The Membrane-anchored Serine Protease Prostasin (CAP1/PRSS8) Supports Epidermal Development and Postnatal Homeostasis Independent of Its Enzymatic Activity*

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Background: Prostasin is a membrane-anchored serine protease with essential functions in epithelial development and homeostasis.

Results: Mice expressing enzymatically inactive endogenous prostasin, unlike prostasin null mice, display normal tissue development and homeostasis.

Conclusion: Essential in vivo functions of prostasin are independent of the catalytic activity of prostasin.

Significance: Prostasin may have a unique role as an allosteric regulator of other membrane-anchored proteases.

The membrane-anchored serine protease prostasin (CAP1/PRSS8) is part of a cell surface proteolytic cascade that is essential for epithelial barrier formation and homeostasis. Here, we report the surprising finding that prostasin executes these functions independent of its own enzymatic activity. Prostasin null (Prss8<sup>−/−</sup>) mice lack barrier formation and display fatal postnatal dehydration. In sharp contrast, mice homozygous for a point mutation in the Prss8 gene, which causes the substitution of the active site serine within the catalytic histidine-aspartate-serine triad with alanine and renders prostasin catalytically inactive (Prss8<sup>Cat−/−Cat−</sup> mice), develop barrier function and are healthy when followed for up to 20 weeks. This striking difference could not be explained by genetic modifiers or by maternal effects, as these divergent phenotypes were displayed by Prss8<sup>−/−</sup> and Prss8<sup>Cat−/−Cat−</sup> mice born within the same litter. Furthermore, Prss8<sup>Cat−/−Cat−</sup> mice were able to regenerate epidermal covering following cutaneous wounding. This study provides the first demonstration that essential in vivo functions of prostasin are executed by a non-enzymatic activity of this unique membrane-anchored serine protease.

Prostasin (also known as channel-activating protease 1, CAP1, and PRSS8) is a phylogenetically conserved membrane-anchored serine protease, encoded by the PRSS8 gene, that is widely expressed in epithelial tissues. Loss-of-function studies in mice have revealed an essential role of prostasin in terminal epidermal differentiation and postnatal survival. Prostasin null (Prss8<sup>−/−</sup>) mice, lacking any prostasin protein, display placental insufficiency that leads to complete or partial embryonic lethality, while mice with epidermal deletion of Prss8 die shortly after birth due to lack of epidermal barrier formation and fatal dehydration (1, 2). Prostasin is also an essential regulator of the epithelial sodium channel in the context of alveolar fluid clearance, lung fluid balance, and intestinal sodium and water absorption (3, 4).

It is now generally recognized that prostasin and the type II transmembrane serine protease, matriptase, form part of a single epithelial proteolytic cascade in the context of placental development, terminal epidermal differentiation, and epithelial tight junction formation (5–10). The specific mechanistic interrelationship between the two proteases, however, has remained unclear, with different studies placing prostasin either upstream or downstream from matriptase depending on the specific context (5–7, 9).

Using a reconstituted cell-based assay, we recently found that prostasin can form complexes with matriptase and can stimulate matriptase autoactivation independent of prostasin’s own catalytic activity. In this regard, a catalytically inactive prostasin mutant was able to both activate matriptase and to stimulate the activity of matriptase toward a physiological target substrate, proteinase-activated receptor-2 (11).

To determine the biological relevance of these observations, we herein generated and characterized knock-in mice with a point mutation engineered into the endogenous Prss8 gene,
which resulted in the synthesis of a catalytically inactive protease. Analysis of these mice unexpectedly revealed that prostasin supports both epidermal development and long term survival independent of its enzymatic activity.

**EXPERIMENTAL PROCEDURES**

*Generation of Catalytically Inactive Prostasin Knock-in Mice*—All experiments were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium following institutional guidelines and standard operating procedures. Prss8<sup>Cat</sup>−/−/Cat− mice were generated by homologous recombination in embryonic stem (ES) cells using a replacement targeting vector. A 2.5-kb fragment containing exon 6 of the mouse Prss8 gene was amplified from feeder-free W4129 S6 ES cell DNA using high-fidelity long range PCR. A NotI site was incorporated at the 5′ end during amplification and an EcoRI site existed at the 3′ end. This fragment was inserted between the EcoRI and NotI sites of pBluescript II KS. A T→G substitution was introduced into exon 6 (corresponding to nucleotide 943 of the mouse Prss8 cDNA, NM_133351.3) by site-directed mutagenesis using a QuiKChange kit (Stratagene, La Jolla, CA). Mutagenesis primers used were as follows: 5′-TTTCTCCTTAGGTAGCTGCT- GGGGGCCC-3′ and 5′-GGGCCCCCCAGCTACCTAAGGAG AAA-3′ (mutation underlined). Following introduction of the desired point mutation, an adjacent 2.5-kb DNA fragment was amplified containing a native EcoRI site at its 5′ end and with both NotI and SalI sites added at the 3′ end. This was inserted into the vector via EcoRI/Sall sites effectively reassembling a homologous 5-kb DNA fragment, which included the introduced T→G substitution in exon 6 of Prss8 and also contained NotI sites at both 5′ and 3′ ends. The final targeting vector was generated in the vector PL452 (12), which contains a homology region. The integrity of the introduced T→G region was confirmed by PCR genotyping using V76R (5′-CCATTACCAACTTCTGGCAGCTGC-3′) and Prss8<sup>S</sup>52 (5′-ACTTACACGACTAAGTGGCAGCTGC-3′) primers.

*RNA Preparation and RT-PCR*—Tissues were collected from newborn mice, snap-frozen in liquid nitrogen, and ground to a fine powder with mortar and pestle. Total RNA was prepared by extraction in TRIzol reagent (Invitrogen) as recommended by the manufacturer. Reverse transcription and PCR amplification were performed using a High Capacity cDNA reverse transcription kit (Invitrogen), per the manufacturer’s instructions. First strand cDNA synthesis was performed using an oligo(dT) primer. The primer pair utilized for Prss8 RT-PCR was as follows: 5′-TTGCTGTAGGAGTCTAGC-3′ and 5′-AAGCTGT GACCATTTCTGC-3′. Annealing temperature for this primer set was 55 °C. Expression levels were normalized to S15 mRNA levels in each sample.

*Western Blot Analysis*—Tissues from newborn mice were homogenized in ice-cold PBS containing 1% Triton X-100, 0.5% sodium deoxycholate and protease inhibitors (Sigma) and incubated on ice for 10 min. The lysates were centrifuged at 20,000 × g for 30 min at 4 °C to remove tissue debris, and the supernatant was used for further analysis as described below. For profilagrin processing analysis, epidermis was homogenized in 50 mM Tris/HCl, pH 8.0, 10 mM EDTA, and 8 M urea. Protein concentrations were determined using a BCA assay to allow loading of equivalent total protein. Samples were mixed with 4× SDS sample buffer (Invitrogen) containing 7% β-mercaptoethanol, boiled for 5 min, separated on 4–12% Bis-Tris NuPage gels (Invitrogen), and transferred to 0.2-μm pore size PVDF membranes (Invitrogen). Membranes were blocked with 5% BSA in 3.5% milk powder in 0.1% Tween-20 and incubated with 1× blocking buffer. Membranes were washed 3 times with 3.5% milk powder in 0.1% Tween-20 and then incubated with 1× blocking buffer. Membranes were washed 3 times with 3.5% milk powder in 0.1% Tween-20 and then incubated with 1× blocking buffer.
Tris-buffered saline containing 0.05% Tween 20 for 1 h at room temperature. Individual PVDF membranes were then probed with primary antibodies diluted in 1% BSA in TBS-T overnight at 4 °C. The antibodies used included mouse anti-human prostasin (BD Transduction Laboratories, catalog no. 612173), rabbit anti-human GAPDH (Cell Signaling, Danvers, MA, catalog no. 2118), and a polyclonal rabbit anti-mouse profilaggrin/filaggrin (Covance, Inc., Chantilly, VA, catalog no. PRB-417P). The next day, membranes were washed 3 × 5 min with TBS-T and incubated for 1 h with alkaline phosphatase-conjugated secondary antibodies (Dako, Carpinteria, CA). After 3 × 5 min washes with TBS-T, the signal was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Pierce). Densitometric scans were quantified using ImageJ software (14).

**Histological Analysis**—Newborn to 24-h-old pups were euthanized by decapitation and prepared for histology as described previously (15). Hair follicle density and diameter were determined on a region-matched area of dorsal skin; all hair follicles within a 1-cm segment were measured. Epidermal thickness (excluding stratum corneum) and stratum corneum thickness were measured in this same 1-cm segment of dorsal skin and were calculated by averaging 100 independent measurements per skin specimen.

**Immunohistochemistry**—Antigens from 5 μm paraffin sections were retrieved by incubation for 20 min at 100 °C in 0.1 M sodium citrate buffer, pH 6.0. The sections were blocked with 2.5% BSA in PBS and incubated overnight at 4 °C with mouse anti-human prostasin (1:125, BD Transduction Laboratories, catalog no. 612173). Bound antibodies were visualized using a biotin-conjugated anti-mouse secondary antibody (1:400, Vector Laboratories, catalog no. 612173). Bound antibodies were visualized using a streptavidin-biotin-conjugated anti-mouse secondary antibody (1:400, Vector Laboratories, catalog no. 612173) using 3,3′-diaminobenzidine as the substrate (Sigma-Aldrich).

**Transepidermal Fluid Loss Assay**—Transepidermal fluid loss assay was performed exactly as described (15).

**Cutaneous Wound Repair**—Full thickness incisional skin wounds (15 mm) were made in the interscapular dorsum, and healing was assessed by daily inspection of wounds by an investigator blinded as to genotype, as described previously (16). Specifically, macroscopic closure of the incisional interface was evaluated both visually and by palpation. At the time of gross inspection, maximal longitudinal wound length was measured, and each wound was photographed. Wound areas were then determined using ImageJ software (14). Wounds were collected for histological examination at days 5, 10, 14, and 21. Histological and morphometric analysis of wounds was performed as described (16).

**RESULTS**

**Characterization of Mice Expressing Catalytically Inactive Endogenous Prostasin**—To generate mice expressing catalytically inactive prostasin, we introduced a c.943T→G nucleotide substitution into exon 6 of the Prss8 gene by homologous recombination in embryonic stem cells (Fig. 1A). This resulted in the substitution of serine 238 of the catalytic histidine-aspartate-serine triad with alanine. Genomic analysis confirmed the introduction of the point mutation in the Prss8 gene (Fig. 1, B and C, the targeted allele hereafter referred to as Prss8\(^{\text{Cat}^-}\)). Analysis of mRNA allele showed that the introduction of the point mutation and a neighboring LoxP site into intron 5 did not affect Prss8 expression (Fig. 1D). Western blot analysis of protein extracts from skin, kidney, and lungs of newborn Prss8\(^{\text{Cat}^-}/\text{-Cat}^-\) pups and wild-type (Prss8\(^{+/+}\)) littersmates demonstrated that the mutant prostasin was expressed at levels similar to wild-type prostasin (Fig. 1, E and F). An additional faster migrating prostasin species was detected in kidney and lung of Prss8\(^{\text{Cat}^-}/\text{-Cat}^-\) mice (red triangle in Fig. 1E, kidney and lung panels, compare lanes 1–3 with 4–6), which may represent an activation site-cleaved mutant prostasin unable to be cleared through serpin or Kunitz-type inhibitor complex formation. Immunohistochemistry of skin and other major organ systems (Fig. 2) showed normal spatial localization of the catalytically inactive mutant prostasin. Expression was predominantly restricted to epithelia, and no differences were identified relative to wild-type prostasin, as published previously (10).

**The Enzymatic Activity of Prostasin Is Dispensable for Mouse Development and Long Term Survival**—We interbred Prss8\(^{+/+}\text{-Cat}^-\) mice and genotyped 111 offspring from a total of 13 litters and found that Prss8\(^{\text{Cat}^-}/\text{-Cat}^-\) pups were born in Mendelian frequency (Fig. 3A, black bars). Surprisingly, no Prss8\(^{\text{Cat}^-}/\text{-Cat}^-\) pups died within the 21-day preweaning period (Fig. 3A, gray bars). Furthermore, no deaths were observed in a prospective cohort of 20 Prss8\(^{\text{Cat}^-}/\text{-Cat}^-\) mice and 24 Prss8\(^{+/+}\) littersmates followed for 56 to 140 days postweaning (Fig. 3B).

One possible explanation for the survival discrepancy between Prss8\(^{\text{Cat}^-}/\text{-Cat}^-\) and Prss8\(^{+/+}\) mice relates to the targeting strategy employed to generate prostasin null mice. Prss8\(^{+/+}\) mice were originally made by the insertion of LoxP sites into the Prss8 gene or a critical regulatory element may have contributed to inadvertent removal of an essential non-protein coding intron. Cre-mediated recombination (1, 2).

Furthmore, no deaths were observed in a prospective cohort of 20 Prss8\(^{\text{Cat}^-}/\text{-Cat}^-\) mice and 24 Prss8\(^{+/+}\) littersmates followed for 56 to 140 days postweaning (Fig. 3B).

One possible explanation for the survival discrepancy between Prss8\(^{\text{Cat}^-}/\text{-Cat}^-\) and Prss8\(^{+/+}\) mice relates to the targeting strategy employed to generate prostasin null mice. Prss8\(^{+/+}\) mice were originally made by the insertion of LoxP sites into introns 2 and 5, followed by Cre-mediated recombination (1, 2). Thus, inadvertent removal of an essential non-protein coding gene or a critical regulatory element may have contributed to the observed postnatal lethality. To investigate this possibility, we generated Prss8\(^{+/+}\) mice via an alternate strategy, using ES cells with a retroviral insertion in intron 2. This insertion resulted in a null mutation by placing a strong splice accep- tor site in intron 2, leading to formation of a truncated Prss8 mRNA fused to a β-geo reporter gene (Fig. 4). Importantly, this targeting strategy did not delete any native DNA sequences. Nevertheless, Prss8\(^{+/+}\) mice generated by this strategy also displayed uniform postnatal lethality (data not shown and Fig. 3D).

It is known that subtle genetic background differences and maternal effects can strongly influence the phenotypic expression of induced mouse mutations (17). We, therefore, next interbred mice carrying Prss8\(^{\text{at}^-}\) and Prss8\(^{\text{alleles to generate breeding pairs capable of producing Prss8\(^{\text{at}^-}/\text{-Cat}^-\) and Prss8\(^{+/+}\) offspring within the same litter. Whereas Prss8\(^{+/+}\) offspring from these crosses died within 48 h after birth, Prss8\(^{\text{at}^-}/\text{-Cat}^-\) littersmates, again, were born in the expected frequency and displayed normal preweaning survival (Fig. 3C). Prss8\(^{\text{at}^-}/\text{-at}^-\) offspring were also born in the expected ratio and 73% survived the preweaning period (Fig. 3C). Similar results were obtained when using a breeding scheme that allowed
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for the generation of Prss8<sup>+</sup>/*, Prss8<sup>Cat</sup>−/−/Cat<sup>−</sup>, and Prss8<sup>Cat</sup>−/− mice on the same genetic background (Fig. 3D).

Prostasin Supports Terminal Epidermal Differentiation through a Non-catalytic Mechanism—The normal survival of Prss8<sup>Cat</sup>−/−/Cat<sup>−</sup> mice indicated that prostasin could support terminal epidermal differentiation and epidermal barrier formation by a mechanism that was independent of its enzymatic activity. In agreement with this, the outward appearance of newborn Prss8<sup>Cat</sup>−/−/Cat<sup>−</sup> pups was indistinguishable from Prss8<sup>+</sup>/* littermates (Fig. 5A). At the histological level, the epidermis of Prss8<sup>+</sup>/* (Fig. 5B) and Prss8<sup>Cat</sup>−/−/Cat<sup>−</sup> (Fig. 5C) mice were also strikingly similar with both exhibiting normal stratum corneum featuring a characteristic “basket weave” pattern with intercorneocyte lacunae formed by a meshwork of interlocking flattened layers of corneocytes connected by desmosomes (18). In contrast, Prss8<sup>Cat</sup>−/− strain corneum was completely compacted (Fig. 5D), presenting with few intercorneocyte lacunae (2). Histomorphometric analysis, revealed a small (18%), but significant, reduction in the thickness of Prss8<sup>Cat</sup>−/−/Cat<sup>−</sup> stratum corneum (Fig. 5, E and F). In accordance with the normal stratum corneum structure, transepidermal water loss of Prss8<sup>Cat</sup>−/−/Cat<sup>−</sup> and Prss8<sup>Cat</sup>−/−/Cat<sup>−</sup> pups was only marginally increased relative to Prss8<sup>+</sup>/* littermates (Fig. 5G), whereas

FIGURE 1. Generation of mice expressing only catalytically inactive endogenous prostasin. A, structure of targeting vector (top), wild-type Prss8 allele (middle), and targeted Prss8 allele with the neomycin cassette removed (bottom). Exons are indicated as blue boxes and intron sequences as black lines. The locations of primers used for PCR screening of ES cell clones and genotyping of mice are indicated by green and black arrowheads, respectively. Homologous recombination in ES cells introduced a c.943T→G substitution into exon 6 and introduced a neomycin selection cassette (orange) flanked by LoxP sites (red triangles) into intron 5. G indicates the position of the serine to alanine codon change in exon 6. B, PCR analysis of DNA from Prss8<sup>+/+</sup> (lane 1), Prss8<sup>Cat</sup>−/−/Cat<sup>−</sup> (lane 2), Prss8<sup>Cat</sup>−/−/− (lane 3) offspring from Prss8<sup>+/+</sup> intercrosses. Positions of amplicons from the wild-type and targeted alleles are shown on the right. Positions of molecular weight markers (bp) are indicated on the right. C, sequence analysis of exon 6 from Prss8<sup>+/+</sup> (left) and Prss8<sup>Cat</sup>−/−/− (right) mice confirms the introduction of the c.943T→G substitution causing the serine 238 to alanine substitution in prostasin (red letters in nucleotide and amino acid sequence). D, RT-PCR analysis of Prss8 mRNA (upper panel) and ribosomal protein S15 mRNA (lower panel) in skin (lanes 1, 5, and 9), large and small intestine (lanes 2, 6, and 10), kidney (lanes 3, 7, and 11), and lung (lanes 4, 8, and 12) of Prss8<sup>+/+</sup> (lanes 1–4), Prss8<sup>Cat</sup>−/−/− (lanes 5–8), and Prss8<sup>Cat</sup>−/−/− (lanes 9–12) mice. A no reverse transcriptase control is included in lane 13. E, prostasin (upper) and GAPDH (lower) Western blots of skin (left panels), kidney, (middle panels), and lung (right panels) from Prss8<sup>+/+</sup> (lanes 1–3), Prss8<sup>Cat</sup>−/−/− (lanes 4–6), and Prss8<sup>Cat</sup>−/−/− (lane 7) mice. Positions of prostasin are indicated with black and green arrowheads at the right. A prostasin species specific for Prss8<sup>Cat</sup>−/−/− is indicated with a red arrowhead. The positions of molecular mass markers (kDa) are indicated at the left, F, densitometric quantification of prostasin protein levels normalized to GAPDH. AU, arbitrary units. p = N.S., not significant; Student’s t test, two-tailed.

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Prss8\textsuperscript{−/−} littermates dehydrated at a rapid pace (Fig. 5G), as described previously (2).

Prostasin is known to be required for the proteolytic processing of the key epidermal polyprotein, profilaggrin, into filaggrin monomers during stratum corneum formation (2). Analysis of epidermal extracts from newborn Prss8\textsuperscript{Cat−/Cat−} pups by SDS-PAGE (Fig. 5H) and by Western blot (Fig. 5I) showed that prostasin-mediated profilaggrin processing did not require the enzymatic activity of prostasin, although the level of processed filaggrin monomer was reduced in Prss8\textsuperscript{Cat−/Cat−} epidermis (Figs. 5, H and I, compare lanes 3–5 with lanes 6–8).

Impaired Hair Follicle Development in Mice Expressing Catalytically Inactive Prostasin—Subtle differences in whiskers and pelage hair were apparent in Prss8\textsuperscript{Cat−/Cat−} mice. Prss8\textsuperscript{Cat−/Cat−} pups were born either without whiskers or with shorter, kink, and curly whiskers (Fig. 6A). The whiskers remained abnormal throughout the observation period (Fig. 6B). The time of pelage hair eruption of Prss8\textsuperscript{Cat−/Cat−} mice was normal (data not shown), as were hair follicle density (Fig. 6C) and hair follicle diameter (Fig. 6D). The coat of newly weaned Prss8\textsuperscript{Cat−/Cat−} mice, however, varied from indistinguishable from Prss8\textsuperscript{+/+} littermates (∼86% of mice, example in Fig. 6E, middle) to markedly thinner and sparser (∼14% of mice, example in Fig. 6E, right). The latter phenotype persisted throughout the observation period in ∼5% of Prss8\textsuperscript{Cat−/Cat−} mice.

Reduced Body Weights of Mice Expressing Catalytically Inactive Prostasin—The body weights of newborn Prss8\textsuperscript{Cat−/Cat−} pups were indistinguishable from Prss8\textsuperscript{+/+} littermates. However, Prss8\textsuperscript{Cat−/Cat−} pups displayed reduced body weight at day 14, which became significant at 3 weeks for females and at 2 weeks for males, and persisted throughout the 11-week observation period (Fig. 6, F and G). The body weights of Prss8\textsuperscript{Cat−/Cat−} mice were 10–26% reduced within the 11-week period, with the greatest weight differences manifesting the weeks prior to and after weaning. Prss8\textsuperscript{Cat−/Cat−} mice presented with no obvious outward phenotype that could explain the lower body weight. Likewise, non-epidermal tissues of Prss8\textsuperscript{Cat−/Cat−} mice were histologically unremarkable when compared with wild-type littermates (Fig. 6H).

Epidermal Barrier Restoration after Incisional Wounding—To determine whether catalytically inactive prostasin could support the restoration of the epidermal barrier after disruption, we generated 1.5-cm full-thickness incisional skin wounds in the mid-scapular dorsal region of Prss8\textsuperscript{Cat−/Cat−} mice and their wild-type littermates. The wounds were left unsutured and undressed and were observed daily by an investigator blinded as to mouse genotype. The wounds were scored as healed based on the macroscopic closure of the incision interface and restoration of epithelial covering (Fig. 7A, representative photographs in Fig. 7D). Interestingly, mice expressing catalytically inactive prostasin were capable of healing their
wounds, although healing was delayed by 26% (mean healing time (days) ± S.D. Prss8+/− = 12.3 ± 1.7, Prss8−/−/−/− = 15.5 ± 0.8, p = 0.003 log-rank test, two-tailed). Histologic examination of the wounds at day 21 showed complete restoration of epidermal covering, including a well-developed stratum corneum in the regenerated epidermis of both Prss8+/− and PrSS8−/−/− mice (Fig. 7, B and C). We next performed a histological analysis of the kinetics of wound healing by analyzing wounds at days 5, 10, and 14 after incisional wounding (Fig. 7E). At day 5 after wounding, 0 of 5 Prss8−/−/−/− mice and 1 of 5 Prss8−/−/−/− wounds had restored epidermal covering. The length of the newly formed epidermal wedges migrating into the wounds in the five Prss8−/−/−/− wounds was 663 ± 152 μm and was 765 ± 277 μm in the four non-reepithelialized Prss8−/−/−/− wounds (p = N.S.,2 Student’s t test, two-tailed). At day 10 after wounding, 2 of 4 Prss8−/−/−/− mice and 3 of 4 control wounds had restored epidermal covering, and at day 14 after wounding, 4 of 4 Prss8−/−/−/− mice and 4 of 4 control wounds had restored epidermal covering. Taken together, these data demonstrate that mice expressing only catalytically inactive prostasin can restore epidermal covering after wounding.

**DISCUSSION**

The PRSS8 gene is conserved in all vertebrate species examined, and it encodes a single-chain protease zymogen that can be proteolytically converted to an active two-chain trypsin-like serine protease. Aligned with this high conservation, prostasin is essential for mouse survival and promotes key proteolytic processes in epithelial tissues (reviewed in Ref. 19). The results of the current study, which show that prostasin can support both epidermal development and long-term mouse survival independent of its catalytic activity, are therefore unexpected. Particularly striking are the normal interfollicular epidermal differentiation and the formation of a functional epidermal barrier in mice engineered to express only catalytically inactive endogenous prostasin, Prss8−/−/−/− mice, as compared with the lack of terminal epidermal differentiation and uniform lethality observed in mice lacking prostasin protein, Prss8−/−/−/− mice. Furthermore, Prss8−/−/−/− mice were able to restore their epidermal covering after wounding, albeit with increased healing times.

At present, it is not possible to determine whether the reduction in profilaggrin processing, the marginal impairment of epidermal barrier function, and the increased skin wound healing times observed for Prss8−/−/−/− mice are due to the loss of catalytic activity or instead due to subtle changes in the trafficking, subcellular localization or turnover of the mutant prostasin. What is clear, however, is that prostasin can support these activities independent of its enzymatic activity.

The specific mechanism by which catalytically inactive prostasin supports interfollicular epidermal development remains to be established and is beyond the scope of the current study. However, in a cell-based assay, we recently found that catalytically inactive prostasin, was capable of stimulating both matriptase auto-activation and cleavage of a physiological matriptase substrate (11). Furthermore, in a reconstituted Xenopus oocyte system, catalytically inactive prostasin has been reported to stimulate the activation of the epithelial sodium channel by inducing proteolytic cleavage of the epithelial sodium channel γ-chain (20, 21). These findings, in conjunction with the in vivo observations presented herein, lend support to the hypothesis that an allosteric interaction of prostasin with another membrane-bound serine protease, matriptase, may be required for normal epidermal barrier formation.

Proteolytic cleavage of single-chain prostasin to two-chain prostasin has been proposed to be the key function of matriptase in epidermal development (5). It follows, logically, that if this assertion is correct, then the non-catalytic function by which prostasin supports terminal epidermal differentiation will be expressed only after conversion of single-chain prostasin to two-chain prostasin. In this respect, prostasin would be similar to hepatocyte growth factor and macrophage stimulating protein, both of which are trypsin-like serine protease-like proteins that are competent to execute their biological functions only after canonical activation site cleavage (22). Importantly,
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FIGURE 4. Generation of Prss8<sup>−/−</sup> mice by retroviral insertion. A, structure of targeting vector (top), wild-type Prss8 allele (middle), and targeted Prss8 allele (bottom). Exons are indicated as blue boxes, and intron sequences are shown as black lines. The β-globin cassette is indicated as an orange box and retroviral long terminal repeat (LTR) as filled green arrows. SA indicates the position of the engrafted splice acceptor site, and pA indicates the position of the polyadenylation site. The locations of primers used for genotyping of mice by PCR are indicated by black arrowheads. The locations of primers used for Prss8 transcript analysis are indicated by green arrowheads. Insertion of the retroviral targeting construct into intron 2 of Prss8 leads to fusion of Prss8 exon 2 to the β-globin gene, resulting in a null mutation. B, RT-PCR of Prss8 (top panel) and ribosomal protein S15 (bottom panel) mRNA isolated from skin (lanes 1 and 5), intestine (lanes 2 and 6), kidneys (lanes 3 and 7), and lungs (lanes 4 and 8) of newborn Prss8<sup>+/+</sup> (lanes 1–4) and Prss8<sup>−/−</sup> (lanes 5–8) littermates using primer pairs amplifying mRNA sequences derived from exons 1 through 4 (see position of green primers in A). No reverse transcriptase was added to the reaction in lane 9. C, Western blot of proteins extracted from the placenta of two Prss8<sup>−/−</sup> embryos (lanes 1 and 2) and two Prss8<sup>+/+</sup> littermates (lanes 3 and 4). Position of prostasin is indicated at the right, and the positions of molecular mass markers (kDa) are indicated at the left. fwd, forward; rev, reverse.

FIGURE 5. Terminal epidermal differentiation and epidermal barrier formation require prostasin, but not prostasin’s enzymatic activity. (A) Representative photographs of newborn Prss8<sup>+/+</sup> (left, and Prss8<sup>Cat−/Cat−</sup> (right) littermates demonstrating similarity in size and outward appearance. B–D, H&E staining of dorsal skin from newborn Prss8<sup>+/+</sup> (B), Prss8<sup>Cat−/Cat−</sup> (C), and Prss8<sup>−/−</sup> (D) mice. The position of the stratum corneum is indicated to the right of each panel. Note the abnormally compacted Prss8<sup>−/−</sup> stratum corneum, and the normal morphology of Prss8<sup>Cat−/Cat−</sup> stratum corneum. Scale bars are 50 μm. E and F, thickness of the epidermis (excluding the stratum corneum) (D) and the stratum corneum (F) of Prss8<sup>+/+</sup> (black circles, n = 11) and Prss8<sup>Cat−/Cat−</sup> (purple squares, n = 14) littermates. Horizontal bars indicate medians (*, p < 0.05, Student’s t test, two-tailed). G, rate of epidermal fluid loss from newborn mice was estimated by measuring reduction of body weight as a function of time. The data are expressed as the average % of initial body weight for Prss8<sup>+/+</sup> (black circles; n = 10), Prss8<sup>Cat−/Cat−</sup> (green squares; n = 9), Prss8<sup>Cat−/−</sup> (yellow triangles; n = 5), and Prss8<sup>−/−</sup> (red diamonds; n = 3) pups in the same genetic background. Error bars indicate S.D. *, p < 0.001 and **, p < 0.0001, relative to Prss8<sup>+/+</sup> (Student’s t test, two-tailed). Note that Prss8<sup>−/−</sup> littermates have impaired barrier function and dehydrate rapidly, whereas the epidermal barrier of Prss8<sup>Cat−/Cat−</sup> mice is functional. H and I, profilaggrin processing was assessed by performing SDS-PAGE with Coomassie Brilliant Blue staining (H) and profilaggrin/filaggrin Western blotting (I) on epidermal protein extracts from Prss8<sup>+/+</sup> (lanes 1 and 2), Prss8<sup>−/−</sup> (lanes 3–5), and Prss8<sup>Cat−/Cat−</sup> (lanes 6–8) mice. Position of the processed filaggrin monomer is indicated at the right, and the positions of molecular mass markers (kDa) are indicated on the left.
prostasin differs from these two growth factors, which are devoid of proteolytic activity, by having non-enzymatic functions while at the same time being an enzymatically active serine protease.

Mice expressing catalytically inactive prostasin presented with a distinct whisker and pelage hair phenotype. It is known that prostasin expression in the mouse hair follicle is restricted to Henle’s layer of the inner root sheath of the non-proliferative compartment where it co-localizes with matriptase (10), and the observed phenotype for Prss8<sup>Cat-/-Cat-</sup> mice is quite similar to the phenotype observed in both matriptase hypomorphic mice (23) and in the spontaneous mutant mouse strain, frizzy (24). The latter mouse strain carries a point mutation in the Prss8 gene (Prss8<sup>fr/fr</sup>) that results in a non-conservative V170D amino acid substitution in the prostasin protein. We have reported previously that recombinant soluble V170D prostasin has a low residual enzymatic activity, and we attributed the frizzy pelage hair and whisker phenotype to this reduced proteolytic activity (6). The V170D mutation in prostasin, however, is predicted by in silico modeling to result in a
2.4-kcal energy loss and was shown experimentally to display abnormal glycosylation (3). V170D prostasin, therefore, may also display non-enzymatic activity-associated deficiencies, which could cause the pelage hair and whisker phenotype. The S238A prostasin that is expressed by \( \text{Prss8}^{-/-} \) mice, however, can be presumed to be deficient solely in its enzymatic activity, which provides additional support for prostasin proteolysis, possibly dependent on matriptase, being important for hair follicle development. Nevertheless, we cannot completely exclude that the pelage hair and whisker phenotype of \( \text{Prss8}^{-/-} \) mice may be due to a “gain of function” acquired by the S238A mutation. Arguing strongly against this, however, the pelage hair phenotype displayed greater penetrance in \( \text{Prss8}^{-/-} \) mice, when compared with \( \text{Prss8}^{-/-} \) mice.

In conclusion, this study reveals that critical biologic functions of prostasin in epithelial development and homeostasis are independent of the enzymatic activity of this protease and adds further support to the hypothesis that an allosteric interaction of prostasin with matriptase is a mechanistic requirement for normal epidermal barrier formation.

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