Epilysin, a Novel Human Matrix Metalloproteinase (MMP-28) Expressed in Testis and Keratinocytes and in Response to Injury*

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We have cloned a new human matrix metalloproteinase (MMP-28, epilysin) from human keratinocyte and testis cDNA libraries. Like most MMPs, epilysin contains a signal sequence, a prodomain with a PRCGVTD sequence, a zinc-binding catalytic domain with an HEIGHTGLGLTH sequence, and a hemopexin-like domain. In addition, epilysin has a furin activation sequence (RRKKKR) but has no transmembrane sequence. The exon-intron organization and splicing pattern of epilysin differ from that of other MMP genes. It has only 8 exons, and 5 exons are spliced at sites not used by other MMPs. Another novel feature of epilysin is that exon 4 is alternatively spliced to a transcript that does not encode the N-terminal half of the catalytic domain. Northern hybridization of tissue RNA indicated that epilysin is expressed at high levels in testis and at lower levels in lungs, heart, colon, intestine, and brain. RNase protection assay with various cell lines indicated that epilysin was selectively expressed in keratinocytes. Recombinant epilysin degraded casein in a zymography assay, and its proteolytic activity was inhibited by EDTA and by batimastat, a selective MMP inhibitor. Immunohistochemical staining showed expression of epilysin protein in the basal and suprabasal epidermis of intact skin. In injured skin, prominent staining for epilysin was seen in basal keratinocytes both at and some distance from the wound edge, a pattern that is quite distinct from that of other MMPs expressed during tissue repair. These findings suggest that this new MMP functions in several tissues both in tissue homeostasis and in repair.

The matrix metalloproteinases (MMPs)1 compose a family of enzymes that share several common structural features and that function both in the turnover and degradation of extracellular matrix proteins and in the processing, activation, or deactivation of a variety of soluble factors (1). MMPs, or matrixins, are a subgroup of the much larger metalloproteinase superfamily, which also includes astacin and ADAM proteinases, among others. To date 23 different MMPs have been cloned, and additional members continue to be identified (2).

To be classified as a matrix metalloproteinase, a protein must have conserved features of two domains, namely the prodomain and the catalytic domain. The prodomain of a typical MMP is about 80 amino acids, and all MMPs, except MMP-23 (3), contain the consensus sequence PRGXXPD. As for all metalloproteinases, the catalytic domain contains an active site Zn2+ that binds three conserved histidines in the sequence HEXHXXGHXH(S/T)XXXXXM, which also contains a conserved methionine to the carboxyl side of the zinc-binding site (metzincins) (4). In an inactive state, the conserved cysteine residue in the prodomain provides the fourth coordination site for the catalytic zinc ion. In addition, with the exception of matrilysin (MMP-7), endomastematrilysin-2 (MMP-26), and MMP-23, MMPs have a hinge region, which is often proline-rich, and a so-called hemopexin-like C-terminal domain (3, 5, 6). Other domains found in MMPs are specialized to subgroups of enzymes. For example, four membrane-type MMPs (MMP-14, -15, -16, and -24) have transmembrane and cytosolic domains, whereas MT4-MMP and MT6-MMP (MMP-17 and -25, respectively) have C-terminal hydrophobic extensions that act as a glycosylphosphatidylinositol-anchoring signal (7–9). The two gelatinases (MMP-2 and MMP-9) have gelatin-binding domains. MMP-23 lacks the hemopexin domain and has a novel cysteine array motif and an immunoglobulin-like C2-type fold domain (3, 10). In addition to a common domain structure, MMPs share a similar gene arrangement suggesting that they were generated by duplications of an ancestor gene. At least eight of the known human MMP genes (MMPs 1, 3, 7, 8, 10, 12, 13, and 20) are clustered on chromosome 11 at 11q21–23. Other known MMP genes are scattered along chromosomes 1, 8, 12, 14, 16, 20, and 22 (3, 11, 12).

MMPs are secreted or bound or anchored to the cell membrane, and all function extracellularly or within the secretion pathway. As demonstrated in defined in vitro studies, almost all MMPs can cleave or degrade some protein components of the extracellular matrix, and many are able to act on a wide variety of proteins (13). Notable exceptions to this rule are stromelysin-3 (MMP-11) and MMP-23, which have no known extracellular matrix substrates (3, 14). In addition and quite importantly, MMPs can process or degrade nonmatrix proteins. For example, matrilysin is responsible for activation of the pro-form of α-defensins (15), a class of secreted antimicrobial peptides, and several MMPs can cleave and inactive the serpin α1-proteinase inhibitor (16, 17), which is an in vivo substrate.
for gelatinase-B (MMP-9) (18). In addition, several MMPs, such as MMP-1, -2, -3, -7, and -11, among others, directly modulate the activity of several growth factors, such as tumor necrosis factor-α, insulin-like growth factor-1, epidermal growth factors, and fibroblast growth factors (19–24). Thus, matrix degradation is neither a sole nor a common functional feature of MMPs.

Many of the secreted MMPs, including MMPs 1, 3, 9, 10, 11, and 13, are not expressed in normal, healthy resting tissues, and with some exceptions, their production and activity are maintained at nearly undetectable levels. In contrast, some level of MMP expression is seen in any repair or remodeling process, in any diseased or inflamed tissue, and in essentially any cell type grown in culture (25, 26). Although the qualitative pattern and quantitative levels of MMPs vary among tissues, diseases, tumors, inflammatory conditions, and cell lines, a reasonably safe generalization is that activated cells express MMPs. Some MMPs, including MMPs 7, 19, 24, 25, and 26, are expressed in healthy tissues (27–31).

In the present study, we report on the cloning and initial characterization of a novel human MMP, MMP-28, which we call epilysin. We isolated the cDNA for this protein from keratinocyte and testis libraries, and we show that it has the essential domains of a prototypic MMP, as well as several unique features. Because of its ability to degrade a protein substrate was fully inhibited by EDTA and a hydroxamate MMP inhibitor, epilysin, is indeed a metalloenzyme. Our data suggest that epilysin is expressed in intact tissues and up-regulated in response to injury. Thus, this new MMP may function in both tissue homeostasis and tissue repair.

EXPERIMENTAL PROCEDURES

Cloning of Human Epilysin cDNA, Exon/Intron Mapping—A search of the GenBank TM data base with the peptide string FDKXXXXLA-HAXXXGXXGDXHDXDXXXW, which is conserved among MMPs, returned a homologous sequence within an 82-kb human genomic DNA clone (GenBank TM accession number AC006237). Nested primers were designed to amplify a 161-bp cDNA fragment by RT-PCR using HT-1080 human fibrosarcoma line RNA as a template. The amplified product corresponds to bases 726–886 (see Fig. 1). By screening a human foreskin, a kidney cDNA library (HL110b, CLONTECH, Palo Alto, CA) by plaque hybridization with the 32P-labeled 161-bp cDNA fragment, we obtained three positive clones, which were then sequenced. The longest clone of 1.5 kb contained exons 3–8 of epilysin (see below for exon numbering). This cDNA clone was then used to screen a pooled human testis cDNA library (HL5033t, CLONTECH). Among the more than 20 positive clones was a clone that contained the coding regions of exons 1 and 2. This cDNA was infected with the construct and BaculoGold DNA (PharMingen), to produce recombinant baculovirus. Following two rounds of virus amplification, High Five insect cells (Stratagene) were infected with baculovirus protein gp67 to direct secretion of the fusion protein. Sf9 cells (Invitrogen) were transfected with the construct and BaculoGold DNA (PharMingen) to produce recombinant baculovirus. Following two rounds of virus amplification, High Five insect cells (Stratagene) were infected and harvested along with conditioned medium 5 days later. Cell pellets were lysed in 10 mM Tris-HCl buffer, pH 7.5, containing 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM Na3P, and 10 mM NaGTP. The resulting expression construct codes for a fusion protein of Schisotoma japonicum glutathione S-transferase (GST) and amino acid residues 123–520 of epilysin corresponding to the putative furin-activated enzyme. This vector also provides the signal peptide from the baculovirus protein gp67 to direct secretion of the fusion protein. Sf9 cell pellets were lysed in 10 mM Tris-HCl buffer, pH 7.5, containing 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM Na3P, and 10 mM NaGTP. The resulting expression construct codes for a fusion protein of Schisotoma japonicum glutathione S-transferase (GST) and amino acid residues 123–520 of epilysin corresponding to the putative furin-activated enzyme. This vector also provides the signal peptide from the baculovirus protein gp67 to direct secretion of the fusion protein. Sf9 cell pellets were lysed in 10 mM Tris-HCl buffer, pH 7.5, containing 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM Na3P, and 10 mM NaGTP. The resulting expression construct codes for a fusion protein of Schisotoma japonicum glutathione S-transferase (GST) and amino acid residues 123–520 of epilysin corresponding to the putative furin-activated enzyme. 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Cell Culture—Human foreskin fibroblasts, immortalized human keratinocytes (HaCaT) (35), and human fibrosarcoma HT-1080 cells (CCL-121, American Type Culture Collection, Manassas, VA) were grown to confluence in Eagle’s minimal essential medium containing 10% heat-inactivated fetal calf serum (Life Technologies, Inc.), 100 IU/ml penicillin, and 50 μg/ml streptomycin. Human colon adenocarcinoma HT-29 cells (HTB38, ATCC) were maintained in RPMI medium. MMP expression was stimulated by treatment with 16 nM phorbol ester from a single bacterial colony was diluted 1:10 and incubated at 37 °C for 2 h. Expression of the fusion protein was then induced by adding isopropl-1-thio-β-galactopyranoside (0.5 mM final concentration) followed by further incubation for 24 h. Recombinant protein obtained in inclusion bodies was solubilized by sonication in the presence of N-lauryl sarcosine and affinity-purified with glutathione-Sepharose as described (38). Recombinant fusion protein bound to glutathione-Sepharose was then digested with PreScission protease according to manufacturer’s instructions (Amersham Pharmacia Biotech) to remove the GST tag. Between the GST domain and epilysin, the

R. P. Siegel, S. A. Fiers, and R. L. Hill, unpublished results.

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fusion protein has the recognition sequence (LEVLFQGP) for PreScission Protease. Recombinant epilysin (pro- and catalytic domains) was then eluted with 50 mM Tris-HCl buffer, pH 7.5, containing 500 mM NaCl, 5 mM dithiothreitol, and 0.1% Brij-35. Caseinolytic activity was measured by zymography using a 4–16% SDS-PAGE blue casein zymogram gel (NOVEX, San Diego, CA) according to manufacturer’s protocol. After electrophoresis, the zymogram gel was washed and incubated in 50 mM Tris-HCl buffer, pH 7.5, containing 1–2.5% Triton X-100 and either 1) 10 mM EDTA, or 2) both 5 mM CaCl2 and 1 mM ZnCl2, or 3) 10 mM batimastat in the presence of 5 mM CaCl2 and 1 mM ZnCl2.

Preparation of Antibodies and Immunoblotting Assay for Epilysin—An 8-chain branching multiple antigenic peptide of 16 amino acids, DQDERWSLSRRRGRNL, corresponding to the middle of the catalytic domain of epilysin (amino acid residues 219–234, see Fig. 1), was used as an antigen (Research Genetics, Huntsville, AL). Rabbits were first immunized with 0.5 mg of the peptide in complete Freund's adjuvant, and three booster injections with 0.5 mg of the peptide in incomplete Freund's adjuvant were given 2, 6, and 8 weeks later by a commercial operation (Research Genetics). Antibodies were purified from whole serum, harvested at 10 weeks after primary injection, by affinity chromatography with the peptide coupled to N-hydroxysuccinimide-Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech). For immunoblotting, confluent cultures of HaCaT cells were washed with serum-free medium and were incubated under serum-free conditions for an additional 48 h. The medium was then collected and concentrated 70-fold using a Centricon microcentrifuge (Amicon, Beverly, MA). 10 µl of concentrated conditioned medium was mixed with an equal amount of Laemmli sample buffer containing 10% β-mercaptoethanol and resolved by SDS-PAGE through a 4–15% gradient gel. Recombinant baculoviral GST-epilysin fusion protein was used as a positive control. Proteins were then electrophoretically transferred to nitrocellulose (Schleicher & Schuell) using a semi-dry blotting apparatus at 2.5 mA/cm2 for 30 min. Membranes were blocked with 5% milk in PBS/Triton X-100 (0.5%) and incubated with 0.15 mg/ml of affinity-purified antibodies in 50 mM Tris-HCl buffer containing 500 mM NaCl, 0.1% Tween 20, and 0.1% bovine serum albumin, pH 8.5. After five washes in the same buffer, the bound antibodies were detected using biotinylated anti-rabbit IgG antibodies and peroxidase-conjugated streptavidin (Dakopatts, Copenhagen, Denmark) and enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech) as described (39).

Expression of Epilysin in CHO Cells, Immunofluorescence Staining—

**FIG. 1.** Nucleotide sequence of the human epilysin (MMP-28) cDNA and its deduced amino acid sequence. The deduced amino acid sequence is shown below the DNA sequence. The first ATG and the termination codon TGA are in bold. Numbers on the right and left refer to the positions of nucleic acids and amino acid residues, respectively. Pre-sequence PRCGVTD and zinc-binding site HEIGHTLGLTH are inverted, and the furin recognition sequence RRKKR is boxed. Predicted signal peptide cleavage site is indicated with an arrow, and the furin cleavage site is marked with an arrowhead. Two potential N-glycosylation sites are underlined. Vertical bars indicate the exon limits, and exons are numbered as indicated in Table I. Our cDNA clones cover the sequence between bases 1 and 1817. First consensus polyadenylation signal AATAAA is found about 500 bp downstream in the genomic sequence (GenBank™ accession number AC006237), and the sequence between bases 1818–2332 is derived from this deposited sequence and represents the most probable 3’ end of epilysin mRNA. The nucleotide sequence data are in the GenBank™ nucleotide sequence data base with the GenBank™ accession number AF219624.
Chinese hamster ovary (CHO) cells were transfected with a cDNA construct coding for epilysin with a C-terminal 10-aa influenza virus hemagglutinin tag under transcriptional control by cytomegalovirus promoter in pcDNA3 vector (Invitrogen, San Diego, CA) using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals). 24 h after transfection, transfected cell clones were selected for neomycin resistance as described (40). For immunofluorescence staining, a pool of transfected cells was plated on glass coverslips, and 3 days later, the cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS, 0.14M NaCl in 10 mM phosphate buffer, pH 7.4). After fixing the coverslips were washed three times with PBS and blocked with 5% bovine serum albumin in PBS for 30 min. Affinity-purified epilysin antibody was then added (1:100 dilution) in 0.5% bovine serum albumin in PBS and incubated 1 h at room temperature. After three washes with the same buffer, fluorescein isothiocyanate-conjugated anti-rabbit IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA) were added and incubated for 1 h. Coverslips were then washed 5 times with PBS and mounted on glass slides using Vectashield anti-fading agent (Vector Laboratories, Inc., Burlingame, CA).

Immunohistochemistry—Individual, 4-mm-wide, full thickness biopsies of human skin used for keratinocyte culture were placed into the wells of 6-well cluster dishes and covered with Dulbecco's modified Eagle's medium containing antibiotics. 24 h later, tissues were fixed in 10% buffered formalin and processed for paraffin embedding. Deparaffinized 5-μm sections were processed for immunohistochemistry using alkaline phosphatase as described (40). For immunofluorescence staining, a pool of transfected cells was plated on glass coverslips, and 3 days later, the cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS, 0.14M NaCl in 10 mM phosphate buffer, pH 7.4). After fixing the coverslips were washed three times with PBS and blocked with 5% bovine serum albumin in PBS for 30 min. Affinity-purified epilysin antibody was then added (1:1000 dilution) in 0.5% bovine serum albumin in PBS and incubated 1 h at room temperature. After three washes with the same buffer, fluorescein isothiocyanate-conjugated anti-rabbit IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA) were added and incubated for 1 h. Coverslips were then washed 5 times with PBS and mounted on glass slides using Vectashield anti-fading agent (Vector Laboratories, Inc., Burlingame, CA).

RESULTS

Cloning and Sequencing of a cDNA Encoding Human Epilysin, Comparison with Other MMPs—To identify undiscovered MMPs, we searched the GenBank™ data base using the TBLASTN program and a peptide query sequence FDGXXXX-XXXX-LAHA-XXXXXX-PG-XXXX-GD-XHFD-XXXXXE. Among the more than 100 hits was a human genomic DNA clone (GenBankTM accession number AC006237) that was submitted by Whitehead Institute/MIT Center for Genome Research as a part of the Human Genome Project sequencing chromosome 17. There was no annotation that the sequence would code for proteins. After translation of the genomic DNA in three forward reading frames, several peptide sequences typical of MMPs, including a propeptide sequence PRCGVTD and a catalytic domain sequence HEIGH, were identified, and these sequences were separated by putative intronic sequences.

To assess if this genomic region was transcribed to an mRNA, two sets of primers were designed; the forward and reverse primers were directed to different suspected exons. As a source of RNA, we used the human fibrosarcoma cell line HT-1080, as these cells are known to express a wide variety of MMPs (40, 42). cDNA was synthesized using random hexamer primers and was amplified by PCR. Two-stage PCR with nested primers produced an amplified DNA fragment of expected size (161 bp), and the nucleotide sequence of this fragment was identical to that of presumed exonic portions of the genomic sequence (data not shown).
To obtain the full-length cDNA for this novel MMP, we screened a human keratinocyte cDNA library using the PCR product as a probe. (RNase protection analysis of various cell lines revealed that epilysin is expressed in cultured human keratinocytes; see Fig. 6.) Among the three positive clones, we isolated and characterized a 1.5-kb cDNA that contained sequence coding for part of the prodomain, the entire catalytic and hemopexin-like domains, a stop codon TGA, and 85 bp of 3′-untranslated region. To determine the 5′ end of this MMP transcript, we screened a testis cDNA library with a probe corresponding to the 5′ end of the 1.5-kb keratinocyte cDNA insert. (Hybridization of tissue-RNA blots revealed that epilysin is expressed in human testis at high levels; see Fig. 5.) Of the positive clones, we identified one containing a cDNA insert that coded for the missing portion of the prodomain and for about 170 bases of 5′-untranslated region. The open reading frame, starting from the first ATG codon, contains 1560 nucleotides and codes for a 520-aa protein with a calculated molecular mass of 59 kDa (Fig. 1). We named this new MMP epilysin. By using the accepted consecutive number nomenclature, epilysin would be assigned MMP-28.

The domain structure and organization of epilysin is predictable for an MMP (Figs. 1 and 2). By using an analysis program available at the SignalP server (33), we identified a typical hydrophobic signal sequence of 22 amino acids at the N terminus of epilysin (Fig. 1). The signal sequence is followed by a prototypic MMP prodomain with the conserved cysteine-switch sequence PCI CVTD (Figs. 1 and 2). In this sequence, a proline that is present in all other human MMPs except MMP-19 (43) is replaced by a threonine (Fig. 2). In addition, after the cysteine switch sequence, there is an 11-aa insertion, which is not present in other known MMPs, followed by an RRKKR furin recognition sequence (Figs. 1 and 2). The catalytic domain is highly conserved relative to other MMPs, and as for secreted MMPs, an 8-aa insertion present only in MT-MMPs (e.g. MMP-14) is lacking from epilysin (Fig. 2). The catalytic center with three histidine residues, HEIGHLGLTH, is unique in that no other MMP has threonine within this sequence. A 39-aa hinge region is followed by a typical hemopexin-like domain. There is no hydrophobic transmembrane sequence typical of membrane-inserted MMPs or a hydrophobic extension typical of glycosylphosphatidylinositol-anchored proteins. In addition, epilysin has two putative N-glycosylation sites as follows: one in the N-terminal part of the catalytic domain and another in the second pexin-like repeat of the hemopexin domain (Fig. 1). The calculated molecular mass of the proenzyme without the signal sequence is 56 kDa; the active, furin-processed enzyme is estimated to be 45 kDa. These weights do not include any contribution in mass by glycosylation.

Comparison of the epilysin amino acid sequence with other MMPs by ClustalW program (Fig. 2) and construction of a phylogenetic tree on the basis of the catalytic domain sequences (Fig. 3) indicate that epilysin is most closely related to several other recently cloned MMPs, including MMP-19, MMP-23, MT4-MMP (MMP-17), MT6-MMP (MMP-25), and stromelysin-3 (MMP-11) (3, 8, 43–45). The number of identical and similar residues with MMP-19 catalytic domain is 46 and 60%, respectively.

**Structural Organization of the Human Epilysin Gene**—We mapped the exon/intron junctions and determined an exon/intron map of the gene by comparing the cDNA and genomic sequences (Fig. 4A). Exon/intron boundaries and the sizes of exons and introns are summarized in Table I. All exons were contained within the genomic BAC clone except exon 1, and hence, we do not yet know the size of intron 1 (Table I). The exon/intron structure of epilysin is unique compared with other MMP genes. Whereas most MMP genes have 10 exons, the epilysin gene has only 8 exons, similar to that of stromelysin-3 (MMP-11) (Fig. 4B) (46). Furthermore, only three of the seven splice sites (splice sites between exons 1 and 2, 5 and 6, and 6 and 7) are at positions conserved among most MMP genes. None of the unique splice sites are similar to those other “nontraditional” MMP genes characterized to date, such as MMP-7, MMP-11, or MMP-14. Overall, the organization of the epilysin gene is similar to that of MMP-19, with one overt difference being that exon 8 of epilysin corresponds to exons 8 and 9 of MMP-19 (Fig. 4B) (47). The exon/intron boundaries conform to the GT/AG rule for splice sites (48) (Table I).

**Analysis of Epilysin Expression in Human Tissues and Cell Lines**—To analyze the expression of epilysin in different human tissues, we hybridized a Northern blot containing mRNA from various human tissues with a 1.5-kb epilysin cDNA probe. At least three different transcripts of 2.6, 2.0, and 1.2 kb were detected in many tissues (Fig. 5), and this heterogeneity is likely due to alternative splicing (see below). The 2.6-kb transcript was most abundant in all tissues, and among tissues, the relative levels of three transcripts were about the same. Epilysin mRNA is highly expressed in testis and at lower levels in lungs, heart, colon, intestine, and brain (Fig. 5).

To determine which cell types express epilysin, we screened RNA from several cell lines known to actively express a variety of MMPs. To obtain maximal specificity and sensitivity, we developed an RNA protection assay for epilysin mRNA. The human cells we used included HT-29 colon carcinoma cells (49), U937 monocytic-like cells (50), HT-1080 fibrosarcoma cells (42), neonatal foreskin fibroblasts (51, 52), immortalized HaCaT keratinocytes (53), and normal primary keratinocytes (53). Because the transcription of many MMP genes is strongly up-regulated by PMA, some cells were treated with this agent for 24 h before RNA was isolated. U937 cells were treated with a combination of PMA and lipopolysaccharide, which mediates...
differentiation to a macrophage-like phenotype accompanied by a potent induction of several MMPs (37, 50).

Although epilysin is expressed in several tissues, of the cell types tested, epilysin mRNA was detected only in keratinocytes (Fig. 6). HaCaT keratinocytes showed the highest expression of epilysin mRNA, and primary keratinocytes, which were grown on collagen to induce MMP expression (54), had a somewhat lower level of expression (Fig. 6). All other cell lines were negative for epilysin expression. Because we could amplify a portion of epilysin cDNA from HT-1080 RNA by RT-PCR, these fibrosarcoma cells express epilysin at low levels.

To determine whether epilysin mRNA is translated into a protein, we generated rabbit polyclonal antibodies against a 16-amino acid multiple antigenic peptide in the middle of the catalytic domain. Anti-epilysin antibodies were affinity-purified using the same peptide. The specificity of the antibody was tested by immunofluorescence staining of transfected CHO cells and by immunoblotting of conditioned medium (Fig. 7). CHO cells transfected with an epilysin expression construct showed a predictable range of recombinant protein production. Whereas some clones showed prominent fluorescence for epilysin, other selected clones had no staining (Fig. 7A). In addition, we used immunoblotting to assess if epilysin protein is released by HaCaT keratinocytes. As a positive control, we used a fusion protein of *S. japonicum* glutathione S-transferase and amino acid residues 123–520 of epilysin, corresponding to the putative furin-activated enzyme. A strongly immunoreactive band of about 58 kDa was detected in HaCaT-conditioned medium, and the predicted 75-kDa band was seen in the fusion protein preparation (Fig. 7B). In addition to the 58-kDa band, we detected in HaCaT-conditioned medium a slightly smaller band of about 55 kDa of much lower intensity (Fig. 7B). As discussed below, this smaller band could be a product of alternative splicing or alternative glycosylation. Together, these findings indicate the epilysin mRNA codes a secreted protein that is produced and released by keratinocytes.

**Detection of Alternative Splicing**—During the cloning of epilysin, we generated a probe by RT-PCR using primers complementary to sequences in exons 3 and 5 (see "Experimental Procedures"). In addition to the expected PCR product, we obtained a shorter cDNA of nearly equal intensity and a weak band of intermediate size (Fig. 8A). Similar amplified products were generated using a different forward primer in exon 3, and all PCR products were positive in Southern blotting (Fig. 8A). We gel-purified and sequenced the amplified DNA, and we found that the longer amplification product contained sequences for exons 3–5, whereas the shorter amplification product represented an mRNA species that contained exons 3 and 5 but lacked exon 4. To assess the relative abundance of the different splice forms, another PCR product was cloned and used to generate an RNA probe for RNase protection analysis.

**FIG. 4.** Organization of the human epilysin gene. Comparison with other human MMP genes. A, organization of the epilysin gene was drawn based on the comparison of the cDNA sequence with the sequence of the genomic BAC clone hRPC.161_P_9. Exons are numbered from the 5’ end of the gene and depicted by black boxes. The noncoding regions of the first and last exons are depicted by open boxes. The size of the first intron is unknown; it was not present in the genomic BAC clone. The positions of the transcription start site (ATG), stop codon (TGA), pro-sequence (PRCGVTD), furin cleavage site (RRKKR), and the catalytic zinc-binding site (HEIGHTLGLTH) are indicated below the gene graph. Base positions in the BAC clone are indicated above the gene graph. B, comparison of exon and domain structures of members of MMP family. The exons in human epilysin (MMP-28), gelatinase A (MMP-2), collagenase-1 (MMP-1), stromelysin-1 (MMP-3), matrilysin (MMP-7), stromelysin-3 (MMP-11), membrane type-1 matrix metalloproteinase (MT1-MMP, MMP-14), and MMP-19 are shown as boxes, with their sizes in nucleotides below. Open boxes indicate untranslated sequences. Filled boxes indicate different domains of the matrix metalloproteinases as follows: signal peptide, prodomain, catalytic domain, hinge region, hemopexin-like domain, transmembrane domain, and intracellular domain. FN, fibronectin-like domain of gelatinase A. The locations of the exon-intron splicing sites in the epilysin gene differ markedly from other MMPs. Only the splice sites between exons 1 and 2, 5 and 6, and 6 and 7 are at conserved positions among most MMP genes, whereas all the splice sites of epilysin gene are utilized also in theMMP-19 gene.
Human Epilysin Gene

Exon/intron boundaries were determined by comparing the MMP-27 cDNA sequence to a human genomic BAC clone hRPC.161_P_9, GenBank™ accession number AC006237. The nucleotide sequence of each exon (uppercase letters) and intron (lowercase letters) at the exon/intron boundaries is shown. The deduced amino acid sequence at the intron-exon boundaries is indicated under the nucleotide sequence. Exon 1 is not present in the BAC clone, and the size of intron 1 is therefore not known. UTR, untranslated region.

| Exon | Intron–Exon–Intron junction | Exon size | Intron size | Genomic DNA bases |
|------|-----------------------------|-----------|-------------|------------------|
| 1    | ATG GTC GCG ... GAG GCG GAG | xxx       | >169, 5'-UTR| ? Not present in BAC |
|      | Met Val Ala ... Glu Ala Glu |           | 111 coding | >280 total |
| 2    | tctcccgccag GCA TTC CTA ... GAT GCC ATC AG | 80        | 168        | 8691–8770 |
|      | Ala Phe Leu ... Asp Ala Ile Ar(g) |           |            | |
| 3    | ctcctgacag A GCC TTT CAG ... GCA AAG CAA G | 188       | 5483       | 8939–9126 |
|      | (Ar(g) Ala Phe Gln ... Ala Lys Gln G(ly)) |           |            | |
| 4    | tgggtgcag GT AAC AAA TGG ... GAT GCC CCA G | 225       | 2853       | 14,610–14,834 |
|      | G(ly) Asn Lys Trp ... Gsp Gly Pro G(ly) |           |            | |
| 5    | ccacgtgacag GG GCC GCC CTG ... AGC CTG TAT G | 246       | 1687       | 17,688–17,933 |
|      | (G(ly) Gly Ala Leu ... Ser Leu Tyr G(ly)) |           |            | |
| 6    | actcacaag GG AAG CCC CTA ... GAT ACT G TA G | 150       | 310        | 19,621–19,770 |
|      | Gtaaaggt | | | |
| 7    | ctctggcag AC AGG CCA CAG ... TTC TTC AAA G | 165       | 857        | 20,081–20,248 |
|      | (G(ly) Lys Pro Leu ... Ile Thr Val A(sp)) |           |            | |
| 8    | ttccacagc AC AGG CCA CAG ... TTC TTC TGA | 21,106–22,100 | | |
|      | (G(ly) Gly Arg Cys ... Ala Leu Phe stop) |           | 995, total | |

*Genomic DNA base positions are derived from BAC clone hRPC.161_P_9, GenBank™ accession number AC006237.

In the genomic sequence of BAC clone hRPC.161_P_9, a C appears at base 14,668, which causes a frame shift. Sequencing of testis and keratinocyte cDNAs confirmed that a G belongs at 14,668 and that the C resides at 14,669, restoring the open reading frame. Because of the addition of this single base, the numbers shown for the end of exon 4 (14,833) through exon 8 (22,099) are increased by one compared with the base positions shown in the GenBank™ AC006237 access file.

FIG. 5. Northern blot analysis of epilysin expression in a variety of human tissues. 2 μg of poly(A) RNA from the indicated tissues were analyzed by hybridization with the cDNA for human epilysin. Migration of RNA size markers is shown on the left. Filters were subsequently hybridized to a human β-actin probe to control the loading of RNA. At least three different transcripts of 2.6, 2.0, and 1.2 kb were detected.

FIG. 6. Expression of epilysin in cultured cells. RNase protection analysis. Confluent cultures of colon adenocarcinoma cells (HT-29), histiocytic lymphoma cells (U937), human fibrosarcoma cells (HT-1080), human foreskin fibroblasts, immortalized human keratinocytes (HaCaT), and primary keratinocytes were treated with PMA (40 nM) and lipopolysaccharide (5 μg/ml) for 24 h where indicated. Total RNA was then extracted and analyzed by RNase protection for the presence of epilysin mRNA as described under “Experimental Procedures.” Protected RNA fragments were fractionated by 5% TBE-PAGE containing 6 μl urea and visualized by autoradiography. Undigested probe (257 nt) and protected fragment (161 nt) are indicated on the left. Migration of RNA size markers is shown on the left. Specific signal for epilysin could be detected only in HaCaT and keratinocyte samples. Full-length probe is also protected to a minor extent because of residual template DNA. Equal loading of the RNAs was confirmed by separate RNase protection analysis for cyclophilin mRNA (cyclo).
catalytic domains of epilysin. Because the prodomain may be necessary for the correct re-folding of the recombinant protein, this region was included in the construct. SDS-PAGE analysis and Coomassie Blue staining of proteins bound to glutathione-Sepharose revealed a single major protein of the expected size (56 kDa) and a minor 33-kDa band (Fig. 9, lane 1). After treatment with PreScission protease, the 56-kDa protein was cleaved into two proteins with apparent molecular masses of 34 and 32 kDa (lane 2), which were identified by immunoblotting as epilysin and GST, respectively (data not shown). After PreScission cleavage, recombinant epilysin was eluted from the column matrix with high salt concentration (500 mM NaCl) (lane 3). Zymogram analysis with 4–16% SDS-PAGE impregnated with blue casein indicated that both the 56-kDa fusion protein and the 34-kDa free epilysin had caseinolytic activity. This proteolytic activity was completely inhibited by exclusion of calcium and zinc and addition of 10 mM EDTA in the incubation buffer, indicating dependence on divalent cations, calcium, and zinc (Fig. 9). Incubation of the casein zymogram gel in the presence of 10 μM batimastat, a specific MMP inhibitor, also completely inhibited the caseinolytic activity (data not shown). This compound is a substrate-based inhibitor containing a hydroxamic acid moiety that chelates the active site zinc cation and renders all MMPs catalytically inactive. PreScission protease (46 kDa) did not have any detectable caseinolytic activity (Fig. 9, lane 2).

Expression in Human Skin—Because epilysin mRNA and protein were detected in cultured human keratinocytes, we assessed if this MMP is expressed in human skin. For this study, we incubated small, uniformly sized pieces of normal adult human skin for 24 h in culture medium and then fixed and processed the samples for immunohistochemistry using affinity-purified antibody. During the incubation, epidermal cells migrated down the edge of the cut surface of the biopsy in an attempt to heal the “wounded” tissue. In other studies, we have demonstrated that the expression of MMPs in this ex vivo model mirrors that seen in vitro. In the center of the tissue specimens, at some distance (about 2 mm) from the wound edge, staining for epilysin protein was seen in the intact epidermis (Fig. 10A). The staining intensity was strongest in basal keratinocytes and progressively weaker in suprabasal cells. A different pattern of staining was seen at the wound edge (Fig. 10C). Here, intense staining for epilysin was seen in migrating keratinocytes at the wound edge and in stationary basal keratinocytes several cells behind the wound front. In contrast to intact epidermis, epilysin was not detected in suprabasal keratinocytes near the wound front (Fig. 10C). No dermal cells were stained for epilysin protein (Fig. 10, A and C), and no reactivity was detected in samples processed with preimmune serum (Fig. 10, B and D).
Here we report the identification, gene, domain organization, and tissue expression of a new member of the matrix metalloproteinase gene family. Based on the sequential numerical nomenclature, this new protein would be designated MMP-28. Because of its prominent expression in the epidermis and its catalytic activity as an endopeptidase, we call this new MMP “epilysin." Epilysin has all the key domains of a typical MMP as follows: a signal peptide, a conserved cysteine-containing prodomain, a conserved histidine-containing catalytic domain, a hinge, and a hemopexin domain. It degrades casein, and its proteolytic activity requires divalent cations and is inhibited by a synthetic MMP inhibitor. In contrast, epilysin does not include domains characteristic of other metalloproteinases such as the disintegrin and thrombospondin-like domains found in ADAMs and tsADAMs, respectively, or a transmembrane domain as is found in most membrane-type MMPs (55).

The unique exon-intron structure suggests that epilysin diverged early from other MMPs. However, the splicing pattern is very close to that of MMP-19, to which epilysin is also most closely related at the amino acid sequence level. A notable difference between MMP-19 and epilysin is that MMP-19 has no furin recognition site between its pro- and catalytic domains. Unlike many MMP genes, which are clustered on the long arm of chromosome 11, the locus for epilysin is present on chromosome 17. In addition, we have recently isolated the cDNA for mouse epilysin (data not shown). Comparison of the amino acid sequences of the catalytic domains indicates that the coding regions of the mouse and human epilysin genes are highly conserved (97% identical residues), suggesting an important function of this region for enzyme activity.

The predicted molecular mass of secreted pro-epilysin is 56 kDa, and if it is cleaved in the secretion pathway at its furin-recognition site, the released protein would be about 45 kDa. These sizes, however, do not account for additional mass contributed by glycosylation. Indeed, by immunoblotting analysis of HaCaT keratinocyte-conditioned medium, we detected a protein of about 58 kDa and a less prominent band of about 55 kDa. These two bands may be due to differential glycosylation, such as characteristic of collagenase-1 (56), or they may reflect two distinct isoforms. By using RT-PCR and RNase protection assays, we determined that epilysin is transcribed into at least two different mRNAs, one of which lacks exon 4. Because exons 3–5, among others, end with split codons for glycine, splicing exon 4 would not affect the amino acid sequence coded by exon 5 (Table I). Omission of exon 4 would reduce the predicted molecular mass of pro-epilysin by about 8 kDa, within the range of the size difference we detected by immunoblotting. Because our antibody was raised against a recognition site, the released protein would be about 45 kDa. These two bands may be due to differential glycosylation, such as characteristic of collagenase-1 (56), or they may reflect two distinct isoforms. By using RT-PCR and RNase protection assays, we determined that epilysin is transcribed into at least two different mRNAs, one of which lacks exon 4. Because exons 3–5, among others, end with split codons for glycine, splicing exon 4 would not affect the amino acid sequence coded by exon 5 (Table I). Omission of exon 4 would reduce the predicted molecular mass of pro-epilysin by about 8 kDa, within the range of the size difference we detected by immunoblotting.
activation state of the pro-enzyme.

In our Northern hybridizations we could detect at least three different MMP-28 transcripts of about 2.6, 2.0, and 1.2 kb. Whereas the longest transcript of 2.6 kb most probably corresponds to the sequence presented in Fig. 1 with some more 5′-untranslated region and poly(A) tail added, the other transcripts remain uncharacterized. They could be either products of alternative splicing or different utilization of polyadenylating sites. At least the 1.2-kb transcript is too short to code for a full-length enzyme. During the screening of the libraries we isolated clones containing intronic sequences (data not shown). Whether they are cloning artifacts or real functional transcripts remains to be shown.

Another unusual feature of epilysin is that it is expressed in normal, intact tissues, such as testis, intestine, lung, and skin, and this pattern of expression suggests that this MMP may serve a role in tissue homeostasis. Similarly, matrilysin (MMP-7) is expressed by the epithelium of intact mucosal tissues (31, 49, 57), and we recently reported that matrilysin functions in innate immunity, a homeostatic function, by activating prodefensin peptides (15). Although matrilysin is expressed by mucosal epithelium, including that of small intestine, injured colon, airways, and exocrine glands, it is not expressed in the epidermis (31, 49). It is tempting to speculate that epilysin participates in host defense in intact epidermis by processing antimicrobial proteins. Indeed, because it is expressed by basal and suprabasal keratinocytes, released epilysin may not encounter a matrix substrate in intact skin. Thus, in normal, intact tissues, such as testis, intestine, lung, and skin, we recently reported that matrilysin (MMP-7) is expressed by the epithelium of intact mucosal tissues (31, 49, 57), and we recently reported that matrilysin functions in innate immunity, a homeostatic function, by activating prodefensin peptides (15). Although matrilysin is expressed by mucosal epithelium, including that of small intestine, injured colon, airways, and exocrine glands, it is not expressed in the epidermis (31, 49). It is tempting to speculate that epilysin participates in host defense in intact epidermis by processing antimicrobial proteins. Indeed, because it is expressed by basal and suprabasal keratinocytes, released epilysin may not encounter a matrix substrate in intact skin. Thus, although epilysin is a member of the matrix metalloproteinases gene family, we cannot yet conclude that matrix components are physiologic substrates for this enzyme.

In addition to tissue homeostasis, epilysin may serve a distinct and additional role in repair of cutaneous wounds. In response to injury, several MMPs are produced by the epidermis in functionally distinct subpopulations of keratinocytes (58). For example, collagenase-1 (MMP-1), stromelysin (MMP-3), and gelatinase-B (MMP-9) are produced by basal keratinocytes at the migrating front, whereas stromelysin-1 (MMP-3) is expressed by the hyperproliferative cells just behind these migrating cells (59–63). Distinct from the localization of these MMPs, prominent staining for epilysin was seen in basal keratinocytes at the migratory front and in all cells behind the wound edge. Again, this pattern is similar to that for matrilysin in wounded epithelium. Although it is not found in cutaneous wounds (58), matrilysin is expressed by migrating and stationary epithelial cells in wounds and ulcerations of mucosal tissues, such as lung and intestine (64, 65). Demonstrating an essential role for matrilysin in mucosal repair, airway epithelial wounds do not repair in MMP-7-null mice (65). Thus, matrilysin serves at least two distinct roles in mucosal tissues as follows: one in innate defense and the other in epithelial repair and migration. Epilysin may have equally critical roles in skin. Our future studies will be directed at determining the function of this new MMP.

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Note Added in Proof—After our article was published in the “JBC papers in press” on December 19, 2000, we learned that another manuscript, “MMP-28, a New Human Matrix Metalloproteinase with an Unusual Cysteine-Switch Sequence Is Widely Expressed in Tumors” by George N. Marchenko and Alex Y. Strongin, had been accepted for publication in Gene. The gene identified and characterized in the Gene paper is identical to that described in the current publication.
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