Mouse Homologue of Skin-specific Retroviral-like Aspartic Protease Involved in Wrinkle Formation*

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Retroviral proteases are encoded in the retroviral genome and are responsible for maturation and assembly of infectious virus particles. A number of retroviral protease sequences with retroviral elements are integrated in every eukaryotic genome as endogenous retroviruses. Recently, retroviral-like aspartic proteases that were not embedded within endogenous retroviral elements were identified throughout the eukaryotic and prokaryotic genomes. However, the physiological role of this novel protease family, especially in mammals, is not known. During the high throughput in situ hybridization screening of mouse epidermis, as a granular layer-expressing clone, we identified a mouse homologue of SASPase (Skin ASpartic Protease), a recently identified retroviral-like aspartic protease. We detected and purified the endogenous 32-kDa (mSASP32) and 15-kDa (mSASP15) forms of mSASP from mouse stratum corneum extracts and determined their amino acid sequences. Next, we bacterially produced recombinant mSASP15 via autoprocessing of GST-mSASP32. Purified recombinant mSASP15 cleaved a quenched fluorogenic peptide substrate, designed from the autoprocessing site for mSASP32 maximally at pH 5.77, which is close to the pH of the epidermal surface. Finally, we generated mSASP-deficient mice that at 5 weeks of age showed fine wrinkles that ran parallel on the lateral trunk without apparent epidermal differentiation defects. These results indicate that the retroviral-like aspartic protease, SASPase, is involved in prevention of fine wrinkle formation via activation in a weakly acidic stratum corneum environment. This study provides the first evidence that retroviral-like aspartic protease is functionally important in mammalian tissue organization.

Proteases play an important role in many physiological processes by regulating the activation, synthesis, and turnover of proteins (1). Proteases are classified into five distinct classes as follows: aspartic, metallo-, cysteine, serine, and threonine proteases. Of these, aspartic proteases, also designated as acidic proteases, are a widely distributed family of proteolytic enzymes known to exist in vertebrates, invertebrates, fungi, plants, and retroviruses (2, 3). Eukaryotic aspartic proteases are ~330-residue monomeric enzymes that consist of two homologous domains. Each domain contains an active site centered on a catalytically essential aspartic residue. These enzymes are synthesized aszymogens that are subsequently proteolytically processed. On the other hand, retroviruses are known to encode aspartic proteases in the viral genome (4). This retroviral aspartic protease is expressed as part of a large polypeptide precursor, with the mature protease released via autoprocessing activity. The primary structure of the enzyme corresponds to a single domain that undergoes autoprocessing in viral particles to produce an active one that exists as a homodimer.

Recent analysis of human, mouse, and rat genomes have revealed that 553, 628, and 626 genes, respectively, are encoded as proteases or protease homologues (5, 6). In the human genome, 21 genes are eukaryotic aspartic proteases, and more than 150 sequences are related to aspartic proteases that are embedded in endogenous retroviral elements as human endogenous retroviruses (5). Most of these human endogenous retroviruses are believed to have been acquired 10–100 million years ago (7). Previously, Krylov and Koonin (8) described new subfamilies of predicted retroviral-like aspartic proteases that include several human paralogues. These genes encode enzymes with some similarity to retroviral aspartic proteases, although they are not embedded within endogenous retroviral elements. They included the DNA damage-inducible UbL-Uba proteins Ddi1p and Ddi2p, neuron-specific nuclear receptors NIX1, NRIP2, and NRIP3, and other Ddi1p-related aspartic proteases (5, 6, 9–12). They were encoded not only in eukaryotes but also prokaryotes.

Recently, Bernard et al. (13) have separated total protein extracts of human reconstructed epidermis and identified a novel retroviral-like aspartic protease, SASPase (Skin ASpartic Protease). Human SASPase (hSASP) was expressed in the granular layer of human epidermis, and immunoblotting of

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¶ The abbreviations used are: hSASP, human SASPase; mSASP, mouse SASP; Nma, 2-(N-methylamino)benzoyl; Dnp, 2,4-dinitrophenyl; RT, reverse transcription; PBS, phosphate-buffered saline; EST, expressed sequence tag; GST, glutathione S-transferase; pAb, polyclonal antibody; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; nano-ESI-MS/MS, matrix-assisted laser desorption/ionization tandem mass spectrometry; MS, mass spectrometry; HIV, human immunodeficiency virus; SC, stratum corneum; SCE, stratum corneum extracts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rmSASP, recombinant mSASP; ES, embryonic stem.
human epidermal extract revealed the expression of two forms of the enzyme, the 28- and 14-kDa forms. Similar to other retroviral proteases, recombinant SASPase undergoes auto-activation processing in vitro, and subsequently a 14-kDa protein is generated. The amino acid sequences of this recombinant 14-kDa product (hSASP14) were determined and shown to correspond with the protease domain. However, it was not clarified whether hSASP14 possessed protease activity, and its optimum pH was not determined. More importantly, the physiological role of SASPase is uncertain.

We have previously performed high throughput in situ hybridization screening against sections of mouse foot sole epidermis using an equalized mouse back skin cDNA library (14). Among them, we identified a mouse homologue of SASPase as a clone specifically expressed in the granular layer of epidermis. Here we report the purification of processed forms of endogenous mouse SASPase (mSASP) from mouse stratum corneum extracts (SCE) and the determination of their amino acid sequences. From these sequences, we succeeded in producing active recombinant protein in bacteria and performed biochemical analysis. Furthermore, to study the physiological role of SASPase, we generated SASPase-deficient mice. They unexpectedly formed fine skin wrinkles. Our data are the first evidence that genome-integrated retroviral-like aspartic protease is functionally important in mammalian tissue architecture.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotide primers were purchased from Proligo Japan (Kyoto, Japan). N-terminal amino acid sequence analysis and mass spectrometric analysis were performed by ApoScience Co. Ltd. (Tokushima, Japan). Peptide (Nma-LFANSMG-K(Dnp)r-NH₂) was synthesized and purified by high pressure liquid chromatography by Peptide Inc. (Osaka, Japan) and dissolved in Me₂SO. FRET-25-STD1 and FRET-25-STD2 were also purchased from Peptide Inc. Generation of knock-out mice was performed by Kurabo, Inc. (Osaka, Japan).

All animal studies have been approved by the Institutional Review Board of the KAN Research Institute, Inc.

**In Situ Hybridization**—In situ hybridization was performed as described previously (14, 15).

cDNA Cloning and Recombinant Protein Expression—Mouse back skin total RNA was isolated from 8-week-old female BALB/c mice according to the method described by Chomczynski and Sacchi (16). First strand cDNA was prepared by Superscript II reverse transcriptase (Invitrogen) from mouse back skin total RNA. The DNA fragment encoding open reading frame of the mouse SASPase 32-kDa form (mSASP32) was amplified from a mouse back skin cDNA library by PCR using 5'-EcoRI-mSASP32 primer (5’-ATATGAAATTCGCCACC-ATGCCCACCCCGGTAGCG-3’) and 3’-NotI-mSASP32 primer (5’-AATTGGCGCCCTTAGTGGGACCGCTCCG- GTG), respectively. These primers were designed using the sequence from GenBank accession number BC057938. After digestion with EcoRI and NotI, the cDNA was subcloned into EcoRI-NotI sites of pGEX4T-1 (GE Healthcare) to yield pGEX-mSASP32. Protease-dead mutant, pGEX-mSASP32(D210A), was generated by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) from pGEX-mSASP32 by mutating the active aspartic acid residue (amino acid 210) to alanine.

To prepare antibodies against mSASP, the cDNA fragment encoding residues 182–300 of mSASP32(D210A) (mSASP-(182–300)(D210A)) was amplified from pGEX-mSASP32(D210A) by PCR using 5’-Sall-mSASP-PR1 primer (5’-ATATGAAATTCGCCACC-ACCGAAGAGATTTGTGGCACCAGC-3’) and 3’-NotI-mSASP-PR1 primer (5’-AATTCGGCCGGTGCCGGTGGTT- CGAAGTCCAGCAC-3’). To produce GST fusion protein of mSASP-(182–300)(D210A), the PCR product was digested with Sall and NotI and then subcloned into Sall-NotI sites of pGEX4T-3 to yield pGEX-mSASP-(182–300)(D210A). To produce His-tagged mSASP-(182–300)(D210A), the NotI site was introduced between Xhol and Pstl sites of pRSET-A (Invitrogen) to yield pRSET-A(NotI), and the Sall/NotI-digested PCR-product was subcloned into Xhol-NotI sites of pRSET-A(NotI) to yield pRSET-mSASP-(182–300)(D210A).

Recombinant protein, GST-mSASP32, GST-mSASP32-(D210A), GST-mSASP-(182–300)(D210A), and His₆-mSASP-(182–300)(D210A) were produced and purified according to the manufacturer’s instructions. Purified GST-mSASP-(182–300)(D210A) was cleaved with thrombin to remove the GST to yield the mSASP-(182–300)(D210A) protein by the thrombin cleavage capture kit (Novagen, Madison, WI).

Northern Blotting and Quantitative Real Time RT-PCR—Northern blotting and quantitative real time RT-PCR were performed as described previously (14). The primer set was as follows: mouse mSASP (BC057938), forward primer (5’-AGA-GGCTATTATTGGCACAGACGTC-3’) and reverse primer (5’-GGAGCAGGCGAATCTTCC-3’). Antibodies—The pAb specific for the C-terminal domain of mSASP32 was produced in rabbits against synthetic peptide (EDFLELIEEEGGSSAPEGHS) corresponding to the amino acids 320–339 of mSASP32 (mSASP-C pAb). The mSASP-C pAb was affinity-purified on this peptide covalently coupled to thiopropyl-Sepharose 6B (GE Healthcare). This pAb specifically recognized mSASP32 by immunoblotting.

We raised two rabbit antisera specific for the protease domain of mSASP, mSASP-PR1 and mSASP-PR2 pAbs (where PR stands for protease domain). The mSASP-PR1 pAb was raised against the mSASP-(182–300)(D210A) protein and was affinity-purified on His₆-mSASP-(182–300)(D210A) covalently coupled to a Hitrap NHS-activated HP 1-ml column (GE Healthcare). This pAb specifically recognized both mSASP32 and mSASP15 by immunoblotting. mSASP-PR2 pAb was raised against GST-mSASP32(D210A) and was affinity-purified on His₆-mSASP-(182–300)(D210A) as described above. This pAb specifically recognized both mSASP32 and mSASP15 by immunoprecipitation. The flow-through fraction of antiserum during affinity purification of mSASP-PR2 pAb was used for immunoabsorption of mSASP32 (mSASP-N pAb antiserum). Mouse monoclonal antibody to keratin 1/10 (clone K8.60) was purchased from Sigma. Rabbit antibodies to keratin 14, keratin 1, involucrin, filaggrin, and loricrin were purchased from Covance (Denver, PA). A rabbit antibody against desmoglein 1 was purchased from Santa Cruz Biotechnology (Delaware, CA).

Preparation of SCE from Nude Mice—Mice (8-week-old female BALB/c nude mice) were first anesthetized, and the sur-
face of the skin was washed with 10 ml per mouse of a SCE buffer (50 mM sodium phosphate buffer, pH 7.0, 5 mM EDTA, 150 mM NaCl and 0.1% Tween 20 supplemented with protease inhibitor mixture (Nakalai Tesque, Tokyo, Japan)). Thereafter, the washed area was scraped with the edge of a microscope slide and continuously rinsed with SCE buffer. The buffer containing corneocytes was collected in a container placed below the mice. Corneocytes were removed by centrifugation at 3,000 × g for 30 min at 4 °C and then ultracentrifuged at 100,000 × g for 1 h at 4 °C. The supernatant was passed over a Millex-GV (0.45 μm; Millipore, Billerica, MA) and then over a Millex-HV (0.22 μm; Millipore). The resulting SCE was ~0.25 mg/ml.

**Immunoprecipitation of mSASP32/15 and N-terminal Amino Acid Sequence Analysis**—First, 0.3 ml of protein G-Sepharose 4B fast flow (GE Healthcare) was incubated with 0.3 mg of mSASP-PR2 pAb for 1 h at 4 °C. After washing three times with SCE buffer, beads were incubated with 15 ml (3.75 mg of protein) of nude mouse SCE for 1 h at 4 °C. The beads were then extensively washed with PBS. The bound proteins were eluted by boiling the beads in an SDS sample buffer (62 mM Tris-Cl, pH 6.7, 3% SDS and 5% glycerol) for 5 min. The samples were separated by SDS-PAGE and stained by 2D Silver Stain II (DAIICHI Pure Chemicals, Tokyo, Japan) or Coomassie staining solution (Bio-Rad). The mSASP32 and mSASP15 bands were excised from the gel and subjected to N-terminal amino acid sequencing.

**Purification of mSASP15 from Mouse SCE and C-terminal Amino Acid Analysis**—5 mg of mSASP-PR2 pAb was coupled to 0.8 ml of Hitrap NHS-activated HP column (GE Healthcare) according to the manufacturer’s instruction and equilibrated with SCE buffer (mSASP-PR2 pAb column). 3 ml of mSASP-N pAb antiserum was incubated with 1 ml of protein G-Sepharose 4B fast flow for 4 h at 4 °C and equilibrated with SCE buffer. 30 ml of mouse SCE was incubated with these beads for 1 h at 4 °C to immunodeplete mSASP32. The absence of mSASP32 was confirmed by immunoblotting with mSASP-C pAb. After removal of mSASP32-bound beads, mSASP32-depleted SCE was then applied to the mSASP-PR2 pAb column and washed with 50 mM sodium phosphate, pH 7.0, containing 1 mM NaCl, and bound mSASP15 was eluted with 0.1 M glycine, pH 2.0. Eluted mSASP15 was immediately neutralized with 1 M Tris-Cl, pH 9.5, and concentrated with an Ultrafree-0.5 centrifugal filter and into BioMax-5K NMWL; Millipore) and then dialyzed against 20 mM Tris-Cl, pH 7.5.

**Mass Spectrometry**—Molecular mass of purified mSASP15 was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra in a Voyager-DE STR (Applied Biosystems), operated in the linear mode. For determination of C-terminal amino acid sequence of mSASP15, purified mSASP15 was subjected to proteolysis of lysyl endopeptidase at 35 °C for 20 h. This digest was desalted on a C18 ZipTip (Millipore), eluted in 2,5-diiodohydroxybenzoic acid, and subjected to MALDI-TOF MS analysis. The rest of the lysyl endopeptidase digest was also desalted on C18 ZipTip and eluted in 50% acetonitrile containing 1% formic acid and subjected to nano-electrospray ionization mass spectrometry (nano-ESI MS) in a Q-TOF2 mass spectrometer (Waters Micromass, Manchester, UK). The selected precursor ion was further analyzed by nano-electrospray ionization tandem mass spectrometry (nano-ESI MS/MS). In collision-induced dissociation experiments (collision gas argon, collision energy 30–40 eV), product ions were analyzed by the orthogonal TOF analyzer.

**Immunofluorescence**—Mouse side skin was fixed in 2% paraformaldehyde/PBS for 1 h at room temperature. Next, samples were incubated with 10% sucrose in PBS for 3 h at 4 °C and then 20% sucrose in PBS overnight at 4 °C. Finally, samples were mounted in Tissue-Tek O.C.T. compound (Sakura Fine-technical, Tokyo, Japan) and frozen on dry ice. Frozen samples were cut into 5-μm thick sections (10-μm sections for confocal imaging) on a cryostat and put on silan-coated glass slides and air-dried. These samples were soaked in Block-Ace blocking solution (Dainippon Pharmacy, Osaka, Japan) for 1 h at room temperature and subsequently incubated in primary antibodies for 1 h at room temperature. Sections were washed three times with PBS and incubated with secondary antibodies for 30 min at room temperature. Alexa 488-labeled goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) and SYTOX Green (Molecular Probes) were used as secondary antibodies. Samples were washed three times with PBS and mounted in PBS containing 50% glycerol. Photographs were recorded using a photomicroscope with an Olympus IX70 through a cooled CCD camera (model ORCA-ER; Hamamatsu Photonics K.K., Hamamatsu, Japan) controlled by Aquacosmos software (Hamamatsu Photonics). Confocal imaging was performed using LSM510 confocal laser scanning microscope (version 2.3; Carl Zeiss Inc., Jena, Germany).

**Purification of Recombinant mSASP15 (rmSASP15)**—All procedures were performed at 4 °C, and chromatography was carried out on an AKTAexplorer 10S chromatography system (GE Healthcare). We produced rmSASP15 via autoprocessing of GST-mSASP32 expressed in Escherichia coli. 500 ml of E. coli culture expressing GST-mSASP32 was collected by centrifugation, and cell pellets were dissolved in 9 ml of buffer H (50 mM sodium acetate, pH 5.5, 1 mM EDTA, 0.15 M NaCl, 0.1% Triton X-100) containing protease inhibitor mixture (Nakalai Tesque, Japan). After sonication, samples were centrifuged at 100,000 × g for 1 h, and the supernatant was then frozen at ~80 °C. 2 ml of supernatant was thawed and dialyzed against buffer A (50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA) by using NAP-10 (GE Healthcare). The sample was passed over a HiTrap SP HP, 1 ml (GE Healthcare), equilibrated with buffer A at a flow rate of 1 ml/min. After washing with 3 column volumes of buffer A, bound rmSASP15 was eluted with a 6-ml linear gradient of NaCl (0–0.25 M) in buffer A, and fractions of 0.4 ml each were collected. Fractions containing rmSASP15 (fractions 1–16) were pooled and dialyzed against buffer B (50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA) and concentrated with Ultrafree-0.5 centrifugal filter and tube (BioMax-5K NMWL; Millipore) and then dialyzed against 20 mM Tris-Cl, pH 7.5.

**Mass Spectrometry**—Molecular mass of purified mSASP15 was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra in a Voyager-DE STR (Applied Biosystems), operated in the linear mode. For determination of C-terminal amino acid sequence of mSASP15, purified mSASP15 was subjected to proteolysis of lysyl endopeptidase at 35 °C for 20 h. This digest was desalted on a C18 ZipTip (Millipore), eluted in 2,5-diiodohydroxybenzoic acid, and subjected to MALDI-TOF MS analysis. The rest of the lysyl endopeptidase digest was also desalted on C18 ZipTip and eluted in 50% acetonitrile containing 1% formic acid and subjected to nano-electrospray ionization mass spectrometry (nano-ESI MS) in a Q-TOF2 mass spectrometer (Waters Micromass, Manchester, UK). The selected precursor ion was further analyzed by nano-electrospray ionization tandem mass spectrometry (nano-ESI MS/MS). In collision-induced dissociation experiments (collision gas argon, collision energy 30–40 eV), product ions were analyzed by the orthogonal TOF analyzer.
FIGURE 1. Identification of mouse SASPase. A, in situ hybridization signal obtained with the SK082D11 probe in sections of adult mouse foot pad epidermis. The antisense SK082D11 probe gives an intense signal in the granular layer of epidermis (Antisense), whereas no signal is detected with the sense probe (Sense). HE, hematoxylin-eosin staining. A dashed line represents the border between the epidermis and dermis. Scale bar, 100 μm. B, mouse EST containing the SK082D11 sequence. One mouse EST (BC057938) contained the whole sequence of SK082D11. Comparison with genomic sequence revealed that 5’–17 nucleotides of BC057938 did not exist in the mouse genome; thus we corrected the 5’-end of BC057938 (Corrected BC057938). The open reading frame (ORF) is represented as a box. C, amino acid sequences of mouse and human SASPase. Mouse SASPase protein was deduced from the corrected BC057938. Putative retroviral type aspartic protease sequences predicted by the PROSITE search are underlined. Active site aspartic acids are denoted by squares. Putative transmembrane domains are underlined with dashed lines. Other possible first methionines are denoted by circles. The sequence data for mouse SASPase has been submitted to GenBank[1] under the accession number DQ841260. The determined processing sites of endogenous mouse SASPase purified from mouse SCE are denoted by arrowheads (see Fig. 2). Processing sites of recombinant human SASPase determined from the in vitro autoprocessing product of recombinant protein are also denoted by arrows (13). Note that the processing sites on either side of the protease domain are completely conserved between mouse and human SASPase.
Samples were then passed over a Hitrap SP HP, 1 ml equili-
brated with buffer C, and washed with 3 column volumes of
buffer C. Bound rmSASP15 was eluted with a 6-ml linear gra-
dient of NaCl (0–0.25 M) in buffer C. Purified rmSASP15 (frac-
tions 13–22) was pooled and concentrated as described above.

For gel filtration, purified rmSASP15 was fractionated (0.5
ml/min) by Superdex 75 HR 10/30 gel filtration chromatogra-
phy (GE Healthcare) and equili-
brated in buffer C, and fractions of
0.5 ml each were collected.

**Peptide Cleavage Assay**—For
determination of specific activity,
purified rmSASP15 (130 pmol) was
incubated with 0.1 mM peptide
substrate (Nma-LFANSMG-K(D-
np)rrr-NH₂) in 100 μl of buffer A
(50 mM sodium acetate, pH 5.29,
0.15 M NaCl), buffer B (50 mM
sodium phosphate, pH 7.65, 0.15 M
NaCl), or buffer C (50 mM sodium
acetate, pH 5.38, 0.7 M NaCl) for
indicated times at 37 °C. For deter-
mination of the optimum pH, puri-
fied rmSASP15 (25 pmol) was incu-
bated with 0.1 mM peptide substrate
in 100 μl of buffer D (50 mM sodium
acetate, pH 4.32 or pH 4.96, 0.7 M
NaCl) or buffer E (50 mM sodium ace-
tate, pH 5.41–7.59, 0.7 M NaCl) for
60 min at 37 °C. All buffers contained 1
mM EDTA and protease inhibitor
mixture (Nakalai Tesque) that con-
sisted of 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, E-64,
and leupeptin to completely inhibit the
activities of serine and cysteine prote-
ase. After incubation, fluorescence
was measured using a VICTOR2
(PerkinElmer Life Sciences) with the
excitation and emission wavelengths
at 355 and 460 nm, respectively. Spe-
cific activities were calculated accord-
ing to the fluorescence of FRETS-25-
STD1 and FRETS-25-STD2 (Peptide
Inc.).

**Generation of Knock-out Mice**—
The mSASP gene is encoded by a
single exon. The 2.2-kb fragment
upstream of mSASP was amplified by
genomic PCR using the following
primers: 5'-AATTCCGCGGAT-
CCTGGGCTGTAGCGTGAGTTT-CAG-3'
and 5'-ATATCCCGGATCTTGAACTTCAGGGAGCGACGTCCT-3'
from

**FIGURE 2.** Expression patterns of mouse SASPase mRNA. A, Northern blotting. Nylon membranes blotted with total RNA (20 μg) from various tissues of 8-week-old female BALB/c mice were probed with digoxigenin-labeled cRNA fragment of the SK082D11 cDNA (Mouse SASPase). Control GAPDH-specific probe was also hybridized (GAPDH). mSASP is expressed in the stomach and skin and weakly in the lung. B, quantitative RT-PCR analysis of the expression of mSASP in various epithelial tissues. Total RNA was prepared from various epithelial tissue of 8-week-old female BALB/c mice, and SYBR green-based quantitative real-time RT-PCR was performed with specific primers for mSASP. All data were normalized to an internal GAPDH mRNA control. Data represent means of three independent experiments. - , +, ++, ++++, +++++, ++++++ and ++++++++ represent expression levels as ratios to GAPDH (× 10⁻³) of 0–0.1, 0.1–1, 1–10, 10–100, 100–1000, 1000–2000, and 2000–3000. C, expression of mSASP during mouse embryonic development. Mouse embryo full stage blot (Seegene, Seoul, Korea) was probed with digoxigenin-labeled cRNA fragment of SK082D11. mSASP (arrow) begins to be expressed at embryonic day 15.5 (E15.5). The lower panel is the ethidium bromide-stained membrane.
exon of mSASP to the pgk-neo cassette, respectively. J1 ES cells were electroporated with the targeting vector and selected in the presence of G418. The G418-resistant colonies were removed and screened by Southern blotting with the 3' H11032 external probe (Fig. 5A). Correctly targeted ES clones were identified by an additional 2.7-kb band together with the 6.8-kb band of the wild type allele when digested with EcoRI. The targeted ES cells obtained were injected into C57BL/6 blastocysts, which were in turn transferred into BALB/c foster mothers to obtain chimeric mice. Male chimeras were mated with C57BL/6 females, and agouti offspring were genotyped to confirm the germ line transmission of the targeted allele. The littermates were genotyped by Southern blotting. Heterozygous mice were then interbred to produce homozygous mice.

Removal of Hair—The mice were anesthetized, and hair was removed by shaving, and hair remover (Kanebo, Tokyo, Japan) was rubbed on. After 10 min, the hair remover was rubbed off, and Acid Care Lotion (Kanebo) was applied to the surface of the skin.

**FIGURE 3.** Localization, purification, and amino acid analysis of mSASP in mouse SC. A, frozen sections of back skin (a–d), foot pad skin (e–h), forestomach (i–k), and vagina (m–p) of 8-week-old female BALB/c mice were doubly stained with anti-keratin 1/10 monoclonal antibody (green; a, e, i, and m) and mSASP-C pAb (red; b, f, j, and n) or anti-keratin 1/10 monoclonal antibody (green; c, g, k, and o) and anti-loricrin pAb (red; d, h, l, and p). Nuclei were counterstained with bisbenzimide (blue). mSASP32 and loricrin is detected exclusively in the granular layer of epidermis, whereas keratin 1/10 is localized in the differentiated layer. Dashed lines represent the border between the epidermis and dermis. Scale bar, 50 μm. B, immunoblotting of mSASP with anti-mSASP-specific antibody. 5 μg of nude mouse SCE was subjected to SDS-PAGE and immunoblotted with mSASP-PR1 (left) and mSASP-C (right) pAbs, respectively. Note that mSASP32 (arrowheads) is detected by both antibodies, whereas mSASP15 (arrow) is only detected by mSASP-PR1 pAb. C, immunoprecipitation (IP) of mSASP32 and mSASP15. mSASP32 and mSASP15 were immunoprecipitated with mSASP-PR2 pAb, and precipitated sample was subjected to SDS-PAGE and silver-stained (Silver staining). The asterisk denotes IgG (IgG). The mSASP32 and mSASP15 were subjected to N-terminal amino acid sequencing and determined as SKEGR and SMGKG, respectively. D, immunoaffinity purification of mSASP15 from nude mice SCE. mSASP15 was purified by immunoaffinity column of mSASP-PR2 from mSASP32-depleted SCE (see “Experimental Procedures”), subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue (Coomassie). Purified mSASP15 was then subjected to MALDI-TOF analysis. The rest of the purified mSASP15 was further treated with lysyl endopeptidase and subjected to both MALDI-TOF and nano-ES-MS/MS analysis. From these analyses, it was deduced that the amino acid sequence of mSASP15 ended with EFDLE. E, schematic diagram of processing of mSASP in mouse SC. mSASP is expressed as mSASP32 (91–339 amino acids) in mouse SC, and mSASP15 (189–324 amino acids) is cleaved out. mSASP15 only consists of a protease domain.
SASPase Knock-out Mice

A

GST-mSASP32

GST-mSASP32 (D210A) (+, IPTG)

mSASP-PR1 pAb

immunoblotting

B

1 2 3 4 5

(kDa)

Silver staining

C

mSASP32

PR

91

339

185

188

189

192

Leu - Phe - Ala - Asn - Ser - Met - Gly - Lys

D

0.7 M NaCl

pH5.38

Relative fluorescence intensity

0.15 M NaCl

pH5.29

Time (min)

E

20000

pH5.77

0.7 M NaCl 60 min

Relative fluorescence intensity

4.0

5.0

6.0

7.0

8.0

pH
Preparation of Cornified Envelopes—For preparation of cornified envelopes, the tip of the ear was cut, placed in water containing 25 mM dithiothreitol and 2% SDS, heated to 100 °C for 15 min, and centrifuged. The pellet was resuspended in 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA. Envelopes were examined in a hemocytometer under phase microscopy.

Epidermal Protein Extraction and Immunoblot Analysis—The epidermis and dermis were separated by heating the skin for 5 min at 54 °C in 5 mM EDTA in PBS. Separated epidermis was extracted in a buffer containing 62.5 mM Tris-Cl, pH 6.8, 2% glycerol, 1% SDS, 5 mM EDTA, and a protease inhibitor mixture (Nakalai Tesque) followed by sonication on ice 5 times for 3 s and centrifuged at 15,000 × g for 20 min at room temperature. The supernatant was used as epidermal extract. Proteins were separated by SDS-PAGE on 15% acrylamide gels. After electrophoresis, proteins were electrophoretically transferred from gels onto nitrocellulose membranes that were then incubated with the first antibody. Bound antibodies were visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG and the appropriate substrate as described by the manufacturer (GE Healthcare).

RESULTS

To identify genes involved in epidermal differentiation, we previously performed “high throughput in situ hybridization screening” of ~10,000 genes expressed in mouse skin and identified 116 unique clones that were expressed in a layer-specific manner in mouse foot pad epidermis (14). Among them, we identified a novel cDNA fragment, SK082D11, of which the in situ hybridization signal was specifically detected in the granular layer (Fig. 1A). To obtain a full-length cDNA clone, the publicly available mouse EST data base was searched, and an EST was identified (BC057938) that contained the entire sequence of SK082D11 (Fig. 1B). Close comparison of BC057938 to the mouse genome data base revealed that 17 nucleotides of the 5’-end were not present. The deduced amino acid sequence of the corrected BC057938 revealed that it encoded 339 amino acids. Searching the publicly available data bases with these predicted amino acids revealed that it was a mouse homologue of human SASPase, a recently identified retroviral-like skin-specific aspartic protease (13). The PROSITE search also predicted a retroviral type aspartic protease signature with aspartic acid 210 as the putative active amino acid site. At the amino acid sequence level, mSASP and hSASP showed 72% identity (Fig. 2A).

Activity of mSASP15. A, autoproCESSing activity of GST-mSASP32 in E. coli. E. coli lysates expressing GST-mSASP32/mSASP32(D210A) before (−) and after (+) 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) induction were immunoblotted with mSASP-PRI pAb. Note that the 15-kDa form (mSASP15; arrowhead in left panel) is produced in lysates expressing GST-mSASP32 (arrow in left panel) but not in GST-mSASP32(D210A) (arrow in right panel). B, purification of mSASP15 from GST-mSASP32-expressing bacterial lysates. Fractions (10 µl) from all steps of the purification process were subjected to SDS-PAGE and followed by silver staining (Silver staining; lanes 1–5) and Coomassie Brilliant Blue staining (Coomassie; lane 6). Lane 1, supernatant after ultracentrifugation; lane 2, eluate of Hitrap SP column chromatography, pH 6.6; lane 3, eluate of Hitrap Q column chromatography, pH 7.0; lane 4, eluate of Hitrap SP column chromatography, pH 6.0; lane 5 and 6, eluate of Superdex 75 gel filtration column chromatography. mSASP15 was purified to near-homogeneity (arrows). C, structure of fluorogenic substrate, Nma-LFANSMG-K(Dnprr)-NH2. The fluorogenic peptide substrate was designed from the cleavage site between Asn188 and Ser189 of mSASP32. Arrow denotes the cleavage sites. Three d-amino acid Lys residues are included to increase solubility of the peptide. D, time course of the hydrolysis of the fluorogenic peptide substrate. The purified mSASP15 (130 pmol) was incubated with 0.1 mM fluorogenic peptide substrate in 50 mM sodium acetate buffer, pH 5.29, containing 0.15 M NaCl (filled circles), 50 mM sodium acetate buffer, pH 5.38, containing 0.7 M NaCl (triangles), and 50 mM sodium phosphate buffer, pH 7.65, containing 0.15 M NaCl (open circles) for indicated times at 37 °C, and hydrolysis of the peptide was measured. All the buffers contained 0.1 mM EDTA and protease inhibitors. Under the same ionic strength (0.15 M NaCl), mSASP15 shows higher protease activity at pH 5.29 than at pH 7.65. Under similar pH conditions (pH 5.29 and 5.38), mSASP15 shows higher activity in 0.7 M NaCl than in 0.15 M NaCl. E, effect of pH on the activity of mSASP15 against the fluorogenic peptide substrate. Purified rmSASP15 (25 pmol) was incubated with 0.1 mM fluorogenic peptide in 50 mM sodium acetate (pH 4.32 and 4.96) or sodium phosphate (pH 5.41–7.59) buffers containing 0.7 M NaCl and 1 mM EDTA for 60 min at 37 °C, and the hydrolysis of the peptide was measured. The maximal rate of hydrolysis was achieved at pH 5.77.
depleted SCE was passed over an mSASP-PR2 pAb immunoaffinity column, and bound mSASP15 was eluted and revealed to be purified to near-homogeneity (Fig. 3D). Purified mSASP15 was subjected to MALDI-TOF MS analysis. MALDI-TOF MS spectra showed a protonated molecule at m/z 14601.63. This signal could only correspond to 189–324 amino acids of mSASP (data not shown). To further confirm these results, purified mSASP15 was completely digested with lysyl endopeptidase and then subjected MALDI-TOF analysis and simultaneously to ESI-Q-TOF MS/MS analysis (data not shown). These analyses also showed that the amino acid sequence of purified mSASP15 is 189–324 amino acids (Fig. 3, D and E).

FIGURE 5. Generation of mSASP-deficient mice. A, restriction maps of the wild type allele, the targeting vector, and the targeted allele of the mSASP gene. The targeting vector contained the pgk-neo cassette in its middle portion to delete the entire exon in the targeted allele. The position of the probe for Southern blotting is indicated as a bar (Probe). S, SalI; E, EcoRI; RV, EcoRV. B, genotype analysis by Southern blotting of EcoRI-digested genomic DNA from wild type (+/+), heterozygous (+/−), and homozygous (−/−) mice for mSASP gene allele. Southern blotting with the probe indicated in A yields 6.8- and 2.7-kb bands from the wild type and targeted allele, respectively. C, loss of mSASP protein in the epidermal extract of mSASP−/− mice examined by immunoblotting. Anti-mSASP immunoblotting of the epidermal protein extracts of mSASP-deficient mice. Epidermal protein extracts (10 μg) from mSASP1/1 (+/+), mSASP1/2 (+/−), and mSASP1/− (−/−) mice were immunoblotted with mSASP-C pAb. In the wild type and heterozygous epidermal extract, mSASP32 bands are detected, whereas in the homozygous one, this band is not detected. D, loss of mSASP protein in the epidermis of mSASP−/− mice examined by immunofluorescence microscopy. Frozen sections of back skin of mSASP1/1 (+/+) and mSASP1/2 (−/−) mice were stained with mSASP-C pAb (red). Nuclei were counterstained with SYTOX-green (green). Images were visualized by confocal laser scanning microscope. In the wild type epidermis, mSASP protein is expressed in the granular layer of epidermis, whereas in the homozygous epidermis, these signals become undetectable. Dashed lines represent the border between the epidermis and dermis. BF, bright field. Scale bar, 50 μm.
Retroviral proteases are first translated from the viral genome as part of large polyprotein precursors, gag or gag-pol (4). The retroviral protease itself is processed via autoprocessing at either side of the protease domain from polyprotein precursors. These precursors are further proteolytically processed by this retroviral protease to yield the mature viral proteins (17). When retroviral proteases with processing sites were expressed in bacteria, the protease domain was cleaved via autoprocessing, and dimeric catalytically active proteases could be produced (18, 19). Therefore, we examined whether bacterially expressed mSASP possesses autoprocessing activity and produces a catalytically active form of protease. Because mSASP32 contained both sides of the processing sites of mSASP15, we expressed mSASP32 (91–339 amino acids of mSASP) as a fusion protein with GST (GST-mSASP32) in E. coli. As a negative control, site-directed mutagenized GST-mSASP32(D210A) was expressed, in which the active-site aspartyl residue (Asp210) was changed to alanine. As shown in Fig. 4A, immunoblotting with mSASP-PR1 pAb detected a recombinant 15-kDa band (rmSASP15) in lysate expressing GST-mSASP32. The production of rmSASP15 was abolished in lysate expressing GST-mSASP32(D210A), suggesting that rmSASP15 is an autoprocessing product of GST-mSASP32. Next, we examined whether rmSASP15 was identical to mSASP15 purified from SCE as shown in Fig. 3. To determine the amino acid sequence of rmSASP15, we purified rmSASP15 from GST-mSASP32-expressing bacterial lysate. The rmSASP15 was separated from this lysate by several ion exchange column chromatographies (Fig. 4B). The fraction containing rmSASP15 was finally separated by Superdex 75 gel filtration chromatography, and rmSASP15 was purified to near-homogeneity. The molecular mass of the purified rmSASP15 was estimated to be 23.5 kDa on gel filtration, and a single band of about 15 kDa was obtained by SDS-PAGE, suggesting that rmSASP15 forms a homodimer, which is a feature of retroviral aspartic protease (20, 21). The purified rmSASP15 was subjected to the N-terminal amino acid sequence and MALDI-TOF analysis. The N-terminal amino acid sequence of rmSASP15 was determined as SKEGR, which is identical to purified mSASP15. MALDI MS spectra also showed protonated spectra at m/z 14,900.23, which is also close to that of purified mSASP15 (m/z 14,901.63). Thus, we concluded that the amino acid sequence of rmSASP15 is 189–324 amino acids, which is identical to purified mSASP15 in mouse SCE. Bernard et al. (13) also demonstrated that incubation of recombinant GST-hSASP25 results in autoprocessing and production of hSASP14. Taken together, these results suggest that mSASP15 in the SCE is the autoprocessing product of mSASP32 in vivo.
For kinetic analysis of HIV protease, oligopeptides containing the amino acid sequences of natural cleavage sites in the HIV polyprotein have been used as substrates for protease assay in a previous study (22). To examine the protease activity of rmSASP15, we designed an intramolecularly quenched fluorescent peptide substrate, Nma-LFANSMG-K(Dnp)rrr-NH₂ containing the autoprocessing site (Asn188–Ser189) of mSASP (Fig. 4C). rmSASP15 cleaved Nma-LFANSMG-K(Dnp)rrr-NH₂ in a time-dependent manner at pH 5.29 (0.15 M NaCl), pH 5.38 (0.7 M NaCl), and pH 7.65 (0.15 M NaCl) (Fig. 4D). The specific activity of each reaction was estimated to be 42.5 microkatal/kg (pH 5.29, 0.15 M NaCl), 150 microkatal/kg (pH 5.38, 0.7 M NaCl), and 24.5 microkatal/kg (pH 7.65, 0.15 M NaCl). The reaction rate at pH 5.29 (0.15 M NaCl) was 1.7-fold faster than that at pH 7.65 (0.15 M NaCl), and it was 3.5-fold lower than at pH 5.38 (0.7 M NaCl). These results suggested that the activity of rmSASP15 was higher at pH 5.3 than at pH 7.7 and was affected by ionic strength. To examine the effect of pH on rmSASP15 activity, we incubated rmSASP15 with Nma-LFANSMG-K(Dnp)rrr-NH₂ at different pH values at a NaCl concentration of 0.7 M. As shown in Fig. 4E, rmSASP15 had a maximum activity at pH 5.77 (Fig. 4E). These results suggested that rmSASP15 is the active form of SASPase and has a weakly acidic pH optimum that is close to the physiological pH of the stratum corneum (SC) (23, 24).

As described above, activated SASPase (mSASP15) is possibly activated and degrades certain substrates in the weakly acidic conditions of SC. To investigate the physiological function of mSASP in vivo, we produced knock-out mice. We constructed a targeting vector to delete the mSASP gene by homologous recombination, replacing the single exon, including the translation initiation codon (ATG) (Fig. 5A). We intercrossed heterozygous mice (mSASP+/−), which were phenotypically indistinguishable from wild type littermates (mSASP+/+), to generate homozygous mice (mSASP−/−). Southern blotting analysis confirmed the correct targeting of mSASP gene (Fig. 5B). Immunoblotting and immunofluorescent staining of mutant mouse skin showed that the expression of mSASP protein was abolished (Fig. 5, C and D).

When heterozygous mutant mice were interbred, wild type, heterozygous, and homozygous mutant mice were produced in Mendelian ratios (data not shown). No obvious phenotype was apparent in these mice before they were 5 weeks old. At 5 weeks of age, a large number of linear grooves running parallel to the body appeared in homozygous mice (Fig. 6A, upper panel). These grooves were enhanced by stretching the hind legs backwards (Fig. 6A, lower panel). To examine whether these grooves were derived from a wrinkled skin surface, the hair was removed. After removal of hair, many wrinkles were clearly visible in mSASP−/− mice when hanged by their tails (Fig. 6B). These results indicate that the linear grooves on the fur surface in mSASP−/− mice are caused by a wrinkled skin surface. To further analyze the wrinkled skin of mSASP−/− mice, we subjected the skin to histological examination that showed all skin layers were present (Fig. 7A). The morphology of other tissues that contain stratified epithelia, such as hair, ear, esophagus, forestomach, urinary bladder, etc., appeared normal (data not shown). The cornified cell envelope from the ear was purified, and no obvious change was observed (Fig. 7B). Next, to analyze the epidermal differentiation of mSASP−/− mice, the expression of various epidermal differentiation markers was examined. As shown in Fig. 8A, immunostaining of keratin 14, keratin 1, involucrin, filaggrin, loricrin, and desmoglein 1 revealed that their expression level and localization were not altered. Immunoblotting of side skin lysates also confirmed no alteration in the expression of keratin 14,
keratin 1, loricrin, and desmoglein 1 (Fig. 8B). These results suggest that mSASP−/− mice have wrinkles at the side of their body without any apparent epidermal differentiation defect.

**DISCUSSION**

The skin epidermis is composed of basal, spinous, granular, and cornified cell layers. Epidermal differentiation consists of a multiple step process accompanied by gene expression and concomitant morphological changes in each cell layer (25–27). Gene expression is completed in the “living” granular layer by high throughput in situ hybridization screening (14). Similar to the tissue distribution of hSASP, mSASP is expressed primarily in stratified epithelia. Our biochemical analysis revealed that the 32-kDa (mSASP32) and 15-kDa (mSASP15) form of mSASP corresponded to the 25-kDa (hSASP25) and 14-kDa (hSASP14) form of hSASP, respectively (13). Interestingly, at the amino acid sequence level, mSASP15 and hSASP14 showed 92% identity, which is much higher than between mSASP32 and hSASP25 (72% identity), implicating that because of its functional importance, this protease domain is evolutionally conserved between human and mouse species.

HIV, type 1, synthesizes its retroviral proteins as polyprotein precursors that consist of several tandem-like linked proteins (4). To yield the mature viral proteins, these polyproteins are cleaved to their component proteins through the action of HIV protease, but only after this enzyme has excised itself from a polyprotein (17). These HIV proteases are responsible for maturation and assembly of infectious virus particles (28). Both the polypeptide and the excised protein of HIV protease are activated by a concentration-dependent dimerization that takes place during the budding of the immature virus within the “limited space” of the viral particle (20, 21). In stratified epithelia, the claudin-based continuous tight junctions at the top of the granular layer cells have a crucial role in the epidermal barrier function (29). In other words, the extracellular space of the SC is surrounded by continuous tight junctions in the granular layer, and intracellular lipids in the cornified cell layer that together form the limited space. It is well known that this space shows a weakly acidic pHe, the so-called acidic mantle (23, 24). Considering that mSASP15 has a weakly acidic pHe optimum, SASPase precursor (mSASP32) is possibly autoprocessed by a concentration-dependent dimerization within the weakly acidic limited space of the SC, and subsequently the active form of mSASP (mSASP15) was produced, which is then able to cleave certain substrates in prevent dehydration of the body and form a physical and biochemical barrier against pathogens, which is the primary function of skin. Therefore, the “granular layer-expressing genes” have important roles in the final stage of epidermal differentiation, cornification, and barrier function. In this study, we reported the identification of the mouse homologue of SASPase as a granular layer-expressing gene by high-throughput in situ hybridization screening (14).
SASPase Knock-out Mice

the SC. So far, several proteases have been found to be involved in epidermal differentiation (30–32). In particular, the role of extracellular proteases in the SC has been well studied. A number of serine, cysteine, and aspartic proteases have been reported to be localized in the SC and suggested to play a role in desquamation by the degradation of corneodesmosommal proteins such as desmoglein 1, desmocollin 1, plakoglobin, and corneodesmosin. It is possible that SASPase partially degrades these proteins and regulates SC functions. This hypothesis will be described elsewhere.

As discussed above, the rate of SASPase activation is thought to be dependent on the concentration of SASPase in the SC, suggesting that its activity is largely dependent on the turnover rate of keratinocyte differentiation. In support of this concept, high expression of hSASP in the SC of psoriasis patients was observed by immunoblotting and immunofluorescence (13). There are several other keratoderma in which the turnover rate of keratinocyte differentiation has changed, such as ichthyosis and atopic dermatitis (33). In these pathological conditions, the amount of activated SASPase, hSASP14, could have possibly been altered. Furthermore, pH elevation has been observed at the epidermal surface in several diseases like seborrheic dermatitis, atopic dermatitis, and xeroderma (34). The increased pH activates serine proteases such as the stratum corneum chymotryptic enzyme (SCCE/KLK7/hK7) and the stratum corneum tryptic enzyme (SCTE/KLK5/hK5) resulting in abnormal proteolysis of the SC that leads to barrier disruption (35). Under these abnormal pH conditions, SASPase may not be activated. Inactivation of SASPase in these diseases may help to understand the molecular mechanism of disease progression.

In this study, we generated mice lacking mSASP by homologous recombination. These mSASP−/− mice were born normally but showed a characteristic wrinkled appearance. So far, the cause of these mSASP−/−-dependent wrinkles is not clear. In general, the primary cause of wrinkle formation is thought to be due to a loss of flexibility in every layer of the skin, such as dermis and epidermis. Dermis-derived wrinkles (deep wrinkles) are reported to be caused by changes in elastic fibers and collagen fibers (36–40). On the other hand, the formation of epidermis-derived wrinkles (fine wrinkles) is mainly ascribed to deficient water conditions in the SC of epidermis (41–42). For example, loose and saggy skin mice models, fibulin-5/DANCE-deficient mice and Saggi/+ mice, have been reported (43–45). These mice showed loose skin hanging in folds, and once extended, these loose skin folds remained extended in the mutant mouse. mSASP−/− mice showed equal amounts of loose skin and showed small linear grooves along the body axis. Considering that SASPase is localized in the SC, mSASP−/−-derived skin resembles fine wrinkles caused by epidermal dryness. Mating with BALB/c nude mice may enable us to further examine this in the future. Identification of an endogenous substrate for mSASP may also help to explain the cause of increased wrinkles in mSASP−/− mice. To date, there is no mouse model for fine wrinkle formation caused by an epidermal single gene disruption. Thus, mSASP−/− mice might be useful for the analysis of mechanisms for fine wrinkle formation.

To date, in the mammalian genome, several genome-integrated retroviral elements have been identified (8). mSASP−/− mice have provided the first evidence that one of these protease family members regulates the morphology of mammalian tissue. Our biochemical and knock-out mouse approach can also be utilized for analyzing the fine wrinkle-like aspartic proteases in mammals.

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