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Short Communication

Analyzing the protease web in skin: meprin metalloproteases are activated specifically by KLK4, 5 and 8 *vice versa* leading to processing of proKLK7 thereby triggering its activation

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Abstract

The metalloproteases meprin α and β are expressed in several tissues, leukocytes, and cancer cells. In skin, meprins are located in separate layers of human epidermis indicating distinct physiological functions, supported by effects on cultured keratinocytes. Meprin β induces a dramatic change in cell morphology and a significant reduction in cell number, whereas *in vitro* evidence suggests a role for meprin α in basal keratinocyte proliferation. Meprins are secreted as zymogens that are activated by tryptic proteolytical processing. Here, we identify human kallikrein-related peptidases (KLKs) 4, 5, and 8 to be specific activators of meprins. KLK5 is capable of activating both metalloproteases. Interestingly, KLK4 and 8 cleave off the propeptide of meprin β only, whereas in contrast plasmin exclusively transforms meprin α to its mature form. Moreover, we show that proKLK7 is processed by meprins. N-terminal sequencing revealed cleavage by meprin β two amino acids N-terminal to mature KLK7. Interestingly, this triggering led to an accelerated activation of the serine protease in the presence of trypsin, but not of other tryptic KLKs, such as KLK2, 4, 5, 8, or 11. In summary, we demonstrate a specific interaction between meprin metalloproteases and kallikrein-related peptidases, revealing possible interactions within the proteolytic web.

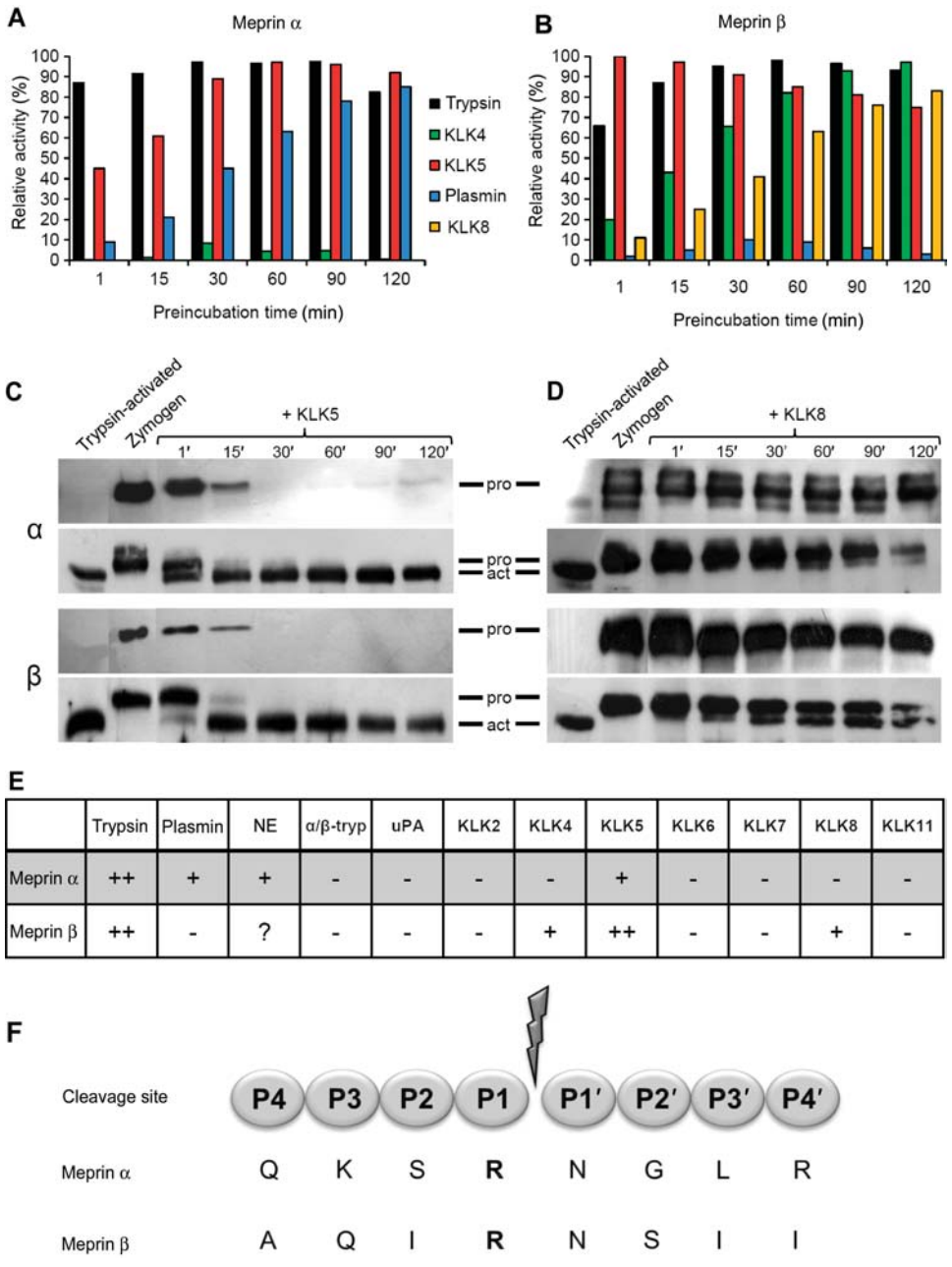
Keywords: astacin; metzincin; proteolysis; serine protease; skin.

The barrier function of the skin is mainly performed by the keratinocytes of the epidermis which are continuously produced by proliferating stem cells of the *stratum basale*, ending in terminal differentiation after 14 days (Candi et al., 2005).

The importance of a precise epidermal differentiation is reflected by the appearance of several skin disorders and diseases caused by defects in the process of epidermal differentiation and desquamation (Descargues et al., 2005, 2006; Komatsu et al., 2006; Alef et al., 2009). Hence, these processes need to be tightly regulated, with many players involved, including several proteases and their inhibitors.

Two of these players are the metalloproteases meprin α and meprin β, members of the astacin family of zinc endopeptidases. In human epidermis, meprin α is expressed exclusively in the keratinocytes of the *stratum basale* where cell division and proliferation occurs. In contrast, meprin β is restricted to the cells of the *stratum granulosum*, the layer where cornification takes place and the epidermal barrier is established. The fact that the two meprins are expressed separately within the epidermis implies different roles for keratinocyte differentiation. Cell culture experiments on human keratinocytes revealed an apoptotic effect of meprin β, whereas meprin α had no negative influence on cell viability (Becker-Pauly et al., 2007). Thus, meprin β induces terminal cell differentiation, whereas meprin α is involved in the regulation of keratinocyte proliferation. The different functions of meprin α and β are supported not only by their different location but also by their strikingly different substrate specificities as well as their specific activation (Rösmann et al., 2002; Becker et al., 2003; Bertenshaw et al., 2003; Kruse et al., 2004; Becker-Pauly et al., 2007).

Here, we demonstrate novel interactions between meprin metalloproteases and kallikrein-related peptidases, illustrating a possible proteolytic network in epidermal physiology. For instance, meprin α, but not meprin β (Becker et al., 2003), is activated by plasmin (Rösmann et al., 2002; Figure 1A,B), a difference that can be as a result of exosite interactions, as there is no obvious preference for plasmin regarding the amino acid sequence within the cleavage sites of both meprins (Rawlings et al., 2008) (http://merops.sanger.ac.uk/). Recently, KLK4, which is also expressed in the *stratum granulosum* (Komatsu et al., 2003), was identified as a specific activator for meprin β, but interestingly not for meprin α (Becker-Pauly et al., 2007; Figure 1A,B). This is most likely based on the cleavage specificity of KLK4, with a clear preference for Arg and Lys in P1 position, also tolerating Gly, Gln, and Asn (Debela et al., 2006b). Additionally, KLK4 prefers the polar amino acid Gln over the hydrophobic Val,



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	Trypsin	Plasmin	NE	α/β-tryp	uPA	KLK2	KLK4	KLK5	KLK6	KLK7	KLK8	KLK11
Meprin α	++	+	+	-	-	-	-	+	-	-	-	-
Meprin β	++	-	?	-	-	-	+	++	-	-	+	-

Figure 1 Promeprin α and β are specifically activated by kallikreins.
Recombinant promeprin α (A) and β (B), expressed and purified as described previously (Becker et al., 2003; Becker-Pauly et al. 2007), were activated using 100 nM trypsin (black), or 300 nM KLK4 (light gray), KLK5 (white), KLK8 (gray), and plasmin (dark gray) at 37°C for indicated times. Activity was measured using azocasein as a substrate for meprins, whereas the relative activity is calculated on the optical density at 340 nm. After preincubation, the serine proteases were inhibited by the addition of 5 mM Pefabloc. All assays were repeated at least two times and the deviation was below 5%. Recombinant KLKs were cloned, expressed, and purified as described elsewhere (Debela et al., 2006a,b). To visualize the proteolytic cleavage of the zymogens during activation, promeprin α and β were incubated with 300 nM KLK5 (C) or 300 nM KLK8 (D) at 37°C for 1–120 min, analyzed by immunoblotting. Untreated zymogens and trypsin-activated meprins served as controls. Anti-Strep-tag (meprin α) and anti-His-tag antibodies (meprin β) were used to detect the zymogens (upper panel). A general astacin antibody, generated against recombinant LAST_MAM (B4F320; Becker-Pauly et al., 2009) detecting the zymogens (pro) and the active forms (act) of human meprins, was applied in the lower panel. (E) Overview of activators for promeprin α and β (NE: neutrophil elastase; α/β-tryp: α- and β-tryptase; uPA: urokinase plasminogen activator; ?=not tested). (F) Schematic presentation of the cleavage sites of meprin α and β. The nomenclature is derived from Schechter and Berger (1967). Amino acids are designated by the one-letter code.

Leu, Thr, and Pro in P2 position with the exclusion of large aromatic and basic side chains at this position. The study by Debela and coworkers revealed that all amino acids from P1 to P4 position within the cleavage site of meprin β (Figure 1F, lower panel) are more preferred by KLK4 than those of meprin α (Figure 1F, upper panel). However, a specificity profile of the primed site residues would be advantageous to explain the complete absence of activation for meprin α.

By searching for potential activators of promeprin α and β in skin, we were also able to identify KLK5 and 8 as excellent candidates (Figure 1A–D). KLK5 (human stratum corneum tryptic enzyme) is expressed in the upper cell layers of human epidermis (Brattsand and Egelrud, 1999) and exhibits the strongest preference for Arg over Lys in P1 with the exclusion of substrates with Phe or Tyr in this position (Michael et al., 2005; Debela et al., 2006b). Additionally, KLK5 has a preference for small and polar amino acids such as Ser, Thr, and Asn in P2 position (Debela et al., 2006b). Activity assays, using azocasein as substrate for activated meprins, showed that KLK5 is able to cleave off the propeptides of both meprin α and β resulting in the release of the active enzymes (Figure 1A,B, white column) with almost the same efficiency as pancreatic trypsin (Figure 1A,B, black column; Figure 1E). These results were verified by Western blot analysis (Figure 1C). Incubation of promeprins with KLK5, followed by immunodetection with antibodies against the propeptide or the full-length enzyme, revealed protein bands corresponding to the zymogen and active forms of meprin α and β, decreasing or increasing in intensity, respectively. During activation, the propeptides including an N-terminal His-tag are cleaved off, demonstrated by a diminished signal using the anti-His-tag antibody (Figure 1C, upper panel). The antibody detecting the complete catalytic domain revealed both the pro- and active enzymes, visualized by signals increasing in intensity upon prolonged incubation with KLK5 (Figure 1C, lanes 3–8). Promeprin α and β are almost completely processed and transformed into their mature forms after 15 min as indicated by the band-shift,

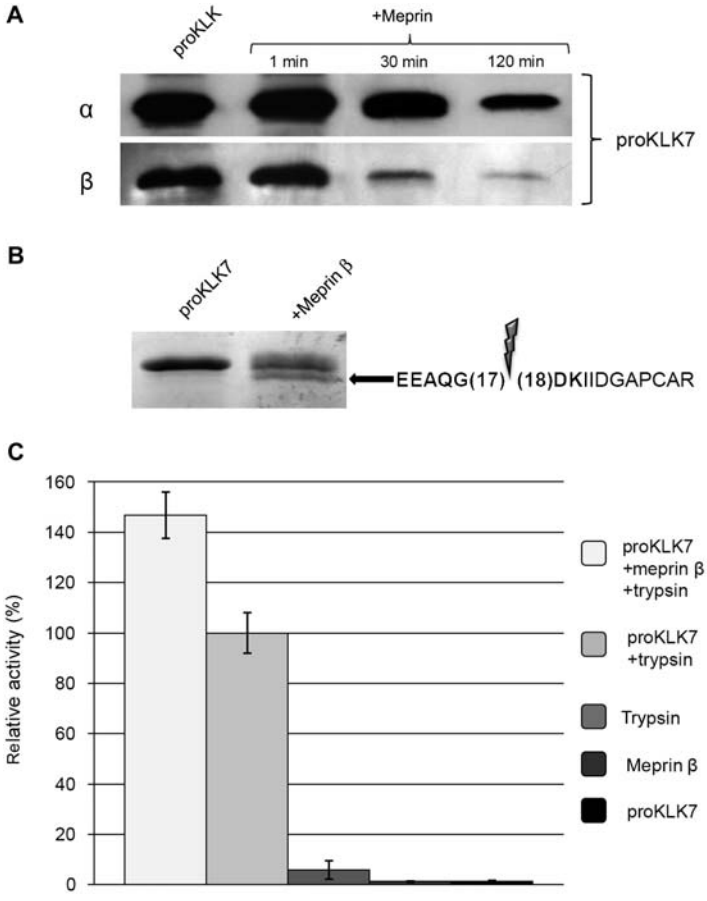

Figure 2 Meprin triggered activation of proKLK7.
The time-dependent processing of proKLK7 (A) by meprin α and β is visualized by immunoblotting using the anti-His-tag antibody. ProKLK7 was incubated with meprin β for 120 min and subjected to SDS electrophoresis (B). N-terminal sequencing of the cleavage product revealed the cleavage site for meprin β which is indicated by the gray lightning. The sequence of the propeptide is highlighted in bold letters. (C) Recombinant proKLK7 was activated by preincubation with 300 nM meprin β for 30 min at 37°C followed by an addition of 100 nM pancreatic trypsin (porcine). KLK7 activity was measured by monitoring the hydrolysis of the chymotryptic substrate (Suc-Leu-Leu-Val-Tyr-AMC). The fluorescence was detected every 5 s over 1.5 h with 380 nm excitation and 460 nm emission wavelengths. The substrate incubated with proKLK7, meprin β, trypsin, and trypsin-activated proKLK7 served as controls.

comparable to trypsin-activated meprins (Figure 1C, lane 1). As a control, untreated promeprin α and β were loaded, respectively.

KLK8 is expressed in the upper layers of human epidermis as well as in the inner lumen of sweat gland ducts and hence is expected to be secreted into sweat (Komatsu et al., 2005). The serine protease is involved in keratinocyte proliferation and desquamation through degradation of desmoglein 1 and corneodesmosin (Kishibe et al., 2007). A cleavage specificity screen (M. Debela, E.L. Schneider, C.S. Craik, unpublished results) revealed that KLK8 has a strong preference for Arg in P1, and Arg and Lys in P3 position. Furthermore, KLK8 prefers hydrophobic residues in P2 and small hydrophobic residues in P4 position. Surprisingly, although all amino acids within the cleavage site of meprin α in P1–P4 position correspond to this specificity profile, particularly Lys in P3 (Figure 1F), KLK8 is only able to activate meprin β but not meprin α, as verified by the azocasein activity assay and Western blot analysis (Figure 1A,B,D).

Hence, the cleavage specificity of KLK8 (and certainly also other enzymes) depends on both the primed and non-primed site. Yet, owing to the interaction of exosites, prediction of cleavage sites for potential substrates remains difficult.

The specific activation of meprin metalloproteases by KLKs subsequently leads to increased proteolytic activity in epidermal skin, which has further influence on proliferation and differentiation of keratinocytes (Becker-Pauly et al., 2007), although the physiological substrates are not known yet. In the case of meprin β, cell adhesion molecules are promising candidates, as cell attachment is diminished by this protease (Becker-Pauly et al., 2007; Huguenin et al., 2008). However, the question arises whether meprins might also be able to activate other proteases. The physiological relevant activation of KLKs for example is still unclear. Utilization of a cellular mass spectrometry-based substrate screen revealed the cleavage of proKLK7 by meprin β in keratinocytes (C. Becker-Pauly and C. Overall, unpublished results). Follow-up *in vitro* analysis of proKLK7 incubated in the presence of recombinant meprin α and β confirmed this finding.

Once activated, meprins are able to cleave within the N-terminal region of proKLK7. This processing was verified by Western blot analysis using an anti-His-tag antibody, revealing a decreased signal upon prolonged incubation as a result of propeptide cleavage (Figure 2A). ProKLK7 incubated with meprin β was subjected to SDS electrophoresis exhibiting a distinct band-shift (Figure 2B). N-terminal sequencing of this product revealed a cleavage site two amino acids N-terminal to mature KLK7, starting with Asp28. Interestingly, this triggering led to an accelerated activation of KLK7 (approximately 150%) in the presence of trypsin (Figure 2C), but not of tryptic KLKs, such as KLK2, 4, 5, 8, or 11 (data not shown). To analyze the activation of

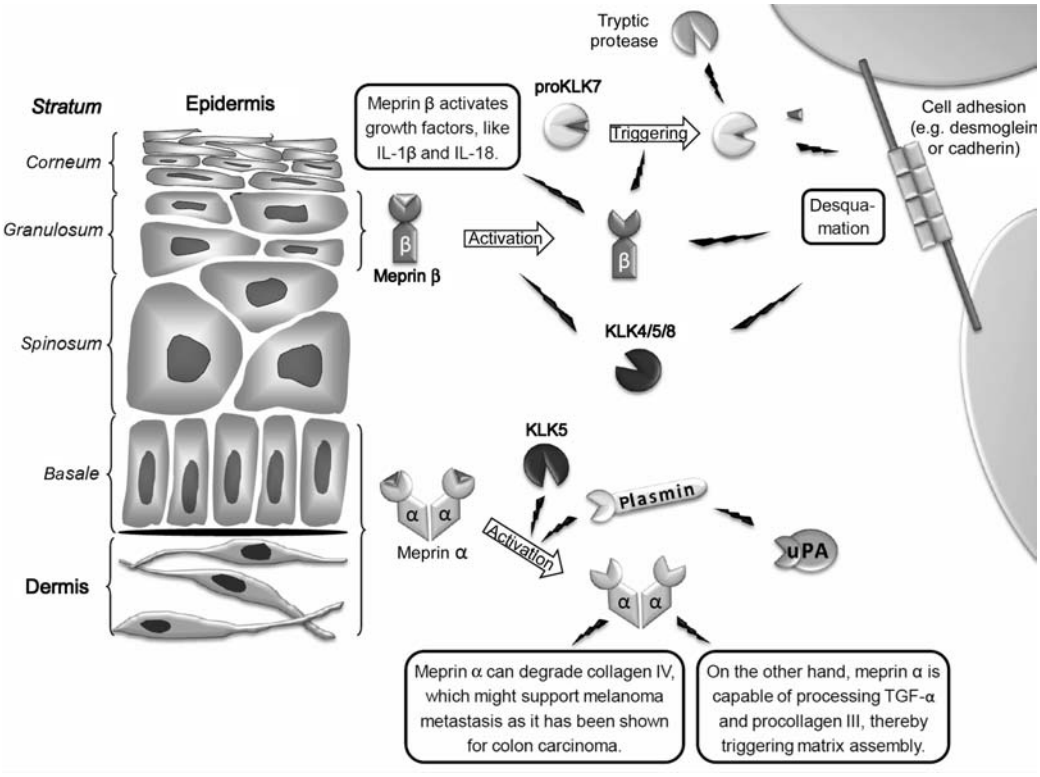

Figure 3 Proteolytic network of meprin metalloproteases and human kallikrein-related peptidases in skin.
The illustration displays possible cascades between proteases and potential substrates regarding skin physiology. Lightning indicates proteolytic cleavage.

proKLK7, we preincubated the zymogen with meprin β, added pancreatic trypsin and measured the hydrolysis of the chymotryptic substrate (Suc-Leu-Leu-Val-Tyr-AMC) by monitoring the resulting fluorescence (Figure 2C). The substrate was incubated with proKLK7, meprin β, trypsin or trypsin-activated proKLK7 in control experiments.

Indeed, pancreatic trypsin is not the physiological relevant protease in skin, but clearly shows that tryptic activity is needed for final activation of proKLK7. Regarding the substrate screen mentioned above it is likely that another tryptic enzyme present in the epidermis is transforming proKLK7 to its mature form after meprin β cleavage.

With regard to a study published by Tye and coworkers, who showed that dipeptidyl peptidase I (DPPI or cathepsin C) can activate proKLK4 (Tye et al., 2009), we evaluated the potential for this cysteine protease (R&D systems, #2336-CY) to cleave off the remaining two amino acids of proKLK7 after meprin incubation. Tested under several conditions and with different substrates, we found no indication at all that DPPI can clip off the N-terminal dipeptide to result in generation of mature KLK7 (data not shown). Notably, DPPI is mainly an intracellular protease, located in lysosomes, and only active under reducing conditions and low pH. Although DPPI can, in part, be secreted (Wolters et al., 1998), the physiological relevance of this interaction is ambiguous.

Hence, to understand physiological and pathophysiological processes, it is essential to study not only the individual players but also their relations within the entire proteolytic web. This study, the interaction between meprin metalloproteases and human kallikrein-related peptidases, reveals just a glimpse of this scenario (Figure 3) and is a base for an extensive study on protease cascades in skin and other tissues.

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