Kallikrein-related Peptidase 5 Functions in Proteolytic Processing of Profilaggrin in Cultured Human Keratinocytes*

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Background: Filaggrin is a skin barrier function-related factor processed from profilaggrin. The identity of human profilaggrin-processing enzymes remains unclear.

Results: The protease kallikrein 5 (KLK5) specifically processed a recombinant human filaggrin fragment fused to a linker.

Conclusion: KLK5 is potentially a key molecule in human profilaggrin maturation.

Significance: KLK5 may function in formation of the skin barrier.

Filaggrin protein is synthesized in the stratum granulosum of the skin and contributes to the formation of the human skin barrier. Profilaggrin is cleaved by proteolytic enzymes and converted to functional filaggrin, but its processing mechanism remains not fully elucidated. Kallikrein-related peptidase 5 (KLK5) is a major serine protease found in the skin, which is secreted from lamellar granules following its expression in the stratum granulosum and activated in the extracellular space of the stratum corneum. Here, we searched for profilaggrin-processing protease(s) by partial purification of epidermal extracts and found KLK5 as a possible candidate. We used high performance liquid chromatography coupled with electrospray tandem mass spectrometry to show that KLK5 cleaves profilaggrin. Furthermore, based on a proximity ligation assay, immunohistochemistry, and immunoelectron microscopy analysis, we reveal that KLK5 and profilaggrin co-localize in the stratum granulosum in human epidermis. KLK5 knockdown in normal cultured human epidermal keratinocytes resulted in higher levels of profilaggrin, indicating that KLK5 potentially functions in profilaggrin cleavage.

In mammals, the skin barrier protects the body from physiological and biological assaults. Epidermis, the major superficial component of the skin, is characterized by terminally differentiated epidermal keratinocytes and is vital for the formation of the skin barrier. It is composed of four layers, namely (from the inside to outside) the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. Impaired skin barrier formation results in disorders such as dryness, infection, and increased hypersensitivity, as seen in atopic dermatitis.

Filaggrin (filament-aggregating protein; NCBI number NM 002016), a precursor of natural moisturizing factor (1, 2), mediates skin barrier formation and is required for aggregation of keratin intermediate filaments during epidermal terminal differentiation (3–7). Its precursor, profilaggrin, is a large (>400 kDa), highly phosphorylated protein that is relatively insoluble in water and undergoes proteolysis to form multiple 37-kDa filaggrin monomers (8–10). Human profilaggrin consists of four components: an N-terminal domain exhibiting an S100-like calcium binding domain, 10–12 repeats of the filaggrin monomer domain and linker segment, and a C-terminal domain. Profilaggrin is both synthesized and processed in terminally differentiating granular cells, and it is a component of keratohyalin granules (11, 12). To date, the specific enzyme(s) for processing of profilaggrin in human skin remains unclear.

The linker domain of profilaggrin, which consists of eight amino acid residues, conjugates filaggrin monomer domains. The linker amino acid sequence is highly conserved in mice and humans, strongly suggesting that a highly similar enzyme processes profilaggrin biochemically to release monomeric filaggrins. Skin-specific retroviral-like aspartic protease (SASPase)2 reportedly processes recombinant human filaggrin by cleaving the linker domain (13). Furthermore, targeted inactivation of SASPase in mouse skin markedly reduces profilaggrin cleavage (13). However, no specific enzymes that mediate profilaggrin processing during terminal differentiation have been found in human skin or in cultured human epidermal keratinocytes.

KLK5, a major serine protease in skin, cleaves corneodesmosomal components (14) and the PAR-2 (proteinase-activated receptor 2) with electrospray tandem mass spectrometry; NHEK, normal human epidermal keratinocyte; MCA, 4-methylcoumaryl-7-amide; rhKLK5, recombinant human KLK5.

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receptor-2) ligand (15). Thus far, it has been assumed that KLK5 is expressed in lamellar granules and secreted from cytoplasmic micro-organs in the stratum granulosum. KLK5 has been suggested to act in the acidic environment of the stratum corneum extracellular space (14, 16, 17). However, because the optimal pH for KLK5 activity is 7.4 (18), it is likely that KLK5 has other physiological functions under neutral pH conditions.

In addition to SASPase, targeted inactivation in mice of either MT-SP1 (matrilase) (19), CAP1/Prss8 (protease, serine, 8)/prostatin (20), or Alox12b (arachidonate 12-lipoxygenase, 12R type) (21) attenuates biochemical cleavage of profilaggrin. In each case, the epidermis cannot produce functional monomeric filaggrins, leading to hyperkeratotic skin conditions. However, it remains unclear whether these enzymes contribute to the filaggrin processing in human skin. Meanwhile, our recent study demonstrated that caspase-14 and bleomycin hydrolase degrade recombinant human filaggrin monomers into free amino acids, which act as natural moisturizing factors (22). Thus, a specific enzyme that triggers profilaggrin processing remains to be elucidated in human keratinocytes.

In this study, we identified KLK5 as a processing enzyme of profilaggrin, based on non-biased assays using HPLC separation of epidermal extracts, and subsequently, LC/MS/MS analyses identified the proteases involved. We show that KLK5 cleaves recombinant filaggrin at the linker domain and processes profilaggrin using cultured human keratinocytes. Moreover, KLK5 and filaggrin are localized in the cytoplasm of epidermal granular cells as well as in the extracellular space of the stratum corneum in human skin. Our results demonstrate, for the first time, that KLK5 plays a physiological role in promoting stratum corneum formation and thus provide novel insight into the mechanisms underlying skin diseases characterized by barrier dysfunction.

EXPERIMENTAL PROCEDURES

Human Tissue Specimens—Human skin specimens were obtained from patients who had undergone skin surgery at Hamamatsu University School of Medicine, University Hospital, and provided prior informed consent. The study was approved by the Ethical Committee of the Hamamatsu University School of Medicine and the Shiseido Committee on human ethics.

Preparation of Skin Samples—For chromatographic fractionation and LC/MS/MS analysis, mouse epidermis was fractionated by incubating total dorsal skin of 1–3-day-old C57BL/6j mice in 1 M NaCl solution overnight at 4 °C. The epidermis was further minced in lysis buffer (0.1 M Tris-HCl, pH 8.0, 0.14 M NaCl, 0.01% Tween 20) using a glass homogenizer. Epidermal extracts were obtained after centrifugation at 15,000 × g for 30 min and filtered through 0.2-µm filters (Millipore, Billerica, MA).

Isolation of Protease Fractions—Ion exchange chromatography was performed with a 2157 automatic sampler, a low pressure mixer, a 2248 pump, a VWM 2141 detector (GE Healthcare), and an Epson LQ-570 recorder. Extracts were applied to an ion exchange (Mono Q) column (1 ml, 5 × 50 mm) and eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM Tris-HCl (pH 8.0). The elution profile was monitored by measuring absorbance at 280 nm.

LC/MS/MS Analysis—Fractionated proteins were loaded onto a fused silica trapping column (100-µm inner diameter × 1 cm, Aqua C18, Phenomenex, Torrance, CA) using an autosampler (SI-2 semimicro-HPLC system, Shiseido Co., Ltd., Kanagawa, Japan). The trapping column was desalted with a gradient starting buffer (0.1% formic acid, 5% acetonitrile) for 30 min. The column was directly connected to a fused silica analytical capillary column (100-µm inner diameter × 12 cm, Aqua C18, Phenomenex (Torrance, CA)) by changing the position of a two-way switching valve. Peptides were separated with a 40-min organic gradient (5–75% acetonitrile). The column flow rate was set to 300–400 ml/min by adjusting the length of split-resistant capillary (50-µm inner diameter × 50–200 mm). Eluted peptides were directly electrospayed into the mass spectrometer (Ceca XP, Thermo Fisher Scientific), and MS/MS spectra were automatically acquired under the control of the Xcalibur data system (Thermo Fisher Scientific). Collected spectra were searched to identify peptides and/or proteins using the SEQUEST algorithm running on BioWorks 3.3 software (Thermo Fisher Scientific). A non-redundant human protein database (NCBI; downloaded in 2007) was used for protein identification. Stringent search criteria were used to minimize false discovery rates (Sf score >0.85, peptide probability >0.001, number of top matches >1).

Measurement of Caspase-14 and Bleomycin Hydrolase Activity in HPLC Fraction—For the caspase-14 activity assay, enzyme fractions were incubated with 1 mM WEHD-4-methylcoumaryl-7-amide (MCA) as a substrate in 0.1 M HEPES, pH 7.5, containing 0.06 M NaCl, 0.01% CHAPS, 5 mM DTT, and 1.5 mM sodium citrate (23). To measure bleomycin hydrolase activity, 0.1 M citrulline-MCA was used as a substrate in 0.1 M Tris-HCl, pH 7.5, containing 10 mM DTT and 5 mM EDTA (24). Enzymatic activity was measured using Fluoroskan Ascent FL (Thermo Electron Co., Wolsam, MA) with 355-nm excitation and 460-nm emission.

Human Filaggrin Cleavage Assay—To assess filaggrin monomer liberation by proteases in fractions, we used recombinant human filaggrin protein as a substrate. Ten microliters of each respective fraction were incubated with 250 ng of recombinant protein in 60 µl of a serine protease buffer (non-reducing conditions) containing 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl or a cysteine protease buffer (reducing conditions) containing 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 5 mM DTT, and 5 mM EDTA for 60 min at 37 °C. After incubation, reactions were stopped by adding 20 µl of 4× SDS-PAGE sample buffer, and 20-µl samples were applied to SDS-PAGE.

Western Blot Analysis—After electroblotting, PVDF membranes (Bio-Rad) were stained with rabbit polyclonal antibodies to human filaggrin C terminus (1:3000, raised by Shiseido, Kanagawa, Japan) or a monoclonal antibody to the His tag (1:1000; Cell Signaling Technology, Inc., Boston, MA). The signal was detected using an ECL Plus Western blot detection system (GE Healthcare).

Preparation of an Antibody Targeting the Human Profilaggrin C-terminal Region—An anti-human profilaggrin IgG antibody directed to the C-terminal domain (CTD) was raised by immunizing rabbits with the synthetic peptide CKASAFGK-DHPRYYATYINKDP. This sequence is specific for human profiles.
filaggrin and shows no homology to mouse or human hornerin (25, 26) or filaggrin-2 (27). Antibodies were purified using antigen-coupled affinity chromatography.

**Construction and Expression of Recombinant Human Filaggrin Fusion Proteins**—Based on the nucleotide sequence of human filaggrin, the C-terminal region was amplified by PCR using the primers 5′-CATATGCTAGGCTCCCACTCTCCTTTATCCTC-3′ and 5′-CTCGAGCTCATGTTATGATATCTC-3′ to obtain an Ndel/XhoI insert. PCR conditions were as follows: denaturation for 2 min at 97 °C followed by 35 cycles of 30 s at 97 °C, 30 s at 51 °C, and 2 min and 30 s at 72 °C and a final extension for 5 min at 72 °C, using Ex Taq DNA polymerase (Takara, Shiga, Japan). The resulting 2187-bp PCR product was cloned into pGEM-T Easy vector (3015 bp) (Promega, Madison, WI) and sequenced with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The construct was digested with Ndel and Xhol, and a 1959-bp filaggrin DNA fragment was cloned into the pET25b vector (5547 bp) (Novagen, Darmstadt, Germany), which encodes a His6 tag. BL21-CodonPlus (DE3)-RP Escherichia coli competent cells (Stratagene, Santa Clara, CA) were transformed with the construct and cultured at 37 °C until the absorbance at 600 nm reached 0.6. After induction of fusion protein expression with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 37 °C, tagged human filaggrin fusion proteins were purified using QIAexpress® nickel-nitrioltriacetic acid Fast Start (Qiagen, Valencia, CA), according to the manufacturer’s protocol.

**Construction of a Mutant Form of the Linker Domain**—The C-terminal region was amplified via PCR using the following primers: the gene-specific sense primer 5′-CATATGCTAGGCTCCCACTCTCCTTTATCCTC-3′, the linker-specific mutant antisense primer 5′-GAGGATGCTCGCAAGATC-3′, and 5′-CTCGAGCTCATGTTATGATATCTC-3′. PCR conditions were as follows: initial denaturation for 2 min at 96 °C and then 35 cycles of 30 s at 96 °C, 30 s at 48 °C, and 2 min and 30 s at 72 °C, followed by extension for 5 min at 72 °C. The 2187-bp PCR product was cloned into pGEM-T Easy vector and sequenced. The construct was further digested with Ndel and Xhol and cloned into the pET25b vector (5547 bp) (Novagen, Darmstadt, Germany), which encodes a His6 tag. BL21-CodonPlus (DE3)-RP Escherichia coli competent cells (Stratagene, Santa Clara, CA) were transformed with the construct and cultured at 37 °C until the absorbance at 600 nm reached 0.6. After induction of fusion protein expression with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 37 °C, tagged human filaggrin fusion proteins were purified using QIAexpress® nickel-nitrioltriacetic acid Fast Start (Qiagen, Valencia, CA), according to the manufacturer’s protocol.

**Biochemical Analysis of Filaggrin Cleavage**—Recombinant human KLK5 (R&D Systems, Minneapolis, MN) was incubated with 2 μM filaggrin fusion proteins in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. Recombinant human KLK7 (R&D Systems) was incubated with 2 μM filaggrin fusion proteins in 50 mM Tris-HCl, pH 8.5, containing 0.15 M NaCl. Reactions were stopped by adding 20 μl of 4 × SDS sample buffer. Twenty microliters of each sample were subjected to SDS-PAGE on Mini-PROTEAN TGX precast gels (Bio-Rad).

**KLK5 Knockdown in Cultured NHEKs**—For lentiviral transduction of KLK5, 1 × 10⁵ NHEK cells were seeded into a 6-well plate. After 24 h, cells were transduced with two KLK5 shRNA-expressing vectors carrying the puromycin resistance gene (TRCN0000073997 and TRCN0000372710; Sigma-Aldrich) or a scrambled (Sigma-Aldrich) shRNA control vector also carrying the puromycin resistance gene at a multiplicity of infection of 3. After 24 h, stable transduction was confirmed by adding puromycin to the medium to select shRNA-expressing cells. Six days later, when cells were 95–100% confluent, 1.2 mM calcium chloride was added to the media to induce NHEK differentiation, and cells were subjected to air exposure under sterile conditions for 10 min to enhance cornification. 5–7 days later, cells were harvested, and proteins were extracted. KLK5 expression levels (by H-55 antibody, 1:1000; sc-20623, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and profilaggrin (by AKH1 antibody, 1:1000; sc-66192; Santa Cruz Biotechnology, Inc.) processing were assessed by Western blot analysis. Keratin 1 (AF87, 1:1000; PRB-149P, Covance (Berkeley, CA)) and keratin 10 (DE-K10, 1:1000; sc-52318, Santa Cruz Biotechnology, Inc.) expression levels were evaluated as differentiation markers. As an internal control, β-actin (13E5, 1:3000; Cell Signaling Technology (Danvers, MA)) expression was assessed. These experiments were approved by the Hamamatsu University recombinant DNA advisory committee.

**Immunohistochemical Staining for hKLK5 and Profilaggrin**—Skin samples were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned, and deparaffinized sections were stained with hematoxylin and eosin. Deparaffinized sections were also incubated with polyclonal rabbit anti-human KLK5 antibodies (1:100; Santa Cruz Biotechnology, Inc.) or anti-profilaggrin CTD antibodies (1:1000) for 1 h at room temperature and further incubated with horseradish peroxidase-conjugated, anti-rabbit IgG antibody (Dako, Glostrup, Denmark). Immunohistochemical localization of KLK5 and profilaggrin was assessed under a microscope equipped with Plan APOCHROMAT (Olympus). For double immunofluorescence analysis, Alexa Fluor 555 and 488 (Molecular Probes, Inc., Eugene, OR) were used as secondary antibodies. DAPI (Molecular Probes, Inc.) was used to visualize nuclei.

**Proximity Ligation Assay**—A proximity ligation assay was performed with the DuoLink system (Olink Bioscience, Uppsala, Sweden) using the monoclonal anti-KLK5 antibody and the polyclonal anti-profilaggrin CTD antibody on deparaffinized sections, according to the manufacturer’s instructions and previous reports (28, 29). Antibody dilutions were 0.5 and 1 μg/ml, respectively. DAPI was used to visualize nuclei.

**Double Immunoelectron Microscopic Analysis**—Normal human skin was fixed in 4% parafomaldehyde, 0.5% glutaraldehyde, 1 m cacodylate buffer and embedded in LR white resin. Sections of selected areas were cut and mounted on nickel grids. Ultrathin sections were incubated for 1 h in 3% bovine...
serum albumin (BSA) in PBS to block nonspecific binding. Mouse anti-human filaggrin antibody (AKH1, 1:50; sc-66192, Santa Cruz Biotechnology, Inc.) and rabbit anti-human KLK5 serum (H-55, 1:50; sc-20623, Santa Cruz Biotechnology, Inc.) served as primary antibodies during a 1-h incubation. As secondary antibodies, 40-nm gold particle-conjugated goat anti-mouse IgG antibody (1:40; EY Laboratories, San Mateo, CA) and 10-nm gold particle-conjugated goat anti-rabbit antibody
were utilized. Finally, samples were stained with 2% uranyl acetate.

RESULTS
Identification of KLK5 as a Profilaggrin-processing Enzyme—We initially hypothesized that human profilaggrin might be cleaved at specific sites, such as the linker domain, during step-wise biochemical processing (Fig. 1A). To identify candidate enzymes that function in processing of profilaggrin to filaggrin monomers, we fractionated epidermal extracts from newborn C57BL/6j mice using ion exchange chromatography (Fig. 1B). A total of 40 isolated fractions were incubated with 53-kDa human recombinant filaggrin encoding a single filaggrin monomer fused to the C-terminal region (Fig. 1C, top). Because we had previously shown that filaggrin monomer contains a site targeted by caspase-14-dependent processing, we subjected...

FIGURE 2. rhKLK5 is a profilaggrin-processing enzyme. A, schematic representation of filaggrin fusion protein with a His6 tag. See “Experimental Procedures” for vector construction. Wild-type linker domain residues are shown above, and mutant residues are displayed below. Substitutions are underlined. B, Western analysis of filaggrin fusion proteins expressed in E. coli. Comparable expression levels of the wild-type (lane 1, arrowhead) and the mutant fusion protein (lane 2) are detected by anti-His6 tag and anti-filaggrin CTD antibodies. Filaggrin fusion proteins are seen as 77-kDa polypeptide. C and D, Western blot analysis by anti-filaggrin CTD antibody indicates that the wild-type filaggrin fusion protein is subjected to biochemical processing following incubation with rhKLK5 in a time-dependent manner (C, lanes 3–7), whereas processing is not seen in the presence of rKLK7 (D, lanes 3–7). Arrows indicate the filaggrin fusion protein (77 kDa), and asterisks indicate the cleavage product (41 kDa).

Role of Kallikrein 5 in Biochemical Processing of Profilaggrin

Identification of KLK5 as a Profilaggrin-processing Enzyme—We initially hypothesized that human profilaggrin might be cleaved at specific sites, such as the linker domain, during step-wise biochemical processing (Fig. 1A). To identify candidate enzymes that function in processing of profilaggrin to filaggrin monomers, we fractionated epidermal extracts from newborn C57BL/6j mice using ion exchange chromatography (Fig. 1B). A total of 40 isolated fractions were incubated with 53-kDa human recombinant filaggrin encoding a single filaggrin monomer fused to the C-terminal region (Fig. 1C, top). Because we had previously shown that filaggrin monomer contains a site targeted by caspase-14-dependent processing, we subjected...
epidermal extracts to a hydrolysis assay using WEHD-MCA, a peptide utilized to investigate caspase-14-like activity, for substrate. Its prominent enzymatic activity was found in fractions 27–31 (Fig. 1B, small closed circles). However, Western blot analysis using an anti-filaggrin antibody showed that a 38-kDa cleavage product (Fig. 1C, bottom, asterisk) was obtained from recombinant human 53-kDa filaggrin protein (Fig. 1C, bottom, arrowheads) with fractions 20–24 under both reducing and non-reducing conditions. Therefore, we concluded that profilaggrin-processing enzymes would be found in fractions 20–24 and that they were not likely to be caspase-type proteases.

Because deiminated/citrullinated filaggrin is degraded by bleomycin hydrolase, we also conducted a citrullinated MCA hydrolysis assay. Significant enzymatic activity was found in fractions 24–30 (Fig. 1B; dashed line, closed diamonds), which corresponded to decreased levels of 53-kDa recombinant proteins seen in Western blot analysis in reducing conditions (Fig. 1C, dashed square). These observations indicate that nonspecific proteolysis is mediated by bleomycin hydrolase in these fractions.

To identify a candidate enzyme responsible for filaggrin processing, we examined fractions ranging from 20 to 24 and from 28 to 31 and subjected each to LC/MS/MS. Analysis using

**FIGURE 3. Filaggrin linker domain is the site for processing.** A, Western blot analysis with anti-filaggrin CTD antibody shows that wild-type and linker mutant forms of filaggrin fusion protein are detected at 77 kDa (lanes 2 and 3, arrowhead). The wild-type protein shows a 41-kDa digestion product in the presence of rhKLK5, whereas the mutant form does not (lanes 4 and 5). B, Western blot analysis following functional blockade of rhKLK5 activity by a neutralizing antibody. Production of the 41-kDa polypeptide is inhibited (lane 3, asterisk), whereas treatment with isotype control IgG2b does not inhibit processing. C, Western analysis of wild-type filaggrin fusion protein incubated with rhKLK5 for 30 or 60 min in the presence or absence of AEBFS, a kallikrein inhibitor. Analysis with anti-filaggrin CTD antibody shows the expected 41-kDa product after incubation with rhKLK5 for 30 min (lane 2, asterisk) or 60 min (lane 3, asterisk), whereas cleavage is blocked in the presence of AEBFS either at 1 mM (lanes 4 and 5) or 10 mM (lanes 6 and 7). Arrowhead, filaggrin fusion protein (77 kDa); asterisk, cleavage product (41 kDa). D, cultured NHEKs were pretreated with high concentration calcium media to induce cellular differentiation after air exposure and examined at different time points. The consequences of KLK5 loss of function were examined using lentivirus-mediated shRNA knockdown. Western blot analysis of whole lysates showed that filaggrin trimers, dimers, and monomers are undetectable at differential time points when cultured NHEKs are transduced with scrambled shRNA (lanes 1–3), indicating that filaggrin is completely processed. On the other hand, KLK5 knockdown by shRNA markedly inhibited processing (lanes 4–9). KLK5 knockdown efficiency was assessed by Western blot analysis using anti-KLK5 antibody. Keratin 1 and keratin 10 expression are comparable in KLK5 knockdown and control groups, indicating that KLK5 loss has no effect on terminal differentiation. As a physiological indicator of the profilaggrin processing, human skin biopsy extracts were also analyzed (lane 1). β-Actin served as an internal control.
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Biochemical Cleavage of the Human Filaggrin C-terminal Region by Recombinant Human KLK5—To examine profilaggrin processing by KLK5, we constructed an expression vector encoding a human wild-type linker bridging a filaggrin monomer with a downstream truncated filaggrin unit C terminus (Fig. 2A, upper sequence) and a corresponding construct expressing a mutant form of the linker domain (Fig. 2A, lower sequence). Western blot analysis using antibodies recognizing the profilaggrin C terminus revealed a 41-kDa digestion product appearing in a time-dependent manner (Fig. 2C, lanes 3–7, asterisk), indicating that the fragment consisted of the truncated filaggrin region, the C-terminal sequence, and the His6 tag domain. By contrast, incubation with rhKLK7, a known chymotryptic enzyme that degrades corneodesmosomal components, failed to process the wild-type fusion protein under the same conditions (Fig. 2D). These results indicate that KLK5 specifically cleaves filaggrin fusion protein.

The Linker Domain Is Required for Filaggrin Processing by rhKLK5—To determine whether the linker domain mediates filaggrin processing by KLK5, we conducted an additional study using a construct containing point mutations in the linker sequence (Fig. 2A, lower sequence). Western blot analysis of mutant and wild-type filaggrin fusion proteins after incubation with rhKLK5 showed 41-kDa fragments in the digests of the wild-type construct (Fig. 3A, asterisk), whereas the band was absent in the digests of the linker mutant protein. Furthermore, the addition of KLK5-neutralizing antibody efficiently inhibited cleavage of the wild-type filaggrin fusion protein as compared with IgG2a control antibody (Fig. 3B, asterisk). Furthermore, AEBSF, a potent organic kallikrein inhibitor, markedly suppressed rhKLK5-induced cleavage of the wild-type filaggrin fusion protein at both 30 and 60 min with the minimum concentration of 1 mM (Fig. 3C, asterisk). Collectively, these results strongly suggest that the linker domain is required for profilaggrin processing by KLK5.

KLK5 Knockdown in Normal Human Epidermal Keratinocytes Perturbs Profilaggrin Processing—To determine whether KLK5 functions in profilaggrin processing in a cellular context, we knocked down KLK5 using lentivirus-mediated shRNA interference in cultured normal human epidermal keratinocytes (NHEKs) and subjected whole cell lysates to Western blot analysis. For lentiviral transduction, 1 × 10⁵ NHEK cells were seeded into 6-well plates. After 24 h, cells were transduced at a multiplicity of infection of 3 with two KLK5 shRNA-expressing vectors that harbored a puromycin resistance gene or a scrambled shRNA control vector also carrying a puromycin resistance gene. After 24 h, stable transduction was confirmed by adding puromycin to culture media to select shRNA-expressing cells. Six days later, when cells were 95–100% confluent, medium was changed to that containing 1.2 mM calcium chloride to induce NHEK differentiation. Cells were also briefly exposed to air (see “Experimental Procedures”) to enhance cornification. 5–7 days later, when profilaggrin expression is normally detectable, cells were harvested for protein extraction.

KLK5 expression levels were significantly decreased in KLK5 shRNA-transduced cells compared with scrambled shRNA controls, whereas keratin 1 and keratin 10 expression levels remained unchanged (Fig. 3D). Levels of profilaggrin, filaggrin trimers, and dimers also remained high following KLK5 knockdown in comparison with non-treated controls (Fig. 3D). These results suggest that KLK5 knockdown in NHEK perturbs profilaggrin processing.

Co-localization of Profilaggrin and KLK5 in the Human Stratum Granulosum—To examine the precise localization of profilaggrin and KLK5 in normal human skin, we undertook histological study. Representative H&E staining in human tissue showed a basophilic linear deposition located in the upper epidermis, demonstrating keratohyline granules beneath the stratum corneum (Fig. 4A). Immunohistochemical study for the profilaggrin C-terminal region confirmed that profilaggrin is localized in the granular layer (Fig. 4B). Positive immuno-
staining of KLK5 was found in the granular layer as well as in the cornified layer (Fig. 4C). Double immunofluorescence staining for profilaggrin (red) and KLK5 (green) revealed an overlapping pattern, indicating that profilaggrin and KLK5 are co-localized in the granular layer of normal human skin (Fig. 4, D–F). Moreover, a proximity ligation assay using antibodies against KLK5 and profilaggrin C terminus indicated a positive reaction at the granular layer (Fig. 4, G and H), suggesting that both proteins are co-localized within 40 nm.

To confirm the co-localization of KLK5 and profilaggrin, we subjected normal human skin to immunoelectron microscopic analysis. Double staining was performed for hKLK5 using 10-nm gold particles and for profilaggrin using 40-nm particles. KLK5 was prominently observed in the extracellular space (Fig. 5B, white arrowheads) and cytoplasm (Fig. 5B, white arrows) of the stratum corneum and in lamellar granules of the uppermost layer of stratum granulosum (Fig. 5C, white arrowheads). Cytoplasmic KLK5 was also detected in conjunction with keratohyalin granules (Fig. 5, D–F), supporting the idea that KLK5 interacts with its substrates in granular cells. No specific staining was observed in negative controls, in which skin specimens were treated with 10- and 40-nm gold particle-conjugated secondary antibodies (Fig. 5, G–I).

DISCUSSION

Our study provides evidence that KLK5 is required for biochemical processing of profilaggrin. We demonstrated, for the first time, that the profilaggrin linker domain is responsible for processing by KLK5 both in the biochemical assays and in the cultured NHEK-based analysis. First, we extracted mouse epidermal proteins and found that the extracts can cleave recombinant human filaggrin proteins that include the linker domain. KLK5 was prominently observed in the extracellular space and cytoplasm (Fig. 5B, white arrowheads) and cytoplasm (Fig. 5B, white arrows) of the stratum corneum and in lamellar granules of the uppermost layer of stratum granulosum (Fig. 5C, white arrowheads). Cytoplasmic KLK5 was also detected in conjunction with keratohyalin granules (Fig. 5, D–F), supporting the idea that KLK5 interacts with its substrates in granular cells. No specific staining was observed in negative controls, in which skin specimens were treated with 10- and 40-nm gold particle-conjugated secondary antibodies (Fig. 5, G–I).
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Several mutations have been found in human filaggrin, particularly in exons encoding monomer sequences and the C-terminal domain. Interestingly, no mutations have been reported in particular to develop diseases, such as atopic dermatitis, has been questioned (31). On the other hand, there are no reports of mutations or polymorphisms in KLK5 thus far. With regard to filaggrin, filaggrin knock-out (FLG−/−) mice results in dry, scaly skin (32). Furthermore, hapten-induced contact hyper-sensitivity is enhanced in FLG−/− compared with wild type mice, indicating that skin barrier formation is markedly impaired. These findings provide an implication that profilaggrin processing is required to maintain the physiological function of the skin barrier in mice.

Profilaggrin cleavage occurs in a stepwise process. Fig. 6 shows our model of filaggrin processing and degradation. We have previously shown that filaggrin is processed by caspase-14...
and calpain 1 and then degraded by bleomycin hydrolase to produce natural moisturizing factor in the stratum corneum (22). Our current study identifies KLK5 as the enzyme that cleaves human profilaggrin at the linker domain at an early processing step. Collectively, we have thus defined enzymes responsible for the two major phases of profilaggrin processing. In addition to the monomeric filaggrin domains within filaggrin, a nonsense mutation in the region of filaggrin that encodes the C-terminal domain was identified in atopic dermatitis patients (33). A normal level of filaggrin mRNA and remarkable reduction of filaggrin protein expression were detected in the epidermis of these patients (33). Thus, further study is required to investigate whether the C-terminal domain is also critical for profilaggrin processing and could constitute a potential therapeutically targetable region in patients with atopic dermatitis.

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Role of Kallikrein 5 in Biochemical Processing of Profilaggrin


