Processing of Procollagen III by Meprins: New Players in Extracellular Matrix Assembly?

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Meprins α and β, a subgroup of zinc metalloproteinases belonging to the astacin family, are known to cleave components of the extracellular matrix, either during physiological remodeling or in pathological situations. In this study we present a new role for meprins in matrix assembly, namely the proteolytic processing of procollagens. Both meprins α and β release the N- and C-propeptides from procollagen III, with such processing events being critical steps in collagen fibril formation. In addition, both meprins cleave procollagen III at exactly the same site as the procollagen C-proteinases, including bone morphogenetic protein-1 (BMP-1) and other members of the tolloid proteinase family. Indeed, cleavage of procollagen III by meprins is more efficient than by BMP-1. In addition, unlike BMP-1, whose activity is stimulated by procollagen C-proteinase enhancer proteins (PCPEs), the activity of meprins on procollagen III is diminished by PCPE-1. Finally, following our earlier observations of meprin expression by human epidermal keratinocytes, meprin α is also shown to be expressed by human dermal fibroblasts. In the dermis of fibrotic skin (keloids), expression of meprin α increases and meprin β begins to be detected. Our study suggests that meprins could be important players in several remodeling processes involving collagen fiber deposition.

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INTRODUCTION

In addition to the nonspecific degradation of protein substrates, proteolysis has been found to be of pre-eminent importance in a variety of physiological functions (Overall and Blobel, 2007; Lopez-Otin and Bond, 2008). In many cases, proteolytic enzymes have been reported to act as highly specific scissors, whose actions are fine-tuned with respect to time and location (Doucet et al., 2008). The multidomain endopeptidases meprins α and β, members of the astacin family of zinc metalloproteinases (MEROPS M12A), are known to be expressed in the brush border membranes of intestinal and kidney tubules, certain cancer cells, and intestinal leukocytes (Sterchi et al., 2008). In addition, differential expression in epidermal layers suggests a role in epithelial differentiation and proliferation (Becker-Pauly et al., 2007). Despite these observations, the physiological functions of meprins α and β remain poorly understood.

Meprins are membrane-bound proteinases that, in the case of human meprin α, can be released from the cell surface after proteolytic cleavage during translocation through the secretory pathway (Kohler et al., 2000); in addition, human meprin β can be released from the cell surface by shedding (Hahn et al., 2003). As such, these proteinases are in contact with the extracellular matrix (ECM), and several reports suggest that they are involved in ECM remodeling and tissue differentiation (Kruse et al., 2004; Sterchi et al., 2008). First, macrophages from meprin β knockout mice have a reduced ability to migrate through the ECM (Crisman et al., 2004). Second, meprin β has been identified as a possible key player in passive Heymann nephritis, revealing a function in the physiological turnover of the glomerular basement membrane in rat kidney (Oneda et al., 2008). Similarly, in colon carcinoma, meprin α is expressed not only apically but also basolaterally, which may increase the potential of tumor cells to break through basement membranes as a result of increased proteolytic activity against ECM proteins (Lottaz et al., 1999; Rosmann et al., 2002). Finally, compared with wild-type mice, meprin α knockout mice show decreased lipopolysaccharide-induced injury in kidney tubules, subsequently revealing the ability of meprin α to cleave adhesion molecules (Yura et al., 2009). The latter has also been confirmed for meprin β in cell culture studies (Carmago et al., 2002; Becker-Pauly et al., 2007; Huguenin et al., 2008). Nevertheless, ECM remodeling by meprin metalloproteinases is not yet understood on the molecular level.

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Abbreviations: BMP-1, bone morphogenetic protein-1; ECM, extracellular matrix; PCP, procollagen C-proteinase; PCPE-1, procollagen C-proteinase enhancer-1

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Meprins α and β differ dramatically not only in their quaternary structure (Becker et al., 2003; Bertenshaw et al., 2003; Becker-Pauly et al., 2009), but also in their cleavage specificity. Although meprin α prefers uncharged residues at the P1’ position in the cleavage site, meprin β shows strong specificity for negatively charged residues at this position (Kruse et al., 2004; Sterchi et al., 2008). Thus, meprin β has a specificity similar to the astacin proteinases of the tolloid subfamily (e.g., bone morphogenetic protein-1 (BMP-1)), for which almost all known substrates have an aspartate residue at P1’ (Hopkins et al., 2007). This leads to the question of whether meprins can also cleave substrates of tolloid proteinases, such as fibrillar procollagens.

In this study we show that both meprins α and β are capable of efficiently releasing the N- and C-propeptides from procollagen III. C-terminal cleavage is shown to occur at exactly the same site as with BMP-1 but, unlike BMP-1, whose activity is stimulated by procollagen C-proteinase (PCP) enhancer-1 (PCPE-1) (Moali et al., 2005), meprins are inhibited in the presence of PCPE-1. Finally, we show for the first time that meprin α is expressed by human dermal fibroblasts and that expression of meprin α in the dermis increases in fibrotic conditions (keloid), in which meprin β also begins to be detected.

RESULTS
Procollagen III is a substrate for meprins α and β
Recombinant procollagen III was first incubated at 37 °C in the presence of meprins α and β (Figure 1a). In both cases, the starting material was cleaved and shorter products were observed by SDS-PAGE. When these products were compared with the products obtained in the presence of BMP-1, the prototypical PCP, and with human collagen III pepsin extracted from placenta (BD Biosciences, Le Pont de Claix, France), we observed that the main cleavage product migrated faster than pN-collagen III obtained with BMP-1 but slightly slower than the commercial collagen III (which lacks the N- and C-telopeptides). This product was analyzed by mass spectrometry after trypsinization (liquid chromatography-tandem mass spectrometry) and was found to correspond to the long triple helix (45% sequence coverage with peptides identified with >95% confidence for meprin α and 44% sequence coverage for meprin β; data not shown). Unfortunately, all attempts to determine the N-terminal sequences of this product failed.

In contrast, we could more easily study the C-terminal processing of procollagen III by meprins using tools developed previously. In particular, we used a mini-procollagen III substrate (Moali et al., 2005) that is made up of an N-terminal myc tag, the last 33 Gly-X-Y triplets of the triple helical region, the C-telopeptide, and the C-propeptide region of the full-length molecule (Figure 1b). Incubation of mini-procollagen III with meprins α or β led to cleavage of the substrate, resulting in a similar cleavage pattern to that obtained with BMP-1 (figure 1c). The band approximately 100 kDa corresponds to the C-propeptide trimer of procollagen III under nonreducing conditions (Figure 1b), which has a theoretical molecular mass of 82,509 Da without glycosylation. N-terminal sequencing of this cleavage product, using either meprin α or β, revealed the sequence DEPMDF that corresponds exactly to the cleavage site identified for BMP-1. As the N-terminal product is often difficult to visualize by SDS-PAGE because of inefficient staining with conventional techniques, we also analyzed cleavage products by western blotting, using antibodies directed against the N-terminal myc tag in mini-procollagen III. In the presence of meprin β, a 45-kDa fragment corresponding to the intact N-terminal fragment (c-myc tag, triple helix, and C-telopeptide, Figure 1d) could also be detected. Interestingly, this band was not observed after incubation with meprin α (Figure 1d). A closer look at the junction of the c-myc-tag and the triple-helical region revealed several potential cleavage sites for meprin α (data not shown). Hence, we assume that the c-myc-tag was cleaved off by the proteinase, preventing detection with the corresponding antibody.

Meprins are more efficient PCPs than BMP-1
To quantify meprin activity on mini-procollagen III, the extent of cleavage was measured by densitometry of gels stained with SYPRO Ruby (Sigma-Aldrich, Munich, Germany) (Figure 2a). When the amount of C-propeptide trimer released was expressed as a percentage of the total conversion to the C-propeptide, both meprins α and β showed higher activities than an equimolar amount of BMP-1. Even in the presence of an optimum concentration of PCPE-1 for stimulating BMP-1 activity (1:1 molar ratio with respect to substrate), the activities of meprins α and β (in the absence of PCPE-1) were still approximately 3-fold higher.

To characterize more precisely the kinetics of the three enzymes, the cleavage of fluorescent mini-procollagen III (Kronenberg et al., 2009) was then monitored as a function of time (Figure 2b). Fitting the curves with the simplified integrated Michaelis-Menten equation (see Materials and Methods) gives an estimation of the kinetic parameters of the enzymes, and shows that indeed meprins are more efficient than BMP-1 in cleaving fluorescent mini-procollagen III (kcat/KM ratios of 2.5 ± 0.5 \times 10^2 M^{-1} s^{-1}, 1.1 ± 0.3 \times 10^5 M^{-1} s^{-1}, and 0.35 ± 0.2 \times 10^5 M^{-1} s^{-1} for meprin α, meprin β, and BMP-1, respectively).

PCPE-1 protects procollagen from cleavage by meprins
Interestingly, when meprins were incubated with mini-procollagen III in the presence of PCPE-1, the activities of both meprins α and β were less than in the absence of PCPE-1 (Figure 2a). Thus, in contrast to BMP-1, which is stimulated several fold by PCPE-1 (Ricard-Blum et al., 2002; Moali et al., 2005), meprins are inhibited in the presence of this tolloid activator. This inhibitory effect of PCPE-1 was measured over a range of concentrations, and was found to increase in a dose-dependent manner (Figure 2c).

PCPE is a substrate for meprins α and β
To test whether the enhancer was diminishing meprin activity by acting as an alternative substrate, we incubated meprins α and β directly with PCPE-1. This resulted in a distinct
Cleavage pattern, with cleavage products in the region of 30 and 15 kDa (Figure 3a). To determine the site(s) of cleavage, we incubated meprins with PCPE-1 fragments, either the CUB1CUB2 fragment, which is known to be sufficient for enhancing activity (Hulmes et al., 1997), or the CUB2NTR region, which has no effect on BMP-1 activity (Kronenberg et al., 2009). As also shown in Figure 3a, CUB1CUB2 was not affected by incubation with meprins α or β. In contrast, both proteinases cleaved CUB2NTR, resulting in similar fragments in the region of the NTR domain as with full-length PCPE-1. These observations therefore localize the site(s) of meprin cleavage to the linker between the CUB2 and NTR domains (Figure 3a and d). N-terminal sequencing of the products of cleavage by meprin β showed one of these sites to be at ESPSAP, some 10 residues before the cysteine at the start of the NTR domain.

In view of these observations, we sought to determine whether these fragments of PCPE-1 were capable of diminishing meprin activity (Figure 3b). The CUB1CUB2 fragment was equally effective as the full-length protein in reducing the

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**Figure 1.** Cleavage of procollagen III and mini-procollagen III by meprins. (a) Cleavage of procollagen III by bone morphogenetic protein-1 (BMP-1) and meprins α and β. Mature collagen III produced by meprin cleavage migrates slower than commercially obtained pepsin-extracted placenta collagen III (pl. coll III; right gel). Analysis by 6–7.5% SDS-PAGE in reducing conditions, and staining with Coomassie Brilliant Blue. (b) Structure of mini-procollagen III (Moali et al., 2005) showing the N-terminal c-myc tag (myc), the triple-helical region, the C-telopeptide (telo), and the C-terminal propeptide (CP III). The arrow indicates the BMP-1 cleavage site. (c, d) Cleavage of mini-procollagen III by BMP-1 and meprins α and β. Analysis by 8% SDS-PAGE under nonreducing conditions, followed by staining with (c) SYPRO Ruby or (d) western blotting using anti-c-myc antibodies. Asterisks indicate the bands analyzed by N-terminal sequencing.
Procollagen Processing by Meprins

D Kronenberg

Procollagen Processing by Meprins

D Kronenberg

activities of both meprins α and β on mini-procollagen III. In contrast, the CUB2NTR fragment was not a potent inhibitor of meprin activity on mini-procollagen III. These observations show that acting as an alternative substrate is not the explanation for the effect of PCPE-1 on meprin activity, as the CUB1CUB2 fragment, which is not a substrate, is equally effective. In addition, CUB1CUB2, but not CUB2NTR, shows PCP-enhancing activity with BMP-1 (Kronenberg et al., 2009), most likely mainly by binding to the substrate. These observations point to a mechanism by which binding of the CUB1CUB2 region to mini-procollagen III prevents access of meprins to the cleavage site. This mechanism is supported by the observation that an inactive mutant of PCPE-1, containing a F90A mutation in the CUB1 domain (Blanc et al., 2007), had no effect on the activities of either meprin α or β (Figure 3c). Unlike wild-type PCPE-1, this mutant does not bind to mini-procollagen III (Blanc et al., 2007), although it is cleaved by meprins in the CUB2NTR linker (Figure 3c).

Meprins are expressed by human fibroblasts

As shown in Figure 4, we found weak but clear expression of meprin α in healthy dermis (Figure 4a, white arrows). This was confirmed by reverse transcriptase-PCR revealing meprin α to be expressed by human adult primary dermal fibroblasts (Figure 4g), and also in normal human dermis (data not shown). These cells are the main source of fibrillar collagens in skin (expression of collagen III is shown in Figure 4g). Moreover, a striking increase in meprin α expression within the dermis was observed under pathological conditions in fibrotic skin (keloid; Figure 4c and e, the latter showing the deeper dermal layers). This probably reflects an increased number of fibroblasts, but may also contribute to the increased amount of collagen deposition, through the mechanisms described above. Whether the accumulation of meprin α at the basolateral side of keratinocytes (Figure 4c, red arrow) promotes the degradation of the basement membrane, such as in colon cancer (Lottaz et al., 1999), requires further investigation.

In the fibroblasts of healthy skin, meprin β could not be detected either by immunofluorescence microscopy or by reverse transcriptase-PCR (Figure 4b and g, respectively). The thickened epidermis of fibrotic skin revealed a more diffuse distribution of meprin β within the epidermis, although with a preference for keratinocytes of the stratum granulosum. In addition, in fibrotic skin, a signal could be observed within the dermis (Figure 4c, the latter showing the deeper layers (Figure 4f), and was confirmed by reverse transcriptase-PCR (Figure 4g).

DISCUSSION

Up until now, three groups of proteinases have been implicated in the maturation of the fibrillar procollagens (I, II, III, V, and IX): the tolloid proteinases (including BMP-1), the furin-like proprotein convertases, and the ADAMTSs (a disintegrin and metalloprotease with thrombospondin-like motifs). In general, tolloid proteinases are involved in C-terminal processing, and ADAMTS-2, 3, and 14 in N-terminal processing, whereas in the case of the minor fibrillar collagens (V and XI), tolloid proteinases and furin-like proprotein convertases are also involved in N- and C-terminal processing, respectively (Hopkins et al., 2007; Moali and Hulmes, 2009). In this study we show that meprins α and β are potential players in procollagen processing, both at N- and C-terminal ends. Importantly, both are able to specifically release the C-propeptide of procollagen III, in vitro, at exactly the same site as BMP-1. Although N-terminal maturation requires further characterization, it
Figure 3. Cleavage of procollagen C-proteinase enhancer-1 (PCPE-1) and effects on meprin activity. (a) Effects of meprins on PCPE-1 and its truncated forms CUB1CUB2 and CUB2NTR. Analysis by 12% SDS-PAGE, non-reducing conditions, Coomassie Brilliant Blue staining. (b, c) Influence of PCPE-1, its truncated forms, and the inactive F/A mutant on meprin C-proteinase activity. The band migrating slightly faster than PCPE-1 in lane 4 of c corresponds to the N-terminal region of mini-procollagen III released by proteinase cleavage. This band is absent from lane 1 as a result of additional cleavage in the junction between the c-myc tag and the triple helix (d), which also affects the migration of the mini-procollagen III substrate. Analysis by 8% SDS-PAGE, Coomassie Brilliant Blue staining. Asterisks indicate bands analyzed by N-terminal sequencing.

seems unlikely that meprins cleave at the same P1\(^1\)Q site as the ADAMTSs. Given the important role played by the telopeptides in the control of collagen fibrillogenesis (Hulmes, 2008), it is possible that slight differences in N-terminal processing might lead to subtle changes in fibril formation and hence possible functional consequences.

In this study we have used procollagen III instead of procollagen I, although the latter is the most abundant fibrillar collagen in human skin. However, collagen III is an important biomarker for skin fibrosis (Busquets et al., 2010). In addition, procollagen III is a well-established substrate used in past biochemical assays characterizing PCPE and BMP-1 (Moali et al., 2005; Kronenberg et al., 2009). With regard to C-terminal processing of the other fibrillar procollagens, given the sequence conservation in the region of the cleavage site, it seems likely that meprins may also have a role, although this remains to be shown. Particularly striking was the efficiency of processing of procollagen III by both meprins, which seems to be greater than that of BMP-1, even in the presence of saturating amounts of PCPE-1.

Surprisingly, although BMP-1 activity was strongly enhanced by PCPE-1, as expected (Moschovich et al., 2001), the latter had an inhibitory effect on meprin activity, on a mini-procollagen III substrate. Using different fragments as well as a site-directed inactive mutant of PCPE-1, we showed that this was because of blocking of the cleavage site by PCPE-1 binding (through its CUB1CUB2 region) to the substrate, and not by acting as an alternative substrate. Importantly, cleavage of PCPE-1 by meprins in the CUB2NTR linker region had no effect on PCPE-1 activity, for which an intact CUB1CUB2 region is a necessary and sufficient condition (Kronenberg et al., 2009). These observations suggest the intriguing possibility that PCPE-1 might control the traffic between alternative processing activities (tolloids or meprins) in different physiological contexts.

Although we have shown that meprins \(\alpha\) and \(\beta\) are capable of removing the propeptides from procollagen III in vitro, the (patho)physiological relevance of these observations will depend on their localization relative to procollagens in vivo, as well as on their status regarding activity (presence of inhibitors or enhancers, competition with other proteinases, interactions with other matrix components, and so on). In a previous study on human skin (Becker-Pauly et al., 2007), we detected meprins \(\alpha\) and \(\beta\) in the keratinocytes of the stratum basale and stratum granulosum, respectively. However, as fibrillar collagens are not found in these regions, these data provided no evidence for an interaction with meprins in vivo. In this study we show that meprin \(\alpha\) is expressed by primary human fibroblasts, along with procollagen III and BMP-1, and is found in normal dermis, whereas meprin \(\beta\) is only expressed in the dermis under pathological conditions (keloid) along with an apparent increase in expression of meprin \(\alpha\). These observations show that procollagens and meprins are indeed likely to encounter one another in vivo, particularly in pathological situations. In this regard, meprins being transmembrane proteins, it will be important to
Figure 4. Distribution of meprins in normal and fibrotic human skin. In normal skin, meprins (a) α and (b) β are expressed in the epidermis, as well as meprin α (a, white arrows), but not meprin β (b), in the dermis, as confirmed by (g) reverse transcriptase PCR (RT-PCR). In fibrotic skin (keloid), meprin α expression increases in the dermis, in both superficial (c; white arrows) and deeper (e) layers, along with increased expression in the basolateral epidermis (c; red arrow). Here also, meprin β is detected in the dermis (d, white arrow; g, particularly in the deeper layers (f), and there is increased expression in the epidermis, particularly in the superficial zone. Bar = 100 μm. fs, fibrotic skin; HDFa, primary human adult dermal fibroblast; ns, normal skin.
determine whether they are active in procollagen processing at the cell surface or after shedding into the ECM.

Previous studies (Pappano et al., 2003), using mouse embryo fibroblasts from Bmp1−/− Tll1−/− mice, have shown the importance of tolloid proteinases in procollagen processing in cell culture. Nevertheless, in these studies, some fully processed procollagen I was detected in the ECM associated with the Bmp1−/− Tll1−/− mouse embryo fibroblast cell layers. As suggested by Pappano et al. (2003), this might be attributable to the activity of mTll-2. However, the data presented in this study suggest the alternative hypothesis that it might also be because of the action of meprins. Data from knockout mouse embryo fibroblasts should be interpreted with caution for several reasons. First, mouse embryonic fibroblast data may not be transposable to human adult tissue. We do not know, for example, how the relative expression levels, by fibroblasts, of meprins and tolloids, vary as a function of age. Furthermore, proteinase and collagen expression by fibroblasts is known to be influenced by the presence of keratinocytes; co-culturing keloid keratinocytes with normal fibroblasts, for example, leads to altered collagen deposition (Lim et al., 2002; Wall et al., 2009). Finally, the presence of serum in some of the experiments with the fibroblast culture model complicates the situation, as this contains α2-macroglobulin, which inhibits BMP-1 (Zhang et al., 2006), as well as other components that are known to inhibit meprins (Hirano et al., 2005).

In conclusion, the data presented in this study suggest a role for meprins in ECM assembly. The inhibitory effect of PCP-E1 and the increased expression of meprins by fibroblasts in keloid tissue suggests that meprins may be involved in C-terminal procollagen processing particularly in pathological situations, because of altered epithelial-mesenchymal cell interactions, or during the early stages of wound repair, when there is a need to rapidly deposit a matrix that subsequently undergoes further remodeling involving tolloid proteinases. Further examination of all the partners involved will be required to test these hypotheses.

MATERIALS AND METHODS

Patients

This study was approved by the institutional review board of the University Hospital of Münster. The Declaration of Helsinki Principles was followed and all patients enrolled gave their written, informed consent. Skin samples from patients with keloids were obtained by routine shave excisions before injection with topical corticosteroids for cosmetic reasons.

Protein expression and purification

Recombinant human meprins α and β (extracellular domains) were expressed in baculovirus-infected insect cells and purified as described (Becker et al., 2003; Becker-Pauly et al., 2007). Full-length human BMP-1-FLAG, procollagen III, and a short form of procollagen III (mini-procollagen III), were expressed in HEK 293-EBNA cells (Moali et al., 2005; Blanc et al., 2007). Recombinant human PCPE-1 with an 8-histidine C-terminal tag, its inactive F90A mutant, and its CUB2-NTR fragment were also expressed in HEK 293-EBNA cells (Blanc et al., 2007; Kronenberg et al., 2009), whereas the CUB1CUB2 fragment was prepared by limited trypsinolysis of the full-length PCPE-1 (Kronenberg et al., 2009). Fluorescent mini-procollagen III, labeled with DyLight 633 (Thermo Scientific, Bonn, Germany) was prepared as described previously (Kronenberg et al., 2009).

Proteolytic activity against procollagen III, mini-procollagen III, and PCPE-1

For activity assays, mini-procollagen III (330 nm) was incubated for 30 minutes at 37 °C with 15 nm proteinase in 50 μl assay buffer (50 nm Tris pH 7.4, 150 nm NaCl, 5 nm CaCl2, and 0.02% Brij35). When present, PCPE-1 was added at equimolar concentrations (with respect to the substrate) and up to 2.5 μM in the case of meprins. Because of its higher sensitivity, the quantitation with SYPRO Ruby was performed with only 164 nm mini-procollagen III and PCPE-1 in the assay, with incubation for 15 minutes. To study proteolytic activity of meprins on PCPE-1, 2 μg of PCPE-1 or its truncated forms were incubated with 15 nm proteinase for 1 hour in a total volume of 50 μl. To test the influence of different PCP-E1 fragments on meprin activity, 2 μg of each fragment was first preincubated with 15 nm enzyme for 1 hour, before adding substrate. For cleavage of full-length procollagen III, 288 nm procollagen was incubated with 15 nm proteinase for 1 hour. All activity assays were performed at 37 °C in the assay buffer described above.

For quantitation of C-proteinase activity using fluorescent mini-procollagen III, substrate (150 nm) was incubated in black Eppendorf tubes at 37 °C with 5.3 nm proteinase in 100 μl assay buffer (50 nm Hepes pH 7.4, 150 nm NaCl, 5 nm CaCl2, and 0.02% Brij35). Aliquots of 10 μl were taken at the indicated time points, then added to 10 μl of Laemmli buffer (without bromophenol blue) and immediately boiled for 5 minutes at 100 °C. Samples were then loaded onto 4–20% gradient acrylamide precast gels (Bio-Rad, Munich, Germany), run in the dark. Protein bands were analyzed using a fluorescence gel reader (STORM 860, GE Healthcare, Munich, Germany) using red laser excitation (λex = 630 nm) and ImageQuant 5.2 for quantitation. Experiments were performed in triplicate. Product formation was plotted against time, and estimates of Kcat/Km were derived from curves fitted by non-linear regression using the simplified integrated Michaelis-Menten equation $P = \frac{S_0 \times (1 - \exp(-Kt))}{K}$, in which $K = \frac{K_{cat}}{K_m} \times E$; $P$ represents product concentration, $E$ the enzyme concentration, and $S_0$ the initial substrate concentration (Devel et al., 2006); fit carried out using Prism 5 from GraphPad Software.

SDS-PAGE, western blotting, and sequencing

In vitro assays containing mini-procollagen III and PCP-E1 were first precipitated with 10% trichloroacetic acid/acetone, resuspended in 7.5 μl 8 M urea, and heated at 100 °C for 5 minutes before addition of sample buffer. Proteins were visualized with Coomassie Brilliant Blue G-250, EZBlue, or SYPRO Ruby (all from Sigma-Aldrich). For the latter, the signal was detected with a Molecular Dynamics STORM 860 Molecular Imager using the blue laser (λex = 450 nm) for fluorescence excitation, followed by protein quantitation using ImageQuant 5.2 software.

For detection of mini-procollagen III using polyclonal rabbit anti-c-myc antibodies (Sigma-Aldrich; diluted 1:7500) proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride
membranes (Immobilon P, Millipore, Schwabach, Germany) by electroblotting. The signal was detected using RotiLumin (Roth, Karlsruhe, Germany) and X-ray film (Hyperfilm ECL, GE Healthcare), following the manufacturer’s instructions. Magic Mark XP (Invitrogen, Karlsruhe, Germany) was used as molecular weight markers.

For N-terminal sequencing, proteins were blotted onto polyvinylidene fluoride membranes, stained with Coomassie Brilliant blue, and then sequenced at the protein microsequencing center of the Institut Fédératif de Recherche (IFR) 128 (Lyon, France).

RNA isolation and reverse transcriptase-PCR
All materials for RNA isolation and reverse transcription were obtained from Peqlab (Erlangen, Germany). Reagents for PCR were obtained from New England Biolabs (Frankfurt, Germany). Primary human dermal fibroblasts, isolated from adult skin (Invitrogen), were cultured following the manufacturer’s instructions. Total RNA was isolated from adherent fibroblasts or normal (n = 6) and keloid dermis (n = 7) using the peqGOLD Total RNA Kit. Equal amounts of RNA (1 μg) were transcribed into complementary DNA in 25 μl reaction mixtures using Moloney Murine Leukemia Virus-reverse transcriptase (200 U μl⁻¹), nonspecific oligo d(T) primers (10 mM; Roth), additional RNase inhibitor (30 U μl⁻¹), and dNTPs (200 μM). After transcription, 1 μl of the newly synthesized complementary DNA was used as a template for PCR, which was performed with 2.5 U of Taq-Polymerase (New England Biolabs), 200 μM dNTP mix, and 0.2 pM of each primer (sense and antisense). Primer sequences are listed in Supplementary Table S1 online.

After initial denaturation at 95 °C for 5 minutes, PCRs were carried out over 30 cycles of 94 °C for 60 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds. Equal amounts of PCR products were visualized by separation on 1% agarose gels containing 0.04% ethidium bromide.

Immunofluorescence analysis
Immunofluorescence assays were performed as previously described (Becker-Pauly et al., 2007). Cryosections from fibrotic (keloids, n = 7) and healthy (n = 15) skin were analyzed using rabbit polyclonal anti-meprin α- or β-specific antibodies (Becker-Pauly et al., 2007). Cell nuclei were stained using 4,6-diamidino-2-phenylindole.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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