The mouse matrix metalloproteinase, epilysin (MMP-28), is alternatively spliced and processed by a furin-like proprotein convertase

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Epilysin (MMP-28) is a recently identified member of the matrix metalloproteinase (MMP) family. To explore the expression of epilysin *in vivo* and to gain insight into its biological functions, we have cloned the mouse epilysin cDNA and determined its expression. The amino acid sequence of the mouse protein is 85 % identical with the human sequence and contains conserved features such as an RKKR furin-activation sequence following the prodomain. Unexpectedly, we found two alternatively spliced forms of the epilysin mRNA lacking 30 and 72 nt at the beginning of the seventh exon coding for part of the haemopexin domain. Expression of recombinant epilysin in HT-1080 fibrosarcoma cells indicated that epilysin was secreted as a major 48 kDa form

and a minor 58 kDa form. Expression of the 58 kDa form was increased by a synthetic furin inhibitor at the expense of the 48 kDa form, suggesting that furin cleaves and activates epilysin. Epilysin mRNA was detected in a number of mouse tissues, with the highest expression in the lung, placenta, heart and uterus, and lower levels in the testis and gastrointestinal tract. The wide expression of epilysin in intact, healthy tissues suggests that this MMP functions in physiological tissue homeostasis and turnover.

Key words: alternative splicing, extracellular matrix, matrix metalloproteinase, proteolysis, tissue expression.

INTRODUCTION

The matrix metalloproteinases (MMPs) comprise a family of at least 21 human endopeptidases capable of degrading most components of the extracellular matrix (ECM) [1]. Cleavage of structural ECM components not only facilitates cell motility, but also affects intracellular signalling and generates biologically active protein fragments. Degradation of structural ECM components is, however, not the sole function of these proteinases, as the involvement of MMPs has been described in a number of other cellular processes. For example, matrilysin (MMP-7) cleaves and activates the proform of α -defensin, a class of secreted antimicrobial peptides [2], and several MMPs can inactivate the serpin α 1-antiproteinase inhibitor [3]. MMPs have also been suggested to play a role in the release of various growth factors from the ECM [4]. MMPs are thus involved in a number of physiological processes such as embryogenesis and wound healing, as well as pathological processes such as tumour angiogenesis and metastasis (reviewed in [5]).

All MMPs share a common domain structure and activation mechanism (reviewed in [1]). The prodomain containing a conserved PRCXXP/TD sequence is found in all MMPs, except MMP-23, and is followed by the catalytic domain with a conserved sequence containing three histidine residues. In most MMPs, the catalytic domain is followed by a proline-rich hinge region and a haemopexin domain. The different MMP subgroups also contain specific domains such as the transmembrane and cytoplasmic domains of the membrane-bound MMPs (MT-MMPs) and the gelatin-binding domain of the gelatinases (MMP-2 and -9).

Many of the secreted MMPs, such as collagenase-1 and -3 (MMP-1 and -13 respectively) and the stromelysins (MMP-3, -10

and -11), are not expressed in normal resting tissues. However, production and activity are up-regulated in response to specific stimuli during tissue injury or inflammation when there is a need for tissue remodelling and degradation [6]. Many tumour cells also express MMPs at various stages of tumour development and invasion. Some MMPs are expressed in resting tissues, such as matrilysin (MMP-7), MMP-19, MT5-MMP (MMP-24), leukolysin (MMP-25) and endometase (MMP-26), suggesting a role for them in tissue homeostasis. Because of the high potential of the MMPs to degrade tissues, their activity is strictly controlled through several mechanisms, most importantly at the level of zymogen activation. Some MMPs contain an RXK/RR sequence inserted between the prodomain and the catalytic domain, which serves as a target sequence for a family of serine proteases, namely the proprotein convertases. Activation of prostromelysin-3 (MMP-11) by furin, a Golgi-associated proprotein convertase [7], indicated a new, intracellular mechanism for activation of MMPs during their transport out of the cell. Furin has also been implicated in the activation of MT1-MMP (MMP-14) [8,9] and MT3-MMP (MMP-16) [10].

Epilysin (MMP-28) is a recently identified member of the MMP family, which we originally cloned from human keratinocyte and testis cDNA libraries [11]. Epilysin mRNA is detected in various human tissues, particularly testis, lung, heart and the gastrointestinal tract, but specific epilysin-producing cells within these tissues have not yet been identified. The only specific human cell type recognized to express epilysin is the basal keratinocyte in the skin, where epilysin expression is up-regulated during wound repair [12]. In the Rhesus monkey, epilysin is expressed by a number of cell types in the placenta during early pregnancy [13]. Epilysin mRNA has also been detected by reverse transcriptase (RT)-PCR in a number of adenocarcinoma cell lines [14].

Abbreviations used: CHO, Chinese-hamster ovary; CMK, decanoyl-Arg-Val-Lys-Arg-chloromethane; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; RT, reverse transcriptase; UTR, untranslated region.

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The nucleotide sequences of the full-length mouse epilysin cDNA and two shorter splice variants have been deposited in the GenBank® Nucleotide Sequence Database under the accession numbers AY065653 (full-length), AY071828 (splice variant A) and AY071829 (splice variant B).

Although epilysin has proteolytic activity [11], no physiological substrates have been identified so far. The aim of the present study was to clone the mouse epilysin gene and examine its expression in normal tissues to gain insights into its biological functions. The mouse is an ideal system for investigation of epilysin *in vivo*, particularly because this species can be genetically manipulated. Our results provide new information on the biochemical properties of this new MMP, including the processing of the enzyme by a proprotein convertase and the presence of mouse-specific alternatively spliced gene products.

MATERIALS AND METHODS

Cloning of mouse epilysin cDNA

To obtain the cDNA sequence of mouse epilysin, a mouse heart cDNA library (Mouse Heart 5'-Stretch Plus cDNA Library; Clontech, Palo Alto, CA, U.S.A.) was screened using a 1.5 kb human epilysin cDNA probe [11] under low-stringency conditions. Positive colonies were selected, and plasmid DNA from these was digested with *EcoRI* to excise the cDNA inserts from the cloning vectors. A 1.5 kb fragment was chosen for further analysis, cloned into the pBSII KS(+) vector (Stratagene, La Jolla, CA, U.S.A.), and sequenced. The fragment was found to contain a part of exon 2, exons 3–8 and approx. 800 bp of the 3'-untranslated region (3'-UTR). The N-terminal sequence was obtained by RT-PCR. Total RNA from GC-1 spg mouse spermatogonia was transcribed to cDNA using Superscript II RT according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD, U.S.A.). PCR was performed using a forward primer corresponding to bases 1–18 at the 5'-UTR and a reverse primer corresponding to bases 348–363 in exon 3 (nucleotides are referred to by their numbers in the GenBank® sequence AY065653). This reaction generated a 363 bp fragment, containing a part of the 5'-UTR, exons 1 and 2 and a part of exon 3. When comparing the mouse cDNA with its human homologue, we found 30 missing nucleotides in the mouse sequence, at the beginning of the seventh exon. RT-PCR was used to determine if the cDNA from the library represents a splice variant of the mouse epilysin and if a corresponding shorter form of the human epilysin also exists. mRNAs from human HaCaT keratinocytes and GC-1 spg mouse spermatogonia were reverse-transcribed to DNA using Superscript II (Gibco BRL). PCR was performed with primers close to the exon border between exons 6 and 7 [human: bases 970–989 (forward) and bases 1153–1174 (reverse); mouse: bases 970–989 (forward) and bases 1217–1241 (reverse)] using AmpliTaq Gold™ (Roche, Mannheim, Germany). PCR products were separated by electrophoresis on a 4–15% (w/v) Tris/borate/EDTA gel (TBE gel; Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Cell culture

Immortalized human keratinocytes (HaCaT) [15] and mouse pSV3-neo transformed spermatogonia (GC-1 spg) [16] were grown in Dulbecco's modified Eagle's medium. HT-1080 human fibrosarcoma cells and Chinese-hamster ovary (CHO) cells were grown in Eagle's minimal essential medium, both containing 10% (v/v) heat-inactivated foetal calf serum (Gibco BRL), 100 international units/ml penicillin and 50 µg/ml streptomycin.

Construction of expression vector, expression of recombinant epilysin, immunofluorescence and immunoblotting

Full-length epilysin cDNA was generated by PCR amplification of the splice variant A from the mouse cDNA library using primers

complementary to bases 317–335 (forward) and 1794–1814 (reverse), and the first and second exons were generated using RT-PCR (see the Cloning of mouse epilysin cDNA section). As the two fragments have an overlap of 46 nt, they were fused using Pfu polymerase in a cycling reaction, where the primers 5'-ggaattccaccATGGTGGCTGGCGTG-3' (forward; epilysin sequence is given in capitals) and 5'-ggaatTCAGAACAGGGC-ACCTCCTGAG-3' (reverse) were added after six PCR cycles had been completed without primers, thus including *EcoRI* sites (sequence underlined) at both ends. The 30 nt (bases 1103–1132) missing in the seventh exon were added to the construct by amplifying the full-length isoform using RT-PCR (see the Cloning of mouse epilysin cDNA section). This PCR product, as well as the PCR products of splice variants A and B, was digested at internal sites with *StuI* (cuts after nt 1014) and *PstI* (cuts after nt 1230), and the internal fragments were exchanged. The full-length and alternatively spliced variants of the epilysin cDNA were then cloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA, U.S.A.), using *EcoRI*, to generate the expression vectors mEpiS (splice variant B; short), mEpiM (splice variant A; medium) and mEpiL (full-length; long), containing the naturally occurring variants of the cDNA coding for mouse epilysin. The full-length cDNA was further cloned into the pEF-IRES-P expression vector [17] to generate the construct pEF-Epi. The constructs were verified by sequencing. Restriction enzymes and ligase were from New England Biolabs (Beverly, MA, U.S.A.).

For transient transfections, the pcDNA3 constructs (and empty vector for controls) were transfected into CHO cells using FuGENE6 (Roche). After 6 h, cells were transferred to serum-free medium and conditioned media, and cell lysates were collected 48 h later.

To generate cell lines with stable, high-level expression of epilysin, pEF-Epi and empty pEF-IRES-P vectors were transfected into HT-1080 fibrosarcoma cells using FuGENE6 (Roche). Puromycin (1 µg/ml; Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to the growth medium 1 day after transfection. During the following 14 days, puromycin concentration was gradually increased to 80 µg/ml, selecting a pool of cells with high expression levels of mouse epilysin or the empty vector. The cells were transferred to serum-free medium lacking puromycin and 10 µM BB-3103 (British Biotech Pharmaceuticals Ltd., Oxford, U.K.) or 75 µM decanoyl-Arg-Val-Lys-Arg-chloromethane (CMK; Bachem, Bubendorf, Switzerland) was added 24 h before collection of the conditioned medium. Cell lysates were prepared as described previously [18]. For deglycosylation experiments, conditioned medium was concentrated 10-fold using Microcon-30 concentrators (Millipore, Bedford, MA, U.S.A.). The proteins were denatured by adding 0.5% SDS and 5% (v/v) 2-mercaptoethanol (final concentrations) at +100 °C for 3 min. Then, 4 vol. of Lac buffer [10 mM EDTA, 0.5% Nonidet P40 and 0.5 µM 4-(2-aminoethyl)benzenesulphonyl fluoride in PBS] were added to all samples and N-glycosidase F (1 unit/sample; Roche) was added to all samples except controls. The samples were then incubated at +37 °C for 1 h.

For immunoblotting, samples of conditioned medium were concentrated 10-fold. Laemmli sample buffer containing 10% 2-mercaptoethanol (final concentration) was added, and the polypeptides were denatured at +100 °C for 3 min before separation by SDS/PAGE (Bio-Rad Laboratories) and transferred to nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) using a semi-dry blotting device (Bio-Rad Laboratories). Immunofluorescence and immunoblotting were performed with an affinity-purified peptide antibody as described previously [11] using the SuperSignal West Dura Extended Chemiluminescence

kit (Pierce Chemical, Rockford, IL, U.S.A.). As the amino acid sequence of the immunogenic peptide used to produce human epilysin antibodies (D219QDERWSLSRRRGRNL234 [11]) differs by only one amino acid from the corresponding mouse epilysin sequence [Q220G (Gln²²⁰ → Gly)], these antibodies recognize the mouse epilysin protein as well.

Extraction of RNA from mouse tissues and RNase protection analysis

Tissue samples were collected from 6–8-week-old male NMRI mice. Female reproductive organs were collected from the corresponding female mice, and embryos from timed pregnant mice. The samples were frozen in liquid nitrogen. For RNA extraction, the frozen tissues were first disrupted using a mortar and further homogenized in TRIzol[®] reagent (Gibco BRL) using a syringe and a 26-gauge needle, and RNA extraction was performed according to the manufacturer's instructions. The RNA samples were analysed by RNase protection assay. To produce an antisense cRNA probe spanning the site of alternative splicing, a 271 nt RT-PCR fragment (see the Cloning of mouse epilysin cDNA section) was subcloned into the plasmid pGEM-T-Easy (Promega, Madison, WI, U.S.A.). This construct was linearized with *Nco*I and transcribed with Sp6 RNA polymerase to synthesize a 370 nt [α -³²P]UTP-labelled antisense cRNA probe, which covers bases 970–1241 of the epilysin cDNA. The probe (100 000 c.p.m./sample) was hybridized with 20 μ g of total RNA from different tissues. After overnight hybridization, unpaired RNA was degraded with a mixture of RNase A and RNase T1 at 37 °C for 1 h, followed by propan-2-ol precipitation (Direct Protect Kit; Ambion, Austin, TX, U.S.A.). Protected RNA fragments were separated by PAGE on a 5 % TBE gel containing 6 M urea, and visualized by autoradiography. An antisense cRNA probe complementary to the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Ambion) was used as an internal control to obtain a 316 nt protected fragment. Epilysin and GAPDH bands were quantified using a phosphorimager, and the epilysin content of each sample was normalized against its GAPDH content.

Northern-blot hybridization analysis

A mouse MTN[®] blot with 2 μ g of mRNA from different mouse tissues was purchased from Clontech. A 1.5 kb DNA fragment covering the entire coding area of the epilysin mRNA was labelled using [α -³²P]dATP and the Strip-EZ DNA[™] labelling kit (Ambion). Hybridization was performed in UltraHyb (Ambion) using 10⁷ c.p.m./ml labelled probe. After extensive washing of the filter, positive bands were identified by autoradiography.

RESULTS

Cloning and sequencing of the mouse epilysin cDNA

To clone the mouse homologue of the human epilysin (MMP-28), a mouse heart cDNA library was screened using a human epilysin cDNA probe under low-stringency conditions. A 1.5 kb *Eco*RI fragment was selected for further analysis and sequenced. Comparison of the mouse sequence with the human epilysin cDNA indicated that the fragment contained a part of exon 2, exons 3–8 and approx. 800 bp of the 3'-UTR of the mouse epilysin cDNA. The missing N-terminal sequence was obtained by RT-PCR using RNA from GC-1 spg mouse spermatogonia as

a template, a forward primer located in the previously sequenced 5'-UTR of the mouse epilysin gene [19] and a reverse primer in the third exon. When comparing this sequence with the human epilysin cDNA, we found that 30 nt at the beginning of the seventh exon were missing in the mouse cDNA.

To determine whether this sequence represents one of several splice variants expressed in the mouse, we performed RT-PCR using primers on each side of the exon border and mRNA from GC-1 spg mouse spermatogonia as a template. In addition to the expected 241 nt PCR product representing the splice variant found in the library, two other bands were obtained (Figure 1A). Sequencing of the amplification products indicated that the major band of 271 nt represents the full-length epilysin, corresponding to the human form, whereas the 199 nt band represents yet another shorter splice variant with 72 nt missing at the beginning of the seventh exon. For comparison, similar primers complementary to the human cDNA were designed and RT-PCR was performed using mRNA from human HaCaT keratinocytes as a template. In contrast with the mouse RT-PCR product, this reaction produced only one reaction product of 204 nt corresponding to the full-length epilysin (Figure 1A). The full-length mouse cDNA contains an open reading frame of 1560 nt from the first ATG codon, coding for a 520-amino-acid protein with a calculated molecular mass of 59 kDa.

Similar to human epilysin, the predicted amino acid sequence of mouse epilysin has several features characteristic of MMPs (Figure 1B). A hydrophobic signal sequence of 22 amino acids is followed by a propeptide containing the characteristic MMP cysteine-switch sequence. In mouse epilysin, this sequence (P89RCGVAD95) differs from its human homologue in that it contains an alanine residue instead of a threonine residue at position 94. All other described MMPs, except MMP-19, include a proline residue in this motif instead of the threonine/alanine residue found in epilysin. The propeptide also contains a YGYL sequence found in all MT-MMPs [20], but among the secreted MMPs, only in MMP-19 [21]. The propeptide ends in a putative furin-recognition sequence RKKR. The catalytic centre of both human and mouse epilysin, HEIGHTLGLTH, differs from the other MMPs in that no other family member contains a threonine residue within this sequence. The catalytic domain is followed by a 39-amino-acid hinge region and a typical haemopexin domain. Two potential N-glycosylation sites found in the catalytic and haemopexin domains of human epilysin are conserved within the mouse homologue (Figure 1B). The alternative splicing of the seventh exon affects the four-bladed propeller-like haemopexin domain, deleting 10 or 24 amino acids from the second repeat of this domain. In the shortest splice variant, the putative N-glycosylation site within the haemopexin domain is deleted: 85 % of the amino acids of the mouse epilysin are identical in the human protein (Figure 1B). As expected, the highest similarity is found in the catalytic domain (97 % identical residues), whereas the other domains show more variation.

Transient and stable expression of recombinant mouse epilysin

To assess if all the three splice variants of mouse epilysin cDNA are translated to proteins, expression constructs coding for the naturally occurring forms of the enzyme were generated and transfected into CHO cells. Immunofluorescence studies of the transiently transfected CHO cells using epilysin-specific antibodies indicated that all three transcripts were translated to proteins, which could be visualized in the perinuclear area, presumably in the Golgi apparatus (Figure 2A). Immunoblotting analyses of conditioned media from these cells further showed

Figure 1 Identification of the cDNA and amino acid sequences of mouse epilysin

(A) Identification of alternatively spliced forms of epilysin cDNA. Total RNA from GC-1 spg mouse spermatogonia was used as a template in RT-PCR. Primers corresponding to nt 968–989 (forward) and nt 1217–1240 (reverse) in the full-length epilysin cDNA (GenBank® accession number AY065653) were used to generate three different products: one major 271 nt band corresponding to the full-length epilysin cDNA, and two minor bands of 241 and 199 nt corresponding to splice variants A and B respectively. The corresponding area of the human epilysin cDNA was amplified by PCR using cDNA from human HaCaT keratinocytes as a template and using primers covering the nucleotides of the human sequence corresponding to nt 970–989 (forward) and nt 1153–1174 (reverse). This generated only one major product of 204 nt corresponding to the full-length epilysin cDNA. The bands were separated by electrophoresis on a TBE gel and sequenced. Migration of DNA molecular-mass markers is shown on the left. (B) Comparison of the amino acid sequences of mouse and human epilysin. The amino acid sequence of mouse epilysin is given in the one-letter code. Amino acid residues differing in the human protein are shown below the mouse sequence. Different protein domains are indicated above the sequence. The pro-sequence PRCGVAD, the zinc-binding sequence HEIGHTLGLTH and the furin-recognition sequence RKKR are underlined. Two potential N-glycosylation sites are underlined in the mouse sequence. Numbers on the left indicate the positions of amino acids. The sequence missing in both splice variants A and B is underlined with a solid line, and the additional sequence spliced out in variant B is underlined with a broken line.

Figure 2 Expression of different epilysin splice variants

(A) CHO cells were transiently transfected with expression vectors for all three naturally occurring splice variants of epilysin [splice variant B (mEpiS), splice variant A (mEpiM) and full-length epilysin (mEpiL)]. ctrl denotes control cells transfected with the empty expression vector. Cells were permeabilized and incubated with primary antibodies specific for epilysin and FITC-conjugated secondary antibodies. (Upper panel) Epilysin-specific immunofluorescence. (Lower panel) Phase-contrast images. (B) Conditioned medium (left panel) and cell lysates (right panel) from the transiently transfected CHO cells were denatured in reducing Laemmli buffer. Polypeptides of the samples were separated by SDS/PAGE (7.5% gel). Epilysin was immunodetected with specific antibodies. Migration of molecular-mass markers is shown on the left and the bands corresponding to the three different forms of epilysin are indicated by arrows.

that all splice variants were secreted to the growth medium (Figure 2B, left panel). In this transient system, epilysin seems to be expressed only as one specific band, the size of which is approx. 52 kDa for the shortest splice variant, 57 kDa for the intermediate splice variant and 58 kDa for the full-length enzyme. The immunoreactive bands in the corresponding cell lysates were of equal size. The approximate molecular mass of epilysin, based on its cDNA sequence, suggests that these bands correspond to the latent proenzymes.

To analyse the secretion and processing of epilysin, we generated a stable-cell-system overexpressing recombinant mouse epilysin in HT-1080 fibrosarcoma cells. HT-1080 cells were transfected with a eukaryotic expression vector coding for full-length epilysin, and pools of transfected cells were selected for puromycin resistance. Immunoblotting of the conditioned medium using affinity-purified epilysin polyclonal antibodies revealed two bands of approx. 48 and 58 kDa (Figure 3A), with the 48 kDa band being more abundant. Treatment of the cells with a synthetic peptidyl-CMK furin inhibitor increased the levels of the 58 kDa form at the expense of the 48 kDa form. The wide-spectrum MMP inhibitor BB-3103, which also inhibits the catalytic activity of recombinant human epilysin [11], did not affect the expression pattern. No proteins were detected by the epilysin-specific antibody in the conditioned medium from vector-transfected control cells. The specificity of the identified protein bands was further confirmed by preabsorption of the antibody with

Figure 3 Stable overexpression of recombinant mouse epilysin in HT-1080 cells

Recombinant mouse epilysin was overexpressed by stable transfection into HT-1080 cells. The cells were transferred to serum-free medium lacking puromycin, and 10 μ M BB-3103 or 75 μ M CMK was added 24 h before the collection of conditioned medium. ctrl, control cells transfected with the empty expression vector; mEpilysin, HT-1080 cells overexpressing recombinant mouse epilysin. Conditioned media and cell lysates were collected and denatured in reducing Laemmli buffer. Polypeptides of the samples were separated by SDS/PAGE (7.5% gel). mEpilysin was immunodetected with specific antibodies (A, C) and the specificity of the bands was controlled with peptide neutralization as indicated (B, D). Migration of molecular-mass markers is shown on the left. Two epilysin-specific bands of approx. 48 and 58 kDa were detected in the conditioned media (A), whereas only one specific band of approx. 58 kDa was detected in the cell lysates (indicated by an arrow in C).

excess antigenic peptide (Figure 3B). Several immunoreactive protein bands were detected by immunoblotting the cell lysates (Figure 3C). However, immunodetection of only the 58 kDa form (Figure 3C, marked with an arrow) was specifically abolished by peptide competition (Figure 3D) and was not detectable in the vector-transfected control. Some additional bands that appeared to be removed by peptide competition were observed in the cell lysates, but these bands were present in the cells expressing recombinant epilysin as well as in the cells expressing the empty control vector. Therefore these other bands cannot represent recombinant epilysin, but instead may randomly contain peptide sequences similar to the immunogenic peptide. Similar results were obtained using 293T human kidney epithelial cells (results not shown). To investigate if recombinant mouse epilysin is glycosylated, conditioned medium from cells overexpressing epilysin or vector-transfected control cells were treated with N-glycosidase F. Both the 48 and 58 kDa forms migrated faster in SDS/PAGE after enzyme treatment, indicating that epilysin is N-glycosylated (Figure 4).

Analysis of epilysin expression in mouse tissues

To determine the expression levels of epilysin in different mouse tissues, RNA was extracted from tissues of NMRI mice and analysed by RNase protection. Epilysin mRNA was detected using a 370 nt cRNA probe covering the exon border between exons 6 and 7, where alternative splicing occurs (Figure 5A). Three different protected fragments were detected: a major 271 nt fragment, representing the full-length epilysin splice variant, and two minor fragments of 135 and 106 nt, representing the minor splice variant A. In this assay, the minor splice variant B generates

Figure 4 Deglycosylation of recombinant mouse epilysin

Conditioned media from HT-1080 cells overexpressing recombinant mouse epilysin (mEpilysin) or vector-transfected control cells were denatured using 2-mercaptoethanol and SDS at +100 °C for 3 min. Lac buffer (4 vol.) containing the neutral detergent Nonidet P40 was added and the samples were incubated at +37 °C for 1 h with or without N-glycosidase F (1 unit/sample). The reactions were terminated by the addition of reducing Laemmli buffer. Samples were separated by SDS/PAGE and analysed by immunoblotting with epilysin-specific antibodies. Migration of molecular-mass markers is shown on the left.

protected fragments of 135 and 64 nt, of which the latter is too short to be detected under these experimental settings. Most of the analysed tissues expressed detectable levels of epilysin mRNA, and the highest expression levels were seen in the lung and the placenta. The other organs expressing moderate levels of epilysin were heart, salivary gland, thymus, uterus, testis, stomach, small intestine and colon. The full-length mRNA was the predominant form in all tissues, and the ratio of full-length form to shorter splice variants was constant. Expression levels were low in the developing embryo and in the adult liver, kidney and brain. To determine the size of the mouse epilysin transcript, a 1.5 kb cDNA probe covering the entire coding area of the transcript was labelled and hybridized to a Northern blot containing mRNA from various mouse tissues. One transcript of approx. 4.5 kb was detected (Figure 6) and, as in the RNase protection analyses, the highest expression level was seen in the lung.

DISCUSSION

Alternative splicing and processing of mouse epilysin by a furin-like proprotein convertase

Information on the specific tissues and circumstances under which a proteinase is expressed is essential to determine its function. After all, simply characterizing the ability of the enzyme to cleave possible substrates *in vitro* does not necessarily reflect a relevant biological function. To explore the *in vivo* expression and to gain insight into the biological functions of epilysin (MMP-28), we cloned the mouse homologue of the epilysin cDNA and characterized its expression in a variety of tissues. The predicted mouse protein is highly similar to the human enzyme, including potentially critical sequences in the prodomain, the conserved furin-recognition sequence and the unique catalytic site sequence. Alternative splicing of the seventh exon was found in the mouse epilysin mRNA, deleting 10 or 24 amino acids from the haemopexin domain. These splice variants are specific for mouse epilysin since they could not be detected by RT-PCR of mRNA from human HaCaT keratinocytes. As the haemopexin domain can play a role in substrate recognition, this alternative splicing may alter substrate-binding capacity or preference.

To investigate if the mouse epilysin cDNA is translated into a protein, we expressed recombinant full-length mouse epilysin in HT-1080 cells (Figure 3). Immunoblotting analyses revealed that epilysin protein was expressed mainly as a secreted approx. 48 kDa glycosylated form. In addition, a minor approx. 58 kDa form was observed. Treatment of the epilysin-expressing HT-1080 cells with a peptidyl-CMK inhibitor of the *trans*-Golgi proprotein convertase furin increased the levels of the 58 kDa

Figure 5 Expression levels of epilysin in different mouse tissues determined by RNase protection assay

(A) (Upper panel) Total RNA from mouse tissues was hybridized with a 32 P-labelled antisense cRNA probe reaching from nt 1174 to 970 in Figure 1(A), covering the alternatively spliced exon border between exons 6 and 7. Subsequently, the non-hybridized probe was degraded by RNase digestion. Protected RNA fragments were fractionated by PAGE on a 5% TBE gel containing 6 M urea and were visualized by autoradiography. The major 271 nt protected fragment and the shorter protected fragments representing the alternatively spliced forms (135 and 106 nt) are indicated on the left. Migration of RNA molecular-mass markers is shown on the right. (Lower panel) A cRNA probe covering 316 nt of the mouse pTRI-GAPDH mRNA was used as an internal standard. (B) The epilysin and GAPDH contents of each sample were quantified using a phosphorimager and the epilysin values from each sample were normalized against its GAPDH value. The amount of epilysin in the first sample of the series (E10) was arbitrarily set to 1, and the other samples were compared against it.

form at the expense of the 48 kDa form. Only one specific 58 kDa band was identified in the corresponding cell lysates. These findings suggest that epilysin is translated as a latent approx. 58 kDa enzyme, which is glycosylated and secreted. During the secretion process, the prodomain of epilysin seems to be cleaved by furin or another proprotein convertase, thereby generating the approx. 48 kDa form of the enzyme, and possibly activating it. As furin activates stromelysin-3 [7] and MT1-MMP [9,22] and probably a number of other MMPs, this pathway is emerging as an increasingly important means of zymogen activation.

The shorter splice variants were also translated into proteins that were secreted into the growth medium (Figure 2). Although the shorter splice variants are translated into proteins *in vitro*, the biological situation might be different, especially since no corresponding forms could be detected from human sources. When transiently overexpressing recombinant epilysin in CHO cells, only the latent form of the enzyme could be detected, whereas in the stably transfected HT-1080 cells both the latent and processed forms of the enzyme could be observed. This difference is quite

Figure 6 Identification of epilysin mRNA by Northern-blot analysis

A mouse multi-tissue mRNA blot was hybridized with a radioactively labelled 1.5 kb cDNA probe covering the entire coding area of the epilysin mRNA. Migration of molecular-mass markers is shown on the right. The 4.5 kb epilysin mRNA is indicated by an arrow on the left.

probably due to the limited capacity of the furin-like enzyme of the transfected CHO cells to process the excess recombinant protein. Alternative splicing of the mRNA is an uncommon feature among the MMPs, as only a few MMP splice variants have been reported [23–25]. It will, therefore, be interesting to investigate the catalytic activity and substrate specificity of the alternatively spliced forms of epilysin towards a biologically relevant substrate.

Expression of epilysin in several tissues in the mouse under physiological conditions

Tissue expression of epilysin in adult, healthy NMRI mice was determined by RNase protection and Northern-blot hybridization (Figures 5 and 6). Epilysin mRNA was detected in a number of tissues and the highest expression levels were in the lung. Epilysin mRNA was also found in the placenta, heart, uterus, testis and gastrointestinal tract. RNase protection showed a constant ratio of full-length epilysin message relative to the shorter splice variants in all the tissues examined, excluding a possible tissue-specific expression pattern of the shorter splice variants. The tissue distribution of mouse epilysin mRNA resembles that of human epilysin with one notable exception: in adult human tissues, the highest expression level was found in the testis [11], whereas the overall expression levels in the adult mouse testis were found to be only moderate.

The wide expression of epilysin in a number of tissues under physiological conditions distinguishes this novel MMP from most previously characterized family members, as many MMPs are expressed only under exceptional conditions such as those related to injury, inflammation or disease (reviewed in [1]). The expression pattern suggests a role for epilysin in normal tissue homeostasis and turnover and thus sets a basis for future efforts to characterize the biological functions and substrates of this enzyme.

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