Distinct Developmental Functions of Prostasin (CAP1/PRSS8) Zymogen and Activated Prostasin*

Received for publication, November 25, 2015, and in revised form, December 18, 2015
Published, JBC Papers in Press, December 30, 2015, DOI 10.1074/jbc.C115.706721
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The membrane-anchored serine protease CAP1 (CAP1/PRSS8) is essential for barrier acquisition of the interfollicular epidermis and for normal hair follicle development. Consequently, prostasin null mice die shortly after birth. Prostasin is found in two forms in the epidermis: a one-chain zymogen and a two-chain proteolytically active form, generated by matriptase-dependent activation site cleavage. Here we used gene editing to generate mice expressing only activation site cleavage-resistant (zymogen-locked) endogenous prostasin. Interestingly, these mutant mice displayed normal interfollicular epidermal development and postnatal survival, but had defects in whisker and pelage hair formation. These findings identify two distinct in vivo functions of epidermal prostasin: a function in the interfollicular epidermis, not requiring activation site cleavage, that can be mediated by the zymogen-locked version of prostasin and a proteolysis-dependent function of activated prostasin in hair follicles, dependent on zymogen conversion by matriptase.

Prostasin (also known as channel-activating protease-1, CAP1, and PRSS8) is a glycosylphosphatidylinositol-anchored trypsin-like serine protease that is widely expressed in epithelial tissues, including both the interfollicular and the follicular compartments of the epidermis. Loss-of-function genetic studies in mice have uncovered critical functions of prostasin in both the formation of epidermal barrier formation and the formation of whiskers and pelage hair (1, 2). Prostasin is synthesized as an inactive proform (zymogen) that is converted to a catalytically active protease by a single endoproteolytic cleavage in the conserved activation cleavage site (3–6). Prostasin zymogen conversion in the epidermis requires the membrane-activated serine protease matriptase (7–9). This observation, combined with the observation that both matriptase-deficient and prostasin-deficient mice display identical epidermal phenotypes, led to the formulation of the hypothesis that prostasin exerts its functions in this tissue as part of a matriptase-prostasin cell surface zymogen activation cascade (8). Several observations made during the last decade suggest, however, that prostasin may also execute biological functions independent of its own proteolytic activity. For example, in a reconstituted Xenopus oocyte system, prostasin can activate the epithelial sodium channel (ENaC) by inducing proteolytic cleavage of its γ subunit to release an inhibitory domain. However, this activation, which can be inhibited by the broad-spectrum serine protease inhibitor, aprotinin, can also be efficiently executed by mutant prostasin variants that lack the catalytic histidine-aspartate-serine triad (10–12). Likewise, catalytically inactive prostasin mutants can stimulate the activation of protease-activated receptor-2 in a reconstituted mammalian cell-based system (13). Strong support for a non-proteolytic function of prostasin in vivo has been gained from the observation that mis-expressed catalytically inactive prostasin induces severe skin pathology in transgenic mice (13, 14), and in particular, by our recent demonstration that mice expressing only catalytically inactive endogenous prostasin, unlike prostasin null mice, display normal long-term survival (15).

The above findings have raised a number of unanswered mechanistic questions regarding prostasin and its functions in epidermal development. We addressed four of these questions in the present study. (a) Does prostasin need activation site cleavage to perform its epidermal functions, analogous to trypsin-like serine protease-like growth factors, such as hepatocyte growth factor and macrophage-stimulating protein (16, 17)? (b) Do prostasin zymogen and activated two-chain prostasin have different functions in distinct epidermal compartments? (c) Does matriptase exert its essential epidermal functions solely through the conversion of the prostasin zymogen? (d) Does prostasin zymogen stimulate matriptase activation in the epidermis?

Experimental Procedures

Generation of Prostasin Zymogen-locked Knock-in Mice—A 2000-bp DNA donor fragment homologous to the genomic sequence of mouse Prss8, except for the desired point mutations CGC to CAG, changing the arginine 44 to a glutamine, was purchased from Blue Heron (Bothell, WA). This fragment spans 1000 bp upstream and 1000 bp downstream from the desired point mutations. The donor DNA additionally contained two synonymous base pair changes to minimize the unspecific binding/cleavage of the zinc finger nuclease to the donor DNA. A custom zinc finger nuclease (ZFN)2 specific for cleaving murine Prss8 was procured from Sigma-Aldrich. The ZFN was designed to bind the following sequence (small letters

* This work was supported by the NIDCR Intramural Research Program (to T. H. B.) and by The Harboe Foundation, The Lundbeck Foundation, and the Foundation of 17.12.1981. (to S. F.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the author and does not necessarily represent the official views of the National Institutes of Health.

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2 The abbreviations used are: ZFN, zinc finger nuclease; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol.
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indicate the cleavage site): 5′-TGCCGTCATCCAGCCacgca-TCACGGTGTGGTGGCGTG-3′. The linearized donor DNA and ZFN mRNA were microinjected into the male pronucleus of FVB zygotes, which were implanted into pseudopregnant mice. The offspring were screened for the point mutation using the following primers: WT forward, 5′-CCGTCAATCCAGCCACTACC-3′; MUT forward, 5′-CCGTCAATCCAGCCCCAG-3′; WT+MUT reverse, 5′-TAGATCTGGACTGACCATCGTACG-3′; reverse, 5′-TAGATCTGGACTGACCATCGTACG-3′.

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, and RNA was purified using the RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription and PCR amplification were performed using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), 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′-TTCCGCAAGTTCACTACC-3′ and 5′-CGGCC- CGGCTAGGCTTACG-3′. The annealing temperature for this primer set was 60 °C. Expression levels were compared with S15 mRNA levels in each sample.

Protein Extraction from Mouse Tissue—The tissues were dissected, snap-frozen on dry ice, and stored at −80 °C until homogenization. The tissues were homogenized in ice-cold lysis buffer containing 1% Triton X-100, 0.5% sodium deoxycholate in PBS plus Protease Inhibitor Cocktail (Sigma) and incubated on ice for 10 min. The lysates were centrifuged at 20,000 × g for 20 min at 4 °C to remove the tissue debris, and the supernatant was used for further analysis. The protein concentration was measured with a standard BCA assay (Pierce).

Western Blotting—Samples were mixed with 4× LDS sample buffer (NuPAGE, Invitrogen) containing 7% β-mercaptoethanol and boiled for 10 min, unless otherwise indicated. The proteins were separated on 4–12% BisTris NuPAGE gels and proteins were separated on 4–12% BisTris NuPAGE gels and nondenatured secondary antibodies (Thermo Scientific). After three 5-min washes with TBS-T, the signal was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Pierce).
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**Zymogen-locked Prostasin Supports Interfollicular Epidermal Development—Homozygosity for Prss8 null mutations causes defects in hair follicle development, as well as postnatal lethality, due to loss of barrier function of the interfollicular epidermis (1, 2, 15). To determine to which extent zymogen-locked prostasin can support distinct aspects of epidermal...**
development, we interbred Prss8+/zym mice and genotyped 69 offspring from a total of eight litters. This analysis showed that Prss8zym/zym pups were born in a frequency that did not significantly deviate from the expected Mendelian frequency (Fig. 2A, black bars). Surprisingly, none of 18 Prss8zym/zym pups died within the 21-day preweaning period (Fig. 2A, gray bars). Furthermore, no deaths were observed in a prospective cohort of 10 Prss8zym/zym mice and 8 Prss8+/+ littermates followed for 6 months postweaning (data not shown). These findings indicated that prostasin locked in the zymogen conformation suffices to induce epidermal barrier formation. Compatible with this notion, the interfollicular epidermis of newborn Prss8zym/zym pups was

**REPORT:** Epidermal Functions of Prostasin Zymogen

![Graph A](image1)

**Distribution at birth**

|       | Distribution at day 21 |
|-------|------------------------|
| P rss8 | 21                     |
|       | 21                     |
| zym/+  | 30                     |
| zym/zym| 18                     |

![Graph B](image2)

% initial body weight

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

![Graph F](image6)

![Graph G](image7)

![Graph H](image8)

![Graph I](image9)

![Graph J](image10)

![Graph K](image11)

![Graph L](image12)

![Graph M](image13)

![Graph N](image14)

![Graph O](image15)

![Graph P](image16)

![Graph Q](image17)
outwardly normal in appearance (Fig. 2B), and newborn Prss8<sup>zym/zym</sup> skin presented with only a mildly compacted, slightly more immature, stratum corneum (Fig. 2, C and D). Indeed, direct analysis of transepidermal fluid loss rates revealed only a minimal increase in newborn Prss8<sup>zym/zym</sup> pups that was much lower than the rapid dehydration rates observed in prostasin null Prss8<sup>+/−</sup> pups (Fig. 2E) (2, 15).

Abnormal Whisker and Pelage Hair Development in Mice Expressing Zymogen-locked Endogenous Prostasin—Although mice expressing only zymogen-locked prostasin displayed normal interfollicular epidermal development and postnatal survival, obvious defects were apparent in hair follicle development. Whiskers, which are present in wild-type mice at birth, were absent in newborn Prss8<sup>zym/zym</sup> pups (Fig. 2F). When the whiskers erupted later in postnatal development, the whiskers were kinked and curly (Fig. 2G). Furthermore, pelage hairs were markedly sparser in adult Prss8<sup>zym/zym</sup> mice (Fig. 2H), correlating with a reduced proliferation of hair follicle cells as compared with littermate controls (Fig. 2, I–N, compare I with J and K with L; results were quantified in M and N).

Epidermal Matriptase Zymogen Conversion Is Only Modestly Stimulated by Wild-type and Zymogen-locked Prostasin—Prostasin and matriptase were previously proposed to be part of a single epidermal proteolytic cascade in which matriptase is upstream of prostasin (7–9). Prostasin, however, is upstream of matriptase in other epithelia (6), and zymogen-locked prostasin supports matriptase auto-activation in cell-based overexpression systems (13). We, therefore, next directly examined the status of matriptase activation in the epidermis of newborn Prss8<sup>+/−</sup>, Prss8<sup>zym/zym</sup>, and Prss8<sup>+/+</sup> pups. We separated skin protein lysates by reducing SDS-PAGE and visualized the matriptase zymogen and activated matriptase by Western blotting using an antibody directed against the serine protease domain. Importantly, activated matriptase was readily found in skin lysates from mice of all genotypes, showing that prostasin is dispensable for epidermal matriptase activation. The matriptase levels varied somewhat between different skin preparations, but we observed no consistent genotype-related difference in overall matriptase expression levels. However, an immunoblot by ProteinSimple, utilizing capillary electrophoresis, which gives a quantitative measure of the amount of both the latent and the cleaved form of matriptase, showed a small increase in the ratio of activated to latent matriptase in Prss8<sup>+/−</sup> and Prss8<sup>zym/zym</sup> skin extracts, as compared with Prss8<sup>+/−</sup> skin extracts (Fig. 2, P and Q). This decrease, however, is unlikely to cause any of the epidermal phenotypes observed in prostasin null mice or in mice expressing zymogen-locked prostasin, as the level of activated matriptase in Prss8<sup>−/−</sup> and Prss8<sup>zym/zym</sup> skin extracts exceeds that observed in matriptase heterozygote (St14<sup>+/−</sup>) mice, which display no epidermal phenotype (18, 19).

The relationship between matriptase and prostasin in the epidermis was initially believed to be a simple one, with matriptase activating prostasin, and prostasin executing the epidermal functions of the cascade through the proteolytic cleavage of specific substrates of unknown identity (8). The findings in this study combined with our recent phenotypic characterization of mice expressing only catalytically inactive prostasin, however, necessitate a significant revision of this model, at least regarding interfollicular epidermal development (18, 19). First, the essentially normal development of the interfollicular epidermis of mice expressing zymogen-locked or catalytically inactive prostasin shows that prostasin requires neither zymogen conversion nor catalytic activity to execute its essential functions in this epidermal compartment. It follows from this observation that matriptase must exert its essential functions in interfollicular epidermal development essentially independently of prostasin zymogen conversion, by cleavage of unidentified substrates or through a non-catalytic mechanism. Likewise the persistence of activated matriptase in the epidermis of prostasin null mice or mice expressing zymogen-locked prostasin shows that prostasin activation of matriptase is of limited importance in the epidermis, although the experimental approach used does not exclude the possibility that matriptase activation in some epidermal sub-compartments may be significantly stimulated by, or even be dependent on, prostasin.

Our study also demonstrates that, although dispensable for interfollicular epidermal development, prostasin zymogen conversion does play a critical role in follicular epidermal compartment. Mice expressing zymogen-locked prostasin displayed delayed whisker eruption, kinky and curly whiskers, and sparse pelage hair. These phenotypes are virtually identical to those of mice expressing catalytically inactive prostasin (15) as well as mice expressing low levels of epidermal matriptase (9). In light of these findings and our recent phenotypic characterization of mice expressing only catalytically inactive prostasin, however, we have determined that matriptase is dispensable for matriptase activation in the epidermis.

![FIGURE 2. Prss8<sup>zym</sup>/zym mice display whisker and pelage hair defects.](image-url)
of the co-expression of matriptase and prostatin in hair shaft-forming keratinocytes of the hair follicle, and the strict requirement of matriptase for prostatin zymogen conversion, it is reasonable to assume that matriptase and prostatin form a more “conventional” proteolytic cascade in this compartment whereby matriptase activates prostatin and prostatin cleaves specific substrates to promote hair morphogenesis.

In summary, our study has demonstrated that prostatin is unique among trypsin-like serine proteases in that it has dual essential in vivo functions as a zymogen that are non-enzymatic, as well as essential proteolytic functions once it is converted to its two-chain enzymatically active conformation.

**Author Contributions**—S. F. did the vast majority of the experiments included in the manuscript. D. H. M. assisted with animal experiments. T. H. B. was the principal investigator and supervisor on this research project.

**Acknowledgments**—We thank Drs. Silvio Gutkind and Mary Jo Dan- ton for critically reviewing this manuscript, and we thank Andrew Cho from the NIDCR Gene Targeting Facility for mouse generation.

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