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Heterologously overexpressed, affinity-purified human meprin α is functionally active and cleaves components of the basement membrane in vitro

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Abstract Meprins are astacin-like metalloproteases of renal and intestinal epithelia and embryonic neuroepithelial cells. The full length cDNA of the human meprin α subunit has been overexpressed in baculovirus-infected insect cells yielding the tetrameric proprotein which could be proteolytically activated and affinity-purified to homogeneity. Recombinant meprin α hydrolyzes the synthetic substrate *N*-benzoyl-tyrosyl-*p*-aminobenzoic acid (PABA-peptide) and cleaves by limited proteolysis the basement membrane constituents laminin 1 and laminin 5. This supports a concept that meprin α, when basolaterally secreted by human colon carcinoma epithelial cells, increases the proteolytic capacity for tumor progression in the stroma.

Key words: Meprin; Astacin family; Expression; Laminin cleavage; Baculovirus

1. Introduction

Meprins (EC. 3.4.24.18) are zinc-metalloproteases of the astacin family and of the metzincin superfamily [1–3]. Astacin family members such as tolloid from *Drosophila* [4], *Xenopus* [5] and zebra fish [6] are involved in embryonic pattern formation or play an important role in tissue remodeling as in the case of the human bone morphogenetic protein 1. The latter is identical to the procollagen C-proteinase, a key player in the biosynthesis of collagen [7–9].

Meprins are mainly expressed both as membrane bound or secreted forms in intestinal and kidney brush border epithelial cells [10,11]; minor expression has been reported in embryonic neuroepithelial cells and leukocytes of the lamina propria [12,13]. They exist as two homologous multidomain subunits, α and β, each comprising an N-terminal propeptide followed by the catalytically active protease domain, the meprin, A5 protein, receptor protein-tyrosine phosphatase μ (MAM)-domain, the meprin and TRAF homology (MATH) and after-MATH domains, an epidermal-growth-factor-like domain (EGF), a transmembrane region and a short cytoplasmic tail [14,15]. Apart from the protease domain the function of the other domains are not yet understood in detail, but they most likely mediate protein-protein interactions [16]. In addition, the α subunit alone possesses an inserted domain (I-domain) which is responsible for different processing of the subunits along the secretory pathway; physiological cleavage of the α subunit within the I-domain results in loss of the membrane anchor and subsequent secretion [17,18]. The β subunit of human meprin on the other hand is cleaved only to a minor degree at a different site [19] and predominantly remains membrane bound. Therefore, membrane localization of the α subunit is solely due to an association with β by intermolecular disulfide bonding between the MAM domains [18,20]. Two dimers are clustered together by non-covalent interactions to form tetrameric complexes [21].

Meprins cleave a wide range of biologically active peptides such as bradykinin, substance P, neurotensin, parathyroid hormone or gastrin in vitro [10,22–24]. Furthermore, they are capable of degrading extracellular matrix components and were identified as the main matrix degrading activity in mouse kidney [25]. Despite this rather broad range of potential substrates the basic physiological function remains speculative. Generally, meprins are sorted to the apical plasma membrane [18]. However, under certain pathological conditions they also appear basolaterally. This was shown for meprin α in mouse kidney after reperfusion of the tissue following epithelial cell injury [26] and for meprin α in the human colon carcinoma cell line Caco-2 [27]. Furthermore, in isolated human colon carcinoma tissue, meprin α activity was shown to be increased. Hence, missorting and subsequent stromal localization of meprin could increase the proteolytic capacity in the stroma and thereby contribute to the migration and invasion of tumor cells [27].

To further characterize the protein-protein interaction of meprin with other proteins and possible substrates and for structural analysis, a substantial amount of the enzyme is required. So far, no heterologous expression has yielded sufficient amounts of active protein for such investigations. Here we report on an expression system for the production of human promeprin α in baculovirus transfected insect cells and its subsequent purification by an inhibitor based, active site directed affinity chromatography. This purification has allowed us to characterize the cleavage specificity towards isolated compounds of the basement membrane.

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Abbreviations: EGF, epidermal growth factor; MAM, meprin, A5 protein, receptor protein-tyrosine phosphatase μ; MATH, meprin and TRAF homology; PAA, polyacrylamide; PABA-peptide, *N*-benzoyl-tyrosyl-*p*-aminobenzoic acid; PBS, phosphate-buffered saline; SF, *Spodoptera frugiperda*

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2. Materials and methods

Cloning was performed following standard procedures [28]. Full length human meprin α cDNA was excised from pSG5 (Stratagene, La Jolla, CA, USA) using *Bam*HI and *Spe*I restriction sites and ligated into pFastBac (Gibco Life Technologies, Paisley, UK), resulting in pFastBac/humepα. A truncated version of human meprin α containing only the catalytic domain (corresponding to amino acids 1–263 of human meprin α) was produced by polymerase chain reaction using the primers:

mep-sense 5′ CTCGGATCCAGCAATGCTTGGATTAGA
mep-antisense 5′ CTCGAATTCCTAAGTGTGAGTTGTGGTG-CAATT

thereby introducing a stop codon at the end of the catalytic domain and *Bam*HI/*Eco*RI restriction sites. This fragment was ligated into pFastBac resulting in pFastBac/protease. The integrity of the insert was verified by DNA sequencing. Creation of recombinant viral DNA was performed using the Bac-to-Bac expression system (Gibco Life Technologies, Paisley, UK). Shortly, the foreign cDNA is ligated into the cloning plasmid pFastBac that contains an expression cassette including the strong viral polyhedrin promoter from *Autographa californica* nuclear polyhedrosis virus. The complete expression cassette is transposed into the viral genome that is localized on a F-plasmid (bacmid) which can be propagated in and isolated from *Escherichia coli* cells. By this procedure, three different types of recombinant viral DNA termed bac/humepα, bac/protease and bac/mock were created and used to transfect insect cells.

2.1. Cell culture and transfection of insect cells

Spodoptera frugiperda (SF)21 and SF9 insect cells were cultured adherently growing at 27°C in Grace's insect media supplemented with 10% fetal bovine serum, 4 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were split 1:20 every five days and media was changed every second day. SF21 insect cells in suspension were cultured at 27°C in Grace's insect media supplemented with 5% fetal bovine serum, 4 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and 1% (v/v) pluronic lipid additive. Cells were kept in glass spinner flasks at a constant stirring speed of 80 rpm. They were seeded at a density of 2×10^4 cells/ml and cell growth was monitored regularly (all media were purchased from Gibco Life Technologies, Paisley, UK).

For transfection with recombinant viral DNA, SF21 insect cells were seeded at a density of 2×10^5 cells/ml in 75 cm^2 flasks. At 80% confluency cells were transfected in media omitting any supplements by using the Lipofectin transfection reagent (Gibco Life Technologies, Paisley, UK) according to manufacturer's manual. Protein expression was analyzed after 72 h.

Transfected cells secreting recombinant baculoviruses were used to infect fresh insect cells. To obtain a high virus titer, harvested baculoviruses after transfection were amplified twice in adhesion cultures of SF9 at 80% confluency. For large scale expression of meprin constructs, 500 ml suspension cultures of SF21 insect cells were infected at a density of 5×10^5 cells/ml with a twice amplified virus stock. Expression was stopped after 90 h and media were stored at −20°C until further use.

2.2. Analysis of protein expression

Heterologously expressed promeprin α was activated by limited proteolysis with bovine trypsin (20 μg/ml, 10 min at 37°C). Meprin α activity was detected using *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (PABA-peptide) as a substrate [29] and analyzed as described previously [10]. To check recombinant protein expression, media containing secreted proteins and cell lysates were analyzed by immunoblotting or PABA-peptide hydrolase activity. Media were used without further purification. Cell lysates were prepared from 1 ml aliquots of cell culture by centrifugation for 10 min at $500\times g$. The cell pellet was washed twice with 0.02 M phosphate-buffered saline (PBS) pH 7.4 and finally resuspended in 300 μl of the same buffer containing 1% Triton X-100, 10 mM pefabloc, 50 μM leupeptin, 10 μM pepstatin, 10 μM aprotinin and 17.4 μM benzamidine. The solution was incubated on ice for 30 min and then centrifuged for 10 min at 12 000 rpm. The protein concentration of the supernatant (cell lysate) was determined according to Bradford [30] using the Bio-Rad protein assay kit (Bio-Rad, Hercules, USA) and all samples were diluted to the same protein concentration.

SDS-PAGE was performed as described by Laemmli [31]. For immunoblot analysis proteins were blotted in a semi-dry blotting apparatus onto PVDF membranes. The membranes were blocked in 5% low fat milk powder for 2 h at room temperature and incubated with polyclonal anti meprin rabbit antisera overnight at room temperature. After removal of the antisera and washing in TBS, the membrane was incubated with horseradish peroxidase linked goat anti rabbit secondary antibody for 1 h. Visualization of the bands was realized using the ECL western blot detection kit according to manufacturer's manual (Amersham Life Sciences, Braunschweig, Germany).

2.3. Purification of meprin

To the media containing secreted promeprin α-ammoniumsulfate to 60% saturation was added. After a 2 h incubation at 4°C the precipitate containing the enzyme was harvested by centrifugation at $11\,000\times g$. The pellet was dissolved in 1/20 volume of 0.1 M Tris pH 8.0 and loaded on a Sephacryl S-300 (Pharmacia Biotech, Uppsala, Sweden) gel filtration column (240 ml). Samples were collected on a Biologic FPLC System (Bio-Rad, Hercules, USA) in fractions of 8 ml and the specific meprin α activity was measured in each fraction using the PABA-peptide assay after activation of the proenzyme with trypsin. Fractions containing the highest meprin activity were pooled and used for affinity purification or stored at −20°C.

The K_i value for meprin inhibition by Pro-Leu-Gly-hydroxamate [32] was determined by incubating the enzyme with the inhibitor in the concentration range between 0.1 μM and 10 μM and fitting the data to the equation [33]

$$\frac{v_i}{v_0} = 1 - \frac{E_0 + I_0 + K_i - \sqrt{(E_0 + I_0 + K_i)^2 - 4\times E_0 \times I_0}}{2\times E_0}$$

(v_0 = initial rate in the absence of inhibitor; v_i = initial rate in the presence of inhibitor; E_0 = enzym concentration; I_0 = inhibitor concentration). Non-linear regression analysis was achieved using GRAFIT (version 4.0, Erithacus Software, UK).

Promeprin α was activated with trypsin, dialyzed against 0.1 M Tris–HCl, pH 7.5 and further purified on an affinity column carrying Pro-Leu-Gly-hydroxamate (480 mg; Bachem, Heidelberg, Germany) immobilized on 5 g of CH-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to manufacturer's instructions. Fractions were collected in vials prefilled with 0.1 M Tris pH 3.5 for immediate neutralization.

2.4. Laminin cleavage

Laminin 1 isolated from Engelbreth-Holm-Swarm tumor cells was kindly provided by Dr. Rupert Timpl (Martinsried, Germany). Human laminin 5 isolated from squamous carcinoma cells was a gift from Dr. Johannes Eble (Münster, Germany). 11 μg of laminin 1 and 8 μg of laminin 5, respectively, were incubated with 0.7 μg of affinity-purified human meprin α for 20 h at 37°C in 20 mM Tris–HCl, 15 mM NaCl, 40 mM $CaCl_2$, pH 7.4.

3. Results

3.1. Protein expression

Two meprin α constructs were designed for expression in baculovirus-infected insect cells. Since SF9- and SF21-cells do not express endogenous meprin subunits, it was expected that meprin α be secreted to the media in soluble form (because the α subunit should be membrane bound only when coexpressed with β). Successful transposition of the bac/humepα, bac/protease and bac/mock constructs into the viral bacmid was verified by PCR using a primer pair flanking the integrated cDNA (data not shown).

After transfection of SF21 insect cells with the recombinant viral DNA, cells were incubated for 72 h at 27°C. Expression and secretion of the proteins was verified by SDS-PAGE and immunoblot analysis (Fig. 1A). No signals were detected in wild type cells and cells that were transfected with the construct bac/mock. In contrast, transfection with bac/protease

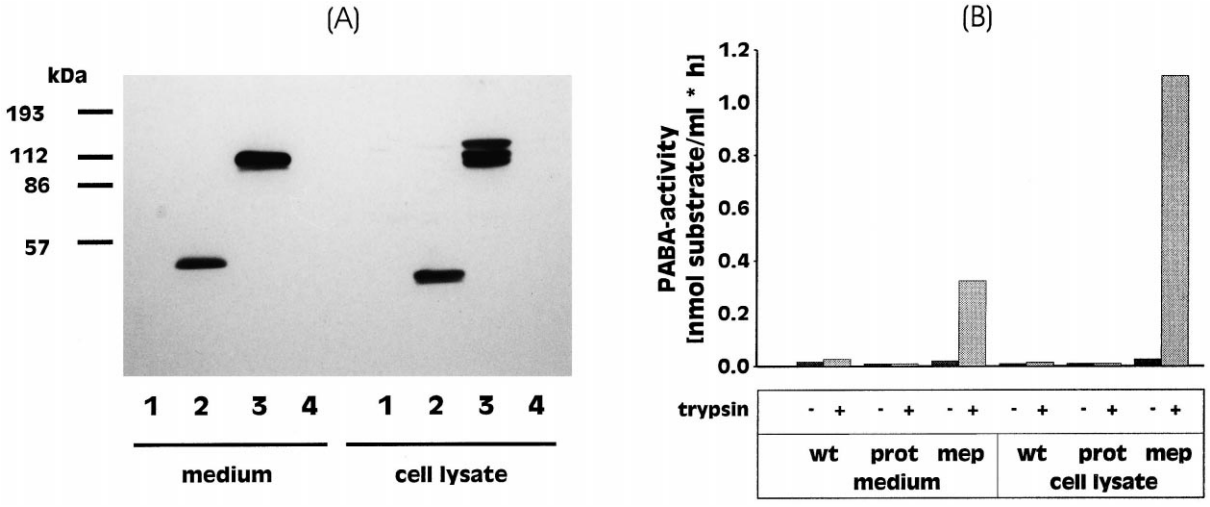

Fig. 1. A: Immunodetection of recombinant proteins. Protein expression after transfection of SF21 insect cells with recombinant viral DNA of the designed constructs bac/humepα (lane 3), bac/protease (lane 2) and bac/mock (lane 1) was analyzed by SDS-PAGE (7.5% PAA) under reducing conditions and subsequent immunoblotting with a meprin specific antibody. Samples of media and cells were taken 72 h after transfection. Wild type cells (lane 4) were transfected under identical conditions but without DNA. 10 μg of total protein from cell lysates and 20 μl of media were loaded. B: Enzymatic activity of heterologously expressed protein. Samples were taken 72 h after transfection with recombinant viral DNA bac/protease (prot) and bac/humepα (mep). Wild type cells (wt) were treated identically. 100 μl of cell culture media and 100 μl of cell lysates were incubated with or without 20 μg/ml trypsin for 10 min at 37°C before 100 μl of PABA-peptide was added. After 2 h reactions were stopped and activities determined.

resulted in the expression of a protein of 36 kDa in the cell lysate and a 44 kDa protein in the media. Transfection with bac/humepα resulted in the expression of protein species with molecular size of 90 kDa and 105 kDa in the cell lysate and 92 kDa in the media. The efficiency of the expression system depends on the ability of the transfected insect cells to produce recombinant baculoviruses that can be used to infect fresh cells and induce a high level expression of the desired protein. For this purpose the cell culture media was removed 72 h after transfection and applied to a fresh culture. These cells again expressed proteins of sizes identical to those in Fig. 1A.

Both the full length meprin α and the protease domain were expressed as inactive proenzymes containing N-terminal propeptides which need to be removed proteolytically for activation. Incubation of the expressed proteins with 20 μg/ml trypsin for 10 min at 37°C resulted in a shift in molecular size of the secreted form of full length meprin α from 92 kDa to 85 kDa, which would be expected due to removal of the propeptide (data not shown). The enzymatic activity of the

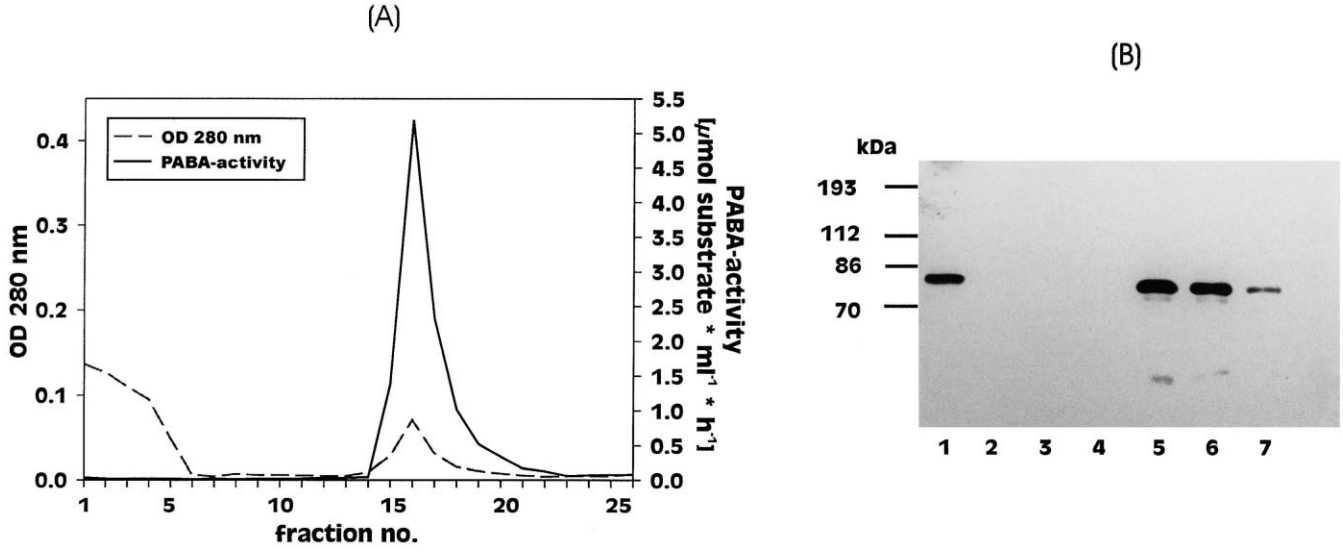

Fig. 2. A: Elution profile of the affinity chromatography on immobilized PLG-NHOH. Protein concentration and PABA-peptide hydrolyzing activity are indicated for each fraction. Proteins were loaded on the column in 0.1 M Tris–HCl pH 7.5 and washed with 0.1 M Tris–HCl, pH 7.5/0.5 M NaCl (fractions 1–6). Elution was performed with 0.1 M Tris pH 10.4 (fractions 7–26). B: Characterization of the affinity-purified samples. The starting material (5 μl, lane 1), the non-binding material (10 μl, lane 2) and fractions 4 (10 μl, lane 3), 10 (10 μl, lane 4), 16 (2.5 μl, lane 5), 18 (10 μl, lane 6) and 21 (10 μl, lane 7) from the affinity chromatography column (Fig. 2A) were analyzed by SDS-PAGE (7.5% PAA) under reducing conditions, blotted and immunodetected using a meprin specific antibody.

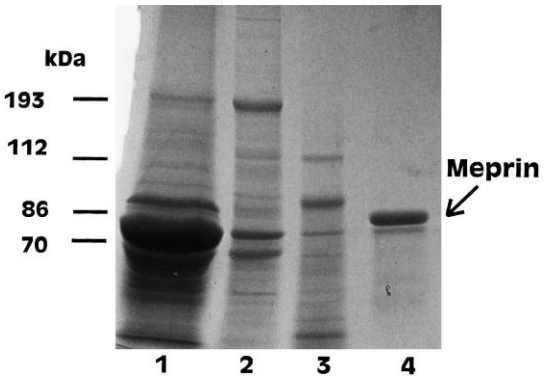

Fig. 3. Efficiency of the steps of meprin purification. Samples from the cell culture media after meprin expression (2 μl, lane 1), the meprin containing fraction after gelfiltration (4 μl, lane 2), the washing fraction 4 from the affinity chromatography (100 μl after precipitation with 10% TCA, lane 3) and the meprin containing fraction 16 from the affinity chromatography (40 μl, lane 4) were analyzed by SDS-PAGE (7.5% PAA) under reducing conditions and stained with Coomassie.

heterologously expressed proteins were determined by using PABA-peptide. Without trypsin incubation, no activity above background level could be detected in media or lysates of transfected cells and wild type cells. PABA-peptide hydrolysis after incubation with trypsin could only be observed in lysates and media of cells that were transfected with bac/humepα. Under these conditions, no activity above background level was detectable for lysates and media of wild type cells and bac/protease transfected cells (Fig. 1B)

3.2. Purification of secreted meprin

For purification of the expressed protein only secreted full length meprin was used since this form of the protein underwent all posttranslational processing steps and was proteolytically active after trypsin treatment. Meprin accumulation in the media was monitored regularly and was highest at 90 h after transfection. No further increase of yield occurred upon longer incubation (data not shown). Upon Sephacryl S300 gelfiltration of the precipitated material (50 mg of the total protein) promeprin α eluted in a high molecular mass fraction and was separated from the bulk of other proteins corresponding to a molecular mass of ~320 kDa (data not shown). This indicates that meprin is produced by SF21 cells as an oligomeric complex. An affinity chromatography was used for further purification with an immobilized inhibitor that selectively binds the active form of meprin. Therefore, meprin containing fractions from gelfiltration were activated with trypsin and loaded on a CH-Sepharose affinity column with immobilized Pro-Leu-Gly-NHOH ($K_i = 0.45$ μM ± 1 nM for human meprin α). Meprin activity was completely retained on the column while the bulk of the protein passed through. Elution of the bound protein responsible for PABA-peptide hydrolyzing activity was achieved by elevating the pH above 9.0 (Fig. 2A). The identity of the eluted activity with meprin was verified by immunoblotting utilizing a meprin specific antiserum (Fig. 2B). This purification procedure is highly efficient and yields homogeneous protein after only two column purification steps (Fig. 3). The weak bands on the Coomassie stained gel at lower molecular mass represent C-terminally truncated forms of meprin after trypsin incubation that are also recognized by the meprin specific antibody on the immunoblot (Fig. 2B). The yield of heterologously expressed meprin α is 2.5 mg/l culture media and 750μg/l after purification. The specific activities of the purified material can be seen in Table 1.

The purified and activated recombinant human meprin α was tested for activity against isolated laminins (Fig. 4). Laminin 1 is cleaved exclusively within the α1 chain resulting in smaller fragments of 350 and 300 kDa, respectively. Laminin 5 is cleaved within the α3 chain resulting in a fragment of 130 kDa. These fragments point to a high specificity of meprin towards individual laminin subunits. Control samples of laminins 1 and 5 incubated in the absence of enzyme under otherwise identical conditions did not exhibit evidence for proteolytic degradation.

4. Discussion

A heterologous system for overexpression of promeprin α was established that allows the subsequent purification of significant amounts of protein for functional and structural studies. Due to the complex structure of meprins which includes covalent and non-covalent association of subunits into a tetrameric complex [21] and a posttranslational proteolytic cleavage that removes the membrane anchor of the α subunit intracellularly [18] many established expression systems turned out to be unsuitable for larger scale production of meprins. For example the expression of human meprin α in yeast cells resulted in the synthesis of an inactive protease (own unpublished results).

Both full length human promeprin α and its C-terminally truncated proprotease domain were expressed in SF21 insect cells. The full length promeprin α is processed similarly as the native enzyme [18], losing its membrane anchor during passage through the secretory pathway and being secreted into the media as a tetrameric enzyme. Secreted promeprin α can be activated by limited proteolysis with trypsin, which results in a protein capable of cleaving the synthetic PABA-peptide

Table 1
Purification of recombinant human meprin α

Purification step	Total protein (mg)	Volume (ml)	Specific activity (U/mg)	Enrichment
Insect cell culture media	9000	1000	–[a]	–
Precipitation	4000	50	0.0006	1
S300 gelfiltration	360	240	0.0055	9.25
Affinity chromatography	0.75	14	0.667	120

1 Unit (U) is the enzymatic activity that hydrolyzes 1 μmol of PABA-peptide/min. Specific activities for human meprin or meprins from other species against PABA-peptide have not been published so far.
[a]Meprin activity could not be detected in the crude media, probably due to the presence of an inhibitory activity in the insect cell culture medium containing calf serum proteins.

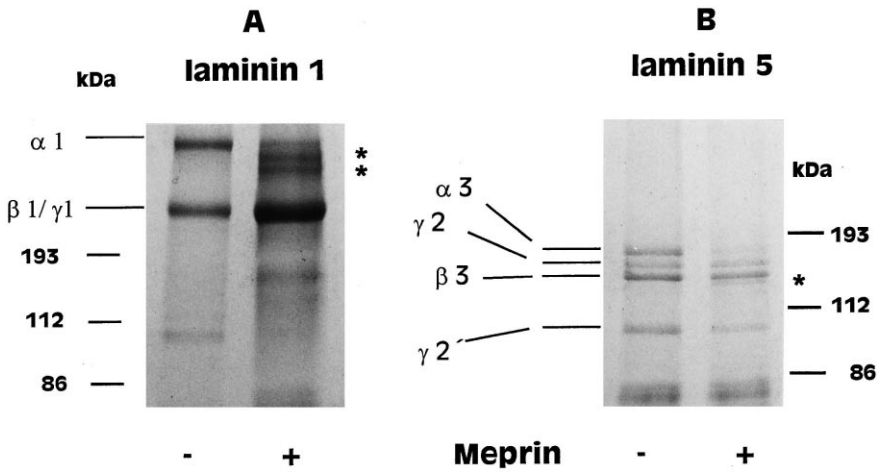

Fig. 4. Proteolytic processing of isolated laminins by affinity-purified human meprin α. 11 μg of laminin 1 (A) and 8 μg of laminin 5 (B) were incubated with 0.7 μg of purified human meprin α for 20 h at 37°C. Control samples were incubated under identical conditions without meprin. After incubation, proteins were precipitated with 20% TCA and 0.15% Triton X-100. For SDS-PAGE under reducing conditions (5–12% PAA), the samples were resuspended in sample buffer. Proteins were detected by Coomassie staining. Subunits of laminins and their physiological cleavage products are indicated by Greek letters following current conventions. Asterisks indicate the resulting fragments of laminin incubation with purified meprin. Bands at lower molecular masses correspond to antibodies used for laminin purification.

and, most specifically, components of the extracellular matrix. Taken together, these data demonstrate that heterologously expressed full length meprin resembles the native enzyme in its typical features.

The shorter version of promeprin α comprising merely the catalytic domain and the N-terminal propeptide was also secreted by the cells, but proved to be inactive. Tsukuba and Bond [34] observed that truncated forms of mouse meprin α lacking the MAM domain did not fold properly, turned out to be inactive and were not secreted by the cells. These differences in intracellular transport could be caused by species specific properties of the meprins from human and mouse. So far, there has been no report on a successful expression of the pure protease domain of meprins in a catalytically active form.

Full length human promeprin α expressed in SF21 insect cells can be purified to homogeneity with only two purification steps. By gel filtration, the bulk of low molecular mass proteins is removed from tetrameric promeprin α for which a molecular mass of 320 kDa was determined. By applying a selective affinity chromatography step, meprin α can be extracted specifically and eluted in pure form. The used inhibitor PLG-NHOH originally developed for collagenase [32] has already been adopted successfully to purify recombinant astacin from *E. coli* inclusion bodies [35]. The K_i of 0.45 μM for the inhibition of meprin is lower than the corresponding values for astacin (16 μM) and collagenase (40 μM). A reason for the considerably stronger binding of the inhibitor to human meprin α could be that the human enzyme has specific features in its substrate binding cleft which are not seen in rat and mouse meprin α or meprin β. As shown by X-ray crystal structure analysis of astacin complexed to PLG-NHOH [36] and by molecular modelling of various members of the astacin family and of the metzincin superfamily, the binding site for the Pro-Leu-Gly-NHOH peptide is considerably conserved [35]. Most remarkably, the inhibitor's proline ring is bound into a niche of the so-called edge β-strand which runs antiparallel to the inhibitor (or substrate). This conserved niche is shaped by a triplet of residues, (Trp/His/Tyr)-(Ser/Ala)-(X). In many metzincins including mouse and rat meprin α, the X residue is Tyr. However, in human meprin α X is Glu, whose negatively charged side chain could interact with the charged imino nitrogen of proline and thereby increase the binding affinity of this inhibitor. Another important feature unravelled by the described binding of the inhibitor to human meprin α is that only the trypsin activated enzyme binds to the affinity column, the proenzyme does not. This provides evidence that the propeptide which comprises 54 residues might sterically hinder the inhibitor's access to the active site.

So far, purification of meprin from various natural tissues yielded a mixture of α and β subunits. The heterologous expression of meprin α without contamination by β allows the assessment of the function of a single subunit. It is known that both subunits are differentially expressed in a tissue-specific manner [13], and that not only are their substrate binding regions different [3], but also their substrate specificities strongly deviate [24]. This is the first proof that purified human meprin α cleaves isolated laminin 1 and 5 at distinct sites within the α1 and α3 chains, respectively, and thus further evidence that its possible stromal localization in colon cancer tissue [27] could contribute to the degradation of matrix components in vivo. It has been shown that cleavage of laminin 5 by matrix metalloprotease-2 induces the presentation of a protein domain that acts as an attractant for epithelial cells in tissues that undergo remodeling [37]. The work presented here indicates that activated meprin α capable of cleaving matrix components in a comparable manner as matrix metalloprotease-2 [37] may cause an imbalance of proteolytic activities in stromal areas and thus may contribute to tumor progression.

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