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Evidence for a Matriptase-Prostasin Proteolytic Cascade Regulating Terminal Epidermal Differentiation*

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Recent gene ablation studies in mice have shown that matriptase, a type II transmembrane serine protease, and prostasin, a glycosylphosphatidylinositol-anchored membrane serine protease, are both required for processing of the epidermis-specific polyprotein, profilaggrin, stratum corneum formation, and acquisition of epidermal barrier function. Here we present evidence that matriptase acts upstream of prostasin in a zymogen activation cascade that regulates terminal epidermal differentiation and is required for prostasin zymogen activation. Enzymatic gene trapping of matriptase combined with prostasin immunohistochemistry revealed that matriptase was co-localized with prostasin in transitional layer cells of the epidermis and that the developmental onset of expression of the two membrane proteases was coordinated and correlated with acquisition of epidermal barrier function. Purified soluble matriptase efficiently converted soluble prostasin zymogen to an active two-chain form that formed SDS-stable complexes with the serpin protease nexin-1. Whereas two forms of prostasin with molecular weights corresponding to the prostasin zymogen and active prostasin were present in wild type epidermis, prostasin was exclusively found in the zymogen form in matriptase-deficient mice. Furthermore, we present histological, biochemical, and genetic evidence that matriptase acts upstream of prostasin, and that matriptase is an essential epidermal activator of the prostasin zymogen.

EXPERIMENTAL PROCEDURES

Mice—Experiments followed institutional guidelines. Matriptase knock-out and β-galactosidase-tagged matriptase knock-in mice were described (8, 11).

Histological Stains—X-gal and immunohistochemical stains were performed as described (11). The mouse prostasin antibody has been described (13).

Generation of Soluble Recombinant Prostasin—HEK-293T cells were transfected with pCMV-SPORT6 expression vector containing full-length mouse prostasin (I.M.A.G.E clone 32941).
of matriptase with prostasin was analyzed by immunohistochemistry of serial sections or, when staining intensity permitted, by immunohistochemical staining of X-gal-stained sections with prostasin antibodies to simultaneously visualize the two proteases. As described recently (11), X-gal staining of the skin of matriptase+/E16β-gal mice showed that matriptase expression was confined to the uppermost living layer of the interfollicular epidermis of 7-day-old mouse pups (Fig. 1A). Interestingly, immunohistochemical staining of interfollicular epidermis revealed a similar localization of prostasin (Fig. 1B).

Combined X-gal staining for matriptase and immunohistochemical staining for prostasin revealed overlapping expression in interfollicular epidermis at this age (Fig. 1C) as well as in newborn pups (Fig. 1E), demonstrating that the two membrane-associated proteases have the potential to physically interact in vivo. To determine the time of onset of expression of matriptase and prostasin in the developing epidermis, combined X-gal staining and immunohistochemistry of matriptase+/E16β-gal embryos at embryonic day (E) 14.5 to E16.5 was performed. At E14.5 and E15.5 no expression of either of the two serine proteases could be detected (Fig. 1, F and G). At E16.5, however, both matriptase and prostasin were expressed (Fig. 1H), temporally correlating with stratum corneum formation and the onset of acquisition of the epidermal barrier (15). X-gal staining combined with immunohistochemical staining for the marker of basal keratinocytes, cytokeratin-14, demonstrated the clear suprabasal expression of the two proteases at this developmental stage (Fig. 1I).

Matriptase Activates the Prostasin Zymogen in a Cell-free System—Previous studies have shown that the matriptase zymogen undergoes autoactivation during synthesis (16, 17). In contrast, the prostasin zymogen is unable to undergo autoactivation and a physiological activator of prostasin has not been identified (18). Activation of the prostasin zymogen occurs by endoproteolytic cleavage after Arg12 within the amino acid sequence QPR12-ITG, a cleavage reaction that could be mediated by matriptase, based on studies of matriptase specificity (19). These observations suggested that matriptase would act upstream of prostasin if the two proteases were part of the same zymogen cascade. To test this, we expressed prostasin in HEK-293T cells. The recombinant prostasin was released from the cell surface with PI-PLC to generate a soluble form of prostasin that presented as a dominant 40-kDa species and two minor species with slightly higher and lower electrophoretic mobility when analyzed by SDS-PAGE and Western blotting under reducing conditions (Fig. 2, lane 1). Prostasin generated this way appeared to be predominantly in the zymogen form, as it did not form complexes with the cognate serpin PN-1, which forms SDS-stable complexes with active prostasin (13) but not with the prostasin zymogen (Fig. 2, compare lane 2 with lanes 4 and 6). This suggested that the faint higher and lower molecular weight species both could represent glycosylation variants of the zymogen (13). Activation of the prostasin zymogen leads to the formation of active two-chain prostasin, which can be distinguished from the prostasin zymogen by a small increase in electrophoretic mobility in high-percentage SDS-PAGE gels after reduction of the single disulfide bridge that links the two chains (13). Exposure of soluble prostasin zymogen to either 1
or 10 nM active matriptase protease domain led to the formation of a prominent immunoreactive band with the mobility expected for active prostasin (Fig. 2, compare lane 1 with lanes 3 and 5). The percentage of prostasin zymogen converted to this higher mobility species by matriptase varied between prostasin preparations and was never complete, even with very high matriptase concentrations (data not shown). This suggested that not all recombinant prostasin released from HEK-293T cells by PI-PLC was in a conformational state that permitted the activation by matriptase. To confirm that the cleavage of the prostasin zymogen by matriptase leads to the formation of active prostasin, we incubated untreated and matriptase-treated prostasin with PN-1 and detected prostasin-PN-1 complexes by Western blotting using anti-prostasin antibodies. In the absence of preincubation with matriptase, no prostasin-PN-1 complex was observed (Fig. 2, lane 2). However, when prostasin zymogen was first exposed to matriptase and then incubated with PN-1, a prominent 85-kDa molecular mass complex that was immunoreactive with anti-prostasin antibodies was observed (Fig. 2, compare lane 2 with lanes 4 and 6). Taken together, these data show that the matriptase catalytic domain is capable of converting the zymogen of prostasin to active, serpin-reactive prostasin in a cell-free system.
Lack of Proteolytically Processed Prostasin Zymogen and Prostasin Zymogen Accumulation in Matriptase-ablated Epidermis—The striking phenotypic similarities between matriptase- and prostasin-deficient mice (Table 1), when combined with our data presented above, strongly suggested that matriptase could be a physiological activator of the prostasin zymogen during terminal epidermal differentiation and predicted the existence of a matriptase-prostasin zymogen cascade in epidermal differentiation. To definitively test this, we determined the state of activation of epidermal prostasin in the presence and absence of matriptase. Protein lysates were prepared from the epidermis of newborn wild-type mice and their matriptase-deficient littermates (8), and prostasin processing was analyzed by the separation of the protein lysates by reducing SDS/PAGE on high percentage gradient gels, followed by Western blotting using prostasin antibodies (Fig. 3A). In wild-type epidermis, prostasin was found in two forms: a 39-kDa form, compatible with the apparent molecular mass of the prostasin zymogen, and a 37-kDa form, compatible with activated prostasin (Fig. 3, lanes 3–5). In contrast, in matriptase-ablated epidermis, prostasin was exclusively found in the higher molecular mass 39-kDa zymogen form. Furthermore, the 39-kDa form of prostasin was frequently more abundant in matriptase-deficient epidermis (Fig. 3, lanes 6–8, and data not shown). This increase in prostasin zymogen did not appear to be caused by a corresponding increase in the steady state level of prostasin mRNA as judged by real-time PCR analysis (data not shown), suggesting that the accumulation was caused by loss of zymogen activation by matriptase. To quantitatively assess the formation of active prostasin in matriptase-sufficient and -deficient epidermis, protein extracts from the epidermis of five matriptase-sufficient and five matriptase-deficient littermates were analyzed by Western blot. The fraction of total prostasin presenting as active prostasin was determined by densitometric scanning of the blot (Fig. 3B). In matriptase-sufficient epidermis, 40–51% of prostasin was in the active two-chain form, while the amount of active prostasin in matriptase-deficient epidermis was below the level of detection. Taken together, these data provide definitive evidence that matriptase is essential for the proteolytic processing of prostasin in the epidermis.

The coordinated expression and co-localization of matriptase and prostasin in the epidermis and the activation of prostasin zymogen by matriptase in vitro and in vivo, when combined with the identical phenotype of matriptase and prostasin-deficient mice, provides compelling evidence for the existence of a matriptase-prostasin zymogen activation cascade regulating terminal epidermal differentiation. This suggests that loss of profilaggrin processing, defective corneocyte maturation, and abnormal intercorneocyte lipid extrusion in matriptase-deficient epidermis may all be secondary to loss of prostasin zymogen activation. Increasing evidence indicates that terminal epidermal differentiation is regulated by a sophisticated cascade of serine proteases and serine protease inhibitors that all become expressed in transitional layer cells during stratum corneum formation and undergo sequential activation during stratum corneum maturation and shedding (8, 11, 20–29). The serine proteases currently proposed to be critical for stratum corneum formation include matriptase, prostasin, stratum corneum tryptic enzyme, stratum corneum chymotryptic enzyme, furin, and profilaggrin processing endopeptidase1. Additionally, serine protease inhibitors, including the Kunitz-type serine protease inhibitor, hepatocyte growth factor...
activator inhibitor-1, and the Kazal-type, multidomain serine protease inhibitor, SPINK5, could have key roles in regulating the activity of one or several of these proteases in both human and mouse epidermis. Perturbations causing increased or decreased serine protease activity in the upper epidermis have serious pathophysiological consequences. Thus, ablation of matriptase or prostasin prevents acquisition of the epidermal barrier by blocking terminal epidermal differentiation (9, 12), while, conversely, SPINK5 deficiency or overexpression of stratum corneum chymotryptic enzyme compromises the epidermal barrier through premature epidermal differentiation and accelerated shedding of the stratum corneum (21–23, 26). It remains to be determined whether a single proteolytic cascade or multiple independent proteolytic cascades are operational during terminal epidermal differentiation. Previously, stratum corneum tryptic enzyme has been proposed to act upstream of stratum corneum chymotryptic enzyme during the desquamation of stratum corneum (25), and our results now show that matriptase acts upstream of prostasin during terminal epidermal differentiation. In addition to loss of epidermal barrier formation, matriptase deficiency also severely impairs stratum corneum differentiation. In addition to loss of epidermal barrier formation and matriptase deficiency also severely impairs stratum corneum desquamation (9). These observations suggest the intriguing hypothesis that the four serine proteases and SPINK5 could be part of a single zymogen cascade with a complexity reminiscent of other serine protease zymogen cascades, such as those involved in blood coagulation or digestion.

Matriptase and prostasin both have a fairly wide expression in epithelial tissues and both are frequently dysregulated in epithelial tumors (10, 30–43). The role of matriptase as a prostasin zymogen activator and the potential function of the matriptase-prostasin cascade in other physiological processes and in pathophysiological processes, such as cancer, are clearly important areas for future study.

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