Reactivity of the anti-(pro)filaggrin MoAbs, AHF3 and AHF2, respectively. Gold particles are indicated with open circles in (**A**). Identical results were obtained with AHF1. (**C**) Reactivity of the anti-filaggrin MoAb, AHF7. (**D**) Reactivity of the anti-involucrin serum. Scale bars represent 200 nm.

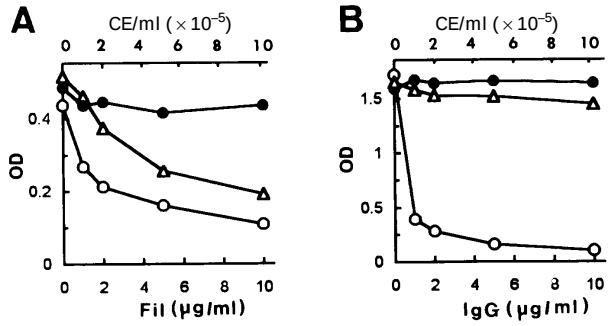

Figure 2 Anti-filaggrin activity of AHF3 is absorbed by plantar CE

AHF3 diluted to 0.1 μg/ml (**A**) and an anti-(mouse IgG) serum diluted to 0.2 μg/ml (**B**) were analysed by ELISA on the affinity-purified filaggrin (Fil) and the IgG fraction of a non-immune mouse, respectively. In both cases, the antibodies were preadsorbed either with phosphate buffer (●), or with increasing concentrations of the related antigen (○), or with increasing amounts of CE (△), highly purified from plantar stratum corneum, as described in the Materials and methods section. Data represent means of duplicates.

amounts of plantar CE (Figure 2A). The dose-dependent absorption observed was similar to that observed with purified filaggrin as the sorbent. Moreover, the anti-filaggrin activity of AHF7 was only slightly absorbed with the highest amount of CE (results not shown). As a control, the anti-IgG activity of a sheep antiserum, analysed by ELISA, was not inhibited at all by pre-incubation with the plantar CE (Figure 2B). The large amount of CE required to totally absorb the AHF3 activity (up to 1 mg/ml) and the previous immunohistological results confirm that a very low amount of filaggrin is closely associated with, if not cross-linked to, plantar CE.

To confirm biochemically that filaggrin epitopes were present in the CE, the anti-(pro)filaggrin MoAbs, AKH1 and AHF1–3, were reacted with the fragments produced by proteolysis of the plantar CE. The highly purified CE samples were incubated for increasing periods of time with V8 protease, and the fragments produced were separated by SDS/PAGE and analysed by immunoblotting (Figure 3). AHF3 was the only anti-(pro)filaggrin MoAb to be reactive on the fragments. It strongly stained high-molecular-mass bands at the top of the gel and more slightly a 67 kDa band. This may indicate that filaggrin is incorporated into large, highly cross-linked homo- or heteropolymers. Several bands stained with Protogold were not detected by AHF3, further confirming the specificity of the reaction. No immunoreactive bands were detected using antibodies to (pro)-filaggrin in the absence of protease cleavage (Figure 3A, lane 1), showing a covalent association. A similar result was observed when the CE samples were sonicated and re-extracted, before proteolysis (results not shown). The filaggrin epitopes recognized on CE by the other AHF MoAbs were probably destroyed during the proteolysis step. Alternatively, the fragments bearing these epitopes were not released as soluble peptides.

A similar result was also observed after immunoblotting of fragments produced by proteolysis of CE purified from breast epidermis (results not shown), showing that CE from another body site also contained filaggrin.

When the products resulting from protease digestion of plantar CE were analysed by immunoblotting with a serum specific for involucrin, a broad immunoreactive smear ranging in molecular mass from 40 to more than 200 kDa, and several discrete immunoreactive bands that migrated at around 150 kDa, were

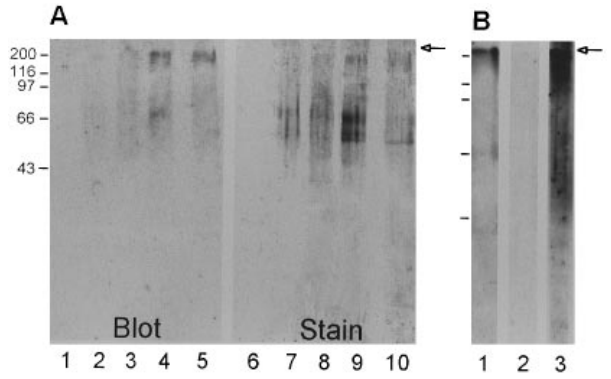

Figure 3 AHF3 reacts with proteolytic fragments of plantar CE

(**A**) CE were highly purified from plantar stratum corneum, as described in the Materials and methods section, and digested with V8 protease for 0 h (lanes 1 and 6), 12 h (lanes 2 and 7), 24 h (lanes 3 and 8), 48 h (lanes 4 and 9), and 72 h (lanes 5 and 10). Solubilized fragments were separated by SDS/PAGE, transferred to nitrocellulose membranes, analysed by immunoblotting with a pool of the anti-(pro)filaggrin MoAbs, AHF1, AHF2, AHF3 and AHF7 (Blot, lanes 1–5), then stained with Protogold (Stain, lanes 6–10). (**B**) The plantar CE samples were digested with V8 protease for 72 h and released fragments were analysed by immunoblotting with AHF3 (lane 1), AHF1 (lane 2) and an anti-involucrin serum (lane 3). Identical results were obtained with AHF1, AHF2, AHF7 and AKH1, but only that corresponding to AHF1 is shown. The position of molecular-mass standards (in kDa) is indicated on the left. The arrows point to the top of the resolving gels.

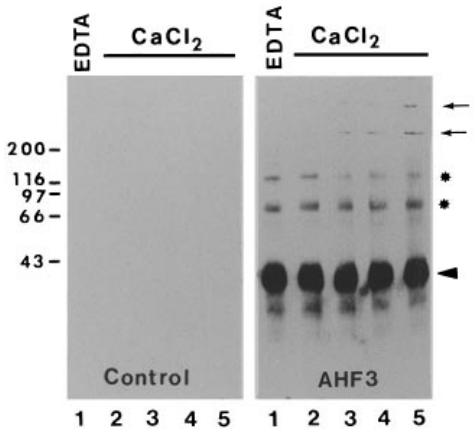

Figure 4 Human filaggrin is a substrate of transglutaminases

A low-ionic-strength homogenate of epidermis was clarified by centrifugation, and the soluble proteins were incubated at 37 °C in the presence of 5 mM EDTA or 10 mM $CaCl_2$, as indicated, for 0 h (lanes 2), 2 h (lanes 3), 4 h (lanes 4), and 6 h (lanes 1 and 5). Proteins were then separated by SDS/PAGE, and analysed by immunoblotting with the control MoAb MOPC (Control) , or with the anti-(pro)filaggrin MoAb, AHF3. The position of molecular-mass standards (in kDa) is indicated on the left. The arrowhead indicates filaggrin, the stars show dimeric and trimeric aggregates of filaggrin, and arrows point to cross-linked high-molecular-mass proteins generated during the incubation in the presence of $CaCl_2$.

observed, thus confirming that human involucrin is a precursor of plantar CE (Figure 3B, lane 3).

In preliminary experiments to test whether human filaggrin is a potential substrate of transglutaminases, a low-ionic-strength homogenate of epidermis containing epidermal transglutaminases and neutral/acidic filaggrin, but not profilaggrin, was prepared. Breast epidermis was used because of the difficulty of obtaining normal plantar epidermis. The extract was incubated under transglutaminase-activating conditions, i.e. with 10 mM $CaCl_2$ and 5 mM dithiothreitol, and compared with controls incubated with 5 mM EDTA. Proteins were then separated by SDS/PAGE and analysed by immunoblotting with AHF3 (Figure 4). Faint but clearly immunoreactive bands with molecular masses of more than 200 kDa (some did not enter the gel) were observed only in the presence of $CaCl_2$. The low amount of *in vitro* cross-linked filaggrin could be related to the fact that *in vivo* only a minor fraction of filaggrin is really incorporated in CE. This result nevertheless supports the prediction that human filaggrin, or at least a fraction of the protein, is a substrate of transglutaminases, as suggested previously for rat filaggrin [20]. Further studies with neutral/acidic, but also with basic, filaggrin will be necessary to test this hypothesis and to define the cross-linked regions more precisely.

Our immunological analysis and previous results [22] show that a very low amount of filaggrin is covalently linked to CE. Therefore, the role of filaggrin as a component of this structure is unclear. This may reflect non-specific cross-linking of abundant structural components of the stratum corneum by transglutaminases, as suggested by the 'dustbin' hypothesis [1]. Alternatively, cross-linking of CE to the corneocyte fibrous matrix may contribute to improving stratum corneum cohesion. Indeed, other components of the matrix, i.e. cytokeratins K1, K2e and K10, are cross-linked to CE in a very specific way, as demonstrated both in human [8,22] and mouse [27] skin. These findings could have far-reaching implications in the understanding of the cohesion mechanisms and barrier function of the stratum corneum.

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