SASPase regulates stratum corneum hydration through profilaggrin-to-filaggrin processing

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The stratum corneum (SC), the outermost layer of the epidermis, acts as a barrier against the external environment. It is hydrated by endogenous humectants to avoid desiccation. However, the molecular mechanisms of SC hydration remain unclear. We report that skin-specific retroviral-like aspartic protease (SASPase) deficiency in hairless mice resulted in dry skin and a thicker and less hydrated SC with an accumulation of aberrantly processed profilaggrin, a marked decrease of filaggrin, but no alteration in free amino acid composition, compared with control hairless mice. We demonstrated that recombinant SASPase directly cleaved a linker peptide of recombinant profilaggrin. Furthermore, missense mutations were detected in 5 of 196 atopic dermatitis (AD) patients and 2 of 28 normal individuals. Among these, the V243A mutation induced complete absence of protease activity *in vitro*, while the V187I mutation induced a marked decrease in its activity. These findings indicate that SASPase activity is indispensable for processing profilaggrin and maintaining the texture and hydration of the SC. This provides a novel approach for elucidating the complex pathophysiology of atopic dry skin.

INTRODUCTION

Skin is the outermost tissue of terrestrial animals. It forms an effective barrier between the organism and the environment, which is indispensable for the prevention of the invasion of microorganisms, chemical compounds and allergens, and the maintenance of moisture levels of the skin. Skin is composed of three layers: the epidermis, dermis, and hypodermis. The epidermis is a stratified squamous, keratinized epithelium composed of the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC) (Watt, 1989). Epidermal cells (keratinocytes) divide and differentiate as they move upward from the SB to the SC. At the SG-to-SC transition, the keratinocytes dramatically transform themselves from three-dimensional living cells to two-dimensional, flattened and dead cells without intracellular organelles containing keratin bundles and lipids to constitute the SC (Candi et al, 2005). During this abrupt and dynamic terminal differentiation, keratinocytes express various proteins such as keratins, profilaggrin/filaggrin, involucrin, small proline-rich proteins,

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loricrin, cystatin A, and elafin, which form the cornified envelope of mature corneocytes (Candi et al, 2005).

It has long been recognized that there is a heritable component for the development of atopic dermatitis (AD) (Barnes, 2010). Recent reports indicate that 'filaggrin' has nonsense mutations in ichthyosis vulgaris (IV) patients and is the major predisposing factor for atopic eczema, asthma, and allergies (Smith et al, 2006; Irvine, 2007; Palmer et al, 2006; Sandilands et al, 2009). These reports further indicate that early onset of AD is caused by outside-to-inside paradigms, namely a primary barrier abnormality (Elias & Steinhoff, 2008), and filaggrin nonsense mutations are carried by nearly 10% of Europeans. However, such filaggrin mutations are found in ~50 and 20% of European and Japanese AD patients, respectively (Barker et al, 2007; Nomura et al, 2008; Sandilands et al, 2007, 2009), indicating that patients with AD who have normal filaggrin alleles are likely affected by other previously unidentified predisposing factors.

Filaggrin is expressed as a profilaggrin of >400 kDa in humans, which is a major component of keratohyalin granules in the SG of the epidermis (Dale et al, 1985; Presland et al, 2006). Profilaggrin is an insoluble phosphoprotein that consists of an amino terminal Ca^{2+}-binding protein of the S-100 family, linked to 10–12 tandem filaggrin monomer repeats (Dale et al, 1985; Harding & Scott, 1983; McGrath & Uitto, 2008). At the SG-to-SC transition, each filaggrin repeat is processed by certain protease(s) to generate the filaggrin monomer (37 and 28 kDa in human and mouse, respectively). The resulting monomer filaggrin has an unusual cationic charge, strongly binds to and bundles the keratin cytoskeleton to form microfibrils, and is believed to contribute to the production of flattened cells in the lower SC (Dale et al, 1978). Additionally, part of the filaggrin–keratin complex is cross-linked by transglutaminase to build the skin barrier (Candi et al, 1998, 2005; Harding & Scott, 1983; Steinert & Marekov, 1995). Keratin-bound filaggrin is citrullinated and further degraded into amino acids, which constitute a part of the natural moisturizing factor (NMF) in the upper SC (Tarcsa et al, 1996; Mechin et al, 2005; Nachat et al, 2005; Ishida-Yamamoto et al, 2002; Denecker et al, 2007; Kamata et al, 2009). Therefore, at the transition of the SG-to-SC, processing from profilaggrin to filaggrin is the rate-limiting, critical step for the profilaggrin processing cascade leading to SC moisturizing.

Several proteases are involved in profilaggrin to filaggrin processing. Calpain I and profilaggrin endopeptidase I (PEP-I) are important for the processing of the linker peptides between the filaggrin monomer repeats to generate the monomeric filaggrin *in vitro* (Resing et al, 1989, 1993a,b, 1995; Yamazaki et al, 1997); furin or convertase are involved in cleavage of the N-terminus from profilaggrin (Pearton et al, 2001); and knockout mice of matriptase/MT-SP1 and prostasin (CAP1/Prss8) show a defect in the conversion of profilaggrin to filaggrin (Leyvraz et al, 2005; List et al, 2003). Although the N- and C-terminal sequences of mouse, rat and human filaggrin have been determined (Resing et al, 1989, 1993b; Thulin & Walsh, 1995; Thulin et al, 1996), whether the linker sequences of profilaggrin are cleaved by matriptase or prostasin remains unclear.

Human and mouse retroviral-like aspartic protease, SASPase (skin aspartic protease; Asprv1), was the first identified stratified epithelia-specific protease expressed exclusively in the SG (Bernard et al, 2005; Matsui et al, 2006). This protease has been cloned as a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced gene (Taps) from mouse back skin epidermis (Rhiemeier et al, 2006). Recently, aberrant SASPase expression in transgenic mice was reported to cause impaired skin regeneration and skin remodelling after cutaneous injury and chemically induced hyperplasia (Hildenbrand et al, 2010). We have previously reported that SASPase-deficient mice showed increased fine wrinkles on the side of the adult body, suggesting that SASPase plays a certain physiological role in the SG and SC, although precise physiological and biochemical analysis was difficult due to the presence of hair. In this report, we produced SASPase-deficient 'hairless' mice. Through physiological and biochemical analyses of the mice, we demonstrate that SASPase activity plays a key role in determining the texture of SC by modulating SC hydration as well as profilaggrin processing. Moreover, we also report the identification of loss-of-function mutation of SASPase in the human genome.

RESULTS

Generation of SASPase-deficient hairless mice

Using high-throughput *in situ* hybridization screening, we previously identified a mouse homologue of SASPase and showed that SASPase-deficient mice displayed fine wrinkles on the side of their bodies (Matsui et al, 2004, 2006). Because SASPase is expressed exclusively in the SG and SC, these wrinkles were hypothesized to be derived from aberrant functions of the SG and SC. To further analyse the effect of a deficiency of SASPase on the epidermal surface, we transferred the ablated allele to a Hos/HR-1 hairless background. Hos:HR-1 mice (BALB/c background) were crossed with $SASP^{-/-}$ mice (C57BL/6J background) using a speed congenic method (Wakeland et al, 1997). Immunoblotting of the skin in $SASP^{+/+}$, $SASP^{+/-}$, and $SASP^{-/-}$ hairless mice showed no expression of SASPase in the $SASP^{-/-}$ epidermis (Supporting information Fig 1A). Frozen sections of $SASP^{+/-}$ and $SASP^{-/-}$ hairless mouse ears were stained with anti-SASP pAb and revealed a loss of the SASPase signal in the SG of the $SASP^{-/-}$ epidermis (Supporting information Fig 1B). Therefore, immunoblotting and immunofluorescence staining of frozen sections did not detect SASPase in SASPase deficient mice, indicating that the epidermis of $SASP^{-/-}$ mice was successively deficient in SASPase protein in the hairless background.

Appearance of $SASP^{-/-}$ hairless mice

Like normal mice, hairless mice (Hos:HR-1) grow hair after birth; however, at 17–23 days, they lose this hair due to hair root atrophy in 3–4 days, starting with the head, and become completely hairless at 3.5 weeks of age. Thereafter, we observed that $SASP^{-/-}$ hairless mice had more fine wrinkles and drier, rougher skin than their $SASP^{+/+}$ and $SASP^{+/-}$ counterparts (Fig 1A left). Female mice tended to show a more marked

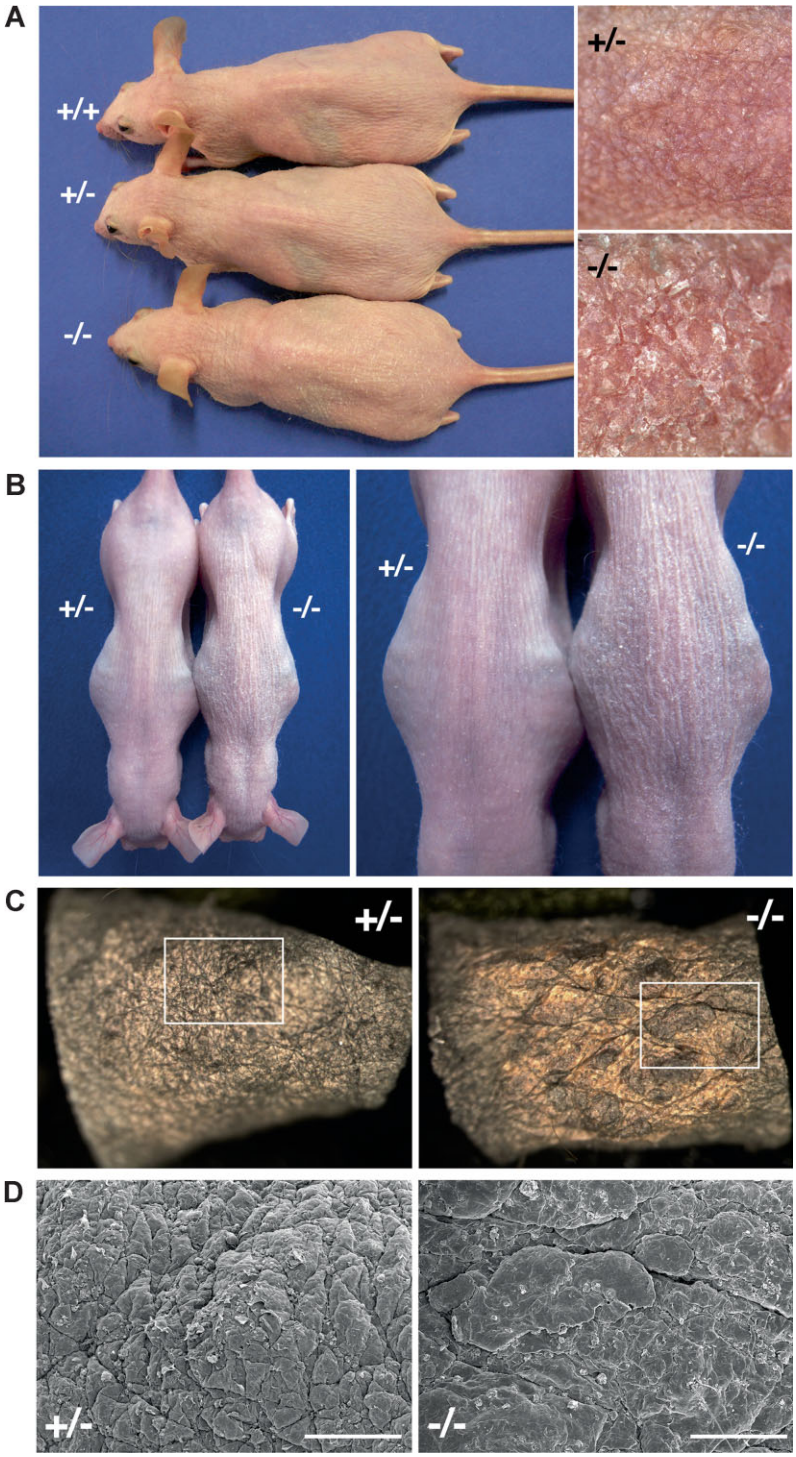

Figure 1. Morphological characterization of the epidermis of SASPase-deficient hairless mice.

A. Macroscopic observation of $SASP^{+/+}$, $SASP^{+/-}$ and $SASP^{-/-}$ hairless mice (left). Note that $SASP^{-/-}$ hairless mice showed drier and rougher skin. Stereomicroscopic examination of $SASP^{+/-}$ and $SASP^{-/-}$ hairless mice epidermis are also shown (right). Note that $SASP^{-/-}$ hairless mice had an epidermis with raised scales composed of many horny cells, whereas $SASP^{+/-}$ hairless mice had smooth, moist-looking skin with fine grooves.

B. Fine wrinkle formation in hung $SASP^{-/-}$ hairless mice. When hung by the tail, more wrinkles appeared on the surface of the back skin in $SASP^{-/-}$ hairless mice compared with $SASP^{+/-}$ hairless mice.

C. Stereomicroscopic examination of critical point dried epidermis of $SASP^{+/-}$ and $SASP^{-/-}$ hairless mice. In the $SASP^{+/-}$ hairless mice epidermis, many fine grooves were observed, whereas in the $SASP^{-/-}$ hairless mice, coarse grooves were observed. White boxes indicate the areas of interest investigated by SEM in D.

D. SEM of the epidermis of $SASP^{+/-}$ and $SASP^{-/-}$ hairless mice. In $SASP^{-/-}$ hairless mice, the intercellular spaces between horny cells could not be readily discerned, whereas individual horny cells were identified in their $SASP^{+/-}$ counterparts. Scale bars: 333 mm (left) and 375 mm (right).

phenotype than male mice (data not shown). Stereomicroscopic examination revealed that $SASP^{+/+}$ and $SASP^{+/-}$ mice had smooth, moist-looking skin with fine grooves, whereas $SASP^{-/-}$ hairless mice had skin with raised scales composed of many horny cells (Fig 1A right). When hung by their tails, more apparent differences were observed (Fig 1B). Specimens were fixed by standard techniques, dehydrated, dried at a critical point and examined employing scanning electron microcopy (SEM). After drying, epithelial scales of the skin of the $SASP^{+/-}$ hairless mice had fallen off, exposing the surface structure and resulting in many fine grooves. In contrast, coarse grooves were observed in the $SASP^{-/-}$ hairless mice (Fig 1C). Similarly, SEM revealed that the individual horny cells were easily identified in $SASP^{+/-}$ hairless mice, whereas the intercellular spaces between the horny cells could not be readily discerned in their $SASP^{-/-}$ counterparts (Fig 1D).

Morphology of SASP$^{-/-}$ mice epidermis

Next, we examined the histological analysis of SASP$^{-/-}$ hairless mice. The epidermis of the SASP$^{-/-}$ mice showed a normal layer organization of keratinocytes, and keratohyalin-granules were observed in both SASP$^{+/+}$ and SASP$^{-/-}$ mice at the level of hematoxylin–eosin (HE)-stained paraffin section images (Fig 2A and B). However, the cornified cells of the SASP$^{-/-}$ SC were compacted more tightly, and the number of layers was increased compared with SASP $^{+/+}$ mice (Fig 2A and B). Ultrathin section electron microscopy confirmed these findings at a higher resolution (Fig 2C–G). The appearance of individual cells from the SB to SS was indistinguishable between the SASP$^{+/+}$ and SASP$^{-/-}$ skin, and the SG contained both F- and L-granules in the SASP$^{-/-}$ SC (Fig 2C and D). Furthermore, the SC of SASP$^{-/-}$ mice showed 7–10 layers of electron dense and tightly compacted cell layers compared with that of SASP$^{+/+}$, which usually exhibited 5–7 layers (Fig 2C–H). These results suggest that a deficiency of SASPase in hairless background mice induced an abnormality in the SC.

Physiological features of SASP$^{-/-}$ epidermis

To physiologically characterize the epidermal surface of the SASP$^{-/-}$ hairless mice, we measured the trans-epidermal water loss (TEWL) and the SC hydration of SASP$^{+/+}$ ($n=7$) and SASP$^{-/-}$ ($n=11$) mice (Fig 3A and B). Although the TEWL was not significantly changed between SASP$^{+/+}$ and SASP$^{-/-}$ mice (Fig 3A), a clear difference was observed in SC hydration. As such, SC hydration of SASP$^{-/-}$ mice was significantly lower than in SASP$^{+/+}$ mice (Fig 3B). These results indicate that SASP$^{-/-}$ hairless mice showed markedly decreased SC hydration without alteration of barrier function.

Aberrant profilaggrin processing in SASP$^{-/-}$ hairless mice

Next, to analyse the epidermal differentiation of SASP$^{-/-}$ hairless mice, the expression of various epidermal differentiation markers were examined. Immunofluorescence staining of frozen sections of back skin epidermis with anti-keratin 14, keratin 1, involucrin and loricrin pAbs revealed normal

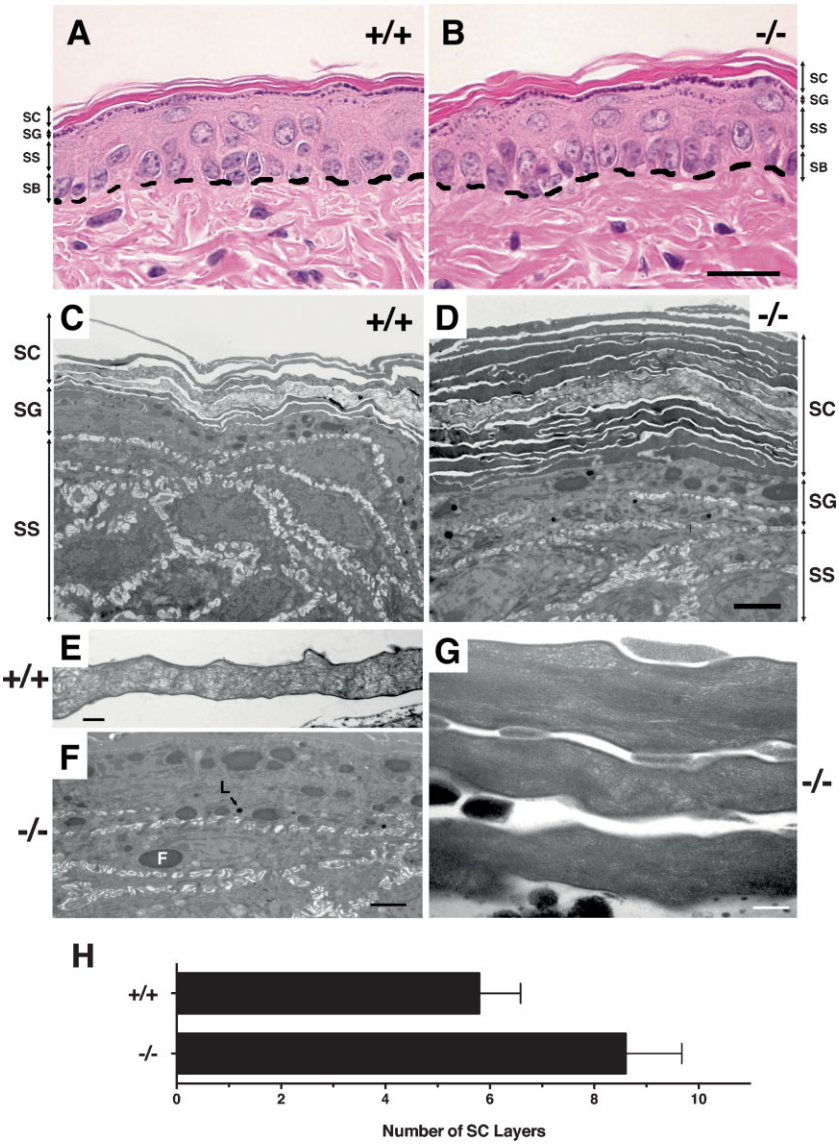

Figure 2. SASP$^{-/-}$ hairless mouse epidermis showed an increased number of layers in the aberrant SC.

A, B. Sections of SASP$^{+/+}$and SASP$^{-/-}$epidermis examined by HE staining. HE staining of SASP$^{-/-}$ hairless mice showed a marked thickening of the SC with an increased number of layers compared with SASP$^{+/+}$ mice. Scale bar: 100 μm.

C, D. Sections of SASP$^{+/+}$ and SASP$^{-/-}$epidermis examined by electron microscopy. TEM analysis of SASP$^{+/+}$ and SASP$^{-/-}$ epidermis showed an increased number of electron dense layers in the SC of SASP$^{-/-}$ mice compared with SASP$^{+/+}$ mice. Scale bar: 4 μm.

E. Typical layers of the SC of SASP$^{+/+}$ hairless epidermis. Scale bar: 500 nm.

F. The granular layer of the SASP$^{-/-}$ hairless mice. Both F-granules (F) and L-granules (L) were observed, suggesting that keratohyalin granule formation normally occurs in SASP$^{-/-}$ hairless mice. Scale bar: 2 μm.

G. Typical electron dense layers of the SC of SASP$^{-/-}$ hairless epidermis. Scale bar: 100 nm.

H. The number of layers in the SC of SASP$^{+/+}$ and SASP$^{-/-}$ epidermis were counted in five independent areas/mouse in the TEM images of two female mice. The average number of layers in the SC was 5.8 ± 0.8 SD and 8.6 ± 1.07 SD in SASP$^{+/+}$ and SASP$^{-/-}$ epidermis, respectively.

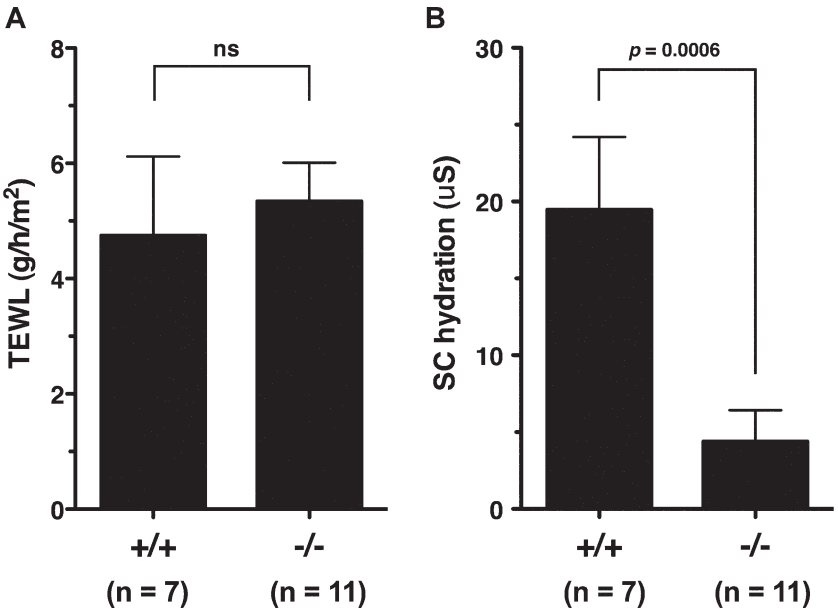

Figure 3. SASPase regulates SC hydration.

A. Trans-epidermal water loss (TEWL) of $SASP^{+/+}$ and $SASP^{-/-}$ hairless mice. TEWL of hairless mice were measured using VAVO SCAN (Asahi Biomed, Tokyo, Japan). The TEWL of $SASP^{-/-}$ hairless mice was not significantly changed. The numbers of animals tested were: $SASP^{+/+}$, $n = 7$ and $SASP^{-/-}$, $n = 11$. ns: not significant (Mann Whitney test on mean values ± SD using Graphpad software).

B. SC hydration levels of $SASP^{+/+}$ and $SASP^{-/-}$ hairless mice. SC hydration levels of hairless mice were measured using ASA-M1 (Asahi Biomed). The SC hydration of $SASP^{-/-}$ hairless mice was significantly lower than that of $SASP^{+/+}$ hairless mice. The numbers of animals tested were: $SASP^{+/+}$, $n = 7$ and $SASP^{-/-}$, $n = 11$. The p value is indicated above the bar (Mann Whitney test on mean values ± SD using Graphpad software).

expressions and localization of epidermal differentiation markers (Supporting information Fig 2A). Immunoblotting of back skin epidermal urea extracts with anti-keratin 14, keratin 1, involucrin and loricrin pAbs also revealed that the corresponding expression levels were not altered (Supporting information Fig 2B). On the other hand, immunofluorescence staining with anti-filaggrin pAb revealed that filaggrin-positive layers of the SC (lower SC) were increased in the back skin epidermis of the $SASP^{-/-}$ hairless mice (Fig 4A and B). To carefully compare the filaggrin in the lower SC, we tape-stripped the SC of the $SASP^{+/-}$ and $SASP^{-/-}$ epidermis. Coomassie brilliant blue (CBB) staining of equivalent amounts of the extracts revealed that all the major bands were decreased, suggesting there were increased concentrations of certain smear proteins (Fig 4C, left). Immunoblotting of the same samples with anti-filaggrin pAb revealed the accumulation of primarily two smear bands below the size of dimeric and trimeric filaggrin and that a mature filaggrin band was rarely detected (Fig 4C, right). These results suggest that a deficiency of SASPase resulted in the accumulation of premature processed dimeric and trimeric filaggrin and that mouse SASPase cleaves the linker sequence of mouse profilaggrin *in vivo*. The processing of mouse profilaggrin in the C57BL/6J mouse was reported to occur in a two-step process via two types of profilaggrin linker sequences with or without FYPV, respectively (Resing et al, 1989). First, a profilaggrin linker sequence containing FYPV may be cleaved, resulting in the accumulation of a two-domain intermediate (2DI) and a three-domain intermediate (3DI). Second, the linker type without FYPV, which connects 2DI and 3DI of monomeric filaggrin, is potentially cleaved by a Ca^{2+} dependent protease (Resing et al, 1989, 1993a). Some amino acid residues are then removed from the exposed sites by further exoprotease activity (Resing et al, 1989). Therefore, accumulation of dimeric and trimeric-like profilaggrin in the $SASP^{-/-}$ epidermis in Hos:HR-1 background suggests that SASPase may be involved in either the first or second processing steps.

SASPase directly cleaves the profilaggrin linker sequence *in vitro*

Accumulation of aberrant dimeric and trimeric profilaggrin in the lower SC of the $SASP^{-/-}$ hairless mice implies that the profilaggrin linker peptide is a direct substrate for SASPase. To confirm this hypothesis, we examined whether SASPase directly cleaves the profilaggrin linker peptide *in vitro*. Because it was difficult to produce recombinant mouse filaggrin with a linker peptide in *Escherichia coli* because of excess degradation, we produced human filaggrin (hFilaggrin) as a fusion protein of maltose binding protein (MBP) connected with the human profilaggrin linker peptide in *E. coli*. Purified MBP-hFilaggrin showed a triple banding pattern. The N-terminal amino acid sequence of the major upper two bands revealed that both proteins had an intact N-terminus of MBP indicating that C-terminally processed filaggrin (MBP-hFilaggrin-ΔC) was copurified with MBP-hFilaggrin. To prepare the active form of SASPase (14 kDa; hSASP14; 191-326aa), we expressed and purified a 28 kDa form of human SASPase (hSASP28; 85-343aa) as a fusion protein with GST (GST-hSASP28) in *E. coli* (Bernard et al, 2005). After complete autoprocessing under weakly acidic conditions (pH 6.0), which is the optimum pH of SASPase, the produced hSASP14 was purified (Fig 5A; Bernard et al, 2005; Matsui et al, 2006). Next, hSASP14 was incubated with MBP-hFilaggrin/-hFilaggrin-ΔC under weakly acidic conditions (pH 6.0). As shown in Fig 5B, MBP-hFilaggrin/hFilaggrin-ΔC was cleaved into mainly 42, 37 and 23 kDa proteins (Fig 5B and C). The 42 kDa protein was recognized by anti-MBP pAb (data not shown), and the N-terminal amino acid sequencing confirmed that the protein at 42 kDa was the MBP protein itself. The N-terminal amino acid sequencing of the 37 and 23 kDa identified the same peptide, 'QVSTH'. This N-terminal amino

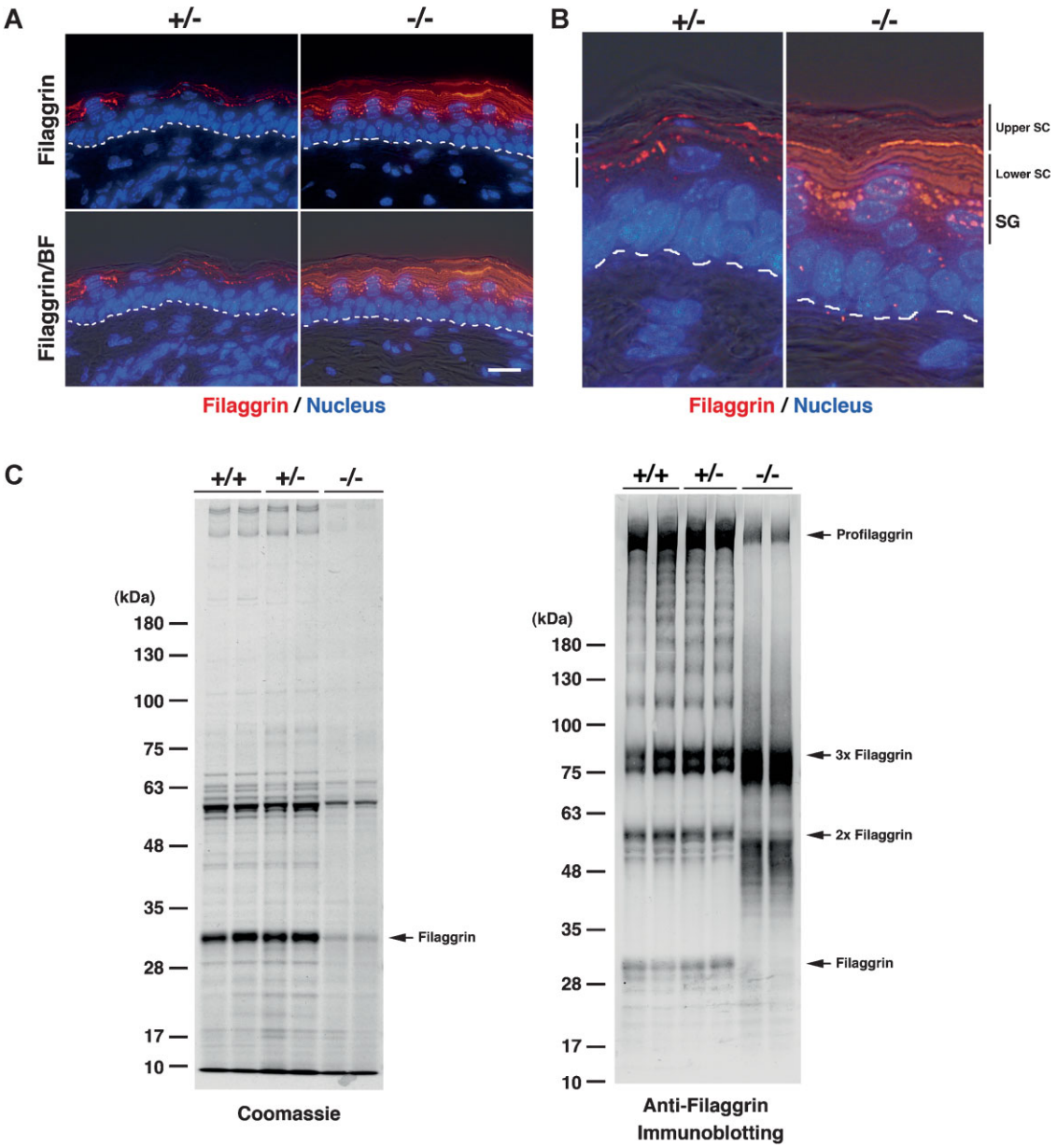

Figure 4. Aberrant expression of filaggrin in SASP$^{-/-}$ hairless mice.

A. Immunofluorescence staining of frozen sections of the back skin of SASP$^{+/-}$ and SASP$^{-/-}$ mice stained with anti-filaggrin antibody (red). Nuclei were counterstained with bisbenzimide (blue). The SASP$^{-/-}$ epidermis showed an increased amount of filaggrin-positive stained lower SC. Dashed lines represent the border between the epidermis and dermis. *BF*, bright field. Scale bar: 10 μm.

B. Enlarged view of A shows an increased amount of lower SC layers in the SASP$^{-/-}$ hairless epidermis.

C. Equivalent amounts of tape-stripped extracts (10 times, 5 μg) from SASP$^{+/+}$ ($n = 2$), SASP$^{+/-}$ ($n = 2$), and SASP$^{-/-}$ ($n = 2$) mice were immunoblotted with anti-filaggrin antibodies. CBB staining of extracts revealed a reduced expression of filaggrin monomer bands and other major SC proteins in the SASP$^{-/-}$ hairless mice epidermis (left; Coomassie). Immunoblotting with anti-filaggrin demonstrated that an accumulation of aberrant filaggrin degradation products (dimer and trimer sized) was detected (right), whereas mature filaggrin was rarely detected. As equivalent amounts of SC extracts were loaded, the intensity of the profilaggrin band was decreased in SASP$^{-/-}$, possibly due to an increase in the concentrations of other smear proteins.

acid sequence corresponded to the previously reported N-terminal amino acid sequence of native monomeric human filaggrin, in which Q is modified into pyrrolidone carboxylic acid (PCA) (Thulin & Walsh, 1995; Thulin et al, 1996). These results indicate that hSASP14 cleaved MBP-hFilaggrin and produced MBP (42 kDa) and hFilaggrin (37 kDa), of which the N-terminus was QVSTH. Furthermore, MBP-hFilaggrin-ΔC was cleaved at the same site and produced MBP (42 kDa) and hFilaggrin-ΔC (23 kDa), of which the N-terminus was QVSTH (Fig 5B and C). Thus, we conclude that hSASP14 cleaved the linker sequence

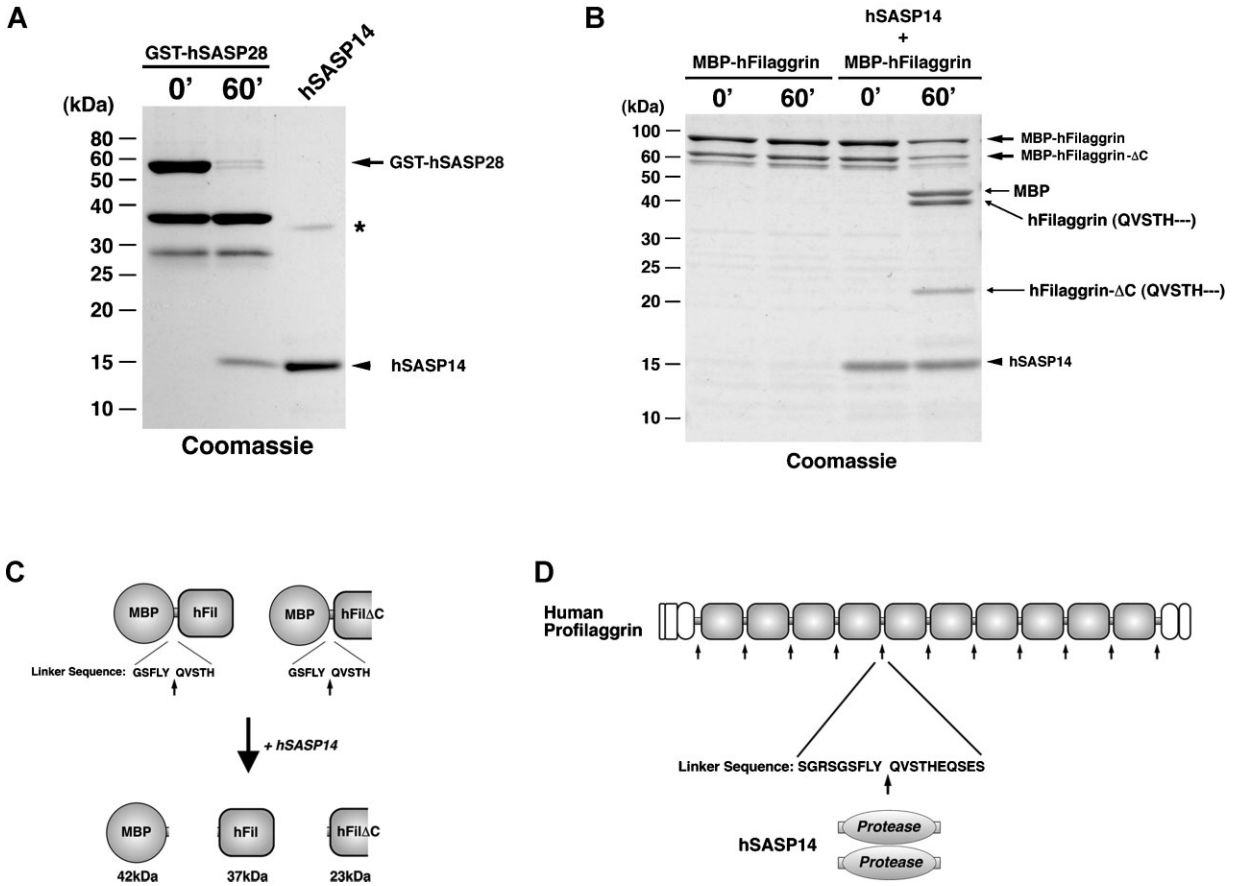

Figure 5. Recombinant hSASP14 directly cleaves recombinant filaggrin *in vitro*.

A. Production and purification of hSASP14 by autoprocessing of GST-hSASP28. Purified GST-hSASP28 (arrow) was incubated for the indicated times (0′, 0 min; 60′, 60 min) with 700 mM NaCl at pH 6.0. GST-hSASP28 underwent autoprocessing and produced hSASP14 (arrowhead). Cleaved GST-fusion proteins were removed by passing through Glutathione Sepharose 4B beads to purify hSASP14 (arrowhead). Asterisk indicates a dimer of hSASP14.

B. Cleavage of profilaggrin linker peptide by hSASP14 *in vitro*. The purified MBP-hFilaggrin/MBP-hFilaggrin-ΔC (arrow) was incubated with or without the purified hSASP14 (arrowhead) with 700 mM NaCl at pH 6.0 for 60 min at 37°C. The linker peptide of profilaggrin between MBP and hFilaggrin in MBP-hFilaggrin/MBP-hFilaggrin-ΔC was cleaved by hSASP14, resulting in the production of MBP (42 kDa), hFilaggrin (37 kDa), and hFilaggrin-ΔC (23 kDa). The N-terminal amino acid sequencing of hFilaggrin (37 kDa) and hFilaggrin-ΔC (23 kDa) protein identified QVSTH amino acids, which corresponded to the linker peptide of profilaggrin.

C. Schematic representation of the mode of processing of MBP-hFilaggrin by hSASP14 as described in B.

D. Schematic representation of the possible cleavage site of profilaggrin by hSASP14. Homodimerized hSASP14 proteins were suggested to primarily cleave between GSFLY-QVSTH in the profilaggrin linker sequence (arrow).

of human profilaggrin between 'GSFLY' and 'QVSTH' *in vitro* (Fig 5C and D).

Normal free amino acid composition in SC of SASP$^{-/-}$ mice

Previous reports suggest that NMFs serve as natural humectants of SC (reviewed in Rawlings & Matts, 2005). To investigate whether aberrant processing of filaggrin had a detrimental effect on the composition of NMFs, we performed free amino acid analysis of tape stripped SC of SASP$^{+/+}$ ($n=7$) and SASP$^{-/-}$ ($n=11$) mice. There were no alterations in the total amount or the composition of free amino acids (Fig 6A and B). These results suggest the NMFs were normal in SASP$^{-/-}$ SC, despite the marked decrease in SC hydration.

Mutations of human SASPase affect autoprocessing and profilaggrin linker cleavage activity

Analysis of our SASP$^{-/-}$ hairless mice indicates that loss of SASPase activity may result in dry skin accompanied by an accumulation of unprocessed profilaggrin. Thus, we investigated whether the human genome possesses SASPase mutations, which consequently affect protease activity, i.e. profilaggrin to filaggrin processing activity. We investigated mutations in the SASPase gene in 28 control subjects and 196 AD-patients (Sasaki et al, 2008). As a result of the mutation search on the human SASPase gene, two types of missense mutations (D232Y and V243A: 3.5% [1/28]) in the control subjects, four types of missense mutations (A54S: 0.5% [1/196],

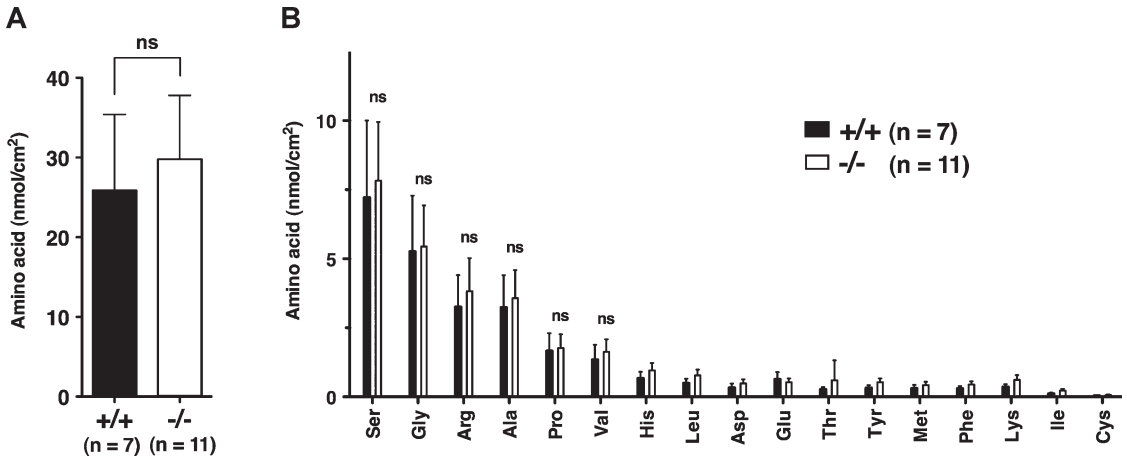

Figure 6. Free amino acids in SC of SASP$^{+/+}$ and SASP$^{-/-}$ mice.

A. Total free amino acid composition analysis. Tape stripped SC of SASP$^{+/+}$ ($n = 7$, filled column) and SASP$^{-/-}$ ($n = 11$, open column) hairless mice were subjected to amino acid analysis. Total free amino acid composition analyses showed no difference between the SC of SASP$^{+/+}$ and SASP$^{-/-}$ mice. ns: not significant (Mann–Whitney test on mean values ± SD using Graphpad software).

B. Individual free amino acid composition analysis. Individual free amino acid composition analyses also showed no difference between the SC of SASP$^{+/+}$($n = 7$, filled columns) and SASP$^{-/-}$ mice ($n = 11$, open columns). ns: not significant (Mann–Whitney test on mean values ± SD using Graphpad software).

I186T: 0.5% [1/196], V187I: 1.5% [3/196], R311C: 0.5% [1/196]), and three types of silent mutations (F101F: 0.5% [1/196], P206P: 3.1% [6/196], N276N: 1.5% [3/196]) in the AD patients were identified (Fig 7A, Supporting information Tables I and II). All mutations were heterozygous. V187I was most frequently identified (identified in three AD patients). A54S and R311C were found in the same patient and in the same allele. The number of patients and mutation types are shown in Supporting information Tables I and II.

To examine whether these mutations affect the protease activity of SASPase, we bacterially expressed and purified GST-hSASP28 bearing the mutations I186T, V187I, R311C, D232Y, or V243A, and performed an *in vitro* autoprocessing assay as previously described (Bernard et al, 2005; Matsui et al, 2006). To compare the autoprocessing activity *in vitro*, we purified GST-hSASP28 mutants (Fig 7B). We could not examine the autoprocessing activity of GST-hSASP28(I186T)/(R311C), because full-length GST-hSASP28(I186T)/(R311C) was barely purified, possibly due to the higher autoprocessing activity in *E. coli*. The rest of the purified GST-hSASP28(WT)/(D232Y)/(V243A) was incubated under weakly acidic conditions (pH 6.0), which is the optimal pH of SASPase (Bernard et al, 2005; Matsui et al, 2006). As shown in Fig 7C, GST-hSASP28(D232Y) showed autoprocessing activity similar to that of the WT, and V243A showed no autoprocessing activity.

We next examined whether the V187I mutation, which was identified most frequently in AD patients (three patients), affected autoprocessing activity *in vitro*. The purified GST-hSASP28(WT)/(V187I) was incubated under weakly acidic conditions (pH 6.0). As shown in Fig 7D, GST-hSASP28(V187I) showed decreased autoprocessing activity in both 300 and 400 mM NaCl. Fig 7E shows the time course by semi-quantification of produced hSASP14. Because V187I is located outside of the cleaved hSASP14, autoprocessed hSASP14 from hSASP28(V187I) should be the same as the WT. Therefore, the initial autoprocessing reaction (1–30 min in 300 mM NaCl) should reflect the difference between WT and V187I. The reaction rate of GST-hSASP28(V187I) at 300 mM NaCl was estimated at 5.6-fold over 15–30 min, which is lower than that of GST-hSASP28(WT). These results indicate that hSASP28(V187I) has substantially reduced activity at pH6.0 *in vitro* compared to WT. Finally, we examined whether the V187I mutation had an effect on the filaggrin linker cleavage activity of GST-hSASP28 *in vitro*. To examine whether the mutation of V187I affected the profilaggrin cleavage activity, we incubated GST-hSASP28(WT) or GST-hSASP28(V187I) with MBP-hFilaggrin/MBP-hFilaggrin-ΔC under weakly acidic conditions (pH 6.0). As expected, the MBP-hFilaggrin cleavage activity by GST-hSASP28 was suppressed in GST-hSASP28(V187I) compared with GST-hSASP28(WT), relative to the production of hSASP14 (Fig 7E). These results indicate that GST-hSASP28(V187I) showed decreased autoprocessing activity at pH 6.0, resulting in decreased profilaggrin linker cleavage activity *in vitro*.

It is important to elucidate whether these heterogenic loss-of-function mutations of SASPase affect human skin physiology. And it is possible there was some dry skin in our non-AD cohort as criteria of this cohort did not discriminate against dry skin (Sasaki et al, 2008). We measured TEWL and SC hydration of a non-AD individual with a mutation (V243A/+) as well as three normal individuals without mutations. The appearance of the skin surface, TEWL and SC hydration of V243A/+ were similar to the three normal individuals (Supporting information Fig 3). These results did not provide conclusive evidence due to the small numbers tested. In the case of complex disorders like AD, it is difficult to prove a direct role of the sequence variant in the

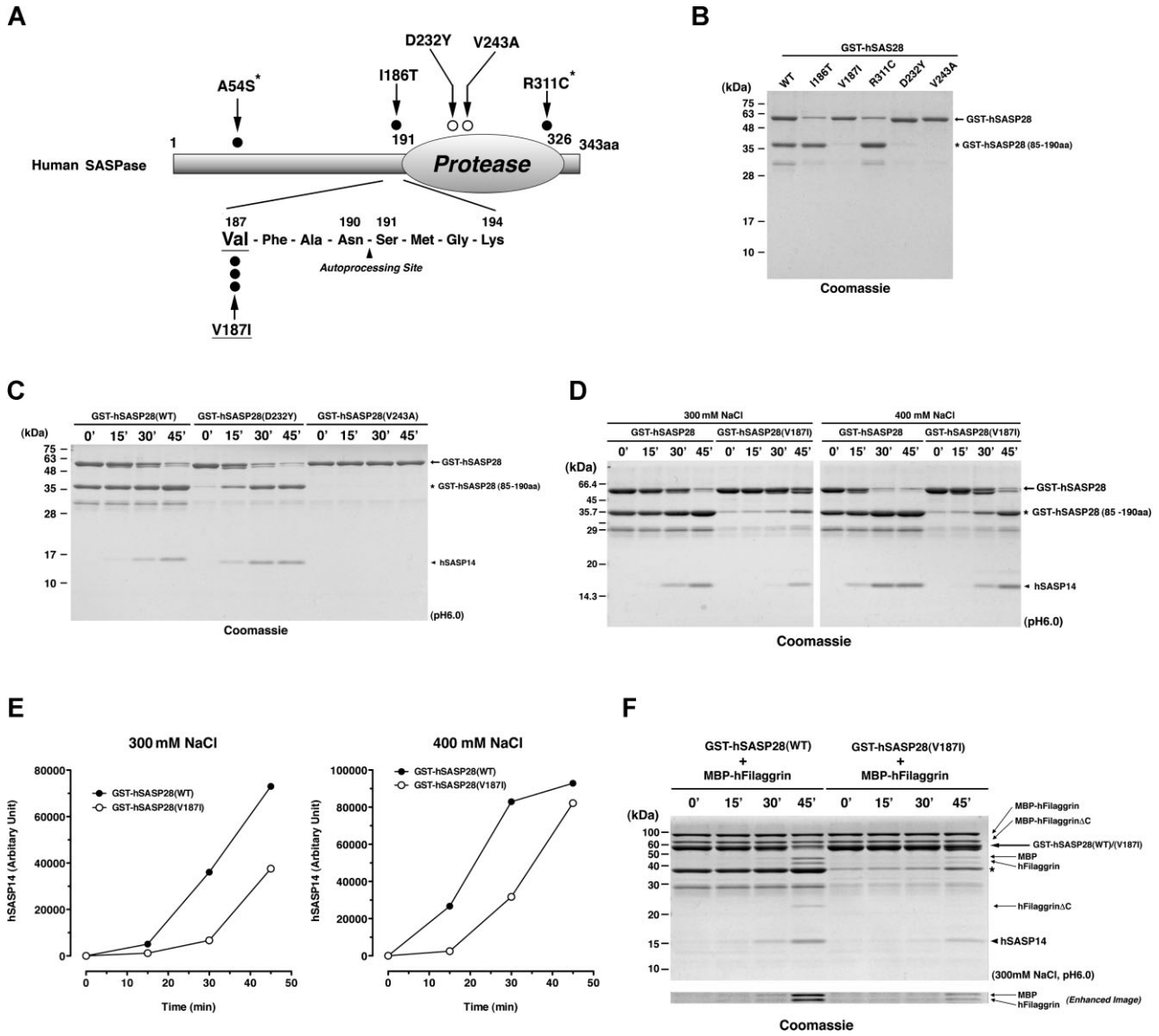

Figure 7. Biochemical characterization of human mutations in SASPase.

A. Schematic representation of human mutations identified in AD patients ($n = 196$; closed circles) and case controls ($n = 28$; open circles). The amino acid sequence of the autoprocessing site (between Asn^{190} and Ser^{191}) is indicated. Asterisks of A54S and R311C indicate that these mutations were found in the same patient and in the same allele.

B. Purification of GST-hSASP28 mutants. Purified GST-hSASP28 (WT)/(I186T)/(V187I)/(R311C)/(D232Y)/(V243A) (1 μg) were subjected to SDS-PAGE and stained with CBB. GST-hSASP28 (V187I) and (V243A) did not produce any partially cleaved products. Arrow indicates GST-hSASP28. Asterisk indicates the partially cleaved product of N-terminal hSASP28 (85-190aa) fused with GST (see A).

C. Comparison of autoprocessing activity among GST-hSASP28(WT), (D232Y) and (V243A). 1.4 mg/ml of GST-hSASP28(WT), (D232Y) and (V243A) were incubated at pH 6.0 for indicated times and subjected to SDS-PAGE and stained with CBB. The autoprocessing activity of GST-hSASP28(D232Y) was comparable to that of GST-hSASP28(WT), whereas GST-hSASP28(V243A) did not show any autoprocessing activity *in vitro*. Arrowhead indicates the hSASP14.

D. Comparison of autoprocessing activity between GST-hSASP28(WT) and (V187I). 0.9 mg/ml of GST-hSASP28(WT) and (V187I) were incubated under 300 or 400 mM NaCl at pH 6.0 for indicated times, subjected to SDS-PAGE, and stained with CBB. The autoprocessing activity of GST-hSASP28(V187I) was weaker than that of GST-hSASP28(WT) in both conditions.

E. Reaction curve of GST-hSASP28(WT) and (V187I) autoprocessing. Autoprocessed hSASP14 from GST-hSASP28(WT) (closed circles) and GST-hSASP28(V187I) (open circles) in Fig 8D was semi-quantified from a gel image. The reaction rate was higher in 400 mM (right) than in 300 mM NaCl (left). V187I mutation affected the initial reaction rate and showed 5.6-fold reduced activity over 15–30 min in 300 mM NaCl.

F. Comparison of profilaggrin linker peptide cleavage activity between GST-hSASP28(WT) and (V187I). 0.9 mg/ml of GST-hSASP28(WT) and (V187I) were incubated with 0.4 mg/ml of MBP-hFilaggrin/MBP-hFilaggrin-ΔC under 300 or 400 mM NaCl at pH 6.0 for the indicated times, subjected to SDS-PAGE, and stained with CBB. The profilaggrin linker cleavage activity was weaker in GST-hSASP28(V187I) than in that of (WT), relative to the production of hSASP14 by autoprocessing. An enhanced image of the MBP and hFilaggrin band areas is also shown.

pathogenesis. In the future, it is necessary to perform a large-scale cohort analysis to clarify the clinicopathological significance of SASPase mutation.

Physiological role of SASPase

Figure 8 summarizes our findings. In normal epidermis, non-phosphorylated profilaggrin is orderly processed into filaggrin and bundle keratin filaments at the 'lower SC', then degraded into free amino acids which constitute most of the NMFs in the 'upper SC'. SASPase deficiency causes incomplete linker cleavage of profilaggrin resulting in an accumulation of trimeric and dimeric profilaggrins slightly degraded from either N- or C-terminal ends in the 'lower SC'. Such aberrant profilaggrin may bind to keratin filaments, finally degrade, and produce a normal composition of free amino acids in the 'upper SC'. Finally, the SC of SASP$^{-/-}$ epidermis has an increased number of layers and produces a wrinkled, dry, rough skin.

DISCUSSION

In our previous study, we reported increased fine wrinkles at the side of the body in SASP$^{-/-}$ mice raised on a C57BL/6J background. However, we were not able to distinguish any morphological changes between SASP$^{+/-}$ and SASP$^{-/-}$ mice, possibly due to the thin epidermis of the back skin (Matsui et al, 2006). In this paper, we generated SASP$^{-/-}$ mice with Hos:HR-1 background. The increased thickness of the epidermis of these mice enabled us to distinguish a clear difference in SC structure and profilaggrin processing pattern in the SC. SASP$^{-/-}$ hairless mice showed a marked decrease in SC hydration and an increased number of electron dense layers in the SC. Such aberrant layers in the SC consisted of an upper SC without filaggrin staining and a lower SC stained by anti-filaggrin Ab with the accumulation of aberrant dimeric and trimeric filaggrin, i.e. premature profilaggrin processing (Figs 5B and 8). Interestingly, the composition

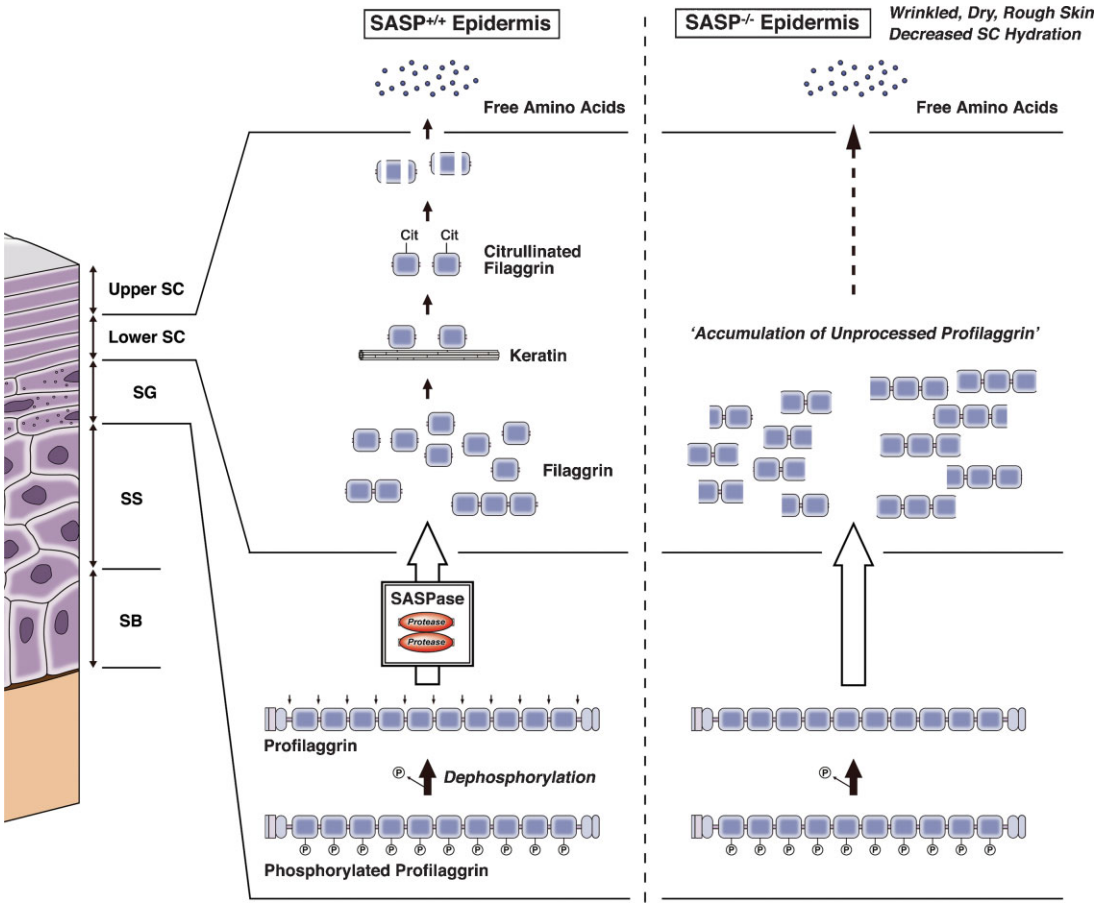

Figure 8. Schematic representation of the possible profilaggrin processing pathway observed in the SASP$^{+/+}$ and SASP$^{-/-}$ epidermis. In the SASP$^{+/+}$ epidermis, highly phosphorylated profilaggrin expressed in the SG is dephosphorylated in the upper most SG. The linker sequence that connects each filaggrin monomer is then cleaved by SASPase at the SG-to-SC transition via production of two kinds of intermediates (2DI and 3DI). Monomeric filaggrin strongly binds to keratin filaments in the lower SC to form bundled keratin filaments. After citrullination, filaggrin is released from keratin and further degraded to form free amino acids, which constitute most of the NMFs in the upper SC. In the SASP$^{-/-}$ epidermis, trimeric and dimeric profilaggrins slightly degraded from either N- or C-terminal ends, accumulate because of incomplete linker sequence cleavage. Aberrantly processed profilaggrin binds to keratin filaments and degrades into free amino acids without the production of monomeric filaggrin. Finally, aberrant SC causes wrinkled, dry, rough skin with decreased SC hydration. SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale.

and quantity of major free amino acids were not altered in the dry skin-like SC of SASP$^{-/-}$ hairless mice (Fig 6).

Hydration of the SC plays an important role in maintaining metabolic activity, enzyme activity, mechanical properties, appearance and barrier function of the skin and is dependent on (i) organization of the tight and semi-permeable barrier of intercellular lamellar lipids, (ii) the diffusion path length created by the SC layers and corneocyte envelopes, and (iii) the presence of NMFs (Rawlings & Harding, 2004; Rawlings & Matts, 2005). NMFs comprise up to 10% of the SC and are reported to be an important natural humectants of the SC because of their hygroscopic features (Rawlings & Harding, 2004; Rawlings & Matts, 2005). Most NMFs are composed of free amino acids derived from the degradation products of filaggrin and its derivatives, PCA and urocanic acid (UCA). There are also non-amino-acid-derived (non-filaggrin-derived) NMFs, such as sugars, hyaluronic acid, urea, citrate, lactate, and glycerol (Fluhr et al, 2008; Rawlings & Harding, 2004; Rawlings & Matts, 2005). Although we did not analyse the PCA or UCA of SASP$^{-/-}$ hairless mice, there were no changes in free amino acids, which suggests that filaggrin-derived NMFs were not altered. The accumulation of aberrantly processed profilaggrin without monomer filaggrin in the 'lower SC' accompanied by a normal free amino acid content in the 'upper SC' in SASP$^{-/-}$ SC implies disruption of several mechanisms for maintenance of SC hydration other than the contribution of NMFs.

As described in the Introduction section, filaggrin is thought to have two major functions: the formation of keratin microfibrils in the lower SC and the production of NMFs in the upper SC (Rawlings & Harding, 2004; Fig 8). It is possible that dry skin of SASP$^{-/-}$ hairless mice is derived from the lower SC where aberrantly processed filaggrin accumulates, but not from the upper SC where the end-products of filaggrin (NMFs) are located. In the normal epidermis of humans, rats and mice, intermediate processing products of mouse filaggrin, 2DI and 3DI, have been reported, and they possess keratin binding activity similar to monomeric filaggrin (Harding & Scott, 1983). Thus, it is suggested that aberrant dimeric and trimeric filaggrins in the SASP$^{-/-}$ SC also possess keratin binding activity. It is widely believed that the SC hydration and physiological and physical mechanics are closely linked and depend on keratin structural organization. Accumulation of premature processed profilaggrin, even if there is keratin binding activity, may alter the cubic-like, rod-packing symmetry of keratin filaments at the SG-to-SC transition and/or at the lower SC, and this may cause alteration of the SC hydration level in the SASP$^{-/-}$ epidermis (Norlen & Al-Amoudi, 2004). The SC of flaky tail mice, in which filaggrin is absent in the cornified cell layers of the epidermis, did not show decreased SC hydration nor an increased number of layers in the SC (Fallon et al, 2009; Presland et al, 2000; Scharschmidt et al, 2009). This suggests that aberrantly processed profilaggrin and a marked decrease of mature filaggrin affect the texture and hydration of the SC. The importance of the amount of mature filaggrin for SC hydration was reported by Ginger et al, who found an inverse relationship between the profilaggrin-12-repeat allele and the occurrence of self-perceived dry skin (Ginger et al, 2005).

SASPase may cleave other as yet unidentified substrates, in addition to profilaggrin, resulting in decreased SC hydration via abnormalities in the intercellular lamellar lipids barrier, the diffusion path length and/or the composition of non-filaggrin-derived NMFs. These possibilities can be examined by crossing SASP$^{-/-}$ mice with filaggrin-deficient mice to examine whether dry skin is derived from aberrant profilaggrin processing.

In normal, healthy controls and AD patients, we identified several missense mutations, which may have affected the activity of SASPase. Among them, we identified a loss-of-function mutation of SASPase (V243A) in non-AD controls. This mutation was located inside the protease domain and no longer showed any activity at pH 6.0. Another missense mutation, V187I, was the most frequently identified in the AD patients (three AD patients). It was located outside of the protease domain at the autoprocessing site, the 'P4' position of the substrate recognition site. A change of the amino acid at P4 resulted in decreased autoprocessing activity *in vitro*. GST-hSASP28(V187I) did not show any autoprocessing activity in *E. coli* (neutral pH), suggesting that this mutation had no activity in the cytoplasm of the SG, resulting in the same loss-of-function effect as SASPase(V243A). SASPase, as a retroviral aspartic protease, must undergo homodimeric formation for its protease activity (Bernard et al, 2005; Matsui et al, 2006). In the HIV protease, a subunit exchange reaction with a catalytically defective protease results in 50% inhibition of enzymatic activity (Darke, 1994). In the case of a heterozygous loss-of-function mutation of bi-allelic expression, half of the molecules are catalytically inactive and induce 'heterodimeric inhibition' of SASPase activity, resulting in a quarter decrease of total activity. The newly found, rare mutations of SASPase (V187I)/(V243A) possibly behave in a dominant negative manner in the person who has the heterozygous mutation.

Of note, the SASP$^{-/-}$ mice showed decreased SC hydration without alteration of TEWL, suggesting a decreased ability of water retention in the SC under normal barrier function. Although the TEWL is an important hallmark for skin barrier function, TEWL is not necessarily correlated with dry skin (Berry et al, 1999; Engelke et al, 1997; Wilhelm et al, 1991). Decreased SC hydration is found in a number of diseases, such as AD, eczema or psoriasis (Harding et al, 2000). There are a number of human epidermal diseases that include the aberrant expression and processing of profilaggrin to filaggrin (reviewed in Dale et al, 1990). Therefore, it is possible that patients who do not have a nonsense mutation of filaggrin, but who exhibit xerosis or AD, might have an aberrant profilaggrin processing pattern. Involvement of SASPase in progression of these diseases from the aspect of the profilaggrin processing pathway should be examined. The profilaggrin degradation pattern could be useful for the diagnosis of xerosis and the early onset of AD.

Collectively, these results indicate that activity of SASPase plays a key role in determining the texture of the SC by modulating SC hydration as well as profilaggrin-to-filaggrin processing. Moreover, these results, in combination with clinicopathological investigations of epidermal diseases derived from the aberrant processing of profilaggrin by SASPase mutation, will provide a novel concept to dissect the complex mechanisms of percutaneous antigen priming in atopic diseases.

The paper explained

PROBLEM:

The SC is the outermost layer of the skin in terrestrial animals and thus acts as a barrier against the external environment. It is hydrated by endogenous substances to avoid desiccation; however, the mechanisms responsible for maintaining hydration of the SC remain unclear at the molecular level. Dry skin is a common phenotype in patients with atopic dermatitis (AD). Recent reports have indicated that the protein filaggrin is mutated in ichthyosis vulgaris patients and is a major predisposing factor for development of atopic eczema, asthma, and allergies. Approximately 50 and 80% of European and Japanese AD patients, respectively, have normal filaggrin alleles, suggesting the presence of previously unidentified predisposing factors.

RESULTS:

We produced 'hairless' mice that were deficient in the enzyme skin-specific retroviral-like aspartic protease (SASPase). The decreased activity of this enzyme in the mice resulted in dry skin with an accumulation of incorrectly processed profilaggrin, a precursor of the filaggrin protein. This incorrectly processed and accumulated profilaggrin subsequently results in a marked decrease of filaggrin production. We also demonstrated that SASPase directly cleaved a profilaggrin linker peptide *in vitro*. Several missense mutations were detected in 5 of 196 AD patients and 2 of 28 normal individuals. Among these, the V243A mutation resulted in complete ablation of protease activity *in vitro*, while the V187I mutation induced a marked decrease in SASPase activity.

IMPACT:

This is the first report demonstrating that a deficiency of the protease SASPase is a likely cause of dry skin *in vivo*. We clarified that the activity of SASPase plays a key role in determining the texture of the SC by modulating SC hydration. This molecular mechanism will provide clues in revealing the role of the SC in terrestrial animals and how they adapted to life on land. Our results also provide novel concepts to assist in determining the complex pathophysiology of atopic dry skin.

MATERIALS AND METHODS

Reagents

Oligonucleotide primers were purchased from Sigma–Aldrich Japan (Kyoto, Japan). N-terminal amino acid sequence analysis was performed by Shimadzu Techno Research (Kyoto, Japan).

Antibodies

For immunofluorescence and immunoblotting, antibodies against keratin 14, keratin 1, involucrin, loricrin and filaggrin were used (Covance, Berkeley, CA). A polyclonal antibody against SASPase, anti-SASP-C, which recognizes the C-terminus of both 28 kDa (human) and 32 kDa (mouse) SASPase, and anti-SASP-PR1 polyclonal antibody, which recognizes both 14 kDa (human) and 15 kDa (mouse) SASPase, have been described previously (Matsui et al, 2006).

Animals

Hairless mice (Hos/HR-1) were obtained from Hoshino Experimental Animal Supply (Ibaragi, Japan). $SASP^{+/-}$ mice of a C57BL/6J background were bred from six generations to a pure Hos/HR-1 background through the speed congenic services of the Central Institute for Experimental Animals (Kawasaki, Japan). Mice heterozygous for a SASPase deletion were interbred, and $SASP^{+/+}$, $SASP^{+/-}$ and $SASP^{-/-}$ littermates were used for experiments. Wild-type (WT, $SASP^{+/+}$), SASPase heterogenic ($SASP^{+/-}$) and SASPase knockout ($SASP^{-/-}$) mice on a Hos/HR-1 backgrounds were used in the study. All mice were maintained under specific pathogen-free (SPF) conditions that are required for maintaining mouse colonies. All animal procedures were approved by the Animal Studies Subcommittee (Institutional Animal Care and Use Committee) of the Tokyo Medical and Dental University and performed in accordance with their guidelines. Basal SC hydration was measured with ASA-M1 (Asahi Biomed, Tokyo, Japan) on the back skin of $SASP^{+/+}$ ($n = 7$) and $SASP^{-/-}$ ($n = 11$) mice. From the same mice, TEWL measurements were taken under basal conditions with a VAVO SCAN (Asahi Biomed). Experiments were performed with adult female mice only (2–5 months old).

Tape-stripped epidermal extract

The dorsal and back skin of $SASP^{+/+}$, $SASP^{+/-}$ and $SASP^{-/-}$ hairless mice were sequentially stripped with Scotch Book Tape 10 times (3M, St. Paul, MN). Corneocytes adherent to the tape surface were eluted by 5 ml of urea-buffer and concentrated by an Amicon-ultra 10 kDa (Millipore) into 50 μl. Protein concentrations were estimated by the Bradford method. Samples were mixed with 25 μl of 3 × SDS sample buffer.

Free amino acid analysis

Surface samples of SC were obtained by adhesive tape striping (Scotch Book Tape) performed five times. Each tape corresponded to a skin area of about $3 \times 10\,cm^2$ derived from anesthetized $SASP^{+/+}$ ($n = 7$) and $SASP^{-/-}$ ($n = 11$) hairless mice. Water-soluble amino acids on the adhesive tapes were extracted with 5 ml 0.1% Triton X-100. After sonication for 30 min at 37 °C, amino acids were quantified using an amino acid analyser (Hitachi model L-8500).

Autoprocessing assay

Fifty microlitres of GST-hSASP28 mutants (1.4 mg/ml or 0.9 mg/ml) in buffer D (50 mM phosphate buffer, pH 6.0, 0.7 M NaCl) containing 1 mM EDTA and protease inhibitor cocktail) was incubated at 37°C. Five-microlitres aliquots were recovered at different times during the

incubation step, and the reaction was stopped by the addition of 20 μl Laemmli buffer. All the aliquots (5 μl) were analysed by SDS-PAGE on 15% acrylamide gels followed by staining with CBB R-250. Semi-quantification of hSASP14 was analysed densitometrically using Adobe Photoshop™ CS3.

Purification of recombinant hSASP14

All procedures were performed at 4°C. Purified GST-hSASP28 was dialysed against buffer D using NAP-10 (GE Healthcare, Japan) and concentrated and frozen at −80 °C. Next, 300 μl of concentrated GST-SASP28 (5 mg/ml) was incubated for 60 min at 37°C to commence autoprocessing. Each sample was diluted in 1 ml of buffer D and passed over 200 μl of Glutathione Sepharose 4B beads (GE Healthcare) twice. The flow-through fraction containing hSASP14 was collected and subjected to the protease assay.

Human filaggrin cleavage assay

Purified hSASP14 (419 pmol) was incubated with 3.6 μM MBP-hFilaggrin in 100 μl of buffer D in the presence of 1 mM EDTA and protease inhibitor cocktail for 60 min at 37°C. After incubation, the reactions were stopped by adding 50 μl of 3 × SDS sample buffer, and 10 μl was subjected to SDS-PAGE. Cleaved fragments were subjected to N-terminal amino acid sequencing.

Author contributions

TM conceived of the study, participated in its design and coordination, carried out the analysis of knockout mice and the biochemical studies and drafted the manuscript; KM and JK carried out the mutation search; AK participated in the design of the study and helped to draft the manuscript; HK and TE conducted the human study; KH and ST helped to analyse the knockout mice; SI carried out the electron microscopic analysis; II and JI participated in the design and coordination of the study and helped to draft the manuscript; MA conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflicts of interest.

For more information

OMIM: SKIN ASPARTIC PROTEASE:
http://www.ncbi.nlm.nih.gov/omim/611765

GeneCards:
http://www.genecards.org/cgi-bin/carddisp.pl?gene=ASPRV1

References

Barker JN, Palmer CN, Zhao Y, Liao H, Hull PR, Lee SP, Allen MH, Meggitt SJ, Reynolds NJ, Trembath RC *et al* (2007) Null mutations in the filaggrin gene (FLG) determine major susceptibility to early-onset atopic dermatitis that persists into adulthood. J Invest Dermatol 127: 564-567

Barnes KC (2010) An update on the genetics of atopic dermatitis: scratching the surface in 2009. J Allergy Clin Immunol 125: 16-29 e11–11; quiz 30-11

Bernard D, Mehul B, Thomas-Collignon A, Delattre C, Donovan M, Schmidt R (2005) Identification and characterization of a novel retroviral-like aspartic protease specifically expressed in human epidermis. J Invest Dermatol 125: 278-287

Berry N, Charmeil C, Goujon C, Silvy A, Girard P, Corcuff P, Montastier C (1999) A clinical, biometrological and ultrastructural study of xerotic skin. Int J Cosmet Sci 21: 241-252

Candi E, Tarcsa E, Digiovanna JJ, Compton JG, Elias PM, Marekov LN, Steinert PM (1998) A highly conserved lysine residue on the head domain of type II keratins is essential for the attachment of keratin intermediate filaments to the cornified cell envelope through isopeptide crosslinking by transglutaminases. Proc Natl Acad Sci USA 95: 2067-2072

Candi E, Schmidt R, Melino G (2005) The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol 6: 328-340

Dale BA, Holbrook KA, Steinert PM (1978) Assembly of stratum corneum basic protein and keratin filaments in macrofibrils. Nature 276: 729-731

Dale BA, Resing KA, Lonsdale-Eccles JD (1985) Filaggrin: a keratin filament associated protein. Ann NY Acad Sci 455: 330-342

Dale BA, Resing KA, Haydock PV (1990) Filaggrins. In: *Cellular and Molecular Biology of Intermediate Filaments*, Goldman RD and Steinert PM, (eds), New York and London, Plenum Press: pp 393-412.

Darke PL (1994) Stability of dimeric retroviral proteases. Methods Enzymol 241: 104-127

Denecker G, Hoste E, Gilbert B, Hochepied T, Ovaere P, Lippens S, Van den Broecke C, Van Damme P, D'Herde K, Hachem JP *et al* (2007) Caspase-14 protects against epidermal UVB photodamage and water loss. Nat Cell Biol 9: 666-674

Elias PM, Steinhoff M (2008) "Outside-to-inside" (and now back to "outside") pathogenic mechanisms in atopic dermatitis. J Invest Dermatol 128: 1067-1070

Engelke M, Jensen JM, Ekanayake-Mudiyanselage S, Proksch E (1997) Effects of xerosis and ageing on epidermal proliferation and differentiation. Br J Dermatol 137: 219-225

Fallon PG, Sasaki T, Sandilands A, Campbell LE, Saunders SP, Mangan NE, Callanan JJ, Kawasaki H, Shiohama A, Kubo A *et al* (2009) A homozygous frameshift mutation in the mouse Flg gene facilitates enhanced percutaneous allergen priming. Nat Genet 41: 602-608

Fluhr JW, Darlenski R, Surber C (2008) Glycerol and the skin: holistic approach to its origin and functions. Br J Dermatol 159: 23-34

Ginger RS, Blachford S, Rowland J, Rowson M, Harding CR (2005) Filaggrin repeat number polymorphism is associated with a dry skin phenotype. Arch Dermatol Res 297: 235-241

Harding CR, Scott IR (1983) Histidine-rich proteins (filaggrins): structural and functional heterogeneity during epidermal differentiation. J Mol Biol 170: 651-673

Harding CR, Watkinson A, Rawlings AV, Scott IR (2000) Dry skin, moisturization and corneodesmolysis. Int J Cosmet Sci 22: 21-52

Hildenbrand M, Rhiemeier V, Hartenstein B, Lahrmann B, Grabe N, Angel P, Hess J (2010) Impaired skin regeneration and remodeling after cutaneous injury and chemically induced hyperplasia in taps-transgenic mice. J Invest Dermatol 130: 1922-1930

Irvine AD (2007) Fleshing out filaggrin phenotypes. J Invest Dermatol 127: 504-507

Ishida-Yamamoto A, Senshu T, Eady RA, Takahashi H, Shimizu H, Akiyama M, Iizuka H (2002) Sequential reorganization of cornified cell keratin filaments involving filaggrin-mediated compaction and keratin 1 deimination. J Invest Dermatol 118: 282-287

Kamata Y, Taniguchi A, Yamamoto M, Nomura J, Ishihara K, Takahara H, Hibino T, Takeda A (2009) Neutral cysteine protease bleomycin hydrolase is essential for the breakdown of deiminated filaggrin into amino acids. J Biol Chem 284: 12829-12836

Leyvraz C, Charles RP, Rubera I, Guitard M, Rotman S, Breiden B, Sandhoff K, Hummler E (2005) The epidermal barrier function is dependent on the serine protease CAP1/Prss8. J Cell Biol 170: 487-496

List K, Szabo R, Wertz PW, Segre J, Haudenschild CC, Kim SY, Bugge TH (2003) Loss of proteolytically processed filaggrin caused by epidermal deletion of Matriptase/MT-SP1. J Cell Biol 163: 901-910

Matsui T, Hayashi-Kisumi F, Kinoshita Y, Katahira S, Morita K, Miyachi Y, Ono Y, Imai T, Tanigawa Y, Komiya T *et al* (2004) Identification of novel keratinocyte-secreted peptides dermokine-alpha/-beta and a new stratified epithelium-secreted protein gene complex on human chromosome 19q13.1. Genomics 84: 384-397

Matsui T, Kinoshita-Ida Y, Hayashi-Kisumi F, Hata M, Matsubara K, Chiba M, Katahira-Tayama S, Morita K, Miyachi Y, Tsukita S (2006) Mouse homologue of skin-specific retroviral-like aspartic protease involved in wrinkle formation. J Biol Chem 281: 27512-27525

McGrath JA, Uitto J (2008) The filaggrin story: novel insights into skin-barrier function and disease. Trends Mol Med 14: 20-27

Mechin MC, Enji M, Nachat R, Chavanas S, Charveron M, Ishida-Yamamoto A, Serre G, Takahara H, Simon M (2005) The peptidylarginine deiminases expressed in human epidermis differ in their substrate specificities and subcellular locations. Cell Mol Life Sci 62: 1984-1995

Nachat R, Mechin MC, Takahara H, Chavanas S, Charveron M, Serre G, 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

Nomura T, Akiyama M, Sandilands A, Nemoto-Hasebe I, Sakai K, Nagasaki A, Ota M, Hata H, Evans AT, Palmer CN *et al* (2008) Specific filaggrin mutations cause ichthyosis vulgaris and are significantly associated with atopic dermatitis in Japan. J Invest Dermatol 128: 1436-1441

Norlen L, Al-Amoudi A (2004) Stratum corneum keratin structure, function, and formation: the cubic rod-packing and membrane templating model. J Invest Dermatol 123: 715-732

Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, Goudie DR, Sandilands A, Campbell LE, Smith FJ *et al* (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

Pearton DJ, Nirunsuksiri W, Rehemtulla A, Lewis SP, Presland RB, Dale BA (2001) Proprotein convertase expression and localization in epidermis: evidence for multiple roles and substrates. Exp Dermatol 10: 193-203

Presland RB, Boggess D, Lewis SP, Hull C, Fleckman P, Sundberg JP (2000) Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris. J Invest Dermatol 115: 1072-1081

Presland RB, Rothnagel JA, Lawrence OT (2006) Profilaggrin and the fused S100 family of calcium-binding proteins. In: *In Skin Barrier*, Elias PM and Feingold KR, (eds) New York, Taylor and Francis: pp 111-140.

Rawlings AV, Harding CR (2004) Moisturization and skin barrier function. Dermatol Ther 17: 43-48

Rawlings AV, Matts PJ (2005) Stratum corneum moisturization at the molecular level: an update in relation to the dry skin cycle. J Invest Dermatol 124: 1099-1110

Resing KA, Walsh KA, Haugen-Scofield J, Dale BA (1989) Identification of proteolytic cleavage sites in the conversion of profilaggrin to filaggrin in mammalian epidermis. J Biol Chem 264: 1837-1845

Resing KA, al-Alawi N, Blomquist C, Fleckman P, Dale BA (1993) Independent regulation of two cytoplasmic processing stages of the intermediate filament-associated protein filaggrin and role of Ca^{2+} in the second stage. J Biol Chem 268: 25139-25145

Resing KA, Johnson RS, Walsh KA (1993) Characterization of protease processing sites during conversion of rat profilaggrin to filaggrin. Biochemistry 32: 10036-10045

Resing KA, Thulin C, Whiting K, al-Alawi N, Mostad S (1995) Characterization of profilaggrin endoproteinase 1. A regulated cytoplasmic endoproteinase of epidermis. J Biol Chem 270: 28193-28198

Rhiemeier V, Breitenbach U, Richter KH, Gebhardt C, Vogt I, Hartenstein B, Furstenberger G, Mauch C, Hess J, Angel P (2006) A novel aspartic proteinase-like gene expressed in stratified epithelia and squamous cell carcinoma of the skin. Am J Pathol 168: 1354-1364

Sandilands A, Terron-Kwiatkowski A, Hull PR, O'Regan GM, Clayton TH, Watson RM, Carrick T, Evans AT, Liao H, Zhao Y *et al* (2007) Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. Nat Genet 39: 650-654

Sandilands A, Sutherland C, Irvine AD, McLean WH (2009) Filaggrin in the frontline: role in skin barrier function and disease. J Cell Sci 122: 1285-1294

Sasaki T, Kudoh J, Ebihara T, Shiohama A, Asakawa S, Shimizu A, Takayanagi A, Dekio I, Sadahira C, Amagai M *et al* (2008) Sequence analysis of filaggrin gene by novel shotgun method in Japanese atopic dermatitis. J Dermatol Sci 51: 113-120

Scharschmidt TC, Man MQ, Hatano Y, Crumrine D, Gunathilake R, Sundberg JP, Silva KA, Mauro TM, Hupe M, Cho S *et al* (2009) Filaggrin deficiency confers a paracellular barrier abnormality that reduces inflammatory thresholds to irritants and haptens. J Allergy Clin Immunol 124: 496-506, 506 e491-496

Smith FJ, Irvine AD, Terron-Kwiatkowski A, Sandilands A, Campbell LE, Zhao Y, Liao H, Evans AT, Goudie DR, Lewis-Jones S *et al* (2006) Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. Nat Genet 38: 337-342

Steinert PM, Marekov LN (1995) The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope. J Biol Chem 270: 17702-17711

Tarcsa E, Marekov LN, Mei G, Melino G, Lee SC, Steinert PM (1996) Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. J Biol Chem 271: 30709-30716

Thulin CD, Walsh KA (1995) Identification of the amino terminus of human filaggrin using differential LC/MS techniques: implications for profilaggrin processing. Biochemistry 34: 8687-8692

Thulin CD, Taylor JA, Walsh KA (1996) Microheterogeneity of human filaggrin: analysis of a complex peptide mixture using mass spectrometry. Protein Sci 5: 1157-1164

Wakeland E, Morel L, Achey K, Yui M, Longmate J (1997) Speed congenics: a classic technique in the fast lane (relatively speaking). Immunol Today 18: 472-477

Watt FM (1989) Terminal differentiation of epidermal keratinocytes. Curr Opin Cell Biol 1: 1107-1115

Wilhelm KP, Cua AB, Maibach HI (1991) Skin aging. Effect on transepidermal water loss, stratum corneum hydration, skin surface pH, and casual sebum content. Arch Dermatol 127: 1806-1809

Yamazaki M, Ishidoh K, Suga Y, Saido TC, Kawashima S, Suzuki K, Kominami E, Ogawa H (1997) Cytoplasmic processing of human profilaggrin by active mu-calpain. Biochem Biophys Res Commun 235: 652-656

