The α and β Subunits of the Metalloprotease Meprin Are Expressed in Separate Layers of Human Epidermis, Revealing Different Functions in Keratinocyte Proliferation and Differentiation

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The zinc endopeptidase meprin (EC 3.4.24.18) is expressed in brush border membranes of intestine and kidney tubules, intestinal leukocytes, and certain cancer cells, suggesting a role in epithelial differentiation and cell migration. Here we show by RT-PCR and immunoblotting that meprin is also expressed in human skin. As visualized by immunohistochemistry, the two meprin subunits are localized in separate cell layers of the human epidermis. Meprin α is expressed in the stratum basale, whereas meprin β is found in cells of the stratum granulosum just beneath the stratum corneum. In hyperproliferative epidermis such as in psoriasis vulgaris, meprin α showed a marked shift of expression from the basal to the uppermost layers of the epidermis. The expression patterns suggest distinct functions for the two subunits in skin. This assumption is supported by diverse effects of recombinant meprin α and β on human adult low-calcium high-temperature keratinocytes. Here, β induced a dramatic change in cell morphology and reduced the cell number, indicating a function in terminal differentiation, whereas meprin α did not affect cell viability, and may play a role in basal keratinocyte proliferation.

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INTRODUCTION

Meprin (EC 3.4.24.18) is a zinc endopeptidase of the astacin family and the metzincin superfamily, originally found in intestinal and renal brush border membranes of humans, mice, and rats (Sterchi *et al.*, 1982, 1983, 1988). Meprins consist of two homologous subunits, α and β, which assemble to homo- or heterooligomeric complexes, whose basic unit is a disulfide-linked dimer (Becker *et al.*, 2003; Bertenshaw *et al.*, 2003; Ishmael *et al.*, 2005). Both subunits are multidomain type 1 membrane proteins composed of an amino-terminal propeptide, an astacin-like protease domain with the extended zinc binding active site motif HEXXHXXGFXHE and a conserved methionine residue in a β-1,4-turn (Met-*turn*) (Stöcker *et al.*, 1993, 1995; Bond and Beynon, 1995), a MAM domain (meprin A5 protein tyrosine phosphatase μ), and a TRAF domain (tumor necrosis factor receptor associated factor), which are thought to mediate protein–protein interactions, followed by an epidermal growth factor-like module, the C-terminal transmembrane domain, and a cytosolic tail. An inserted (I) domain in the α subunit is cleaved on the secretory pathway, resulting in the loss of the membrane anchor and subsequently in the secretion of the enzyme in those tissues, where α is not co-expressed with β, whereas the β subunit predominantly remains membrane bound (Marchand *et al.*, 1995; Eldering *et al.*, 1997). In contrast to the rodent orthologs, the human meprin β subunit contains several *O*-glycosylation sites in the extracellular part near the membrane. Human meprin oligomers may be shed from the cell surface by proteolytic cleavage in this region (Hahn *et al.*, 2003; Leuenberger *et al.*, 2003). Activation of meprins requires the removal of the N-terminal pro-peptide, and is catalyzed by trypsin in the intestinal lumen. Plasmin has also been identified as a meprin α activating enzyme (Rösmann *et al.*, 2002). However, this does not apply to promeprin β, whose activation site appears to be inaccessible for peptidases larger than trypsin (Becker *et al.*, 2003).

There are various *in vitro* observations of a cleavage of basement membrane proteins (e.g., collagen IV, nidogen-1, and fibronectin), protein kinases, growth factors, cytokines

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Abbreviations: HaCaT, human adult low calcium high temperature

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(interleukin-1β), and other bioactive peptides by meprin, but both subunits exhibit markedly different cleavage specificities (Choudry and Kenny, 1991; Kaushal *et al.*, 1994; Chestukhin *et al.*, 1997; Köhler *et al.*, 2000; Bertenshaw *et al.*, 2001; Kruse *et al.*, 2004; Herzog *et al.*, 2005).

Meanwhile, a broader range of sites of meprin expression other than kidney and intestine has been discovered, which varies between species and between developmental stages within a species. The subunits may be co-expressed or expressed individually as shown for the human intestine, where meprin β is expressed in the small intestine only, but meprin α is expressed in both the small and large intestine (Lottaz *et al.*, 1999a). Contrarily, in newborn mice, a more ubiquitous distribution of meprin subunits in the intestine and kidney has been reported (Kumar and Bond, 2001). However, after weaning the mRNA of meprin α was barely detectable in the intestine of ICR and C3H/He mice (Kumar and Bond, 2001). Less described expression sites of meprin are the salivary glands of mice (Craig *et al.*, 1991), the plexus choroideus, the inner ear, the nasal epithelium, and smooth muscle cells of rats (Bunnett *et al.*, 1993; Spencer-Dene *et al.*, 1994). In humans, meprin mRNA was also detected in the liver, skeletal muscle, stomach, and pancreas (http://merops.sanger.ac.uk). The observation that intestinal leukocytes express meprin (Lottaz *et al.*, 1999a) suggests a function in innate immunity. This assumption is supported by the analysis of meprin$^{-/-}$ mice (Crisman *et al.*, 2004), in which these lymphoid cells exhibited a restricted ability to migrate through the extracellular matrix.

Additionally, meprin has also been detected in certain epithelial carcinomas such as colorectal cancer (Matters and Bond, 1999; Lottaz *et al.*, 1999b), where soluble human meprin α is secreted not only apically but also basolaterally, thereby increasing the proteolytic potential of tumor cells for the destruction of the basement membrane (Kruse *et al.*, 2004). An abnormal meprin secretion has also been observed in rats with experimentally induced acute renal failure (Trachtman *et al.*, 1995; Carmago *et al.*, 2002). Taken together, these findings suggest a contribution of meprin to epithelial differentiation, matrix remodelling and cell migration, and also to inflammatory processes, tumor growth and metastasis.

Much less is known on alternative sites of meprin expression in adult mammalian tissues besides the tissues mentioned above. Here we present human skin as a new expression locus of meprin α and β. The fact that each subunit is expressed in a distinct cell layer of the epidermis, namely the stratum basale and the stratum granulosum, demonstrates the uniqueness of these proteases and reveals different physiological functions.

RESULTS

The antisera used for meprin detection are subunit specific and allow for a clear distinction between meprin α and β

To examine the specificity of the polyclonal antibodies used for the immunofluorescences, we tested meprin expressing insect cells (*Sf*9, *Spodoptera frugiperda*). These cells were infected with *Baculo* viruses containing human meprin α or β cDNA as described before (Becker *et al.*, 2003). Non-infected cells were used as a control for unspecific signals. Meprin α and β-expressing cells were detected by the α and β antisera, respectively (Figure 1). Furthermore, an antiserum directed against both subunits provided signals in both transfected cell lines. These results comply with the specificity of the antisera observed in the Western blot analysis (Figure 2j). Not all insect cells show fluorescence signals, which is because not all host cells are infected simultaneously resulting in different meprin levels. However, the selectivity of the antibodies used for the different meprin subunits is clearly evident. To exclude the fact that the antisera bind non-specifically in human tissues, cryosections from human ileum were analyzed. Fluorescence signals could be detected only on the apical side of the intestinal epithelium and on certain cells in the lamina propria, corresponding to leukocytes (data not shown), which is in accordance with published data (Lottaz *et al.*, 1999a).

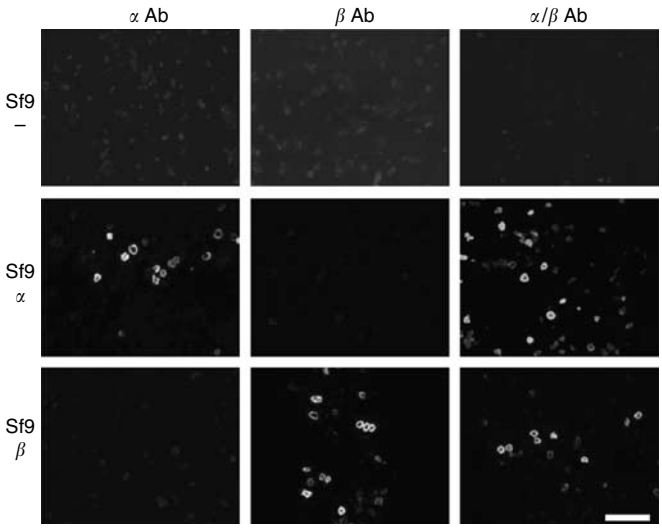

Figure 1. Control assay to test the specificity of the used antisera. Meprin transfected and nontransfected insect cells (*Spodoptera frugiperda, Sf*9) were incubated with antibodies (Abs) generated against meprin α, β, and both subunits. Bar = 50 μm.

Both meprin subunits are detected in human epidermal skin, albeit in different cell layers

The antisera were then used to analyze cryosections of human skin for meprin expression (Figure 2a and b). The fluorescence signals obtained by the α-specific antiserum localize exclusively to keratinocytes of the stratum basale (Figure 2a), the epidermal cell layer, where cell proliferation occurs. The signals appear strictly perinuclear. No fluorescence is observed at the cell membranes or in the extracellular space. By contrast, the data obtained for meprin β show an expression pattern restricted to the stratum granulosum displaying a linear pericellular staining of 3–4 cell layers (Figure 2b). Hence, both meprin subunits are expressed in the epidermis, but they are strictly separated, which confirms their different functionality. To prove further specificity of the antisera, recombinant meprin α and β were preincubated with the used antisera, which resulted in the

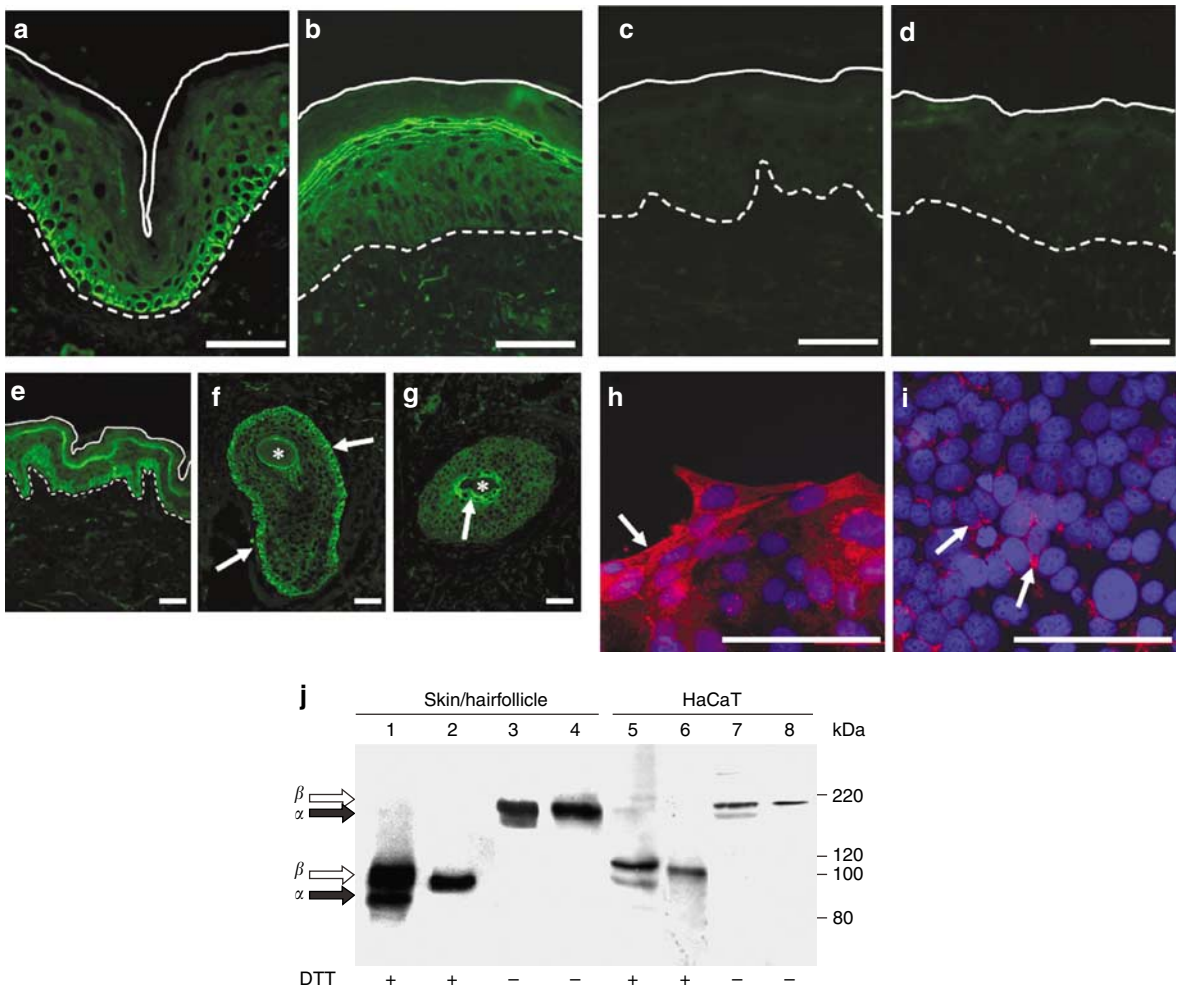

Figure 2. Distribution of meprin α and β in human skin and HaCaT cells. **(a, b)** Immunofluorescences of normal skin for meprin α and β, respectively. (**c, d**) As a control, recombinant antigen was competitively incubated with the antisera, resulting in vanished fluorescence signals. Using the antiserum recognizing both meprin α and β, the signals appear in both cell layers of the epidermis (**e**), corresponding to the results for each subunit alone (**a, b**). In cross-sections of hair follicles, meprin α (**f**) could be detected in the outer root sheath, whereas the β subunit (**g**) is expressed by cells in the inner root sheath. Confocal fluorescence reveals that meprin α (**h**) predominantly occurs at the border of non-confluent cell areas, where the HaCaT cells still proliferate. In contrast, meprin β (**i**) is expressed by confluent cells that are more differentiated. Dotted lines show the relative positions of the basement membrane; straight lines denote the outermost layers of the skin. Asterisks indicate the position of the hair. Arrows point out fluorescence signals. Nuclei of HaCaT cells are marked by 4,6-diamidino-2-phenylindole (**h, i**). Bar = 50 μm. To detect meprin subunits by Western blot analysis (**j**) lysates from skin biopsies, hair roots, and HaCaT cells were run on SDS-PAGE (7.5%) with or without DTT, and transferred to polyvinylidene fluoride membrane. Blots were probed with rabbit anti-human meprin α/β (lanes 1,3,5,7) and meprin β antisera (lanes 2, 4, 6, 8). Signals could be measured due to HRP-coupled secondary antibodies.

absence of meprin-specific fluorescence signals (Figure 2c and d). Moreover, the antiserum recognizing both subunits resulted in fluorescence signals in both the stratum basale and the stratum granulosum (Figure 2e). Meprins could also be detected in hair follicles. The signal for meprin β is present in the region of the inner root sheath, whereas meprin α is localized in the outer root sheath (Figure 2f and g).

Western blot analysis demonstrated the presence of both meprin subunits in cell lysates of healthy human skin as well as in extracts of human hair roots (Figure 2j, lanes 1–4). Under reducing conditions the crossreactive α/β antiserum stained two bands (Figure 2j, lane 1), in the range of about 90 and 110 kDa, corresponding to meprin α and β, respectively. The same lysate analyzed with the β-specific antiserum revealed only the band of the higher molecular weight (Figure 2j, lane 2), indicative of meprin β, which is C-terminally elongated in comparison to the α subunit. Under non-reducing conditions a strong band of about 220 kDa is visible corresponding to disulfide-linked dimers (Figure 2j, lanes 3,4). The detection of more protein in the range of 110 kDa is probably due to still unsecreted and unprocessed promeprin α.

Human keratinocytes express meprin α and β

The expression patterns of meprin in human skin keratinocytes could be confirmed in human adult low-calcium high-temperature (HaCaT) cells. In these cell cultures, meprin α is expressed at the borderline of proliferating cell clusters (Figure 2h), whereas the β subunit was detected only after total confluence was achieved, in cells that have most

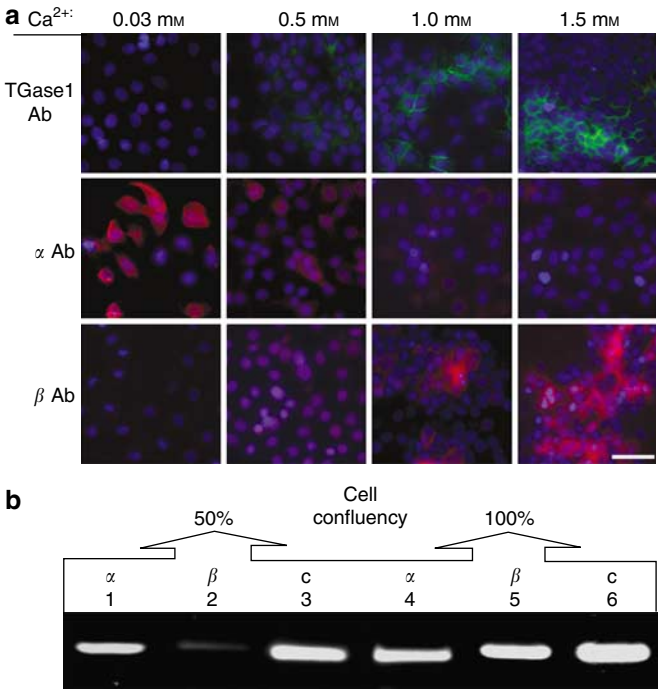

Figure 3. Meprin expression depends on the differentiation level of keratinocytes. (**a**) HaCaT cells were cultured in media containing different Ca^{2+} concentrations (0.03, 0.5, 1.0, and 1.5 mM; from left to right). To evaluate the stage of keratinocyte differentiation, a transglutaminase 1 antibody (TGase1 Ab) was used (green fluorescence). Meprin α and β expression could be detected by subunit-specific antisera α Ab and β Ab, respectively (red signals). Cell nuclei are marked by 4,6-diamidino-2-phenylindole. Bar = 50 μm. To determine meprins on mRNA level in proliferating and more differentiated keratinocytes (**b**), total RNA was isolated from HaCaT cells and transcribed into cDNA by RT-PCR using oligo(dT) primers. Meprin α (lanes 1,4) and β (lanes 2,5)-specific primers were used for PCR, as well as β-actin (lanes 3,6) for control (c). Samples were loaded on an agarose gel (1%) and visualized by ethidium bromide.

probably reached a later stage of differentiation (Figure 2i). This could be confirmed using HaCaT cells, which were treated with different Ca^{2+} concentrations, thereby triggering the differentiation of these cells. At low Ca^{2+} levels (0.03 mM), merely the expression of meprin α could be observed by immunofluorescence microscopy whereas the β subunit was detectable only at higher Ca^{2+} levels (1.0 and 1.5 mM) (Figure 3a). Under these conditions, we also observed the expression of transglutaminase 1, a marker of keratinocyte differentiation (Sayama *et al.*, 2001) (Figure 3a).

These findings are in correlation to the mRNA expression of meprins in HaCaT cells (Figure 3b, lanes 1, 2, 4, 5). The mRNA of the α subunit is detectable in freshly disseminated cells (Figure 3b, lane 1), whereas meprin β mRNA appears only upon complete confluence of keratinocytes (Figure 3b, lanes 4,5). Additionally, the results obtained by immunofluorescence analysis were supported by Western blots (Figure 2j, lanes 5–8), which detected meprin α and β, albeit in lower concentrations as compared to the protein isolated from hair roots.

Recombinant meprin β, but not α, changes the shape and reduces the number of HaCaT cells

In order to investigate whether meprin α or β has an influence on cell growth and proliferation, we added recombinant protein in different concentrations to confluent grown HaCaT cells. Incubation with recombinant meprin α, as active enzyme or zymogen form, did not show any obvious influence on the keratinocytes even at high concentrations (Figure 4a and b). By contrast, incubation with active meprin β caused dramatic changes in cell number and morphology (Figure 4d–h). Increasing the meprin β concentration led to an increase of cell-free areas and to an altered cell shape. After 18 hours incubation, the remaining keratinocytes had a stretched and flattened appearance and gathered to form strands of only a few cell layers thickness (Figure 4h and i). Incubation of HaCaT cells with promeprin β did not show morphological effects except at the highest concentration (10 μg/ml) (Figure 4c); this may indicate that the zymogen form can be activated by secreted proteases from the HaCaT cells. Furthermore, the addition of actinonin, an inhibitor of both meprin subunits (Kruse *et al.*, 2004), to meprin β-treated cells revealed normally grown keratinocytes (Figure 4j).

The viability of the cells after treatment with recombinant meprins was tested in a proliferation assay (Figure 5a). As assumed, only meprin β had a significant influence on cell number. An increase in the concentration of active protein up to 10 μg/ml leads to a decrease of the total cell number down to 10%. Promeprin β reduced the cell number to about 30%. To verify whether this effect on cell viability was due to general cytotoxicity or a more regulated process, an assay was performed testing the binding capacity of annexin V to phosphatidylserine, which is typical for the initial phase of apoptosis. As visualized by immunofluorescence microscopy, incubation with 5 μg/ml active meprin β resulted in increased apoptosis at the border of newly originated cell-free areas (Figure 5d), whereas in confluent areas the cells appeared almost identical to untreated keratinocytes (Figure 5b and c).

Human kallikrein-related peptidase 4 (KLK4) is a potent activator for meprin β

Owing to the lack of pancreatic trypsin outside the gut, we searched for an alternative activator of promeprin β in the skin. In contrast to promeprin α, which can be activated by plasmin outside the intestine (Rösmann *et al.*, 2002), this is not the case for promeprin β owing to sterical reasons (Becker *et al.*, 2003). Therefore, we tested whether kallikrein-related peptidases (Debela *et al.*, 2006b), which are expressed in the skin (Komatsu *et al.*, 2003), are able to activate promeprin β *in vitro*. In the course of these experiments we found that KLK4 was able to transform the zymogen of meprin β to its mature form within the same efficiency as pancreatic trypsin, visualized by an activity assay using azocasein as substrate (Figure 6a). Promeprin β was activated with trypsin or KLK4 at 37°C for different periods of time. Although 100 nM trypsin fully activated the β subunit within 30 minutes, incubation with 300 nM KLK4 generated maximum meprin β activity within 120 minutes. Azocasein degrading activity could be

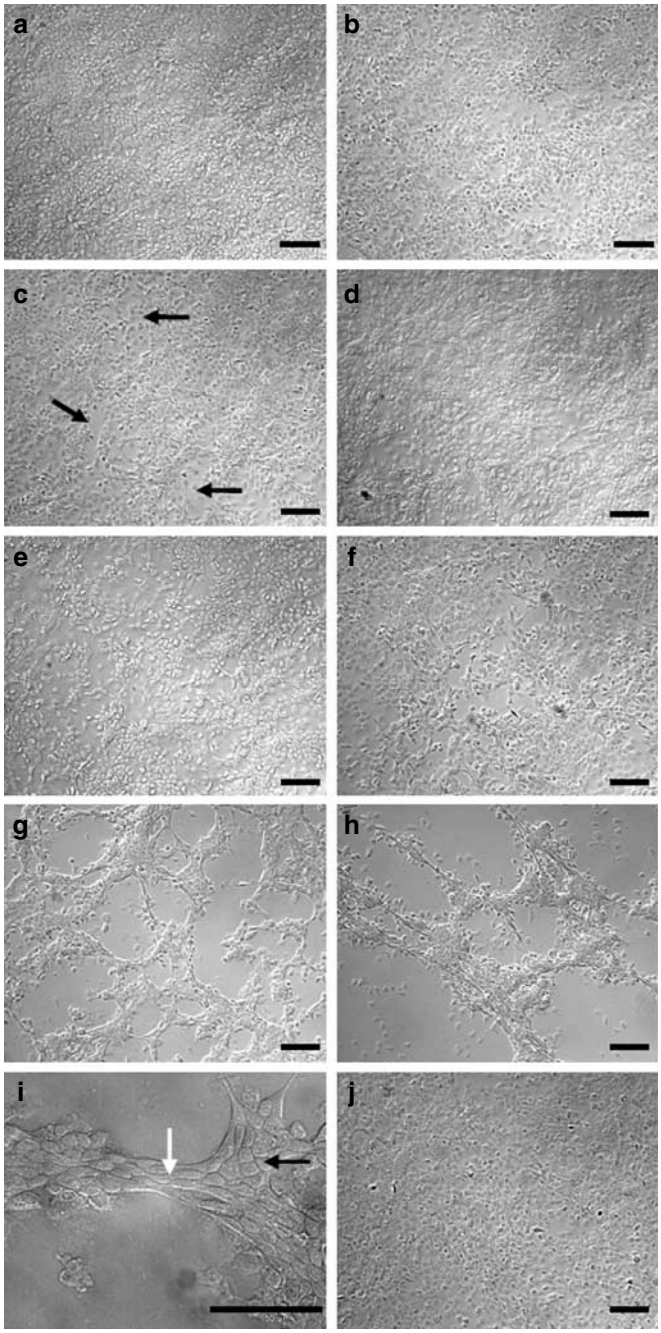

Figure 4. Influence of recombinant meprin on HaCaT cells. After total confluence was achieved, recombinant promeprin α (**a**) and β (**c**), as well as their active forms (**b** and **d–h**, respectively) were added to the keratinocytes in concentrations of 0.1 μg/ml (**d**), 1 μg/ml (**e**), 2 μg/ml (**f**), 5 μg/ml (**g**), and 10 μg/ml (**a–c, h**) followed by incubation in a humidified chamber for 18 hours at 37°C. To visualize the change in cell shape, a typical area is shown at a greater magnification (**i**). The black arrow indicates normally growing roundish HaCaT cells; the white arrow points out keratinocytes, which have a stretched and narrower shape. The inhibitor actinonin (0.5 μM) was added to meprin β (10 μg/ml) incubated keratinocytes (**j**) in order to verify that catalytic activity is responsible for the change of cell morphology. Arrows indicate cell-free areas. Bars = 50 μm.

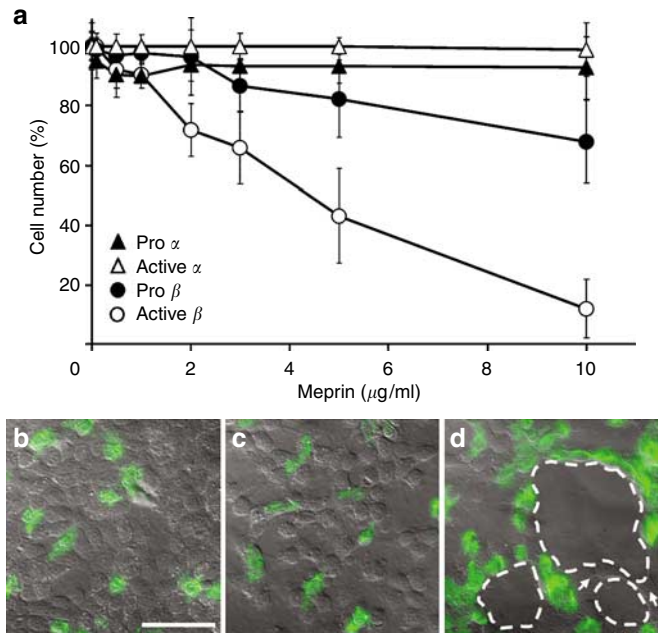

Figure 5. Influence of recombinant meprins on the proliferation of keratinocytes. (**a**) HaCaT cells were incubated with recombinant meprins in the following concentrations: 0.1, 0.5, 1, 2, 3, 5, and 10 μg/ml. To obtain 100% cell viability, an untreated control was analyzed. The percentage of living keratinocytes was quantified using MTT as a substrate for the gluthathione-S-transferase (Invitrogen, Karlsruhe, Germany). The diagram shows the results of threefold assays. To test whether the decreased cell number is due to cytotoxic or apoptotic effects caused by meprin β, an Apoptosis Detection Kit II (Calbiochem/Merck, Darmstadt, Germany) was used. Untreated HaCaTs (**b**) exhibit constitutive apoptotic signals in the same way as meprin β (5 μg/ml)-treated keratinocytes in confluent areas (**c**). Only at the border of cell-free areas (encircled by dotted lines) a strong increase of apoptosis was detected (**d**). White arrows indicate non-apoptotic cells. Bar = 50 μm.

completely inhibited by adding 0.1 mM actinonin (data not shown). These results were verified on the protein level by Western blot analysis (Figure 6b). Incubation of promeprin β with 300 nM KLK4 (Figure 6b, lanes 2–7) and subsequent immunoblotting with different antibodies revealed protein bands corresponding to the zymogen (z) and mature (m) forms of human meprin β. A meprin β-specific antibody (β Ab) recognizing the zymogen and mature forms and an anti-His-tag antibody (His Ab) were used in the upper and lower panel, respectively. It is obvious that human promeprin β is almost completely converted to its active form by limited KLK4 digestion within 90 minutes, as indicated by the bandshift due to the conversion of the zymogen to the mature enzyme (Figure 6b, lanes 2–7, upper panel). The newly generated band shows the same electrophoretic mobility as trypsin-activated meprin (Figure 6b, lane 8, upper panel). As a control, untreated promeprin β was loaded on lane 1. During activation, the propeptide of recombinant promeprin β including the N-terminal His-tag is cleaved. This correlates to the vanishing signal observed with the His Ab. The signal for promeprin β disappears upon prolonged incubation with KLK4 (Figure 6b, lanes 2–7, lower panel).

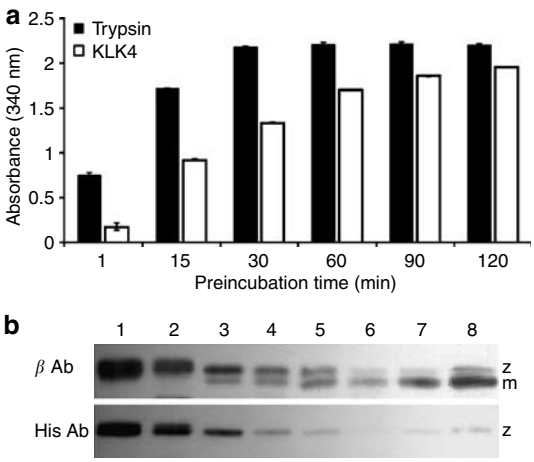

Figure 6. Promeprin β is activated by human kallikrein-related peptidase 4. (**a**) Recombinant promeprin β was activated using 100 nM trypsin (black) or 300 nM KLK4 (white) at 37°C for given periods of time and later tested for azocasein cleaving activity. After preincubation, trypsin and KLK4 were inhibited by the addition of 5 mM Pefabloc. The data represent averages of two independent activity assays (+/− maximum/minimum). Absorbance at 340 nm correlates to proteolytic activity. To visualize the proteolytic cleavage of the zymogen during activation, promeprin β was activated with 300 nM KLK4 at 37°C for 1, 15, 30, 60, 90, and 120 minutes (**b**, lanes 2–7) and aliquots were subjected to SDS electrophoresis and immunoblotting. An antibody (β Ab) detecting the zymogen (z) and the mature (m) form of meprin β was applied in the upper panel, and an anti-His-tag antibody (His Ab) detecting only the zymogen was used in the lower panel. As controls the untreated zymogen (lane 1) and trypsin-activated meprin β (lane 8) were loaded.

Again, this is in agreement with trypsin-activated meprin (lane 8).

The expression pattern of human meprin changes drastically during hyperproliferative hyperkeratosis

To further assess meprin expression, different skin diseases/keratinization disorders were studied: skins from four patients with Netherton syndrome (N.s.), five patients with psoriasis vulgaris (P.v.), and five patients suffering from ichthyosis vulgaris (I.v.) (Figure 7). In I.v. – a model disease for a retention hyperkeratosis, characterized by hypoproliferative epidermis – the expression of meprin α was rather strong, but the distribution of the enzyme was comparable to healthy skin. By contrast, in skin diseases characterized by epidermal hyperproliferation such as Netherton Syndrome and psoriasis, the α subunit was dislocated and expressed in the uppermost layers of the epidermis corresponding to the stratum granulosum. Furthermore, as far as meprin β is concerned, we observed that its expression is no longer restricted to the stratum granulosum, but can also be found in the stratum corneum.

DISCUSSION

With regard to the described expression sites of meprin, the question arose as to whether this enzyme is also present in human skin, the largest epithelial organ. In fact, here we show by immunofluorescence studies that both meprin subunits are constitutively expressed in normal human

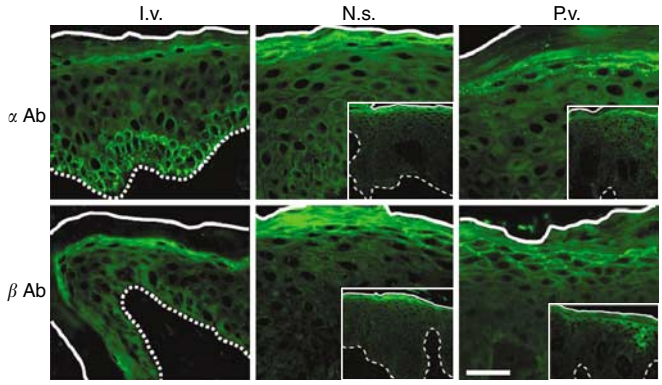

Figure 7. Meprin expression in hyperkeratotic and hyperproliferative skins. Meprin expression (α Ab and β Ab) in pathological situations was analyzed taking skins from patients with N.s. and patients with P.v. as examples of hyperproliferation disorders and from I.v. Although, the expression pattern of meprin in hypoproliferative epidermis (I.v.) was comparable to healthy skin (Figure 2a and b), the situation dramatically changed in hyperproliferative epidermis. Here, the α subunit is relocated and appears at the *S. granulosum* area (N.s. and P.v.). Dotted lines show the relative positions of the basement membrane; straight lines denote the outermost layers of the skin. Smaller pictures give an overview of the entire epidermis. Bar: 50 μm.

epidermis, but not detectable under the same assay conditions in the underlying dermis and subcutis. The epidermis is a constantly proliferating tissue and makes up about 3% of the total skin; it comprises several cell layers piling up to a thickness between 0.02 and 1 mm (Jastrow and Vollrath, 2002). The most amazing and concurrently the most interesting point is that the two meprin subunits are not colocalized. The α subunit is expressed exclusively in the keratinocytes of the basal layer, the stratum basale, where cell division and proliferation occur. By contrast, meprin β could only be identified in the cells of the stratum granulosum, that layer of the epidermis where the cornified envelope is formed and the epidermal barrier is established (Candi *et al.*, 2005).

Meprin α immunofluorescence in the basal layer is mostly perinuclear, whereas meprin β staining is restricted to the cell borders. This subcellular distribution of the two subunits is consistent with their different processing along the secretory pathway. If expressed alone, meprin α is secreted, because it loses its membrane anchor owing to proteolytic cleavage within the I-domain (Marchand *et al.*, 1995; Tang and Bond, 1998). Outside the stratum basale secreted meprin α is diluted by diffusion and therefore escapes detection. This observation is comparable to results from other groups also working with secreted proteases in human skin (Sadowski *et al.*, 2003; Buth *et al.*, 2004). On the other hand, meprin β-producing cells of the stratum granulosum integrate the enzyme within their plasma membrane, because β does not contain a cleavable I-domain and therefore remains predominantly membrane bound (Butler *et al.*, 1987; Hahn *et al.*, 2003). The localization of meprin β limits its activity to the narrow pericellular space.

The observation that both meprin subunits are expressed in different cell layers, implying different roles for keratinocyte differentiation, could be confirmed using HaCaT cells.

When these keratinocytes were cultured in a medium containing low Ca^{2+} concentrations (0.03 mM), they maintained a proliferative basal cell-like phenotype. By elevating the Ca^{2+} concentration to above 0.1 mM, the cells can be induced to differentiate *in vitro* (Watt and Green, 1982). Further elevation of Ca^{2+} concentrations to 1.0 mM or higher induces terminal differentiation and cornification. This differentiation process closely follows the maturation pattern of epidermis *in vivo* (Yuspa *et al.*, 1989). We could show that meprin expression in keratinocytes depends on the particular stage of cell differentiation; hence, the α subunit could be detected in keratinocytes treated with low Ca^{2+} concentrations (0.03 mM), whereas meprin β is expressed by HaCaT cells, which are cultured under high Ca^{2+} levels (1.5 mM).

From the proliferation of keratinocytes in the stratum basale to their cornification in the stratum corneum, the cells are passing through several stages of differentiation (Eckert *et al.*, 1997). The meprin α expressing stratum basale consists of cylindrical keratinocytes with relatively large nuclei, which are separated from the dermis by a basement membrane. In contrast, meprin β expression occurs in the stratum granulosum, the cell layer where keratinocytes undergo a most dramatic change in their morphology and physiology, resulting in cell flattening and loss of the nuclei. Thus, meprin subunits are involved in completely different physiological processes, with α and β being expressed by keratinocytes in their initial and terminal stages of differentiation, respectively. So far there are no data about the downregulation of meprin expression or about the half life of meprin on the protein level. Meprins are not inhibited by the tissue inhibitors of metalloproteases or α_2-macroglobulin.

The presumed functional difference between the two meprin subunits could be visualized in the present work by their influence on cultured human keratinocytes (HaCaT cells). Confluently grown HaCaT cells were obviously not affected by either the zymogen or the active form of meprin α, whereas active meprin β led to a dramatic change in cell number and morphology. This effect on keratinocytes is promoted by the catalytic activity of the β subunit, because the addition of actinonin, a potent inhibitor for meprin, resulted in a behavior comparable to untreated cells. Interestingly, there was also a small but significant decrease in cell number upon addition of inactive promeprin β. We assume that this might be due to zymogen activation by an endogenous protease. The possible mode of activation is discussed below. Concomitant with the decrease in cell numbers, there was a change in cell morphology from round to stretched/flattened cells that exhibit a tendency to form stacks as seen in the cells of the stratum corneum. This suggests that meprin β might be involved in the terminal differentiation of keratinocytes at the junction between the stratum granulosum and the stratum corneum. Moreover, we could show that meprin β-treated cells reacted positively in an assay for the initial phase of apoptosis. This was almost exclusively seen in keratinocytes at the borders of cell-free areas. Terminal epidermal differentiation is associated with cell death in the skin and therefore somewhat resembles apoptosis (Candi *et al.*, 2005). On the other hand, there is evidence that cornified cell envelope formation is distinct from apoptosis in epidermal keratinocytes (Takahashi *et al.*, 2000). Therefore, the issue of a role for meprin β in differentiation processes of human keratinocytes remains intriguing and deserves further attention.

An earlier report by Carmago *et al.* (2002) demonstrated that meprin A (a designation for a mixture of meprin α homooligomers and α/β heterooligomers) has a negative effect on the viability of certain strains of kidney cells (LLC-PK_1, immortalized porcine epithelial cells; MDCK, Madin-Darby canine kidney cells). In the light of our data acquired with homogeneous α and β subunits, it appears most likely that meprin β was responsible for the decrease in cell number, once more indicating that this metalloprotease is capable of inducing epithelial cell differentiation.

By contrast, it is tempting to speculate that meprin α might be involved in the detachment of basal keratinocytes from the basement membrane found beneath the stratum basale in the initial phase of keratinocyte differentiation. This view is consistent with the observation of a possible role of meprin α in certain pathological situations like colorectal cancer, where it has been suggested to be important for the migration of transformed epithelial cells through the basement membrane (Lottaz *et al.*, 1999b). Meprin α is capable of cleaving major components of the basement membrane like collagen IV, nidogen 1, laminin V, and fibronectin (Kruse *et al.*, 2004). In abrasively growing melanoma cells, the degradation of the extracellular matrix is triggered by plasmin, which is activated from its precursor plasminogen by urokinase (Quax et al., 1991a, b; de Vries *et al.*, 1994). We have shown previously that the urokinase/plasminogen system is also an activator for human promeprin α (Rösmann *et al.*, 2002; Becker *et al.*, 2003). Therefore, it is conceivable that meprin is implicated in the pathogenesis of melanoma formation and/or progression. The α subunit could also be involved in wound healing processes as it is described for MMP-19 (Sadowski *et al.*, 2003). This metalloprotease is expressed in basal undifferentiated cells of the epidermis and regulates insulin-like growth factor-mediated proliferation, migration, and adhesion in keratinocytes through proteolysis of insulin-like growth factor binding protein-3.

Additionally, we could show that meprin α is relocated in skin diseases characterized by epidermal hyperproliferation, namely in psoriasis vulgaris and in Netherton syndrome. Here, the basal keratinocytes show an increased mitotic activity, resulting in a thickened epidermis. Surprisingly, meprin α expression is decreased in the stratum basale, but instead is increased in the uppermost layers of the epidermis, showing a dotted pericellular signal. This suggests that the enzyme formed heterooligomers with the β subunit, resulting in membrane-bound protein, as has been described for the kidney and small intestine (Gorbea *et al.*, 1991; Lottaz *et al.*, 1999a). The altered expression pattern of meprin α in hyperproliferative skin diseases such as psoriasis or Netherton syndrome suggests that meprin α is involved in the normal regulation of keratinocyte proliferation.

The different functions of meprin α and β subunits in the skin are reflected by their different location, and could be due

to their strikingly different substrate specificities (Bertenshaw *et al.*, 2003; Kruse *et al.*, 2004) as well as to their modes of zymogen activation, as only α but not β subunits are activated by plasmin (Rösmann *et al.*, 2002; Becker *et al.*, 2003). A potent activator for meprin β outside the gut has not been identified so far. Potential candidates are serine proteases with trypsin-like specificity, which are colocalized in the epidermis. Several enzymes such as human kallikrein-related peptidase 7, HMW-uPa, and tryptases (α1 and β3) (kindly provided by L. Bruckner-Tudermann, Freiburg, Germany and C. Sommerhoff, Munich, Germany) failed to activate human meprin β *in vitro*. Presumably, these enzymes are too large to access the cryptic site of activation. Therefore, smaller members of the serine protease family, such as kallikrein-related peptidases (Lundwall *et al.*, 2006), are more likely candidates as activators of promeprin β (Yousef and Diamandis, 2001; Michael *et al.*, 2005). Here, we could identify human kallikrein-related peptidase 4 (KLK4) as a potent activator for the zymogen of the β subunit. Human kallikrein-related peptidase 4, also known as prostase, is also expressed by cells of the stratum granulosum (Komatsu *et al.*, 2003) and therefore it may represent a physiological relevant regulator of meprin β activity in the skin. Active recombinant KLK4 has a molecular mass of about 24 kDa (Debela *et al.*, 2006a, b). At first glance, the enzyme exhibits tryptic cleavage specificity, but it is more selective in preferring Arg over Lys in the P1 position and also prefers hydrophobic residues in the region between P4 and P2 (Takayama *et al.*, 2001; Matsumura *et al.*, 2005; Debela *et al.*, 2006a, b). Therefore, KLK4 may be involved in a regulatory network rather than in degrading processes.

On activation, meprin β may be involved in the reconstruction of cell–cell contacts at the transition to the stratum granulosum/stratum corneum interface.

Thus, the discovery of the skin as a meprin-expressing tissue opens up a new research field, which offers the possibility to clarify a new intriguing physiological function of the zinc endopeptidase meprin.

MATERIALS AND METHODS

Patients

The study was approved by the Institutional Review Board of the University Hospital of Münster. The Declaration of Helsinki Principles were followed and all patients enrolled gave their written, informed consent.

Chemicals, enzymes

All chemicals were of analytical grade and, if not stated otherwise, obtained from Amersham Bioscience, Freiburg; Applichem, Darmstadt; Serva, Heidelberg; Biorad, Munich; Bachem, Heidelberg; Sigma/Aldrich, Deisenhofen; and Merck, Darmstadt, Germany.

RNA isolation and RT-PCR

All materials were obtained from Peqlab Biotechnology GmbH, Erlangen, Germany, if not stated otherwise. Total cellular RNA from HaCaT cells was extracted using the E.Z.N.A. Total RNA Kit following the manufacturer's instructions. RNA was then reverse transcribed to cDNA with M-MuLV reverse transcriptase and used as a template in PCR. RT-PCR was performed with oligo d(T)16 primers (ROTH, Karlsruhe, Germany), RNAse inhibitor, dNTPs, and DEPC water. In each reaction 3 μg of total RNA was used. For PCR, each incubation mixture was split into three and transferred to new reaction tubes to amplify meprin α, meprin β, and β-actin separately. A negative control without reverse transcriptase was run for each sample of RNA. In addition, a negative control without RNA was run in parallel with the test samples. The following meprin primers were synthesized:

meprin α:	sense 5′-GATGATGACCACAATTGGAAAATTG C-3′
	antisense 5′-ACCTGTCTGTTTTCTACCGGCCACTC-3′
meprin β:	sense 5′-AGAGAGCACAATTTTAACACCTATAGT-3′
	antisense 5′-CTATCGAAATGCATGAAGAAACCAGA-3′
β-actin:	sense 5′-GACATCCGCAAAGACCTGTACG-3′
	antisense 5′-ACTGGGCCATTCTCCTTAGAG-3′

PCR was performed for 40 cycles (denaturing at 95°C for 45 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 60 seconds). PCR products were resolved on 1% agarose gels, followed by staining with ethidium bromide and recording with a digital camera. For sequencing, PCR samples were purified by the E.Z.N.A Cycle-Pure Kit and analyzed at Genterprise (Mainz, Germany).

Cell lysates and Western blot analysis

Frozen tissue biopsies of 10 normal human skin samples were homogenized in a mortar. Twenty scalp hairs were each plucked from ten healthy voluntary individuals and hair roots were transferred into 40 μl lysis buffer. The samples were kept at 4°C for 20 minutes, heated at 99°C for 10 minutes, and then stored at −20°C before Western blot analysis.

The homogenate, as well as hair roots or HaCaT cells, was dissolved in lysis buffer (9.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 135 mM NaCl, 2.7 mM KCl, pH 7.4; containing 1% Triton X-100, 1 μM pepstatin, 1 μM E64, 2 mM pefabloc). For immunoblot analysis proteins were subjected to 7.5% SDS-PAGE (Laemmli, 1970) under reducing and non-reducing conditions and afterwards transferred onto a polyvinylidene fluoride membrane (Immobilon P, Millipore, Eschborn, Germany) by electroblotting (80 mA, 75 minutes). For detection with meprin-specific antibodies the membrane was saturated with 5% dry milk in Tris-buffered saline for 1 hour at room temperature, incubated with the first antibody (polyclonal anti-meprin α or β antibodies, 1:1,000) for 1 hour and afterwards with horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000) for 45 minutes at room temperature. Detection was performed using Rotilumin (ROTH, Karlsruhe, Germany) following the manufacturer's instructions using X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech, Freiburg, Germany).

6 × His-tag marked recombinant promeprin β before and after KLK4 incubation was visualized using Penta-His antibodies (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Cells and culture conditions

The HaCaT keratinocyte cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 (in air) in DMEM (Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% fetal bovine serum and 50 U/ml penicillin and 50 μg/ml streptomycin. For some

experiments, HaCaT cells were grown in a serum-free DMEM to avoid inhibitory effects on the used proteases.

Ca^{2+} switch experiments were performed to study keratinocyte differentiation. For this purpose, HaCaT cells were cultured in Ca^{2+} free DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin at 37°C and in 5% CO_2. After 1 day, post-confluence medium was changed to DMEM basal medium (without growth factors) with 0.03, 0.5, 1.0, and 1.5 mM Ca^{2+} (500 μl per well). Cells were exposed to experimental medium for 24 hours followed by another 48 hours of identical medium. The total experimental period was 96 hours.

Expression and purification of recombinant meprins

Expression, purification, and activation of recombinant meprin β were performed as described before (Becker *et al.*, 2003). For affinity purification of recombinant meprin α, a Strep-*tag* was inserted into human meprin α-cDNA (Köhler *et al.*, 2000) between signal and propeptide. The following primers were used:

sense1: 5′-GGCGGCCTTGCTGTTCTTCTA-3′
antisense1: 5′-GAGAGAATTCCTTTTCGAACTGCGGGTGT GACCACGGTACAGCTGCTATGTGGGCAAAAA-3′
sense2: 5′-GAG AGA ATT CAT TAA GCA TCT TCC TGAA GA-3′
antisense2: 5′-TGG CCT TAT AGG CAC ATC CTT-3′

Primers were synthesized by ROTH (Karlsruhe, Germany). Sequences of constructs were verified by DNA sequencing (Genterprise, Mainz, Germany). Recombinant protein was expressed using the Bac-to-Bac expression system (Gibco Life Technologies, Paisley, UK) following the manufacturer's instructions. All media and supplements were obtained from Gibco Life Technologies. Recombinant baculoviruses were amplified in adherently growing *Spodoptera frugiperda* (*Sf*)9 insect cells at 27°C in Grace's insect medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Protein expression was performed in 500 ml suspension cultures of BTI-TN-5B1–4 insect cells growing in Express Five SFM supplemented with 4 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin in Fernbach flasks using a Multitron orbital shaker (INFORS AG, Bottmingen, Switzerland). Cells were infected at a density of 2×10^6 cells/ml with an amplified viral stock at a MOI of ~10. Protein expression was stopped after 72 hours. Meprin α was further purified from the media by ammonium sulfate precipitation (60% saturation), stirring overnight at 4°C, followed by centrifugation at 11,000 *g* for 2 hours at 4°C. Pellets were dissolved in 1/10 volume of 100 mM Tris-HCl, pH 8.0, 50 mM NaCl and dialyzed against the same buffer. Afterwards the protein solution was loaded on a streptavidin column (IBA, Göttingen, Germany). After a washing step using 20 ml of 100 mM Tris-HCl, pH 8.0, 50 mM NaCl, protein was eluted with 100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2.5 mM D-desthiobiotin under native conditions. Furthermore, protein was concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, Schwalbach, Germany). Activation was carried out as described before for meprin β (Becker *et al.*, 2003).

Immunofluorescence analysis

The used polyclonal antisera from rabbit were generated against the following peptides: the α subunit-specific antibody is directed against a fragment of 420 amino-acid residues including the propeptide, the protease, and MAM domain of human meprin α. The β-specific antibody was obtained using a peptide comprising 150 amino acid residues of the TRAF domain of the human β subunit. The crossreactive antibodies detecting both subunits were generated using a 330-residue-long peptide including the propeptide, the protease domain, and a part of the MAM domain of human meprin β. All antigen peptides were expressed in *Escherichia coli* (Dumermuth *et al.*, 1993; Lottaz *et al.*, 1999a).

Cryosections of normal (10 patients) and hyperkeratotic human skins from four patients with N.s., five patients with P.v., and five patients suffering from I.v., as well as monolayers of keratinocytes (HaCaTs) and insect cells (HighFive) on cover glasses were incubated with normal goat serum (Jackson ImmunoResearch via Dianova, Hamburg, Germany), 10% in phosphate-buffered saline (9.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 135 mM NaCl, 2.7 mM KCl, pH 7.4), to block nonspecific binding. Then the samples were incubated at 4°C for 18 hours with anti-meprin sera or monoclonal anti-human transglutaminase 1 antibody (Biomedical Technologies Inc., Stoughten; 1:1,200 in phosphate-buffered saline). To perform epitope-competition experiments, antibodies were incubated with respective immunogens, namely recombinant human meprin α and β at room temperature for 2 hours before sample incubation. Excess antibodies were removed by washing with phosphate-buffered saline, and the samples were again incubated at room temperature for 2 hours with Alexa 568 (Invitrogen GmbH, Karlsruhe, Germany) or fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch via Dianova, Hamburg, Germany). For HaCaT analyses, nuclei were stained with 4,6-diamidino-2-phenylindole (Applichem GmbH, Darmstadt, Germany). Immunofluorescence detection was carried out by an Axioskop 2 (Carl Zeiss AG, Jena, Germany) or via a confocal laser scanning microscope (Leica TCS NT; Leica Microsystems, Wetzlar, Germany).

Incubation of HaCaTs with recombinant meprins

The HaCaT cells were seeded in chambers of microscope slides (BD Falcon™ CultureSlides; BD Biosciences, Heidelberg, Germany). After total confluence, recombinant meprins were added to the cells at different concentrations (100, 500 ng, 1, 2, 5, and 10 μg) each in a threefold assay. As a control, HaCaTs incubated with recombinant protein in the presence of actinonin (0.5 μM) or without meprin were analyzed. After 18 hours incubation, the chambers were removed and the keratinocytes were analyzed using an Axioskop 2 (Carl Zeiss AG, Jena, Germany).

Cell proliferation assay and apoptosis detection kit

For assessing the proliferation after treatment with recombinant meprins, HaCaT keratinocytes were seeded in serum-free medium into chambers of BD Falcon™ CultureSlides (BD Biosciences, Heidelberg, Germany). After total confluent growth of the cells, recombinant meprins were added at different concentrations (100, 500 ng, 1, 2, 5, and 10 μg) each in triplicate assays. As a control, HaCaTs without meprin were analyzed. After 18 hours incubation, MTT, a component of the Vybrant®MTT Cell Proliferation Assay Kit, was added to the keratinocytes and further treatment was performed using the suppliers' instruction (Invitrogen, Karlsruhe, Germany).

To visualize apoptosis after meprin treatment, HaCaT cells were cultured and incubated with recombinant protein as described

above. Apoptotic cells were detected using Annexin V-fluorescein isothiocyanate Apoptosis Detection Kit II (Merck/Calbiochem, Darmstadt, Germany). This assay has been designed especially for whole cells and depends on the interaction between phosphatidylserine and annexin V. The test was carried out following the providers' instruction.

Azocasein assay

To test whether human kallikrein-related peptidase 4 (KLK4) is able to convert the zymogen form of meprin β to the mature enzyme, an activity assay using azocasein as substrate was established. Recombinant meprin β was incubated with 100 nM trypsin or 300 nM KLK4 at 37°C for different periods of time. After preincubation, trypsin and KLK4 were inhibited by the addition of 5 mM Pefabloc. Low remaining background activity was subtracted from all samples. Although trypsin fully activated the β subunit within 30 minutes, incubation with KLK4 was not as efficient, but maximum activity could be generated within 120 minutes. The addition of 0.1 mM actinonin completely inhibited azocasein degrading activity. Recombinant KLK4 was cloned, expressed, and purified as described elsewhere (Beaufort *et al.*, 2006).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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