The FASEB Journal article fj.10-168658. Published online January 31, 2011.

Hornerin is a component of the epidermal cornified cell envelopes

Julie Henry,* Chiung-Yueh Hsu,* Marek Haftek,† Rachida Nachat,* Heleen D. de Koning,‡ Isabelle Gardinal-Galera,*,§ Kiyotaka Hitomi,‖ Stéfana Balica,§ Catherine Jean-Decoster,¶ Anne-Marie Schmitt,¶ Carle Paul,*,§ Guy Serre,* and Michel Simon*,[1]

*Centre National de la Recherche Scientifique (CNRS)-Toulouse III University UMR5165, Institut Fédératif de Recherche 150 (INSERM-CNRS-Université Paul Sabatier-Centre Hospitalier Universitaire), Toulouse, France; †Lyon I University EA4169, Lyon, France; ‡Department of Dermatology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; §Department of Dermatology, Toulouse University Hospital, Toulouse, France; ‖Graduate School of Bioagricultural Sciences, Nagoya University, Naboya, Japan; and ¶Centre Européen de Recherche sur la Peau et les Epithéliums de Revêtement, Pierre Fabre Dermo-Cosmétique, Toulouse, France

ABSTRACT **A single-nucleotide polymorphism within the gene encoding hornerin (HRNR) has recently been linked with atopic dermatitis (AD) susceptibility. HRNR shares features with filaggrin, a key protein for keratinocyte differentiation, but conflicting reports have been published concerning its expression in the epidermis, and its role is still unknown. To analyze HRNR expression and function in the epidermis, anti-HRNR antibodies were produced and used in Western blot analysis and immunohistochemical, confocal, and immunoelectron microscopy analyses of human skin and of cornified cell envelopes purified from plantar stratum corneum. We also tested whether HRNR was a substrate of transglutaminases. In the epidermis, HRNR was detected at the periphery of keratohyalin granules in the upper granular layer and at the corneocyte periphery in the whole cornified layer. Detected in Western blot analysis as numerous bands, HRNR was relatively insoluble and only extracted from epidermis with urea and/or reducing agents. The presence of HRNR in the purified envelopes was confirmed by immunoelectron microscopy and by Western blot analysis after V8-protease digestion. HRNR was shown to be a substrate of transglutaminase 3. These data demonstrate that HRNR is a component of cornified cell envelopes of human epidermis. Its reduced expression in AD may contribute to the epidermal barrier defect observed in the disease.—Henry, J., Hsu, C.-Y., Haftek, M., Nachat, R., de Koning, H. D., Gardinal-Galera, I., Hitomi, K., Balica, S., Jean-Decoster, C., Schmitt, A.-M., Paul, C., Serre, G., Simon, M. Hornerin is a component of the epidermal cornified cell envelopes. *FASEB J.* 25, 000–000 (2011). www.fasebj.org**

Key Words: atopic dermatitis · skin · keratinocytes · transglutaminase · terminal differentiation · corneocytes

DIFFERENTIATION OF THE EPIDERMIS is an oriented process during which keratinocytes of the basal layer undergo a series of metabolic and structural changes throughout their migration to the surface of the skin. Cornification, the later stages of the process, is a real programmed cell death, and results in the formation of corneocytes. It is the stacking up of these anucleate cells, forming the outermost layer or stratum corneum (also called cornified layer), that enables the epidermis to fulfill one of its main functions: it provides a multiple barrier between the body and its environment by opposing water loss and the penetration of exogenous molecules, UV radiation, and pathogens, and by protecting the body, because of its great mechanical resistance. Alterations in keratinocyte differentiation are involved in the pathophysiology of many skin diseases, *e.g.*, atopic dermatitis (AD; OMIM%603635).

AD is a common chronic inflammatory skin disease affecting 15–20% of children and 1–3% of adults in industrialized countries. It is often linked with other atopic conditions, such as asthma and allergic rhinitis. AD results from complex interactions between genetic and environmental factors. The pathogenesis of the disease is still poorly understood, although immune system dysfunctions, as well as defects in the epidermal barrier, have long been suspected (1–4). A major breakthrough was the discovery (5) that loss-of-function mutations in the gene encoding filaggrin (FLG) on chromosome 1q21 are a major risk factor for AD, with an estimated odds ratio >3 (for a review, see ref. 6). This strengthens the hypothesis of a stratum corneum failure as a primary event, potentially leading to increased penetration of allergens and skin inflammation (1, 2, 7). Indeed, FLG is an essential component of the stratum corneum.

[1] Correspondence: CNRS-UPS UMR5165, CHU Purpan, Pl. du Dr Baylac TSA40031, 31059 Toulouse cedex 9, France. E-mail: michel.simon@udear.cnrs.fr

doi: 10.1096/fj.10-168658

This article includes supplemental data. Please visit *http://www.fasebj.org* to obtain this information.

FLG is a member of the S100-fused protein family. It is a basic 324-aa, histidine-rich protein synthesized by granular keratinocytes as a large precursor called profilaggrin, a component of keratohyalin granules. Profilaggrin consists of 10 to 12 FLG repeats separated by 7 amino acid-linker peptides. The FLG units are flanked by 2 unique sequences, the N-terminal S100 domain showing 2 calcium-binding sites. During cornification, profilaggrin is proteolyzed into FLG subunits. The latter associate with keratin intermediate filaments and facilitate their aggregation and the consequent formation of the intracorneocyte fibrous matrix. In the upper stratum corneum, FLG units are deiminated; they separate from the keratins and then are completely degraded into amino acids, which constitute the natural moisturizing factor, an essential component for stratum corneum hydration and for photoprotection (8). A proportion of FLG is also cross-linked to the cornified cell envelope (CE; refs. 9, 10). CE is a 15-nm-thick, highly insoluble, and complex protein structure, with a monolayer of ω-hydroxyceramides attached to its extracellular surface. CE replaces the plasma membrane of terminally differentiated keratinocytes. It results from the formation of very stable Nε-(γ-glutamyl)lysine isopeptide bonds between various proteins, including involucrin (IVL) and loricrin, in a reaction catalyzed by calcium-dependent enzymes called transglutaminases (TGases; EC 2.3.2.13). CE is critical for the stratum corneum barrier functions since it confers resistance to the layer acting as a shell, and controls the organization of the lipid lamellae in the extracellular spaces (11).

However, a maximum of 1 in 2 patients with AD carries the *FLG*-null allele, even in the cohorts with the most severe cases (6). Moreover, additional AD predisposition genes may exist in 1q21 apart from the association with *FLG* (12). Interestingly, a genome-wide association study in AD has identified a single-nucleotide polymorphism 7 kb downstream of another gene located in 1q21, the hornerin-encoding gene (*HRNR*), as a susceptibility variant (13).

HRNR is a 2850-aa protein with a predicted molecular mass of 280 kDa. Like FLG, it is a member of the S100-fused protein family. Its structural organization has similarities with that of profilaggrin: a repetitive central domain flanked by an N-terminal unique peptide with calcium binding sites, and by a C-terminal unique domain (14, 15). The protein was first identified in mice, where it was shown to be expressed in cornified epithelia, including the epidermis, and to be present both in the cytoplasm of granular keratinocytes, showing colocalization with profilaggrin, and in the stratum corneum (14, 16). This suggests that FLG and HRNR have similar functions. Conflicting results exist concerning HRNR expression in humans. Takaishi *et al.* (15) have reported that HRNR is only expressed in the hyperproliferative epidermis (psoriatic and wounded skin), whereas Wu *et al.* (17) have detected the protein in the healthy epidermis.

This prompted us to reinvestigate the expression of HRNR in normal human skin using highly specific antibodies, and to search for its function. We provide *in vitro* and *in vivo* evidence that HRNR is a component of CE. The reduced expression of HRNR in the epidermis of AD patients, as suggested by Wu *et al.* (17) and demonstrated here might contribute to the epidermal barrier defects observed in the patients.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antisera were generated against a synthetic peptide (HP2) present on each of the repeated units 1A–6A and 1C–5C of human HRNR with an added N-terminal cysteine residue for coupling purposes (CQHGSRSGQSSR; Génosphère, Paris, France), as described previously (18). Antipeptide anti-HRNR antibodies were purified by affinity chromatography against the synthetic peptide covalently bound on a SulfoLink resin (SulfoLink Immobilization Kit for peptides; Pierce, Rockford, IL, USA) as described by the manufacturer. Monoclonal antibodies directed against FLG (AHF3; ref. 19), tetra-His (Qiagen, Venlo, The Netherlands), IVL (SY5; Abcam, Cambridge, UK), corneodesmosin (G36–19; ref. 20), and actin (C4; Millipore, Billerica, MA, USA) were also used.

Alexa Fluor 555-conjugated goat anti-rabbit, Alexa Fluor 488-coupled goat anti-mouse, and Alexa Fluor 555-coupled goat anti-mouse secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA). For immunoblotting analyses, peroxidase-coupled goat anti-rabbit and anti-mouse secondary antibodies were purchased from Zymed Laboratories (San Francisco, CA, USA).

Patients and control volunteers

All experiments were performed according to the principles of the Declaration of Helsinki and with appropriate approval from the University of Toulouse Ethics Committee. Biopsies of lesional and nonlesional skin were obtained from 5 untreated patients with AD after obtaining written informed consent. In addition, human abdominal skin from healthy donors undergoing plastic surgery was used.

Extraction of epidermal proteins

The epidermis was cleaved from the dermis, and epidermal proteins were either sequentially extracted, as described previously (ref. 21; see Supplemental Material for details), or extracted by homogenization in 35 mM Tris-HCl (pH 6.8), 1.5% SDS, 5% glycerol, and 2.5% β-mercaptoethanol. The homogenates were cleared by centrifugation for 15 min at 15,000 rpm, and the supernatants were kept at −20°C until used.

Immunoblotting analyses

Samples were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane by semidry transfer. The blots were probed with primary antibodies and peroxidase-conjugated secondary antibodies. Detection was performed with ECL reagent (GE Healthcare, Little Chalfont, UK). The anti-HRNR, AHF3, and anti-actin antibodies were diluted to 1:200, 1:5000, and 1:10,000, respectively. Peroxidase-coupled secondary antibod-

ies were used according to the manufacturer's recommendations. Immunoreactive bands were scanned and quantified by densitometry using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

Immunohistochemistry was performed on cryosections (5 μm) of normal human abdominal skin. The anti-HRNR antibodies were diluted to 1:50, and detected with the appropriate Histostain-SP kit (chromogen AEC; Zymed Laboratories). Images were taken using a Nikon Eclipse 80i microscope equipped with a Nikon DXM 1200C digital camera and NIS image analysis software (Nikon, Tokyo, Japan).

Indirect immunofluorescence

The anti-HRNR and AHF3 antibodies were diluted to 1:50 and 1:2000, respectively. For the commercially available secondary antibodies, the manufacturer's recommendations were followed.

Colocalization experiments were performed on tissue samples fixed for 24 h in 4% paraformaldehyde, dehydrated for 24 h in 70% ethanol, and embedded in paraffin. Sections (5 μm) were deparaffinized and rehydrated before heat-induced antigen retrieval was performed in 50 mM glycine (pH 3.5) for 40 min at 95°C. Unspecific binding was blocked with 2% fish skin gelatin in PBS. Samples were incubated overnight with rabbit anti-HRNR and mouse anti-FLG antibodies simultaneously. Alexa Fluor-conjugated secondary antibodies were used for primary antibody detection, and samples were mounted in Mowiol (Calbiochem, Darmstadt, Germany). Fluorescence was analyzed using an inverted Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

For indirect immunofluorescence analyses of AD and healthy skin, cryosections were incubated first with fish gelatin, then with either the anti-HRNR or anti-FLG antibodies, and finally with the corresponding Alexa Fluor-conjugated secondary antibodies, as described above. Images were taken using a Nikon Eclipse 80i microscope equipped with a Nikon DXM 1200C digital camera and NIS image analysis software.

Immunoelectron microscopy

Normal human skin and plantar CEs, purified as described below, were fixed in 3% paraformaldehyde in PBS (pH 7.2) for 3 h, low-temperature dehydrated in graded ethanol, and embedded in Lowicryl K4M (Electron Microscopy Services, Hatfield, PA, USA). Postembedding indirect Immunogold labeling was performed using goat anti-rabbit or/and anti-mouse antibodies bound to 15- and 5-nm colloidal gold (GE Healthcare), as described previously (9, 20).

Purification and analysis of CEs

Human CEs were highly purified from plantar stratum corneum, as described previously (ref. 9; see Supplemental Material for details).

For protease digestion, 10^5 purified envelopes were resuspended in 90 μl of 125 mM Tris/HCl (pH 6.8), 0.5% SDS, and 10% glycerol containing 0.1 mg/ml of *Staphylococcus aureus* V8 protease, and incubated for 24 to 72 h at 37°C. The reaction was stopped by boiling in Laemmli's buffer. Samples were centrifuged, and supernatants were separated by SDS-PAGE.

Expression of recombinant N-terminal GST-tagged proteins

Human epidermis cDNA, prepared as described previously (22) and tested for the absence of genomic contamination, was used as a template for PCR experiments with primers derived from the HRNR and FLG-2 gene published sequences. The resulting PCR products were subcloned into pGEX-6P-1 (GE Healthcare), allowing inducible expression of N-terminal GST-tagged proteins in the *Escherichia coli* strain BL21 (DE3; Invitrogen). Recombinant proteins were affinity purified from bacterial lysates on glutathione Sepharose columns (see Supplemental Material for details). A recombinant GST-FLG subunit was produced as described previously (23).

Expression of recombinant C-terminal 6His-tagged proteins

FLG and HRNR cDNAs were amplified by PCR. The resulting PCR products were cloned into TA vector (Invitrogen) and subcloned into pET41b vector (Merck, Darmstadt, Germany), allowing inducible expression of C-terminal 6His-tagged proteins in the *E. coli* strain BL21-Condon Plus (DE3+)-RiL (Stratagene, La Jolla, CA). Recombinant proteins were affinity purified from bacterial lysates on Ni-NTA spin columns (see Supplemental Material for details).

TGase cross-linking *in vitro*

Production of recombinant human TGase 1 has been previously reported (24), and recombinant human TGase 3 is commercially available (R&D Systems, Abingdon, UK). Before cross-linking assays, TGase 3 was activated by proteolysis with thermolysin (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's recommendations. Thermolysin activity was then inhibited by the addition of α2-macroblogulin (2.5 μg/2 μg thermolysin; Biomac, Leipzig, Germany) and 1 mM DTT, and incubation for 30 min at 37°C (25). Recombinant human involucrin (His-IVL) used as a positive control in the cross-linking assay was produced essentially as described above for HRNR-His (see Supplemental Material for details). Recombinant HRNR-His and His-IVL were incubated for 1 to 4 h at 37°C with 10 ng/μl of TGase in 100 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.5 mM monodansylcadaverine (Sigma-Aldrich), and either 5 mM $CaCl_2$ or 25 mM EDTA. Reactions were stopped by the addition of EDTA to 25 mM. Samples were then separated by SDS-PAGE, and incorporation of monodansylcadaverine was revealed by observation under UV illumination (254 nm).

Primary normal human keratinocyte cultures and staining

Primary human keratinocytes were isolated from foreskin and cultured in keratinocyte serum-free medium (K-SFM; Life Technologies; Invitrogen), a low-calcium medium (0.09 mM $CaCl_2$). The calcium concentration was raised to 1.2 mM to induce cell differentiation. For confocal analysis, keratinocytes were fixed in 4% paraformaldehyde for 20 min at room temperature. After fixation, cells were washed with PBS and permeabilized with 0.2% Triton X-100 in PBS solution for 10 min. After washing with 0.05% Tween 20 in PBS (PBST) and incubation in 5% FBS (Life Technologies; Invitrogen) in PBS for 30 min, cells were incubated with primary antibodies as described above and with 4,6-diamidino-2-phenylindole (100 ng/ml; DAPI) in PBS for 30 min at room temperature to stain the nuclei. They were then mounted in Mowiol.

RESULTS

HRNR expression in normal human epidermis

To analyze the expression of HRNR, sera were produced in rabbits against a peptide (HP2) present 11 times in the repeated domain of the protein (**Fig. 1*A***), *i.e.*, in all of the segments A and C, as defined by Takaishi *et al.* (15). Anti-HRNR antibodies were then affinity-purified on the same peptide. Their specificity was tested by immunoblotting on recombinant forms of a FLG subunit, HRNR segments 4A–4C, and of FLG-2, another member of the S100-fused protein (26), all being produced in *E. coli* as GST-tag recombinant proteins. The anti-HRNR antibodies only recognized HRNR (Fig. 1*B*). Their reactivity was completely suppressed by preincubation with HP2 but not with two other synthetic peptides derived from the sequence of the repetitive domain of HRNR (Fig. 1*B*). Moreover, the reactivity of the AHF3 antiprofilaggrin antibody was not altered by preincubation with any of the peptides. Further confirming the specificity of the anti-HRNR antibodies, no reactivity was observed using preimmune sera (data not shown).

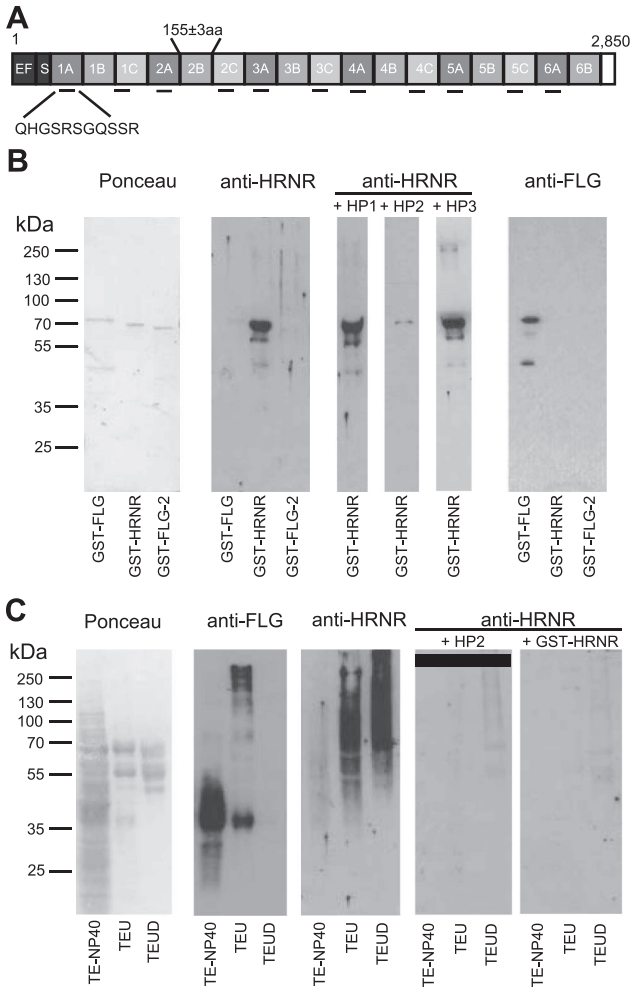

Figure 1. Immunoblotting detection of HRNR in extracts of normal human epidermis. *A*) Schematic representation of human HRNR showing the N-terminal EF-hand domain (EF), the spacer domain (S), and the repetitive segments 1A–6B. Position and sequence of the peptide HP2 used for immunization are indicated, as is the length of each repetitive segment. *B*) Purified recombinant FLG, FLG-2, and HRNR were stained with Ponceau red and analyzed by immunoblotting with the anti-HRNR and anti-FLG AHF3 antibodies. *C*) Epidermal proteins were sequentially extracted in isotonic buffer in the presence of a detergent (TE-Nonidet P-40-buffer extract), 8 M urea (TEU-buffer extract), and a reducing agent (TEUD-buffer extract). Equal amounts of proteins were separated by gel electrophoresis, stained with Ponceau red, and immunoblotted with anti-HRNR and AHF3 antibodies. When indicated, anti-HRNR antibodies were preincubated with either HP2, unrelated peptides HP1 and HP3, or the recombinant GST-HRNR. Molecular mass standards (kDa) are indicated at left.

To characterize the expression and solubility properties of HRNR, normal human epidermis was sequentially homogenized in equal volumes of a Tris/HCl buffer containing EDTA and a detergent (TE-Nonidet P-40 buffer), 8 M urea (TEU buffer), and finally 8 M urea and DTT (TEUD buffer). Extracted proteins were then separated by SDS-PAGE and immunoblotted with the anti-HRNR antibodies and with AHF3 for comparison (Fig. 1*C*). In TEU and TEUD buffer extracts, the anti-HRNR antibodies detected a high-molecular-mass band, corresponding to the expected full size of HRNR, and a number of discrete smaller bands (down to ≈45 kDa). No signals were detected in the TE-Nonidet P-40 buffer extract. The anti-HRNR antibody reactivity was completely lost after preincubation with either the peptide used for immunization or the recombinant HRNR (Fig. 1*C*). This pattern of reactivity is similar to that of AHF3: profilaggrin, intermediates in the processing of profilaggrin to FLG, and basic FLG subunits were extracted in the TEU buffer, whereas deiminated FLG subunits were extracted with the TE-Nonidet P-40 buffer, as previously reported (23).

HRNR localization in normal human epidermis

To localize HRNR in the epidermis, immunohistochemical analysis of healthy skin specimens from 5 Caucasian subjects was performed (**Fig. 2**). Immunostaining of human cryosections with the anti-HRNR antibodies showed the presence of the protein in the upper granular layer and in the entire cornified layer (Fig. 2*A, B*). The labeling was completely suppressed by preincubation with either the peptide HP2 or the recombinant HRNR, but not with the control peptides described above (Supplemental Fig. S1). Because of the similar location of HRNR and profilaggrin, paraffin-embedded skin sections were doubly stained with the anti-HRNR and AHF3 antibodies, and analyzed by confocal microscopy (Fig. 2*C*). In the granular layer, HRNR was shown to be expressed later than profilaggrin, since it was not detected in the lowest granular keratinocytes, in which profilaggrin was already evidenced. In the upper granular cells, HRNR and pro-

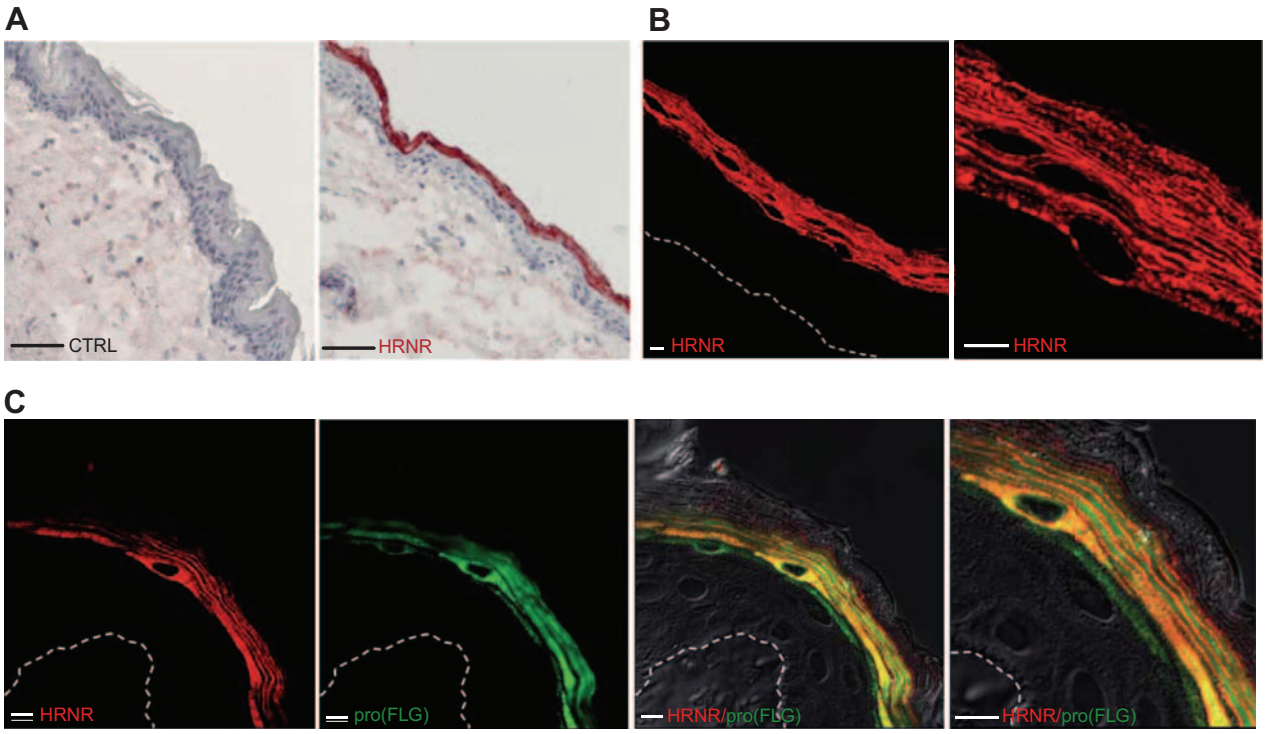

Figure 2. Immunohistochemical detection of HRNR in normal human skin. *A*) Cryosections of human skin were stained with either the anti-HRNR antibodies or a preimmune serum (CTRL) by immunohistochemistry. *B*, *C*) Sections of paraffin-embedded human skin were either labeled with anti-HRNR alone (*B*) or doubly stained with anti-HRNR and anti-profilaggrin [pro(FLG)] AHF3 antibodies (*C*). Scale bars = 50 μm (*A*); 5 μm (*B*, *C*).

filaggrin labelings were granular and largely colocalized, showing that HRNR is present in keratohyalin granules. In the cornified layer, FLG was mainly detected in the intracellular fibrous matrix of the lower corneocytes. In contrast, HRNR was predominantly detected at the corneocyte periphery in the entire cornified layer.

To confirm the presence of HRNR in the superficial stratum corneum, we performed single tape-stripping experiments on healthy human epidermis. Total protein extracts of the samples obtained were analyzed by immunoblotting with the anti-HRNR and AHF3 antibodies. HRNR- but not FLG-derived polypeptides were detected (Supplemental Fig. S2).

To describe more precisely the cellular localization of HRNR, an immunoelectron microscopy analysis was performed with the anti-HRNR and AHF3 antibodies. A monoclonal antibody directed against corneodesmosin, a component of desmosomes and corneodesmosomes, was used as a control (**Fig. 3**). In the upper granular cells, the anti-HRNR antibodies stained the periphery of the keratohyalin granules, highlighted by AHF3 labeling. In the stratum corneum, they labeled the corneocyte periphery along the CE, while AHF3 stained the intracorneocyte matrix. FLG labeling tended to disappear from the fourth stratum corneum layer. The specificity of labeling was underlined by the strict distribution of corneodesmosin within the intercellular junctions.

HRNR is a component of CEs

To confirm that HRNR is a component of CEs, these structures were highly purified from human plantar stratum corneum and incubated for increasing periods of time with protease V8. The fragments produced were analyzed by immunoblotting with the anti-HRNR antibodies (**Fig. 4*A***). The antibodies strongly stained multiple bands, indicating size heterogeneity of the fragments, whereas no reactivity was observed in the absence of envelope proteolysis. The smearing pattern of immunoreactivity was typical of either cyanogen bromide or protease digests of CEs.

Postembedding immunoelectron microscopy confirmed the presence of HRNR in the purified CEs (Fig. 4*B*). The anti-HRNR antibodies strongly labeled the structures on their fibrillar internal surface.

Together, these results demonstrate that HRNR is a component of CEs.

HRNR is a substrate of TGase 3 *in vitro*

CE components, including IVL, are assembled through Nε-(γ-glutamyl)lysine bonds formed by TGases. Three different isoforms are involved in the process (11), the best characterized being TGases 1 and 3. We attempted to identify the TGase responsible for HRNR cross-linking to the envelope. To this end, the recombinant form of HRNR was produced with a His-tag instead of a GST-tag to avoid GST interference. The purified HRNR

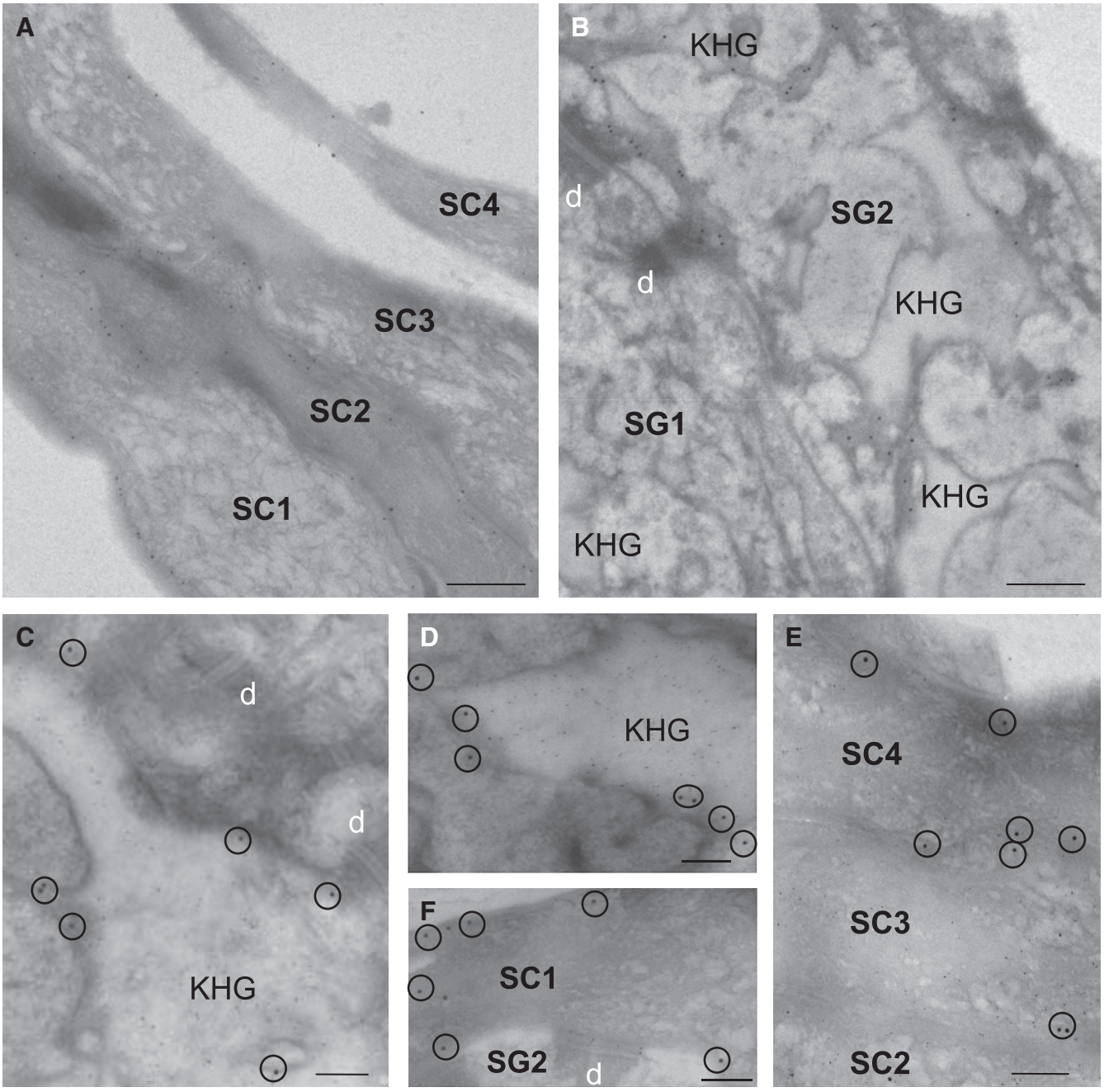

Figure 3. Immunoelectron microscopy localization of HRNR in normal human skin. *A*, *B*) Immunolocalization of HRNR in the stratum corneum (*A*) and the stratum granulosum (*B*). *C–F*) Double labeling with the anti-HRNR antibodies (15-nm gold) and either AHF3 (*C–E*) or anti-corneodesmosin (*F*) antibodies (5-nm gold). *C*, *D*) Details of keratohyalin granules (KHG). *E*, *F*) Localization of HRNR in the stratum corneum. Gold particles corresponding to HRNR are circled. d, desmosome; SG1 and SG2, stratum granulosum layers; SC1–SC4, stratum corneum layers. Scale bars = 500 nm (*A*, *B*); 200 nm (*C–F*).

was incubated for 0 to 4 h at 37°C with either TGase 1 or TGase 3, in the presence of 5 mM $CaCl_2$ and 0.5 mM monodansylcadaverine, as an amine acceptor. Samples were then separated by gel electrophoresis, and gels were observed under UV light (**Fig. 5**). His-IVL was shown to be a substrate of both enzymes. Incubation of HRNR-His with TGase 3 induced a progressive incorporation of monodansylcadaverine, as seen by the increasing intensity of the UV-detected band. No signals were observed in the presence of 25 mM EDTA, showing the specificity of the reaction. Incubations with TGase 1 had no effect.

To further verify that HRNR is cross-linked to the CE by TGase, we performed *in situ* cross-linking assays. Human skin cryosections were incubated for 3 h with the recombinant HRNR in the presence of $CaCl_2$. The recombinant protein His-IVL was used as a positive control. Both recombinant proteins were shown to be incorporated at the level of the upper epidermis where TGases are known to be active.

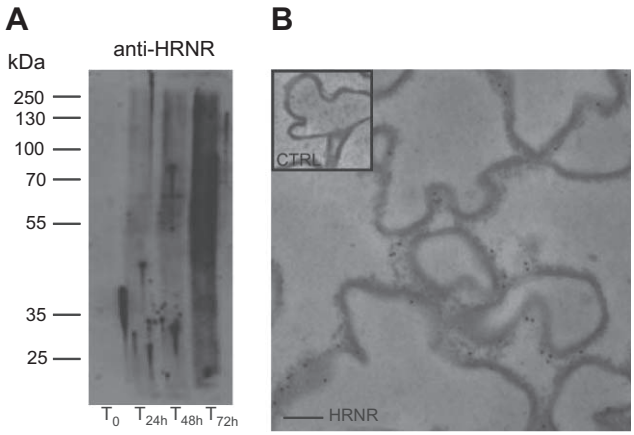

Figure 4. Immunoblotting and immunoelectron microscopy detection of HRNR in CEs. CEs, highly purified from plantar stratum corneum, were analyzed by immunoblotting with the anti-HRNR antibodies following digestion with V8 protease for 0–72 h (*A*), and low-temperature-embedded in Lowicryl K4M (*B*) and analyzed by immunoelectron microscopy with anti-HRNR and control (CTRL) antibodies. Scale bar = 0.25 μm.

Substitution of calcium ions by EDTA prevented the epidermal TGase activity and, consequently, the incorporation of HRNR recombinant form (data not shown).

HRNR is colocalized with IVL in calcium-differentiated human primary keratinocytes

We examined the expression and localization of HRNR in primary cultures of normal human epidermal keratinocytes. The cells were induced to differentiate by increasing the calcium concentration in the culture medium from 0.09 to 1.2 mM. Expression of endogenous HRNR and IVL was tested by immunostaining and confocal microscopy (**Fig. 6**). In low-calcium medium, IVL and HRNR were expressed in few keratinocytes. We also observed that all cells expressing HRNR also expressed IVL but that not all IVL-expressing cells

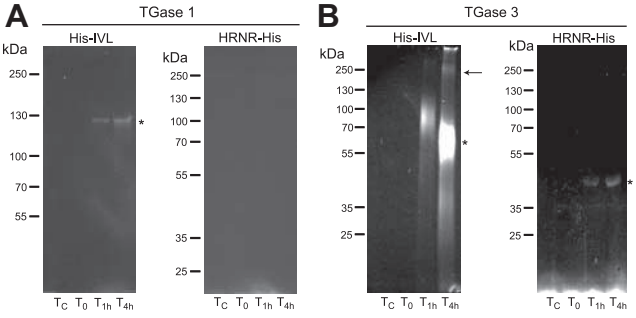

Figure 5. Capacity of HRNR to be a substrate of TGases *in vitro*. Monodansylcadaverine and either the recombinant HRNR-His or the recombinant His-IVL were incubated with TGase 1 (*A*) and TGase 3 (*B*) for 0, 1, and 4 h (T_0, T_{1h}, and T_{4h}, respectively) in the presence of $CaCl_2$ and for 4 h in the presence of EDTA (T_c). Samples were separated by gel electrophoresis, and incorporation of monodansylcadaverine was revealed by UV illumination. Asterisk indicates intrachain cross linking; arrow indicates interchain cross linking.

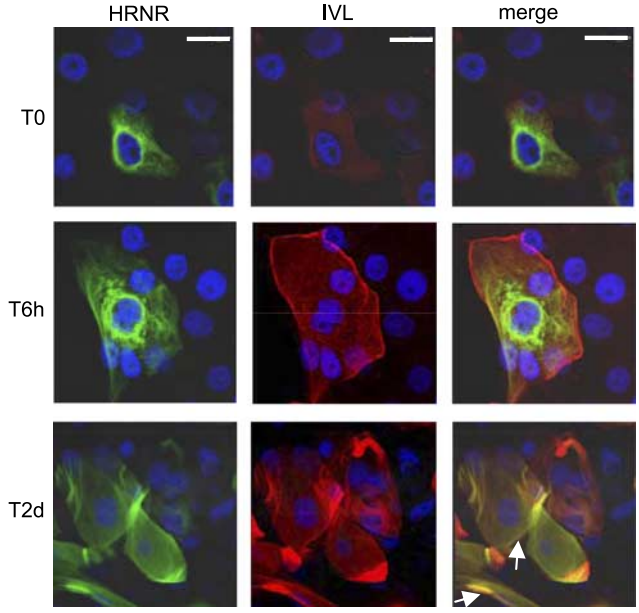

Figure 6. HRNR expression in cultured keratinocytes. Primary normal human epidermal keratinocytes were induced to differentiate by increasing the calcium concentration in the culture medium from 0.09 to 1.2 mM. Expression of HRNR and IVL was tested by confocal microscopy at time 0 (T_0) and after 6 h (T_{6h}) and 2 d (T_{2d}). Arrows indicate colocalization of HRNR and IVL at the periphery of keratinocytes. Scale bars = 20 μm.

expressed HRNR. HRNR was predominantly localized in the cytoplasm and strongly detected at the periphery of the nucleus. When keratinocytes were induced to differentiate in the high-calcium medium, they became flattened. After 6 h of culture in high-calcium medium, IVL was shown to accumulate at the cell periphery, whereas HRNR was mainly detected at the periphery of the nucleus. After 2 d, HRNR and IVL were completely colocalized at the keratinocyte periphery, suggesting that HRNR is incorporated in CEs.

HRNR expression is reduced in patients with AD

Expression level and localization of HRNR were analyzed by indirect immunofluorescence in the lesional and nonlesional skin of 5 patients with AD and 5 matched control subjects (**Fig. 7*A***). A reduced detection of HRNR was observed in the stratum corneum of both the lesional and nonlesional samples, as compared to the normal samples. A reduced expression of profilaggrin was also noticed, which was more pronounced in the lesional skin samples. These results were confirmed by Western blot analysis of total extracts of epidermal proteins obtained by homogenization in the presence of SDS and β-mercaptoethanol (Fig. 7*B*). However, a similar pattern of HRNR proteolysis was observed. Quantification of the signals indicated a mean reduction in HRNR levels of 32 and 52% in the extracts of nonlesional and lesional skin samples, respectively, as compared to normal skin samples.

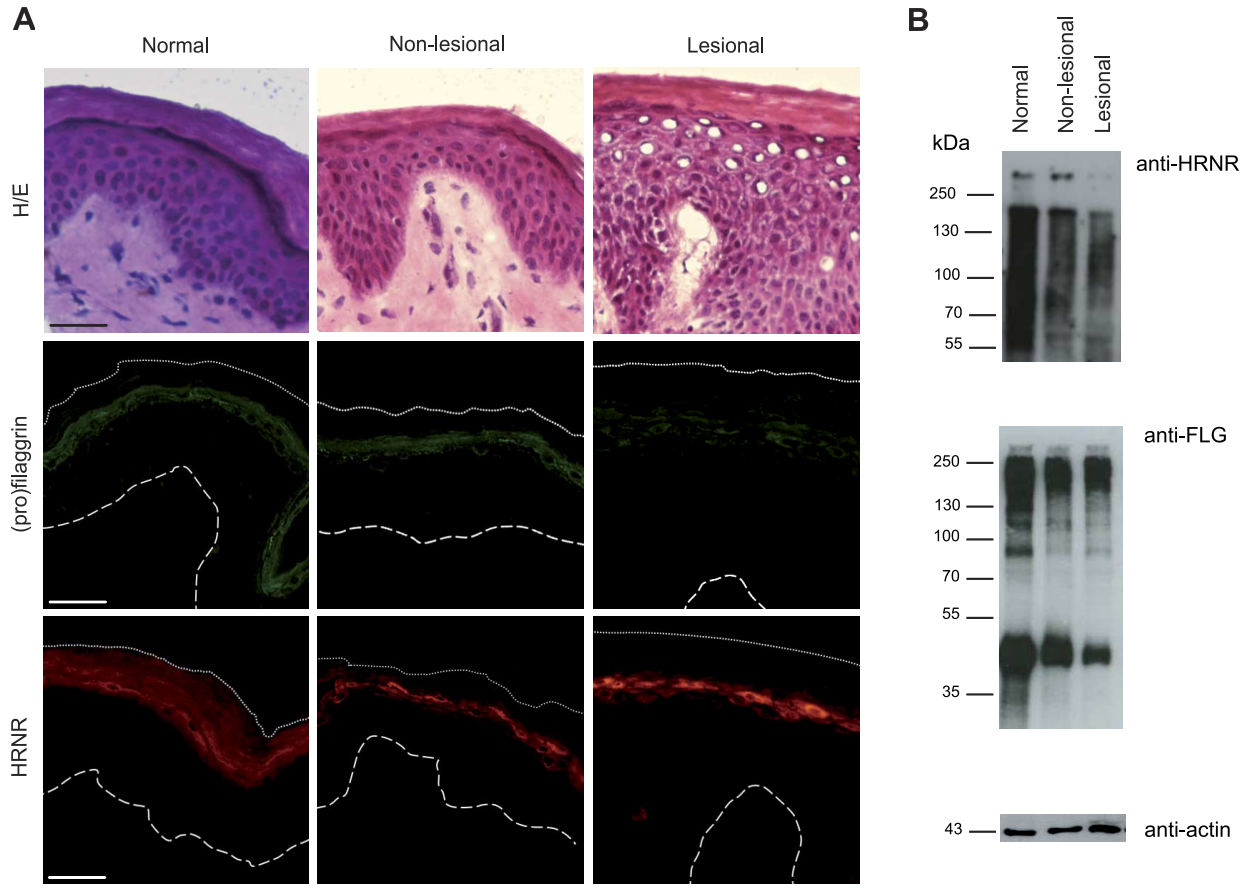

Figure 7. HRNR expression and localization in patients with AD. *A*) Cryosections of nonlesional and lesional skin of a patient with AD and of a healthy control volunteer were stained with hematoxylin/eosin (H/E) and analyzed by indirect immunofluorescence with the anti-profilaggrin AHF3 monoclonal antibody and the anti-HRNR antibodies, as indicated. *B*) Equal amounts of total epidermal proteins, extracted in the presence of SDS and β-mercaptoethanol, were separated by gel electrophoresis and immunoblotted with the anti-HRNR, anti-profilaggrin (anti-FLG) and anti-actin antibodies. Representative results of 5 patients and controls are shown. Scale bars = 20 μm.

HRNR expression on skin barrier disruption in normal skin

To examine the effect of epidermis barrier disruption on the expression level and localization of HRNR, tape stripping of normal skin was performed as described previously (27), and skin biopsies obtained 48 h after stripping were analyzed by indirect immunofluorescence with the anti-HRNR and anti-IVL antibodies. As compared to nonstripped normal skin, both proteins were detected in a higher number of cell layers, the staining extending to the middle spinous layers (Supplemental Fig. S3).

DISCUSSION

In this study, we demonstrated that HRNR is expressed in human epidermal keratinocytes and is a component of CEs by using highly specific antipeptide antibodies. We detected the protein in the granular and cornified layers of the normal epidermis and in primary keratinocytes differentiated *in vitro*. This is consistent with the reported expression of the protein in the granular keratinocytes of stratified squamous epithelia in mice (14, 16). Our observations are at variance with a previous study suggesting that HRNR is only expressed in hyperproliferative human epidermis (15). We therefore investigated whether this discrepancy could be explained by the different ethnic background between our population, composed of Caucasian donors, and that of Takaishi *et al.* (15), composed of Asian donors. We analyzed sections of formalin-fixed paraffin-embedded skin samples from healthy Japanese and Caucasian donors by indirect immunofluorescence using the anti-HRNR antibodies that we produced. The pattern of labeling was the same with all samples (Supplemental Fig. S4), indicating that HRNR is expressed in the normal epidermis, independently of ethnic origin. The difference in findings may be related to differences in experimental conditions.

We showed that HRNR is colocalized with profilaggrin in the cytoplasm of granular keratinocytes. Whereas profilaggrin is distributed uniformly within the granules (28–30), HRNR was predominantly detected at their periphery. Such distributions have al-

ready been observed in the mouse epidermis (16). This suggests that profilaggrin is synthesized at an earlier stage of keratinocyte differentiation than HRNR. This is confirmed by the finding that lower granular keratinocytes contain profilaggrin but not HRNR (Fig. 2*C*).

Taken together, the following points demonstrate that HRNR, at least the repetitive domain, is a component of CEs: HRNR is detected by confocal and immunoelectron microscopy at the periphery of corneocytes; immunoelectron microscopy analysis of purified plantar envelopes shows it to be located on the internal brushy side of these structures; plantar CEs, when treated with V8 protease, release anti-HRNR antibody-reactive polypeptides; HRNR is a substrate of TGase 3 *in vitro*; both HRNR and IVL are colocalized at the periphery of primary keratinocytes differentiated *in vitro*. This is in agreement with the immunodetection of Hrnr at the periphery of corneocytes, probably on cornified envelopes, in the mouse epidermis (16). The repetitive domain of HRNR contains a large number of Gln (>250), which may serve as TGase-reactive residues, but only 5 Lys. This suggests low intrachain cross-linking. Whether the S100 domain is also cross linked to the envelope, like many S100 proteins including S100A7, A10, and A11 (31), remains to be tested.

Since the protein extracts were prepared under conditions designed to minimize nonpecific protein degradation, our data suggest that human HRNR, like profilaggrin, is synthesized as a high-molecular-mass precursor and then proteolytically processed to fragments of roughly 45 kDa during keratinocyte differentiation. This is consistent with the fact that both HRNR and profilaggrin are formed by repetitive domains. The same has been suggested for mouse Hrnr (14). The proteases involved in this process are under investigation.

Recombinant HRNR peptides were recently reported to aggregate *in vitro* and easily form dimers and multimers (17). We never observed such a tendency with the large fragment that we produced. Therefore, only small HRNR-derived peptides could display this aggregation property.

The fused S100 protein family consists of 7 known members: FLG, FLG-2, HRNR, trichohyalin, trichohyalin-like-1, repetin, and cornulin. While they share only a limited amino acid homology outside the S100 domain, they have a number of common features. All are mainly expressed in keratinizing epithelia, including the epidermis. FLG and FLG-2 are synthesized as precursors sequestered in keratohyalin granules and processed later by proteolysis during cornification. Trichohyalin is also sequestered as an inactive form in granules of the inner root sheath of hair follicles (32). We now provide evidence that HRNR, as well, is located at the periphery of keratohyalin granules and produced as a precursor secondarily processed by proteolysis. In addition, several of the fused S100 proteins, namely FLG, trichohyalin, repetin, and cornulin, are components of CEs. We show here that it is also the case for HRNR. As such, HRNR function is probably to reinforce the envelopes. In agreement with this hypothesis, 48 h after barrier disruption using tape stripping, we found an extension of the expression of both HRNR and IVL from the granular layer to the middle spinous layers. The increase in IVL expression is in accordance with previous studies in which barrier disruption was achieved by either acetone or sodium lauryl sulfate exposure (33, 34). Antimicrobial proteins were also previously shown to be upregulated after tape stripping and SDS application (27).

A dysfunction in the epidermal barrier is now considered a key mechanism in the pathophysiology of AD. Although loss-of-function mutations in the gene encoding FLG are associated with the disease, other genes are probably involved, considering that the majority of patients with AD do not carry the mutations. CE-related genes are good candidates, since the structure and integrity of the envelopes are altered in AD skin. In agreement with this concept, the expression of genes encoding some components of CEs, *i.e.*, loricrin and proteins of the LCE and S100 families, has been shown to be down-regulated in a transcriptomic analysis of chronic AD lesions with an acute exacerbation (35, 36). It is therefore tempting to propose that a reduced expression of HRNR, as observed in the skin of 4 patients with AD (17) and demonstrated here for 5 additional unrelated patients, including the nonlesional areas, participates in the alterations in the envelopes and, in turn, contributes to the abnormality of the epidermal barrier associated with AD. The effect of HRNR down-regulation, using RNA interference technology, on the barrier but also epidermal morphogenesis and keratinocyte terminal differentiation could be tested in the near future using reconstructed skin equivalent. FJ

The authors thank Prof. Jean-Pierre Chavoin (Plastic Surgery Department, Hospital of Toulouse, Toulouse, France) and Prof. Nam-ho Huh (Okayama University Medical School, Okayama, Japan) for the human skin samples, Renan Destrade for his technical assistance, and Sophie Allard from the confocal microscopy facility [Institut National de la Santé et de la Recherche Médicale (INSERM), IFR150, Toulouse]. Electron microscopy specimens were observed at Centre de Microscopie Electronique Appliqueé à la Biologie et la Géologie (CMEABG; University of Lyon, Lyon, France). This work was supported by grants from Centre National de la Recherche Scientifique (CNRS), Toulouse III University, Pierre-Fabre Research Institute, and the French Society for Dermatological Research (SRD), and by the European COST program SKINBAD (action BM0903), Toulouse University Hospital, and INSERM. I.G.-G. was supported by the Fondation pour la Dermatite Atopique. The authors are indebted to all the patients who participated in this study.

REFERENCES

1. Taïeb, A. (1999) Hypothesis: from epidermal barrier dysfunction to atopic disorders. *Contact Dermatitis* **41,** 177–180
2. Elias, P. M., Wood, L. C., and Feingold, K. R. (1999) Epidermal pathogenesis of inflammatory dermatoses. *Am. J. Contact Dermat.* **10,** 119–126
3. Cork, M. J., Robinson, D. A., Vasilopoulos, Y., Ferguson, A., Moustafa, M., MacGowan, A., Duff, G. W., Ward, S. J., and Tazi-Ahnini, R. (2006) New perspectives on epidermal barrier

dysfunction in atopic dermatitis: gene-environment interactions. *J. Allergy Clin. Immunol.* **118,** 3–21
4. Bieber, T. (2008) Atopic dermatitis. *N. Engl. J. Med.* **358,** 1483–1494
5. Palmer, C. N., Irvine, A. D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S. P., Goudie, D. R., Sandilands, A., Campbell, L. E., Smith, F. J., O'Regan, G. M., Watson, R. M., Cecil, J. E., Bale, S. J., Compton, J. G., DiGiovanna, J. J., Fleckman, P., Lewis-Jones, S., Arseculeratne, G., Sergeant, A., Munro, C. S., El Houate, B., McElreavey, K., Halkjaer, L. B., Bisgaard, H., Mukhopadhyay, S., and McLean, W. H. (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat. Genet.* **38,** 441–446
6. Rodriguez, E., Baurecht, H., Herberich, E., Wagenpfeil, S., Brown, S. J., Cordell, H. J., Irvine, A. D., and Weidinger, S. (2009) Meta-analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease. *J. Allergy Clin. Immunol.* **123,** 1361–1370
7. Cork, M. J., Danby, S. G., Vasilopoulos, Y., Hadgraft, J., Lane, M. E., Moustafa, M., Guy, R. H., MacGowan, A. L., Tazi-Ahnini, R., and Ward, S. J. (2009) Epidermal barrier dysfunction in atopic dermatitis. *J. Invest. Dermatol.* **129,** 1892–1908
8. Rawlings, A. V., and Harding, C. R. (2004) Moisturization and skin barrier function. *Dermatol. Ther.* **17**(Suppl. 1), 43–48
9. Simon, M., Haftek, M., Sebbag, M., Montezin, M., Girbal-Neuhauser, E., Schmitt, D., and Serre, G. (1996) Evidence that filaggrin is a component of cornified cell envelopes in human plantar epidermis. *Biochem. J.* 317: 173–177
10. Richards, S., Scott, I. R., Harding, C. R., Liddell, J. E., Powell, G. M., and Curtis, C. G. (1988) Evidence for filaggrin as a component of the cell envelope of the newborn rat. *Biochem. J.* **253,** 153–160
11. Candi, E., Schmidt, R., and Melino, G. (2005) The cornified envelope: a model of cell death in the skin. *Nat. Rev. Mol. Cell. Biol.* **6,** 328–340
12. Morar, N., Cookson, W. O., Harper, J. I., and Moffatt, M. F. (2007) Filaggrin mutations in children with severe atopic dermatitis. *J. Invest. Dermatol.* **127,** 1667–1672
13. Esparza-Gordillo, J., Weidinger, S., Folster-Holst, R., Bauerfeind, A., Ruschendorf, F., Patone, G., Rohde, K., Marenholz, I., Schulz, F., Kerscher, T., Hubner, N., Wahn, U., Schreiber, S., Franke, A., Vogler, R., Heath, S., Baurecht, H., Novak, N., Rodriguez, E., Illig, T., Lee-Kirsch, M.-A., Ciechanowicz, A., Kurek, M., Piskackova, T., Macek, M., Lee, Y.-A., and Ruether, A. (2009) A common variant on chromosome 11q13 is associated with atopic dermatitis. *Nat. Genet.* **41,** 596–601
14. Makino, T., Takaishi, M., Morohashi, M., and Huh, N. H. (2001) Hornerin, a novel profilaggrin-like protein and differentiation-specific marker isolated from mouse skin. *J. Biol. Chem.* **276,** 47445–47452
15. Takaishi, M., Makino, T., Morohashi, M., and Huh, N. H. (2005) Identification of human hornerin and its expression in regenerating and psoriatic skin. *J. Biol. Chem.* **280,** 4696–4703
16. Makino, T., Takaishi, M., Toyoda, M., Morohashi, M., and Huh, N. H. (2003) Expression of hornerin in stratified squamous epithelium in the mouse: a comparative analysis with profilaggrin. *J. Histochem. Cytochem.* **51,** 485–492
17. Wu, Z., Meyer-Hoffert, U., Reithmayer, K., Paus, R., Hansmann, B., He, Y., Bartels, J., Glaser, R., Harder, J., and Schroder, J. M. (2009) Highly complex peptide aggregates of the S100 fused-type protein hornerin are present in human skin. *J. Invest. Dermatol.* **129,** 1446–1458
18. Nachat, R., Mechin, M. C., Takahara, H., Chavanas, S., Charveron, M., Serre, G., and Simon, M. (2005) Peptidylarginine deiminase isoforms 1–3 are expressed in the epidermis and involved in the deimination of K1 and filaggrin. *J. Invest. Dermatol.* **124,** 384–393
19. Simon, M., Sebbag, M., Haftek, M., Vincent, C., Girbal-Neuhauser, E., Rakotoarivony, J., Somme, G., Schmitt, D., and Serre, G. (1995) Monoclonal antibodies to human epidermal filaggrin, some not recognizing profilaggrin. *J. Invest. Dermatol.* **105,** 432–437
20. Serre, G., Mils, V., Haftek, M., Vincent, C., Croute, F., Reano, A., Ouhayoun, J. P., Bettinger, S., and Soleilhavoup, J. P. (1991) Identification of late differentiation antigens of human cornified epithelia, expressed in re-organized desmosomes and bound to cross-linked envelope. *J. Invest. Dermatol.* **97,** 1061–1072
21. Simon, M., Montezin, M., Guerrin, M., Durieux, J. J., and Serre, G. (1997) Characterization and purification of human corneodesmosin, an epidermal basic glycoprotein associated with corneocyte-specific modified desmosomes. *J. Biol. Chem.* **272,** 31770–31776
22. Guerrin, M., Ishigami, A., Mechin, M. C., Nachat, R., Valmary, S., Sebbag, M., Simon, M., Senshu, T., and Serre, G. (2003) cDNA cloning, gene organization and expression analysis of human peptidylarginine deiminase type I. *Biochem. J.* **370,** 167–174
23. Girbal-Neuhauser, E., Durieux, J. J., Arnaud, M., Dalbon, P., Sebbag, M., Vincent, C., Simon, M., Senshu, T., Masson-Bessiere, C., Jolivet-Reynaud, C., Jolivet, M., and Serre, G. (1999) The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro)filaggrin by deimination of arginine residues. *J. Immunol.* **162,** 585–594
24. Hitomi, K., Yamagiwa, Y., Ikura, K., Yamanishi, K., and Maki, M. (2000) Characterization of human recombinant transglutaminase 1 purified from baculovirus-infected insect cells. *Biosci. Biotechnol. Biochem.* **64,** 2128–2137
25. Galliano, M. F., Toulza, E., Gallinaro, H., Jonca, N., Ishida-Yamamoto, A., Serre, G., and Guerrin, M. (2006) A novel protease inhibitor of the alpha2-macroglobulin family expressed in the human epidermis. *J. Biol. Chem.* **281,** 5780–5789
26. Wu, Z., Hansmann, B., Meyer-Hoffert, U., Glaser, R., and Schroder, J. M. (2009) Molecular identification and expression analysis of filaggrin-2, a member of the S100 fused-type protein family. *PLoS One* **4,** e5227
27. De Koning, H. D., Kamsteeg, M., Rodijk-Olthuis, D., van Vlijmen-Willems, I. M. J. J., van Erp, P. E. J., Schalkwijk, J., and Zeeuwen, P. L. J. M. (2010) Epidermal expression of host response genes upon skin barrier disruption in normal skin and uninvolved skin of psoriasis and atopic dermatitis patients. *J. Invest. Dermatol.* **131,** 263–266
28. Dale, B. A., and Ling, S. Y. (1979) Immunologic cross-reaction of stratum corneum basic protein and a keratohyalin granule protein. *J. Invest. Dermatol.* **72,** 257–261
29. Lynley, A. M., and Dale, B. A. (1983) The characterization of human epidermal filaggrin. A histidine-rich, keratin filament-aggregating protein. *Biochim. Biophys. Acta* **744,** 28–35
30. Smith, S. A., and Dale, B. A. (1986) Immunologic localization of filaggrin in human oral epithelia and correlation with keratinization. *J. Invest. Dermatol.* **86,** 168–172
31. Ruse, M., Lambert, A., Robinson, N., Ryan, D., Shon, K. J., and Eckert, R. L. (2001) S100A7, S100A10, and S100A11 are transglutaminase substrates. *Biochemistry* **40,** 3167–3173
32. Presland, R. B., Rothnagel, J. A., and Lawrence, O. T. (2006) Profilaggrin and the fused S100 family of calcium-binding proteins. In *Skin Barrier* (Elias, P. M., and Feingold, K. R., eds) pp. 111–140, Taylor & Francis, New York
33. Ekanayake-Mudiyanselange, S., Aschauer, H., Schmook, F. P., Jensen J.-M., Meingassner, J. G., and Proksch, E. (1998) Expression of epidermal keratins and the cornified envelope protein involucrin is influenced by permeability barrier disruption. *J. Invest. Dermatol.* **111,** 517–523
34. Törmä H., Lindberg, M., and Berne, B. (2008) Skin barrier disruption by sodium lauryl sulfate-exposure alters the expression of involucrin, transglutaminase 1, profilaggrin, and kallikreins during during the repair phase in human skin in vivo. *J. Invest. Dermatol.* **128,** 1212–1219
35. Sugiura, H., Ebise, H., Tazawa, T., Tanaka, K., Sugiura, Y., Uehara, M., Kikuchi, K., and Kimura, T. (2005) Large-scale DNA microarray analysis of atopic skin lesions shows overexpression of an epidermal differentiation gene cluster in the alternative pathway and lack of protective gene expression in the cornified envelope. *Br. J. Dermatol.* **152,** 146–149
36. Guttman-Yassky, E., Suarez-Farinas, M., Chiricozzi, A., Nograles, K. E., Shemer, A., Fuentes-Duculan, J., Cardinale, I., Lin, P., Bergman, R., Bowcock, A. M., and Krueger, J. G. (2009) Broad defects in epidermal cornification in atopic dermatitis identified through genomic analysis. *J. Allergy. Clin. Immunol.* **124,** 1235–1244

Received for publication July 28, 2010.
Accepted for publication January 13, 2011.