Kallikrein 8 Is Involved in Skin Desquamation in Cooperation with Other Kallikreins

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Mari Kishibe, Yoshiho Bando, Ryuji Terayama, Kazuhiko Namikawa, Hitoshi Takahashi, Yoshio Hashimoto, Akemi Ishida-Yamamoto, Ying-Ping Jiang, Branka Mitrovic, Daniel Perez, Hajime Iizuka, and Shigetaka Yoshida

From the Departments of 4 Structural Anatomy and Neuroscience and 5 Dermatology, Asahikawa Medical College, Midorigaoka-Higashi 2-1-1-1, Asahikawa 078-8510, Japan and the 6 Department of Immunology, Berlex Biosciences, Richmond, California 94804-0099

Kallikrein type serine proteases, KLK8/neuropsin, KLK6, and KLK7, have been implicated in the proliferation and differentiation of epidermal keratinocytes and in the pathogenesis of psoriasis. However, their mechanistic roles in these processes remain largely unknown. We applied 12-O-tetradecanoylphorbol-13-acetate on the wild type (WT) and the Klk8 gene-disrupted (Klk8-/-) mouse skin, inducing keratinocyte proliferation similar to the human psoriatic lesion. Klk8 mRNA as well as Klk6 and Klk7 mRNA were up-regulated after 12-O-tetradecanoylphorbol-13-acetate application in the WT mice. In contrast, Klk8-/- mice showed minimal increases of Klk6 and Klk7 transcripts, the proteins, and enzymatic activities. Relative to the WT, the Klk8-/- skin showed less proliferation and an increase in the number of cell layers in the stratum corneum. However, overexpression of Klk8 by adenovirus vector in knock-out keratinocytes did not result in an increase in Klk6 or Klk7 mRNA. The inefficient cleavage of adhesion molecules DSG1 and CDSN in Klk8-/- skin contributes to a delay in corneocyte shedding, resulting in the hyperkeratosis phenotype. We propose that in psoriatic lesion, KLK8 modulates hyperproliferation and prevents excessive hyperkeratosis by shedding the corneocytes.

A main function of the skin is to serve as a permeability barrier for keeping moisture in our bodies and to protect physical, chemical, and biological materials entering from the outside. The outermost corneocytes are shed from the epidermal surface as a result of proteolytic degradation of corneodesmosomes by epidermal proteases during the desquamation process. It is important to maintain a balance between proliferation of epidermal cells and shedding of outermost layers. Several stratum corneum proteases are essential for the maintenance of structural and functional barrier of the epidermis and are also involved in keratinocyte desquamation (1).

Human tissue kallikreins comprise of a subgroup of 15 serine proteases encoded by a tightly clustered multigene family on chromosome 19q13.4 (2–4). This region is also synthetic to the locus on mouse chromosome 7 where the murine kallikrein gene family cluster is localized (5), and mouse serine proteases share a high degree of sequence and structural similarity with human homologues (2, 5). In this paper, we use the terms KLK1–KLK15 for human kallikrein genes and hK1–hK15 for the enzyme products from KLK1–KLK15. In addition, we use terms Klk1–Klk15 for mouse homologues of human genes and mK1–mK15 for the products from Klk genes following the nomenclature of kallikreins (6). KLKs have been shown to play critical roles in desquamation as recently reported (7, 8). hK5 and hK7 (previously designated SCTE and SCCE, respectively) have been shown to be involved in skin desquamation through their ability to degrade desmosome and/or corneodesmosome component protein such as desmoglein 1 (DSG1),2 desmocollin 1, and corneodesmosin (CDSN) in vitro (8). Pro-hK7 can be activated by hK5, and pro-hK5 can be activated by hK14 and hK5 itself (7). Furthermore, the expression and activation of hK7 are known to increase in psoriasis and itchy dermatitis (9, 10).

KLK8, also known as neuropsin, has been shown to have a trypsin-like activity (11). In human and mouse skin, hK8 and mK8 are localized from the upper stratum spinosum to the stratum corneum. Increases in KLK8 transcripts and protein were observed in human skin diseases, such as psoriasis vulgaris, lichen planus, and atopic dermatitis (12, 13). These results led to the hypothesis that hK8 is involved in the pathogenesis of inflammatory skin diseases.

There was no remarkable histological difference between the epidermis of wild-type (WT) and Klk8 gene-null (Klk8-/-) mice. However, Klk8-/- mice showed delayed recovery of the epidermis from the UVB-induced inflammation (14). A previous study showed that Klk8 mRNA was induced by an external stimulus, such as the application of 12-O-tetradecanoylphorbol-13-acetate (TPA), which causes epidermal proliferation and hyperkeratosis like psoriasis of human skin (15). In the present study, we used TPA to compare the reaction of WT and Klk8-/- mouse skin. Our results suggest that mK8 is involved

2 The abbreviations used are: DSG1, desmoglein 1; CDSN, corneodesmosin; TPA, 12-O-tetradecanoylphorbol-13-acetate; WT, wild-type; Klk8-/-, Klk8 gene-null; VPR-MCA, Boc-Val-Pro-Arg-MCA; FSR-MCA, Boc-Phe-Ser-Arg-MCA; RPY-pNA, Meo-Suc-Arg-Pro-Tyr-pNA-HCl; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcription; Ab, antibody; GFP, green fluorescent protein; AdGFP, adenovirus containing GFP; MOI, multiplicity of infection.
in desquamation through a protease cascade reaction leading to the degradation of DSG1 and CDSN.

**EXPERIMENTAL PROCEDURES**

**Mice and TPA Application Model**—All of the experiments were performed with Klk8−/− mice with C57BL/6 genetic background (16) and WT C57BL/6 mice. All of the experimental protocols were carried out according to the protocols approved by the Institutional Animal Care and Use Committee of Asahikawa Medical College. The shaved dorsal skin surface was treated once topically with 10 nM TPA (BIOMOL Research Laboratories) in 200 μl of ethanol. The control mice were treated with 200 μl of ethanol. The mice were killed 24 h, 48 h, 72 h, 5 days, and 7 days following the TPA treatments. The treated area of the dorsal skin was removed, frozen in liquid nitrogen, and stored at −80 °C until use.

**RT-PCR**—Total RNA was isolated according to the TRIzol protocol (Invitrogen) and incubated with DNase (Promega) for RT-PCR. Two micrograms of total RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega), and PCR was performed using Taq DNA polymerase (Promega). The primers used were as follows: Klk8 forward sequence, CCCACTGCAAAAAACAGAC; Klk8 reverse sequence, CTCTCATGTGCT; Klk7 forward sequence, GCTGGACAAG-CTCCATTGCTGCT; Klk7 reverse sequence, TGCGGACAAGAGAAAGATT; Klk7 reverse sequence, TGCTACTGCACCCATTGGACA; Klk6 forward sequence, CCCAGATACCATTCACTTCAGT; Klk6 reverse sequence, CGTGGGGGAGAACTGGATG; β-2-microglobulin forward sequence, TGCTACTGCAGCGGCTCCATG; and β-2-microglobulin reverse sequence, TATGTTCCGCTCCTTATGCT. Each reaction was performed at 30 cycles. Real time quantitative PCR was performed following the protocol described for a Light cycler SYBR green kit (Roche Applied Science).

**Histopathology**—Quantification/counting of the number of cell layers in the stratum corneum was performed as previously reported (17). Briefly, 5-μm fresh frozen sections were stained with 1% aqueous solution of safranin for 1 min and flooded with 2% potassium hydroxide aqueous solution. The number of the corneocyte layers was counted at six randomly selected locations/slide.

For immunohistochemistry, fresh frozen sections (5 μm thick) were incubated with 0.3% H2O2 for 5 min to quench the endogenous peroxidase activity followed by blocking with 5% bovine serum albumin in phosphate-buffered saline for 20 min. The sections were incubated with primary antibodies for 1 h at room temperature. The primary antibodies (Abs) used were anti-mK8 Ab (Medical & Biological Laboratories) diluted at 1:1000, anti-mK6 Ab (18) diluted at 1:2000, anti-K67 Ab (Dako Cytomation) diluted at 1:100, anti-DSG1 Ab (BD Bioscience) diluted at 1:100, and anti-CDSN Ab (19), diluted at 1:200. Primary antibodies were detected using a peroxidase conjugated secondary antibody (1:1000 dilution). A Vectastain Elite ABC kit (Vector Laboratories) was used for the detection of peroxidase activity.

For proliferation assay, fresh frozen sections (5 μm thick) were incubated with anti-Ki67 Ab (Dako Cytomation) diluted at 1:100 for 1 h at room temperature. The primary antibody was detected using a Vectastain Elite ABC kit. The number of the Ki67-positive cells was counted at four randomly selected locations/slide.

**Western Blotting**—The incised mouse skin was incubated with 10 mM EDTA in phosphate-buffered saline for 5 min at 56 °C. The epidermis was mechanically separated from the dermis and homogenized in a sample buffer containing 62.5 mM Tris-HCl, 2% glycerol, 1% SDS, 5 mM EDTA, 1 mM PMSF, and a protease inhibitor mixture (Sigma), followed by sonication on ice five times for 3 s and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was used as the epidermal extract. Proteins of the epidermal extract were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated with one of the following antibodies: anti-mK6 Ab, anti-DSG1 Ab (BD Biosciences), anti-CDSN Ab, and anti-α-tubulin Ab (Abcam). The final detection was performed with 1:2000 dilutions of peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies and visualized using the ECL Advance Western blot detection kit (Amersham Biosciences Bioscience) and luminescent Image Analyzer LAS-3000 (Fuji).

**Protease Activity Assays in Mouse Epidermis**—The TPA-applied, shaved back skin was incubated in 1 mM NaCl at 4 °C for 72 h for separating at the dermal-epidermal junction. Each epidermis was homogenized with the extraction buffer containing 60 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, and 5 mM EDTA and centrifuged at 15,000 × g for 20 min at 4 °C. The proteolytic activity of the supernatant was measured as described previously (20, 21) with a slight modification. Briefly, 10 μg of protein of the epidermal extract was incubated with 100 mM synthetic substrate Boc-Val-Pro-Arg-MCA (VPR-MCA) (Peptide Institute), Boc-Phe-Ser-Arg-MCA (FSR-MCA) (Peptide Institute), and Meo-Suc-Arg-Pro-Tyr-pNA-HCl (RYP-pNA) (Chromogenix AB) in a total volume of 1 ml at 37 °C for 2 h with shaking. All of the reactions were stopped by the addition of 1 mM PMSF. Released 7-amino-4-methylcoumarin was measured on a F-4500 fluorescence spectrometer (Hitachi) set at 370 nm for excitation and 460 nm for emission. Released pNA was measured on photometer Ultraspec 2100 pro (Amersham Biosciences) set at 405-nm wavelength. All of the measurements were performed in duplicate.

**In Situ Zymography**—In situ zymography was performed using 5-μm fresh frozen sections as previously described (22). Briefly, the sections were washed with 2% Tween 20 in deionized water and incubated with 2 μg/μl BOPY-FL-conjugated casein (Molecular Probes) at 37 °C for 2 h. PMSF (1 mM) was added to some sections as negative controls. After washing, the sections were examined using fluorescence microscopy.

**Construction of Adenovirus Vectors and Transfection in Klk8−/− Mouse Keratinocytes**—The cDNA fragment encoding Klk8 was cloned into an expression cosmid cassette designated pAXpCAwt, whose foreign gene expression is strongly induced by a CAG promoter. The generated cosmid was used as a transfer vector for adenovirus preparation. For obtaining recombinant adenovirus (AdKlk8), we used Takara adenovirus expression kit, followed by CsCl gradient centrifugation (23). Viral titers (plaque forming unit/ml) were determined using a plaque-forming assay in HEK 293 cells (23). An identical virus,
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**RESULTS**

**KLK8 mRNA Was Up-regulated after TPA Treatment**—We applied TPA on the dorsal skin of WT mice to induce psoriasiform epidermis and investigated the change of Klk8 mRNA with RT-PCR. Klk8 was weakly expressed before TPA application. The expression of Klk8 mRNA was markedly increased after TPA treatment (Fig. 1A). Quantitative RT-PCR revealed that Klk8 mRNA was up-regulated 5-fold, peaking at 48 h after TPA treatment, whereas no up-regulation was observed in control mice treated with ethanol (Fig. 1B). Weak mK8-like immunoreactivity was detected in the stratum granulosum without TPA treatment (Fig. 1C). The mK8-like immunoreactivity was increased in the upper stratum spinosum to the stratum corneum up to 48 h after TPA application (Fig. 1, D–F).

**Number of Proliferating Cells and Cell Layers of the Stratum Corneum of the WT and Klk8−/− Skin after TPA Treatment**—We compared morphologic change of the skin of WT and Klk8−/− mice after TPA treatment. TPA induced acanthosis and hyperkeratosis in the dorsal skin of both genotypes (Figs. 2, C and D, and 3A). TPA-treated epidermal keratinocytes in both genotypes were strongly positive for Ki67, a hyperproliferation marker (Fig. 2, C and D). The number of Ki67-positive cells peaked at 48 h after TPA application. WT skin showed significantly more Ki67-positive cells at 0 h, 24 h, 48 h, and 7 days (Fig. 2E). We next compared the number of cell layers of the stratum corneum. The number of cell layers of the stratum corneum in Klk8−/− mice was significantly higher than that in WT mice without TPA application. Five and 7 days after TPA application, the difference became even more prominent (Fig. 3). Therefore, we hypothesized that Klk8 is involved in proliferation of epidermal keratinocytes and corneocyte shedding at the epidermal surface with or without TPA application.

**Changes of Klk6 and Klk7 mRNA and Proteins in WT and Klk8−/− Skin after TPA Application**—Because kallikreins are thought to function in a cascade reaction with each other, we next investigated the changes of Klk6 and Klk7 mRNA in WT and Klk8−/− epidermis after TPA application. RT-PCR lacking expression of the transgene, but expressing green fluorescent protein (AdGFP) was used as a negative control. Klk8−/− newborn keratinocytes were isolated from 2–4-day-old mice. Primary cultures were initiated and maintained in keratinocyte growth medium containing epidermal growth factor (10 ng/ml), insulin (5 μg/ml), and bovine pituitary extract (50 μg/ml) at 37 °C in humidified atmosphere containing 5% CO2. Keratinocytes were grown until confluent and were infected with adding AdKlk8 or the control virus, AdGFP, to the culture medium from 3 to 30 multiplicity of infection (MOI) ratios. The cells were incubated at 37 °C for 1 h, followed by adding fresh keratinocyte growth medium and incubating at 37 °C for 48 h. The expression of Klk8 mRNA was confirmed by RT-PCR, and Klk8 was detected by Western blot analysis with 10 μl of culture medium. After incubation, total RNA was isolated, and quantitative RT-PCR was performed as described above.

**Statistical Analysis**—Student’s t test was used for statistical analysis. The data are presented as the means ± S.E.

**FIGURE 1.** Expression of Klk8 mRNA after TPA application to WT skin. A, RT-PCR of Klk8 mRNA at indicated times after TPA application. β2-Microglobulin (β2-MG) primers were used as an internal control. B, quantitative real time PCR analysis of Klk8 mRNA in WT epidermis. White bars, the epidermis treated with 10 nM TPA (n = 8). Black bars, the epidermis treated with ethanol (n = 3). The data are presented as the means ± S.E. * (p < 0.05) and ** (p < 0.01) indicate significant differences from 0 h. # (p < 0.05) and ## (p < 0.01) indicate significant differences between experimental groups. C–F, immuno-histochemical analysis of mK8 expression after TPA application. The skin samples were taken indicated time after TPA application. Scale bar, 30 μm.

**FIGURE 2.** Number of Ki67 positive cells of epidermis after TPA application to WT and Klk8−/− mice. A–D, Ki67 staining of WT (A and C) and Klk8−/− (B and D) mice skin at 0 h (A and B) and 24 h (C and D) after TPA application. Positive staining for Ki67 (arrows) was found in the nuclei of epidermal keratinocytes. Scale bar, 30 μm. E, the number of Ki67-positive cells of WT (white bars; n = 6) and Klk8−/− mice (black bars; n = 6) after TPA treatment. The Ki67-positive cells were counted at four randomly selected locations/slide. The data are presented as the means ± S.E. * (p < 0.05) and ** (p < 0.01) indicate significant differences between experimental groups.
revealed that Klk6 and Klk7 mRNAs were weakly expressed before TPA application in both genotypes. Although Klk6 and Klk7 mRNA of the WT epidermis were markedly increased after TPA application, the increase of these mRNAs in the Klk8/H11002/H11002 epidermis was significantly weaker (Fig. 4A). Using the quantitative RT-PCR, we found that Klk6 mRNA of both genotypes was increased, peaking at 24 h after TPA application. However, the fold increase in Klk6 mRNA was significantly different between the genotypes. Klk6 mRNA was increased up to 35 times in WT, whereas Klk8/H11002/H11002 mice showed only 4-fold increase (Fig. 4B). Klk7 mRNA was up-regulated 2.4-fold at 24 h after TPA application in WT mice, whereas no significant change was observed in Klk8/H11002/H11002 mice (Fig. 4C). These results suggest that Klk8 is involved in the up-regulation of both Klk6 and Klk7 mRNA after TPA treatment.

Immunohistochemistry revealed that the expression of mK6 was weak in the epidermis but was high in the hair follicles in both genotypes (Fig. 5A and J). After TPA application, mK6 of WT mice was highly expressed from the stratum granulosum to the stratum corneum (Fig. 5D and G). mK8 was also expressed from the upper layer of stratum spinosum to the stratum corneum (Fig. 5B, E, and H). Merged images revealed that the expression of mK8 partially overlapped with that of mK6 (Fig. 5F and J). In contrast, mK6 was weakly expressed in the epidermis of Klk8/H11002/H11002 mice even after TPA application (Fig. 5K and L).

Western blot analysis detected the increased 30-kDa form of mK6 at 24 h after TPA application in both genotypes (Fig. 5M). In WT mice, the 28-kDa form was detected at 72 h after TPA application but not in Klk8/H11002/H11002 mice. This shorter fragment may represent active mK6 from which pro-sequence had been cleaved off.

We tried but failed to detect mK7 by immunohistochemistry and Western blot analysis using anti-mK7 antibody (Santa Cruz). This may be due to relatively weak expression of mK7 in the control epidermis of mice (9).

Protease Activity Assays in the Epidermis after TPA Application—To investigate the difference of protease activity between WT and Klk8/H11002/H11002 mice, we measured proteolytic activity of epidermal extraction toward VPR-MCA and FSR-MCA, both of which were shown to be good substrates of mK8 and mK6 (11, 24), and toward RPY-pNA, a substrate of mK7 (13). Protease activity to VPR-MCA of WT epidermis gradually increased upon TPA treatment peaking at 72 h, whereas the activity to FSR-MCA increased, peaking at 48 h. The proteolytic activities to VPR-MCA and FSR-MCA of the untreated Klk8/H11002/H11002 epidermis were comparable with that of WT epidermis. However, upon TPA treatment, these protease activities in
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**FIGURE 4. Changes of Klk6 and Klk7 mRNA of WT and KLK8−/− epidermis.** A, RT-PCR of Klk6 and Klk7 mRNAs after TPA application. Klk6 and Klk7 mRNA were highly induced after TPA treatment, but the inductions in Klk8−/− mice were minimal. B and C, real-time PCR analysis of Klk6 (B) and Klk7 mRNA (C). White bars, TPA-treated WT (n = 10). Black bars, TPA-treated Klk8−/− mice (n = 10). The data are presented as the means ± S.E. * (p < 0.05) and ** (p < 0.01) indicate significant differences from 0 h. # (p < 0.05) and ## (p < 0.01) indicate significant differences between the genotypes. β2-MG, β2-microglobulin.

**FIGURE 5. Expression of mK6 and mK8 after TPA application in WT and Klk8−/− mice.** A–I, the expression of mK6 (green; A, D, and G) and mK8 (red; B, E, and H) at 0 h (A and B), 24 h (D and E), and 48 h (G and H) after TPA treatment in the WT mice. Merged images together with 4',6'-diamino-2-phenylindole staining (blue) are shown (C, F, and I). Epidermal-dermal junction is demarcated by dotted lines. J–L, mK6 expression at 0 h (J), 24 h (K), and 48 h (L) in the epidermis of TPA-treated Klk8−/− mice. Scale bar, 10 μm. M, mK6 protein in WT and Klk8−/− mice after TPA application. The arrow shows mK6 (30 kDa). In WT, 28 kDa mK6 (arrowhead) was also detected at 72 h.

Klk8−/− epidermis increased only slightly and were significantly lower than those of the WT (Fig. 6, A and B). In WT epidermis, the proteolytic activity toward RPY-pNA was increased, peaking at 24 h after TPA application. In contrast, Klk8−/− epidermis showed minimal change (Fig. 6C), consistent with the change of Klk7 mRNA (Fig. 4, A and C). In situ zymography also showed that the protease activity of Klk8−/− mice at 72 h was lower than that of WT (Fig. 6, D and E). Released fluorescence from casein in both genotypes was detected from the stratum granulosum to stratum corneum, where mK8 and mK6 were colocalized (Fig. 5J). PMSF, a serine protease inhibitor, inhibited proteolysis activity toward casein in both genotypes (Fig. 6, F and G). These results suggest that serine protease activity of the Klk8−/− mouse epidermis remained low even after treatment with TPA.

Overexpression of mK8 Did Not Up-regulate Klk6 or Klk7 mRNA Expression—To investigate whether mK8 itself can up-regulate other kallikrein genes, especially Klk6 and Klk7, we transfected primary keratinocyte cultures from the skin Klk8−/− with adenoviral vectors containing Klk8 cDNA (AdKlk8) or control AdGFP. Forty-eight hours after the transfection, the total RNA was isolated, and quantitative RT-PCR was performed. The expression of mK8 was confirmed by RT-PCR and Western blot analysis (Fig. 7, A and B). Klk8 expression was prominent in AdKlk8-transfected cells compared with WT keratinocytes as well as Klk8−/− keratinocytes without transfection. Klk6 mRNA was not significantly changed after transfection of Klk8 or GFP compared with Klk8−/− keratinocytes without transfection (p value between 0 and 3 MOI of AdKlk8 was 0.30). Klk7 mRNA was not significantly changed either (p value between 0 and 3 MOI of AdKlk8 was 0.067). Neither was there a difference in Klk6 or Klk7 mRNA between Klk8−/− and GFP-transfected keratinocytes (Fig. 7, C and D).

Reduction in DSG1 and CDSN Processing in Klk8−/− Mice—To address the possible molecular target of the Klk8-related proteases in corneocyte shedding, we investigated the changes of cohesion molecules of the stratum corneum, DSG1 and CDSN, by Western blot analysis and immunohistochemistry. We used anti-DSG1 antibody, which detects the intracellular domain of DSG1, and anti-CDSN antibody, which detects both the mature and proteolyzed forms. Western blot analysis revealed that DSG1 was significantly reduced until 48 h after TPA application in WT epidermis (Fig. 8, A and B). In contrast,
and a previous study showed that TPA increased the expression of Klk8 mRNA (Fig. 1) (15). Our study provides evidence that mK8 is involved in preventing hyperkeratosis. The number of cell layers of the stratum corneum of the Klk8−/− epidermis was significantly higher than that of the WT epidermis, and the difference was prominent after 72 h (Fig. 3B). UVB-radiated epidermis of Klk8−/− mice also showed significantly more thickening of the stratum corneum than that of WT after only 5 days (14). In addition to the function in shedding, mK8 may be involved in keratinocyte proliferation. WT skin had more proliferating cells before and after TPA application as compared with Klk8−/−. These results indicate that mK8 is involved in both proliferation of epidermis and corneocyte shedding. The molecular mechanism remains to be determined. However, hK8 and other kallikreins are also expressed in carcinoma cells (12, 26).

To address the possible involvement of other kallikreins in corneocyte shedding, we investigated the relationship of Klk8 and other Klks using Klk8−/− mice. We found that Klk8 is an important factor for inducing Klk6 and Klk7 (Fig. 4). Previous reports suggested that both hK6 and hK7 are involved in the pathogenesis of psoriasis (10, 13). Cascade reactions of Klks have been suggested to play a critical role in skin desquamation (7, 8). hK5 can activate pro-hK7 to active hK7, which can cleave desmocollin 1 and CDSN. hK5 also has a potential to directly cleave DSG1 and CDSN. In addition, at least eight kallikreins, including hK5, hK6, hK7, and hK8, are thought to be involved in desquamation cascade of the human skin (13).

In the present study, Klk8 mRNA showed parallel up-regulation with Klk6 and Klk7 mRNA after TPA treatment (Figs. 1 and 4), raising the possibility that these enzymes function in an activation cascade. Double immunostaining revealed that mK8 was expressed earlier than mK6 during epidermal differentiation (Figs. 5, 6, and 7). These expressions coincided with the proteolytic activity, namely, WT mice showed stronger proteolytic activity than Klk8−/− mice in the stratum granulosum to the stratum corneum (Fig. 6). However, Klk8 transfection to Klk8−/− keratinocytes did not up-regulate Klk6 or Klk7 mRNA, indicating that mK8 itself is not involved in the transcriptional regulation of Klk6 or Klk7 (Fig. 7). From these findings, we hypothesize that mK8 regulates and activates mK6 post-translationally through a molecular mechanism that remains to be determined.
the differentiation status. Proteolytic activity for substrates of mK6 and mK8 of WT but not Klk8−/− mouse epidermis was gradually increased until 72 h (Fig. 6, A and B). At this time point, Klk8 and Klk6 mRNA and mK6 were decreased over the expression peaks (Figs. 1, 4, and 5). It can be assumed that even after mK8 has reached its expression peak, the activated forms of downstream proteases such as mK6 retain their proteolytic activities. This result again implies that mK8 is located in the upper stream of activation cascade. In the human skin, hK7 can be ascribed to a major chymotrypsin-like kallikrein (13, 27). The proteolytic activity to chymotryptic substrates in the WT epidermis was increased at 24 h (Fig. 6), consistent with the expression of Klk7 mRNA (Fig. 4C). Another kallikrein, hK5 is a major component of the serine proteases of the human skin. We therefore tried, but failed, to detect Klk5 mRNA in the mouse skin (data not shown), suggesting that the expression of Klk5 in the mouse skin may be very low. In addition, there has been no report in the literature on the existence of Klk5 in the mouse skin (data not shown), suggesting that the expression of Klk5 in the mouse skin may be very low. In addition, there has been no report in the literature on the existence of Klk5 in the mouse skin.

The primary mechanism of corneocyte shedding is the cleavage of corneodesmosomes. Therefore, we examined the changes of the molecules of corneodesmosomes. Western blot analysis showed the reduction of uncleaved DSG1 and the appearance of the cleaved CDSN fragment after TPA application in WT mice (Fig. 8), suggesting that mK8 and/or relating enzymes cleaved these corneodesmosome proteins. The same pattern of changes of DSG1 and CDSN was observed when these molecules were incubated with hK5 and hK7 (8).
unpublished observation\(^3\) showed that recombinant mK8 did not cleave DSG1 efficiently, suggesting that mK6 and mK7 are more involved in DSG1 cleavage.

In summary, we showed that Klk8 and mK8 are involved in proliferation of keratinocytes and desquamation possibly through other kallikreins. The assumed protease cascade may result in the cleavage of DSG1 and CDSN. The phenotype of mK8 \(^{-/-}\) skin in this study may be the result of a failure to maintain the balance of proliferation and shedding.

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\(^3\) M. Kishibe and S. Yoshida, unpublished observation.