

Characterization of the soluble, secreted form of urinary meprin

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A soluble form of the kidney membrane metalloendopeptidase, meprin, is present in urine. Urinary meprin is expressed in BALB/C mice with the *Mep-1^{a/a}* genotype (high meprin, expressing meprin- α and meprin- β) but not in BALB.K mice of the *Mep-1^{b/b}* genotype (that only express meprin- β). Western blotting with antisera specific to the meprin- α and the meprin- β subunits established that the only form of meprin present in urine samples was derived from meprin- α . This form of meprin is partially active, and comprises at least three variants by non-reducing SDS/PAGE and by zymography and two protein bands on reducing SDS/PAGE. Sequencing of these two bands established that the N-terminus of the larger protein band begins with the pro-peptide sequence of the α -subunit (VSIKH..), whereas the

smaller band possessed the mature meprin N-terminal sequence (NAMRDP..). Trypsin is able to remove the pro-peptide, with a concomitant activation in proteolytic activity. After deglycosylation, the size of the pro- and mature forms of urinary meprin are consistent with cleavage in the region of the X-I boundary. There is a pronounced sexual dimorphism in urinary meprin expression. Females secrete a slightly larger form, and its proteolytic activity is about 50% of that released by males. The urinary meprin is therefore a naturally occurring secreted form of this membrane-bound metalloendopeptidase and is more likely to be generated by alternative processing pathways than by specific release mechanisms.

INTRODUCTION

The kidney brush-border membrane of rats and mice differs from that of many other species in that it contains large quantities of a potent metalloendopeptidase called meprin (EC 3.4.24.18). Meprin is a zinc metalloendopeptidase that comprises disulphide-linked multimers of two different subunits, termed α and β [1]. All mouse strains express meprin- β but expression of meprin- α is restricted to a subset of inbred mouse strains [2]. The α - and β -subunits from human, rat and mouse have been cloned and fully sequenced, and comprise a series of discrete domains (Figure 1). Both meprin- α and meprin- β contain a hydrophobic signal peptide and an N-terminal pro-sequence of approx. 40 amino acids long, immediately before the 200-amino acid proteolytic astacin domain. This pro-sequence prevents the mature N-terminus of the protein from forming an active-site-stabilizing salt bridge [3]. Meprin- α and meprin- β molecules that retain the pro-sequence are therefore zymogens, and can be activated *in vitro* by limited proteolysis with, for example, trypsin [4,5]; the enzyme(s) responsible for activation *in vivo* are unknown. In the renal membrane-bound form of meprin, the pro-sequence is removed from meprin- α , but remains attached to the β -subunit [6].

The pro-astacin domain is located N-terminally to a long C-terminal segment comprising the domain common to meprin, A-5 protein and receptor protein tyrosine phosphatase μ (MAM-domain), a domain of unknown function (X-domain), an epidermal growth factor (EGF)-like domain and a C-terminal hydrophobic domain that is a transmembrane anchor [1]. Relative to meprin- β , meprin- α also contains a short additional inserted sequence (I-domain), located between the X-domain and the EGF-domain [6]. During maturation of meprin- α , the transmembrane anchor, EGF-domain and I-domain are lost by a proteolytic clip in the region of the X-I boundary and thus, this subunit loses its membrane anchor. Meprin- α (probably as a

dimer) is retained in the membrane by a combination of covalent (disulphide bond) and non-covalent association with meprin- β , which provides the membrane anchoring. Nearly all of the meprin- α can be released from the membrane by treatment with reducing agents [7,8]. A role for meprin is not yet known. The high level of expression of meprin in kidney brush-border membranes seems to be unique to rodents, and might imply a rodent-specific function. Preliminary work from our laboratory has shown that rodent urine contains a soluble metalloendopeptidase activity that is related to meprin [9], although differences in size and stability were noted. In this paper, we characterize urinary meprin and discuss the significance of these data in terms of structure and function of the meprins.

MATERIALS AND METHODS

The inbred strain BALB/C and the congenic strain BALB.K were used. BALB/C mice express both meprin- α and meprin- β and are referred to as 'high meprin' animals, whereas the BALB.K strain only expresses meprin- β ('low meprin' phenotype). Male and female mice of either strain were housed in groups of four to ten using a 12 h dark/12 h light cycle and were given free access to food and water. Pooled urine was collected by bladder massage from unanaesthetized animals. Immediately after collection, the urine was either desalted on 5 ml 'spun columns' of Sephadex G-25, previously equilibrated with 20 mM Hepes buffer, pH 7.6, or concentrated with Centricon (Amicon) centrifugal concentrators with an $M_r = 30000$ cut-off. This step also served to remove low-molecular-mass components in the urine, particularly the major urinary proteins (MUPs), a family of 18–19 kDa proteins secreted in rodent urine.

SDS/PAGE was performed using 5% gels that were stained for protein using Coomassie Blue, or electroblotted on to

Abbreviations used: EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; MAM, a domain common to meprin, A-5 protein and receptor protein tyrosine phosphatase μ ; MUP, major urinary protein; PVDF, poly(vinylidene difluoride).

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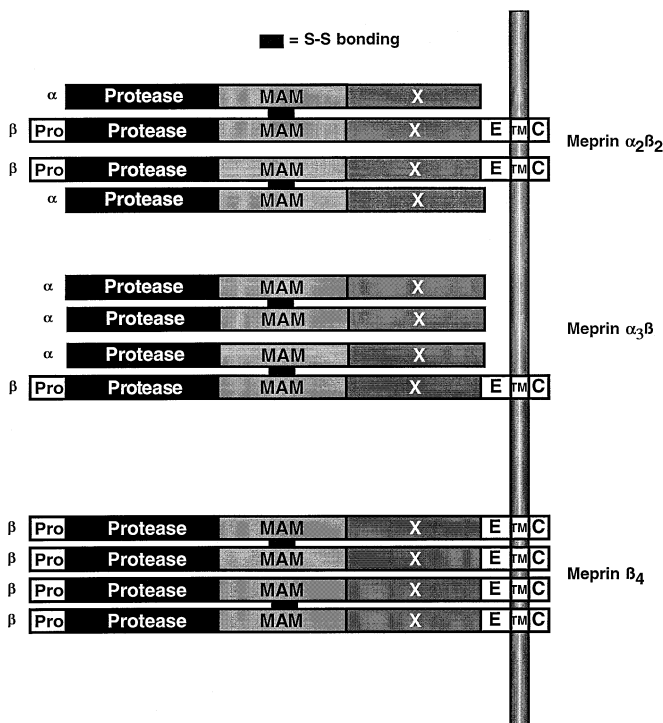


Figure 1 Putative domain, oligomeric and membrane-bound structure of meprin- α and meprin- β

The domain structure of the two meprin species is derived from a combination of experimental data and from sequence motifs and domains inferred from the cDNA sequences. See the text for details. Abbreviations: C, cytoplasmic domain; TM, transmembrane domain; E, EGF-like domain; MAM, MAM-domain.

nitrocellulose before immunoblotting. Proteins were also separated on 5% reducing gels and blotted on to poly(vinylidene difluoride) (PVDF) before sequencing using an Applied Biosystems 476A peptide sequencer. Anion-exchange chromatography was performed using an FPLC system (Pharmacia) fitted with a Mono-Q column ($V_i = 1$ ml). The column was equilibrated with 10 ml of 20 mM Hepes buffer, pH 7.4, before application of 1.0 ml of urine, desalted into the same buffer. Bound protein was eluted from the column using a linear salt gradient (0–1 M NaCl) at a flow rate of 1.0 ml/min.

Meprin activity was determined using casein labelled with fluorescein isothiocyanate (FITC) [10,11]. FITC-casein (35 μ g) was incubated in a final volume of 100 μ l with proteinase samples in 20 mM Hepes, pH 7.5, for periods of up to 16 h. At the end of the incubation period, trichloroacetic acid (200 μ l) was added to a final concentration of 3.3% (w/v). The samples were held at 4 $^{\circ}$ C for 1 h to ensure complete precipitation of undigested FITC-casein before centrifugation at 10000 g for 2 min. The supernatant fraction (200 μ l) was transferred to 2.8 ml of 0.5 M Tris/HCl, pH 9.5, and the fluorescence was measured at 490 nm (excitation) and 525 nm (emission) using a Perkin-Elmer 3000 fluorimeter. The fluorescence readings were converted into μ g of casein solubilized/h.

After zymography on gelatin-containing gels [11], the cleared zones were scanned by laser densitometry, and the 'troughs' in the trace were quantified. The volume of the trough was directly proportional to the amount of meprin-containing sample applied ($r > 0.98$) and provided that the same set of meprin standards

were applied to different gels, the activity in different samples could be quantified and compared using this method [12].

RESULTS AND DISCUSSION

Zymography has been used extensively for detection of matrix metalloendopeptidases. We have established that this technique can also be used to detect active bands in polyacrylamide gels containing 0.5% gelatin (Figure 2), in tissue preparations from 'high meprin' mice. It may be inferred from the high molecular mass of the band and the lack of activity in brush-border membranes from a 'low-meprin' congenic mouse strain (BALB.K) that fails to express meprin- α [13] that the zone of gelatinolytic activity is meprin. BALB/C and BALB.K mice are genetically identical except for a region of chromosome 17 that includes the major histocompatibility complex and the *Mep-1* gene that encodes meprin- α [14,15]. Zymography is only effective under non-reducing conditions; no lytic bands are seen when reducing conditions are used (results not shown). Based on sequence similarities with astacin, meprin is likely to have intramolecular disulphide bonds within the protease domain, and we assumed that the failure to detect monomeric meprin was because the correct disulphide bond arrangement was not reformed. Even after activation of BALB/C or BALB.K kidney preparations by trypsin, no zymogram band was seen that corresponded to meprin- β . This protein is more thermally unstable than the α -subunit [16] and may therefore denature more extensively and fail to refold in the gel.

On zymograms, kidney preparations from BALB/C mice showed a major band of activity, and under conditions of high loading, one or two fainter, equally spaced bands of lower mobility (Figure 2). The major band of activity in urine was larger than the corresponding band in kidney preparations. Previous work on the membrane-bound forms of meprin has provided evidence for homo- and mixed hetero-tetramers of α - and β -subunits, in the stoichiometry $\alpha_3\beta$, $\alpha_2\beta_2$ and β_4 [7]. The multiple bands on the zymogram might represent these different forms of meprin. However, meprin- β is less stable than meprin-

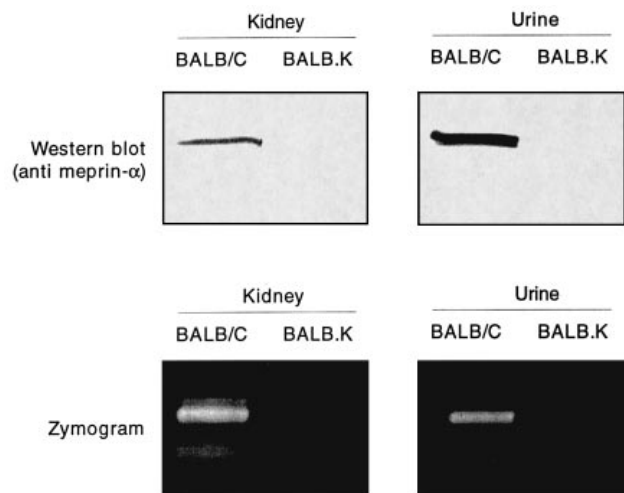


Figure 2 Identification of urinary meprin

Kidney homogenates (50 μ g of protein) or freshly acquired, desalted urine samples (10 μ l) from BALB/C and BALB.K mice were electrophoresed on 12.5% reducing SDS/PAGE for Western blotting and 7.5% non-reducing SDS/PAGE gel containing 0.5% (w/v) gelatin for zymography. On the zymogram, zones of digestion were stained with Coomassie Brilliant Blue.

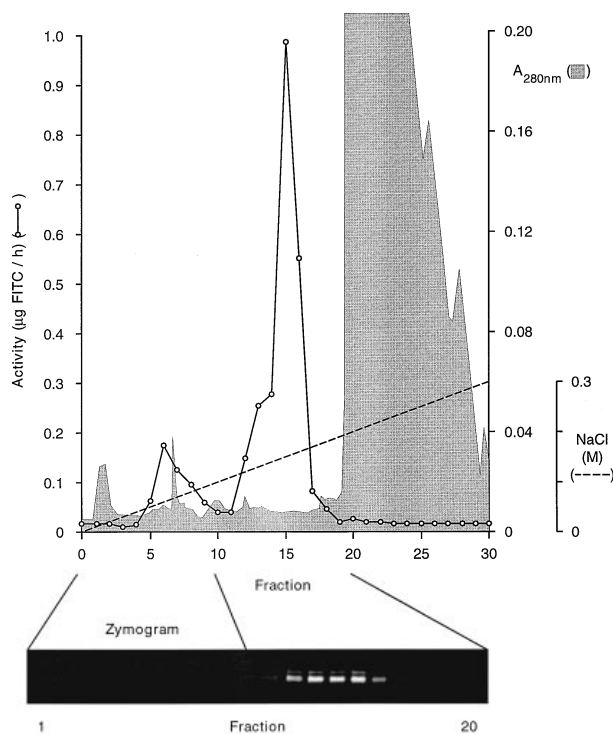


Figure 3 Separation of urinary meprin on anion-exchange chromatography

Freshly acquired urine (1.0 ml) was desalted on Sephadex G-25 and applied to a MonoQ column in 20 mM Hepes, pH 7.4. Proteins were desorbed by a gradient of 0–1.0 M NaCl in the same buffer, eluted at 1 ml/min. Fractions (1 ml) were collected and portions (80 µl) were assayed for proteolytic activity using FITC-casein (○—○). A further portion (10 µl) of fractions (1–20) were analysed by zymography.

α and is not active unless treated with a proteinase such as trypsin. Thus additional meprin- α -containing species must have given rise to three bands on the zymogram. The lack of signal from BALB.K-derived samples confirmed that meprin- β was not activated *in vitro* under the conditions of zymography, and it is likely, therefore, that the multiple bands of activity were variants of meprin- α . Western blots of kidney and urine samples from BALB/C and BALB.K mice were probed with antibodies specific for meprin- α or meprin- β [9]. There was no signal in urine samples with an anti-meprin- β antibody (results not shown). The antibody specific for meprin- α reacted with three bands on non-reducing SDS/PAGE and two bands on reducing SDS/PAGE. Again, the predominant band in urine was larger than the corresponding band in renal tissue.

To characterize the urinary meprin further, urine was collected from BALB/C male mice, desalted and separated by anion-exchange chromatography. The desalted urine sample was applied to a MonoQ column and eluted with a linear gradient of NaCl (Figure 3). Two peaks, the first minor and the second major, of FITC-casein-hydrolysing activity eluted at low NaCl concentration (50 mM and 150 mM) in advance of the residual MUP peaks. The first peak of activity was not sensitive to chelating agents (results not shown) and was therefore not a metalloendopeptidase and was not active on zymograms. The second peak of activity coincided with the appearance of bands on zymography of the same mobility as meprin bands from kidney preparations. On SDS/PAGE, the material collected from the active fractions migrated as a doublet of approx.

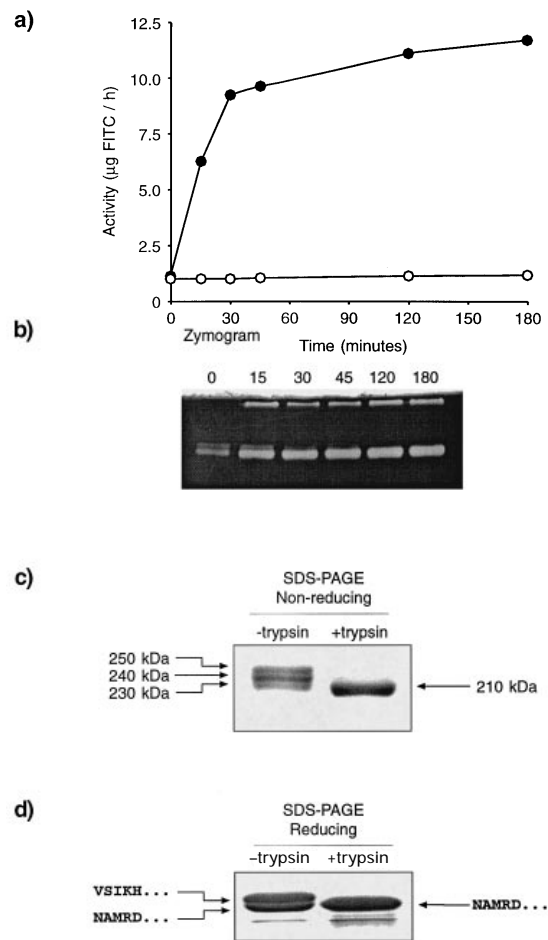


Figure 4 Characterization of urinary meprin

Urinary meprin, purified by MonoQ ion-exchange chromatography (100 µl), was treated at 37 °C with trypsin (10 ng) in a final volume of 200 µl of 20 mM Hepes buffer, pH 7.5. At suitable times, duplicate aliquots (10 µl) were removed, added to 20 ng of soya-bean trypsin inhibitor and frozen before assay. One sample was assayed for proteolytic activity towards FITC-casein (a, ●); the other was analysed by zymography (b). A control incubation, with no added trypsin, was included (○). Other meprin samples were similarly treated with trypsin for 60 min before analysis on non-reducing SDS/PAGE (c). Further samples were separated on non-reducing SDS/PAGE and blotted on to PVDF for automated sequencing. The N-terminal sequences obtained from each band are indicated on the diagram (d).

95 kDa and these were the only protein bands found (results not shown).

Active fractions from MonoQ chromatography were concentrated, separated on non-reducing SDS/PAGE and blotted on to PVDF membrane before five cycles of automated Edman degradation. The upper band yielded the sequence VSIKH..., which is the meprin- α pro-peptide sequence. The product (meprin plus pro-segment) would be approx. 40 amino acids (4–5 kDa) larger, which was consistent with the lower mobility on SDS/PAGE. The lower band had the sequence NAMRD..., which was the start of the mature astacin domain (Figure 4d). Thus urinary meprin is a mixture of meprin- α and pro-meprin- α . The three bands seen on zymography and on non-reducing SDS/PAGE (Figure 4c) are likely to be the three possible hybrid forms: α/α , pro- α/α and pro- α /pro- α . However, the largest species, a homodimer of pro- α , would not be expected to be active. It is possible that it undergoes slight activation during the refolding phase on zymography, or that it is slightly active in its

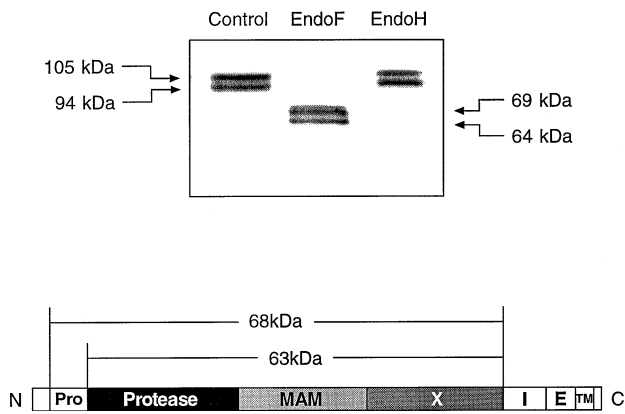


Figure 5 Glycosylation of urinary meprin

Duplicate samples of MonoQ-purified urinary meprin ($2 \mu\text{g}$) were treated with 50 mM EDTA, pH 7.5, 0.1% (w/v) SDS, 1% (w/v) octyl- β -glucopyranoside at 100 °C for 2 min before cooling and addition of endoF (50 m-units) or endoH (1 m-unit) in a final volume of $20 \mu\text{l}$ for 20 h. Samples were then separated on reducing SDS/PAGE and stained with Coomassie Blue. Molecular masses were estimated using BioRad low-molecular-mass standards (not shown).

own right. Further evidence for the latent activity of pro-meprin- α comes from activation by trypsin *in vitro*. When urinary meprin was treated with trypsin, a dramatic increase in activity towards FITC-casein ensued (Figure 4a). At the same time, the two strong bands on zymography become a single band with a very high gelatinolytic activity (Figure 4b). On non-reducing SDS/PAGE, the three bands of intact meprin dimers became a single band after trypsin treatment (Figure 4c) and the two meprin bands were seen, on reducing SDS/PAGE, as a single band (Figure 4d). This band contained the sole N-terminal sequence NAMRDP..., and thus trypsin treatment removed the pro-peptide to create the N-terminus of the mature astacin domain.

Mature meprin- α in the secreted urinary form is smaller than the corresponding form in the brush-border membrane. However, the N-terminal sequence is the same in the two proteins, and the differences in mass must be due to differences either in C-terminal processing or in glycosylation. Urinary meprin (a mixture of α - and pro- α -subunits) was treated with endoH and endoF and the products were resolved on reducing SDS/PAGE (Figure 5). After endoF treatment, two products, at 69 kDa and 64 kDa, were observed but endoH was without effect. These results are entirely consistent with the peptide masses of a protein consisting of pro-astacin-MAM-X (68 kDa) and astacin-MAM-X (63 kDa). If the soluble form of meprin- α has undergone C-terminal processing additional or alternative to the membrane-bound counterpart, the molecular mass differences are slight.

It has previously been noted that membrane-bound meprin from male and female mice differs in mobility on SDS/PAGE. Inasmuch as there appears to be a single gene for meprin, it is likely that this difference reflects sex-specific post-translational processing, probably glycosylation [17]. This difference in size is sustained in the soluble form of the protein. Bands of activity from male BALB/C mice migrated faster than those from female mice. Moreover, the intensity of the signal on zymography was consistently lower than that observed in male animals (Figure 6). At present, we do not know whether this is due to a difference in the release mechanism in male and female mice, or to a difference in stability of the enzymes that might influence the recovery of activity in zymograms. Unlike male mice, MUPs are present in

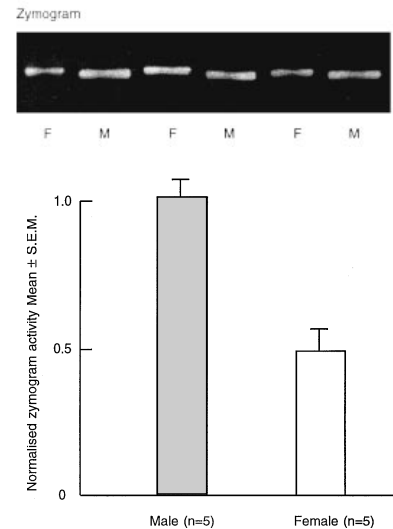


Figure 6 Expression of urinary meprin in male and female mice

Freshly acquired urine samples from male (M) or female (F) mice were desalted on Sephadex G-25 and $10 \mu\text{l}$ portions were separated by zymography on 7.5% non-reducing SDS/PAGE containing 0.5% (w/v) gelatin and analysed by laser densitometry. Further samples ($2 \mu\text{l}$) were analysed in the same way. The activity data (means \pm S.E.M., $n = 5$) were normalized by setting the activity in male urine to 1.0 after calculation of the means.

the urine of female mice at very low levels and it is conceivable that male-derived meprin could be stabilized by the higher protein content of the urine as it is elaborated.

The origin of urinary meprin is enigmatic. It is a dimer that consists of a mixture of the pro- and mature forms of C-terminally processed meprin- α . It is resistant to endoH, implying a mature complement of carbohydrate, and the size is consistent with C-terminal processing and cleavage within the I region, probably close to the X-I boundary. Membrane-bound mouse meprin- α is correctly processed at the C-terminus in constructs that delete the dibasic and furin sites in the I-domain or as much as 50% of the amino acids of the whole I-domain (proximal or distal to the X-domain) [18]. By contrast, correct C-terminal processing of rat meprin- α requires the intact furin site [19]. Since both studies expressed mutagenized meprins in the same cell line (COS-1) it is likely that subtle differences in the sequences in this region of the protein modulate processing. Moreover, the presence of multiple sites of cleavage means that the secreted form might be generated by alternative or sequential proteolytic events. Identification of the site of C-terminal processing is a priority.

Meprin- β is responsible for anchoring meprin- α in the membrane by disulphide and non-covalent interactions but membrane-bound meprin- α is fully converted to the active form that has lost the pro-segment. Soluble meprin- α , on the other hand, is predominantly in a form that retains the pro-segment. Why should secretion be coincident with retention of the N-terminal zymogenic pro-segment? One possibility is that the final maturation of membrane-bound meprin- α , an N-terminal processing, occurs at the brush-border membrane. The enzyme responsible for removal of the pro-sequence could be a trypsin-like enzyme that would be present in the glomerular filtrate. Soluble meprin- α is presumably also exposed to this activity, yet activation is marginal which implies that total exposure time might be important. Alternatively, internalization and recycling of the meprin- β /meprin- α complexes might expose pro-meprin- α to endosomal proteinases that might effect activation. There

must be considerable species or tissue specificity if this is the case, because rat meprin- α /meprin- β co-expressed in Hu293 cells [20] or COS-1 cells [21] results in retention in the membrane of an inactive form of meprin- α that can be activated by trypsin (i.e. pro-meprin- α). Although such cells might be expected to undergo some endosomal processing, they are not bathed in the glomerular filtrate that derives from circulating plasma and may therefore fail to be exposed to the 'pro-meprin- α convertase' activity. Such data suggest that the pro-meprin- α to meprin- α conversion is a late event in the maturation of the membrane-bound form of the enzyme, and may involve an interaction at the brush-border membrane between meprin- β -bound pro-meprin- α and a soluble proteinase in the glomerular filtrate, or between the zymogen and another membrane-bound endopeptidase.

Whether the soluble form of meprin- α has a function is still unclear. Mice and rats excrete a high level of protein in the urine, comprising primarily a group of allelomorphous variants of the mouse MUPs and their rat counterparts. The MUPs are related in sequence [22] and by structure [23] to the lipocalin/calycin family/superfamily of proteins, a group of proteins that fold to form a pocket into which hydrophobic ligands can insert. It has been proposed that the MUPs function to bind odorants in urine, and we and others [24,25] have recently demonstrated that two common mouse pheromones are bound in the MUPs structure. A second class of urinary proteins that might be involved in the specification of the 'odortype' of the individual are the Class I histocompatibility antigens [26]. Soluble Class I molecules which are intact in plasma are fragmented in urine [27], and it is tempting to speculate that meprin is the enzyme responsible for this fragmentation, whether membrane-bound or soluble. Hence, meprin- α joins the growing list of membrane-bound proteins with soluble counterparts [28].

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REFERENCES

- Bond, J. S. and Beynon, R. J. (1995) *Protein Sci.* **4**, 1247–1261
- Beynon, R. J. and Bond, J. S. (1983) *Science* **219**, 1351–1353
- Bode, W., Gomis-Rüth, R. F., Huber, R., Zwilling, R. and Stöcker, W. (1992) *Nature (London)* **358**, 164–167
- Butler, P. E. and Bond, J. S. (1988) *J. Biol. Chem.* **263**, 13419–13426
- Macadam, G. C., Beynon, R. J. and Bond, J. S. (1990) *Int. J. Biochem.* **22**, 989–996
- Gorbea, C. M., Marchand, P., Jiang, W., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Bond, J. S. (1993) *J. Biol. Chem.* **268**, 21035–21043
- Gorbea, C. M., Flannery, A. V. and Bond, J. S. (1991) *Arch. Biochem. Biophys.* **290**, 549–553
- Marchand, P., Tang, J. and Bond, J. S. (1994) *J. Biol. Chem.* **269**, 15388–15393
- Flannery, A. V., Macadam, G. C. and Beynon, R. J. (1991) *Biochim. Biophys. Acta* **1079**, 119–122
- Sarath, G., De La Motte, R. S. and Wagner, F. W. (1989) in: *Proteolytic Enzymes: A Practical Approach* (Beynon, R. J. and Bond, J. S., eds.), pp. 25–55, IRL Press, Oxford
- Beynon, R. J. and Oliver, S. (1996) in: *Methods in Molecular Biology, Humana Press*, Totowa, NJ, in the press
- Beynon, R. J., Oliver, S., Stateva, L., Evershed, R. P. and Robertson, D. (1996) in: *The Astacins* (Stöcker, W. and Zwilling, R., eds.), in the press
- Jiang, W., Sadler, P. M., Jenkins, N. A., Gilbert, D. J., Copeland, N. G. and Bond, J. S. (1993) *J. Biol. Chem.* **268**, 10380–10385
- Reckelhoff, J. F., Butler, P. E., Bond, J. S., Beynon, R. J. and Passmore, H. C. (1989) *ImmunoGenetics* **27**, 298–300
- Bond, J. S., Rojas, K., Overhauser, J., Zoghbi, H. Y. and Jiang, W. (1995) *Genomics* **25**, 300–303
- Kounnas, M. Z., Wolz, R. L., Gorbea, C. M. and Bond, J. S. (1991) *J. Biol. Chem.* **266**, 17350–17357
- Stroupe, S. T., Craig, S. S., Gorbea, C. M. and Bond, J. S. (1991) *Am. J. Physiol.* **261**, E354–E361
- Marchand, P., Tang, J., Johnson, G. D. and Bond, J. S. (1995) *J. Biol. Chem.* **270**, 5449–5456
- Milhiet, P. E., Chevallier, S., Corbeil, D., Seidah, N. G., Crine, P. and Boileau, G. (1995) *Biochem. J.* **309**, 683–688
- Johnson, G. D. and Hersh, L. B. (1994) *J. Biol. Chem.* **269**, 7682–7688
- Milhiet, P. E., Corbeil, D., Simon, V., Kenny, A. J., Crine, P. and Boileau, G. (1994) *Biochem. J.* **300**, 37–43
- Flower, D. R., North, A. C. T. and Attwood, T. K. (1993) *Protein Sci.* **2**, 753–761
- Bocskai, Z., Groom, C. R., Flower, D. R., Wright, C. E., Phillips, S. E. V., Cavaggioni, A., Findlay, J. B. C. and North, A. C. T. (1992) *Nature (London)* **360**, 186–188
- Bacchini, A., Gaetani, E. and Cavaggioni, A. (1992) *Experientia* **48**, 419–421
- Robertson, D. H. L., Beynon, R. J. and Evershed, R. P. (1993) *J. Chem. Ecol.* **19**, 1405–1416
- Singh, P. B., Brown, R. E. and Roser, B. (1988) *J. Exp. Med.* **168**, 195–211
- Singh, P. B., Brown, R. E. and Roser, B. (1987) *Nature (London)* **327**, 161–164
- Ehlers, M. R. W. and Riordan, J. F. (1991) *Biochemistry* **30**, 10065–10071