Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 327 (2005) 328–334
BBRC
www.elsevier.com/locate/ybbrc

Shedding of membrane epithin is blocked without LDLRA4 and its protease activation site

Eun-Gyung Cho[a,b], Ronald H. Schwartz[a], Moon G. Kim[a,*]

[a] *Laboratory of Cellular and Molecular Immunology, NIAID, NIH, Bethesda, MD 20892-0420, USA*
[b] *National Creative Research Initiative Center for Cell Death, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea*

Received 27 November 2004

Abstract

Epithin, a mouse type II transmembrane serine protease, is processed at Gly^{149} and released from the membrane. Here, we report the identification of an epithin isoform, epithin(Δ), containing a 66 amino acid deletion from the full-length epithin, which is missing the 4th LDLRA domain and the protease activation sequence. This truncated isoform showed the same characteristic N-terminal processing at Gly^{149} as the full-length form, however, no protease activity was detected. The N-terminal processed epithin(Δ) short form (Epi(Δ)-S) was not released into the medium under conditions in which the processed epithin short form (Epi-S) is released. This type of epithin shedding was also prevented when serine protease inhibitors were added to cells expressing the full-length form. These results strongly suggest that the serine protease activity is involved in the shedding process. The presence of epithin(Δ) message was detected in multiple tissues and its significance is discussed.
Published by Elsevier Inc.

Keywords: Epithin; Epithin(Δ); Isoform; Type II; Transmembrane; Serine protease; N-terminal processing; Shedding

Most members of the serine protease family are either secreted or sequestered in cytoplasmic storage organelles before signal-regulated release. After release or translocation, they display diverse cellular roles, including participation in blood coagulation, wound healing, digestion, and immune responses, as well as tumor invasion and metastasis. The type II transmembrane serine proteases (TTSPs) are a subfamily of proteolytic enzymes characterized by a short N-terminal cytoplasmic tail, a membrane-spanning region, potential ligand-binding domains, and a C-terminal trypsin-like serine-protease domain [1]. Although the function of each separate domain has not been characterized in detail, it has been suggested that the N-terminal domain also participates in intracellular signal transduction while the extracellular C-terminal domain participates in proteolysis [1].

In our earlier studies, we cloned epithin, a mouse TTSP, from a cDNA library of fetal thymic stromal cells and the thymus of a severe combined immunodeficient mouse [2,3]. We subsequently characterized its N-terminal processing at Gly^{149} [4]. MT-SP1/matriptase, the human ortholog of epithin, was also identified from PC-3 human prostate and breast cancer cell lines [5,6], and described in both membrane-anchored and soluble forms. MT-SP1/matriptase was shown to initiate signaling and a proteolytic cascade through activating protease-activated receptor 2 (PAR2) and the single-chain urokinase-type plasminogen activator (sc-uPA), both of which are important in angiogenesis [7]. Targeted gene deletion in the mouse revealed that epithin plays diverse roles in post-natal survival, epidermal barrier function, hair follicle development, and thymocyte survival [8]. In addition, the early expression of a *Xenopus* homolog

* Corresponding author. Fax: +1 301 402 3184.
E-mail address: mkim@niaid.nih.gov (M.G. Kim).

0006-291X/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.bbrc.2004.12.014

of epithin, XMT-SP1, suggested an additional role in early embryonic development [9].

The members of TTSPs are rapidly expanding [1]. Recently, a paralog of human matriptase called matriptase-2 was reported [10,11]. Although its overall structure with a cytoplasmic tail, two CUB domains, three LDLR domains, and a C-terminal trypsin-like serine-protease domain is highly conserved, it shared only 35% amino acid identity with matriptase and is encoded by a separate gene.

In this paper, we identify and characterize the mouse epithin(Δ) isoform that lacks protease activity. We describe the differences in structure, protease activity, and N-terminal processing between epithin and epithin(Δ). In addition, the mechanism by which epithin(Δ) is generated was examined at the gene structure and message expression levels.

Materials and methods

Cell lines and culture conditions. COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS, Gibco-BRL) in a 10% CO_2 incubator. CHO-K1 cells were cultured in F-12 Nutrient Mixture medium (Gibco-BRL) with 10% FBS in a 5% CO_2 incubator. To obtain the COS7 conditioned medium, cells were cultured in a serum-free medium supplemented with Insulin-Transferrin-Sodium Selenite (ITS) (Roche Diagnostics, Mannheim, Germany). The S2 cell line was grown in a flow hood in Complete DES expression medium with L-glutamine (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FBS, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate (Gibco-BRL) at 23 °C.

Vector constructions. pcDNA3/epithin(Δ): the full-length epithin(Δ) cDNA was ligated into the pcDNA3 vector (Invitrogen). *pMT/BiP/V5-HisB/EpiPD*: the cDNA fragment (nucleotides 1754–2614) was amplified by PCR and ligated into the pMT/BiP/V5-His B vector (Invitrogen). The construction was designed to be in-frame with a C-terminal His tag. The His-tagged LDLRA4 repeat plus protease domain, spanning amino acids 565–851 of epithin, was expressed under the control of the *Drosophila* metallothionein (MT) promoter. This protein was secreted into the culture medium when the *Drosophila* S2 cells were induced with $CuSO_4$. For expression of the epithin(Δ) protease domain, Epi(Δ)PD, epithin(Δ) cDNA was PCR amplified using the same primer pairs.

Antibody preparation. The anti-epithin antibodies used in this paper were described in detail elsewhere [4].

Expression of the epithin protease domain in Drosophila S2 cells. The EpiPD or Epi(Δ)PD constructs were transfected into S2 cells using the calcium phosphate method. For the selection, the cells were maintained in complete medium containing 300 μg/ml hygromycin B (Roche Diagnostics) for 3–4 weeks. For the purification of the protease domain, the stable S2 cells were cultured in 500 ml of DES serum-free medium (Invitrogen). When the cell density reached 2.3×10^5/ml, $CuSO_4$ (500 μM final concentration) was added to the culture medium and the medium was harvested 2 days after induction. After centrifugation at 15,000 rpm for 15 min at 4 °C to remove debris, the supernatant was applied to a 1 ml Ni–NTA–agarose column. The column was then washed with 10 ml of washing buffer (40 mM Tris–HCl, pH 7.9, 1 M NaCl, and 10 mM imidazole) and eluted with 1 ml of elution buffer (2 mM Tris–HCl, pH 7.9, 50 mM NaCl, and 100 mM imidazole). Elution was repeated 5 times. Concentration of the pooled fractions to a final 1 ml volume in trypsin assay buffer was achieved by using a Centricon YM-10 concentration system (Amicon, Millipore, Billerica, MA). The amount of protein was estimated by a dotMETRIC 1 μl Protein Assay (Chemicon, Temecula, CA).

Assay for protease activity. The activity of the purified protease domains, EpiPD or Epi(Δ)PD, was assayed using a fluorogenic peptide substrate: N-t-Boc-QAR-7-amido-4-methylcoumarin (AMC) (Sigma, St. Louis, MO). Forty or one hundred and sixty nanograms of each purified enzyme was added to 0.1 mM of substrate in 20 μl of 5× assay buffer (0.1 M Tris–HCl, pH 8.2, 0.1 M $CaCl_2$) and the final reaction volume of 100 μl was made up by adding distilled water. Samples were incubated for 1 h at 37 °C. The reaction was stopped by adding 50 μl of 30% acetic acid. After adding 850 μl of distilled water, the released AMC was measured with a Spectrofluorophotometer (Shimadzu RF-540, Japan) configured with an excitation wavelength of 380 nm and an emission wavelength of 440 nm. For the determination of parameters, 1 ng of the recombinant protein was used, and the measurements were plotted using Microcal Origin software, version 3.5 (Microcal Software, Northampton, MA).

Transient or stable transfection of epithin or epithin(Δ) cDNAs. The pcDNA3/epithin or epithin(Δ) constructs were transfected into COS7 cells for transient overexpression or into CHO-K1 cells for stable expression by using Lipofectamine Plus reagent (Gibco-BRL). In brief, a complex of DNA (1 μg) and the Plus (6 μl)-Lipofectamine (4 μl) reagent was added to 2.5×10^5 COS7 cells in a 35-mm dish. At 48 h post-transfection, the cell lysates and conditioned medium of COS7 cells were harvested and used for Western blotting and immunoprecipitation. For the selection and maintenance of CHO-K1 cells stably expressing epithin (CHO-K1/epithin(Δ) cells), 700 μg/ml of Geneticin 418 (Gibo-BRL) was added to the culture medium.

Site-directed mutagenesis. Site-directed mutagenesis of the putative N-terminal processing site in pcDNA3/epithin or epithin(Δ) was performed using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following mutagenic primers were used: primer 1, 5′-CTGCCTTCAGTGAGaaCAGTGTCATCGCCTAC-3′ and primer 2, 5′-GTAGGCGATGACACTGttCTCACTGAAGG CAG-3′. Mismatches are indicated by lowercase letters. The mutant constructs, pcDNA3/MuEpithin and MuEpithin(Δ), were verified by DNA sequencing and then transfected into COS7 cells. At 48 h post-transfection, the cells and conditioned medium were used for further experiments.

Test of Epi-S or Epi(Δ)-S release from COS7 cells. The pcDNA3/epithin or epithin(Δ) constructs were transfected into 6×10^5 COS7 cells in 60 mm dishes for transient overexpression using the Lipofectamine Plus reagent. At 48 h post-transfection, cells were washed twice with TBS buffer (20 mM Tris, pH 8.0, 150 mM NaCl) and incubated with 1.5 ml TBS buffer containing ecotin (final 100 mM), leupeptin (final 5 μg/ml), $CaCl_2$ (final 10 mM) or EDTA (final 10 mM) for 1 h. The conditioned media were then clarified by centrifuging at 5000 rpm for 5 min and the supernatants were precipitated by adding TCA (trichloroacetic acid, 100%, 150 μl) for 20 min on ice. After centrifugation at 13,000 rpm for 20 min, the precipitates were dissolved in 25 μl of 1× SDS sample buffer and subjected to SDS–PAGE and Western blotting.

Reverse transcription (RT)-PCR. The 4 types of stringent epithin(Δ) specific primers (SP) were designed: SP1, TGCACCAAATATACC TACCACTTG; SP2, TGCACCAAATATACCTACCACTTGTG; SP3, TGCACCAAATATACCTACCACTTGTGTG; and SP4, TGCACCAAATATACCTACCACTTGTGTGG (underlines indicate the sequences 3′ of the deletion point). Two micrograms of total RNAs from the brain, kidney, and thymus (Ambion) was reacted with a Qiagen OneStep RT-PCR kit (Qiagen). The reaction mixture (25 μl) was 5× Qiagen RT-PCR buffer (5 μl), dNTP mix (10 mM of each dNTP, 1 μl), Epi(Δ)SP1 (10 pmol/μl, 1.5 μl), Epi(2290–2271) primer (sequence: TTGCCAGCAGGGAAGACATG, 10 pmol/μl, 1.5 μl), Qiagen OneStep RT-PCR enzyme mix (1 μl), RNasin (40 U/μl, 0.25 μl, Promega, Madison, WI), anti-*Taq* antibody (0.5 μl, Clontech, Palo

Alto, CA), RNA template (to 2 μl), and DEPC-treated water (12.25 μl). The thermal cycler conditions for both RT and PCR in a GeneAmp PCR System 9700 (PE, Applied Biosystems, Foster City, CA) were 50 °C (30 min), 95 °C (15 min), [94 °C (30 s), 64 °C (30 s), and 72 °C (1 min)] 35 cycles, 72 °C (7 min). Ten microliters of PCR products was analyzed on an agarose gel (1.5% gel). For Southern blotting after transferring onto a Nylon membrane (Schleicher and Schuell, Keene, NH), the end-labeled internal oligo probe Epi(2010–1991) (sequence: AGTCAGGAGAGATGAGCGAG, 10 pmol/μl, 1 μl) 10× T4 polynucleotide kinase buffer (1 μl, New England Biolabs, Beverly, MA) was used in QuikHyb solution for 5 h at 57 °C. After washing twice with 2× SSC containing 0.1% SDS for 15 min each, the membrane was exposed for 24 h and the image was obtained using a phosphoimager (Molecular Dynamics, Piscataway, NJ).

Results and discussion

Comparative domain structures of epithin, epithin(Δ), and matriptase-2

The presence of the epithin(Δ) isoform was first noticed during our original cloning of epithin cDNA [3]. The cloned cDNA lacked 198 base pairs (66 amino acids) compared to the full-length epithin. Prediction of protein domain structure for the 789 amino acid truncated gene is shown in Fig. 1A. The deletion includes the LDLRA4 repeat and the activation site (RVVGG) for the protease (Figs. 1A and B). When it is compared to the paralog matriptase-2, the major difference is in the protease activation sequence which is deleted in epithin(Δ) but not in matriptase-2 (Figs. 1A and B). Matriptase-2 was shown to have protease activity in assays with synthetic peptides [10]. Since the zymogen forms of serine proteases are generally cleaved at an activation site (-K(R)I(V)VGG-), we hypothesized that the protease domain of epithin(Δ) might not reveal protease activity because it could not be activated by cleavage. To test this idea we examined whether epithin(Δ) possesses protease activity.

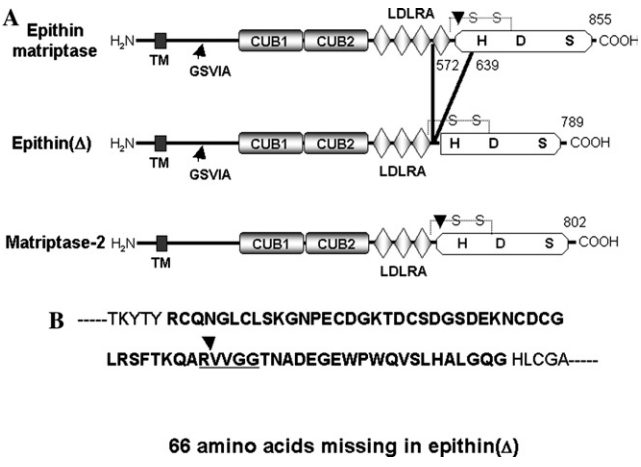

Fig. 1. Structure and sequence of epithin(Δ) form. (A) Domains of epithin, epithin(Δ), and matriptase-2. TM, transmembrane region; CUB, complement subcomponent C1r/C1s, urchin embryonic growth factor, and bone morphogenic protein 1; LDLRA, low density lipoprotein receptor class A, a cysteine-rich repeat. The activation sites for serine protease activity (arrowhead) in epithin (RVVGG) or matriptase-2 (RIVGG), and the connecting disulfide bonds (labeled as S–S) are shown. The catalytic triad for the serine protease (H, D, and S) is also displayed. The N-terminal processing sites of epithin (GSVIA) are indicated by arrows. (B) The amino acid sequence deleted in epithin(Δ) is shown in bold. The activation sequence for the serine protease is underlined and the cleavage site is indicated by an arrowhead.

The protease domain of epithin(Δ) does not show proteolytic activity

We produced soluble recombinant proteins containing the protease domain, EpiPD and Epi(Δ)PD (Fig. 2A), using a *Drosophila* expression system. The purified proteins from metal-chelate affinity chromatography showed the expected sizes of 39 kDa for EpiPD and 32 kDa for Epi(Δ)PD on a reducing SDS–PAGE gel (Fig. 2B, lanes 1 and 2). After each sample was placed in the protease assay buffer, the single 39 kDa band of EpiPD changed to a family of sizes containing a small amount of the uncleaved protein, a large amount of the expected 30 kDa cleavage product, and a low amount of protein with additional cleavages. The changes presumably arise from autoactivation of the zymogen (lane 4 vs 2). In contrast, the 32 kDa band of

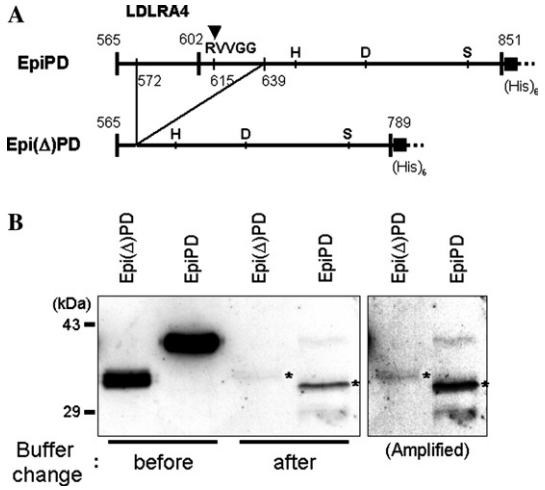

Fig. 2. Production and cleavage of recombinant protease domains of epithin and epithin(Δ). (A) The diagram of constructs containing His-tagged EpiPD and Epi(Δ)PD. The potential cleavage sequence (RVVGG) for protease activation in epithin and the deletion in epithin(Δ) are indicated. (B) Western blotting on a reducing SDS–PAGE gel (12%) of each affinity-purified protease domain before or after buffer-change. Recombinant protease domains in the purification buffer were probed with an anti-epithin antiserum (left panel). The pattern after changing the buffer to initiate the protease activity is shown either at the same intensity as the exposure before activation (center panels) or with the signal intensities enhanced in photshop (Version 6.0) (right panels). Star marks represent each main band from Epi(Δ)PD and EpiPD after activation. EpiPD, epithin protease domain; Epi(Δ)PD, epithin(Δ) protease domain.

Table 1
Comparison of the protease activity between Epi(Δ)PD and EpiPD

Purified protein[a]	Buffer	Amount used (ng)	Released AMC[c] ($\times 10^4$) (pM)
Epi(Δ)PD	Trypsin assay[b]	40	0
		160	0
Epi(Δ)PD		40	42.3
		160	113.6

[a] Epi(Δ)PD, epithin(Δ) protease domain; EpiPD, epithin protease domain.
[b] Trypsin assay buffer (50 mM Tris–Cl, pH 7.6, 5 mM $CaCl_2$).
[c] From substrate N-t-Boc-QAR-AMC.

Epi(Δ)PD stayed at the same apparent molecular mass (lanes 1 and 3) under these conditions.

The catalytic activity of these fractions was then tested using the synthetic peptide substrate, N-t-Boc-QAR-AMC. As expected, EpiPD showed a strong activity for cleaving the substrate and this enzymatic activity was dose-dependent, as shown in Table 1. Epi(Δ)PD, however, did not show any detectable activity on the substrate. Therefore, it is clear that Epi(Δ)PD can neither be processed nor converted to an active protease form, although it maintains the catalytic triad (H, D, and S) that are the core residues responsible for the serine protease activity.

The N-terminal processing occurs normally in epithin(Δ)

Previously, we showed that epithin is cleaved into Epi-L and Epi-S through N-terminal processing at Gly^{149} (G^{149}SVIA, Fig. 1A, arrow) [4]. Because the sequence for the processing is conserved in epithin(Δ), we tested if N-terminal processing occurs at G^{149} in epithin(Δ). When epithin(Δ) cDNA was produced transiently in COS7 cells or stably in CHO-K1 cells, two forms, Epi(Δ)-L (103 kDa) and Epi(Δ)-S (84 kDa), were observed (Fig. 3A, arrowhead and arrow, respectively), indicating that N-terminal processing occurs. As shown previously for full-length epithin [4], both Epi(Δ)-L and-S were pulse-labeled within 10 min and located on the cell surface of COS7 cells (data not shown).

To examine whether or not the N-terminal processing occurred in the same way for both epithin and epithin(Δ), wild type cDNAs, [pcDNA3/epithin or epithin(Δ)] and mutant cDNAs [pcDNA3/MuEpithin or MuEpithin(Δ)], in which Gly^{149} was substituted with Asn, were transfected into COS7 cells. In wild type cDNA-transfected cells, epithin(Δ) was expressed as two forms (Fig. 3B, lane Epi(Δ) in cell lysate), but none was detected in the culture medium (lane Epi(Δ) in medium). In contrast, full-length epithin transfected cells secreted Epi-S into the medium under the same conditions (lane Epi in medium). When the mutant cDNAs were expressed, no short forms were detected in cell lysates or the culture medium and the long forms in the cell lysates were accumulated without secretion (Fig. 3B, lanes MuEpi(Δ) and MuEpi in cell lysate, and MuEpi(Δ) and MuEpi in medium). These results indicate that the N-terminal processing occurs normally in epithin(Δ), but this is not enough to lead to secretion.

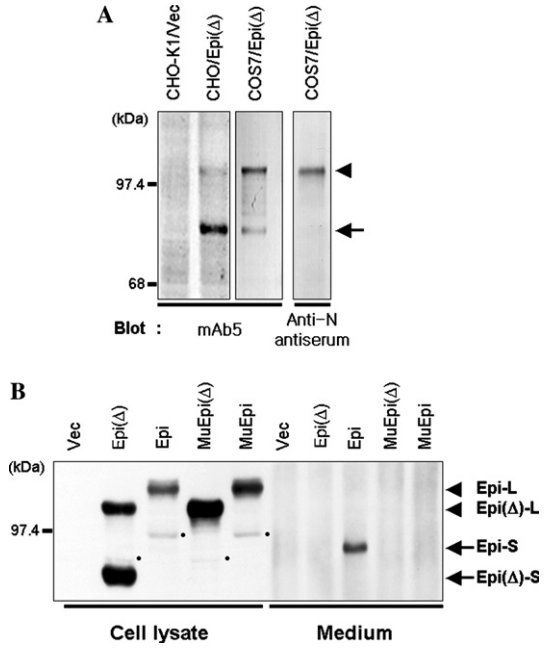

Fig. 3. N-terminal processing occurs normally in epithin(Δ). (A) Epithin(Δ) cDNA was expressed stably in CHO-K1 or transiently in COS7 cells. The lysates were loaded onto a reducing SDS–PAGE (8% gel), and Western blotting was performed using the mAb5 or anti-N antiserum. The arrowhead indicates Epi(Δ)-L [epithin(Δ)-long] and the arrows indicate Epi(Δ)-S [epithin(Δ)-short]. CHO-K1/Vec and CHO-K1/Epi(Δ), are vector and epithin(Δ) cDNA transfected CHO-K1 cells, respectively; COS7/Vec and Epi(Δ), are vector and epithin(Δ) cDNA transfected COS7 cells, respectively; and anti-N, anti-amino terminal antiserum. (B) N-terminal cleavage alone is not enough to induce the release of the short form of epithin(Δ) into the medium. cDNAs of wild-type epithin(Δ) (Epi(Δ)), wild-type epithin (Epi), epithin(Δ) with a point mutation (MuEpi(Δ)), and epithin with a point mutation (MuEpi) were all transfected with corresponding constructs into COS7 cells. Cell lysates and conditioned media were immunoprecipitated with anti-epithin antiserum and Western blotting was performed with the mAb5. In MuEpi or MuEpi(Δ), Gly of G^{149}SVIV was changed to Asn. The small dots indicate the unglycosylated form (data not shown). Vec, pcDNA3 alone-transfected; Epi-L, epithin-long; Epi(Δ)-L, epithin(Δ)-long; Epi-S, epithin-short; and Epi(Δ)-S, epithin(Δ)-short.

The big difference between epithin(Δ) and epithin in these transfection experiments is that epithin(Δ) has a block in the shedding of the protein into the medium. From these observations, we conclude that N-terminal processing occurs independently of the protease activity of epithin, and that the shedding and protease autocleavage are closely linked.

The release of Epi-S is blocked by treatment with serine protease inhibitors

Because epithin(Δ) does not show protease activity and is not shed, we hypothesized that the protease activ-

ity of epithin is important for the release of Epi-S. As one method to test this, serine protease inhibitors such as ecotin and leupeptin were added to epithin-transfected COS7 cells. These two inhibitors were the most potent in their inhibition of purified recombinant epithin in our previous report [4]. As shown in Fig. 4 (lanes 4 and 5), the release of Epi-S was completely blocked by treatment with either inhibitor for 1 h. In contrast, Ca^{2+} and EDTA did not affect the release process (Fig. 4, lane 2 and 3). For epithin, the activity of the protease domain was neither dependent on Ca^{2+} (data not shown) nor blocked by EDTA [4]. These results strongly suggest that the serine protease activity (whether epithin itself or some other serine protease) is an important factor for the release of epithin. However, we cannot formally exclude the possibility that LDLRA4 is also involved in the release process.

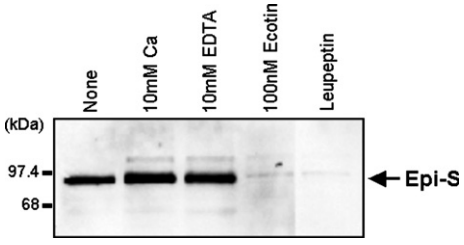

Fig. 4. Release of Epi-S into the medium is inhibited by serine protease inhibitors. Epithin cDNA was transfected into COS7 cells. After 48 h, fresh medium containing each reagent or inhibitor was incubated for 1 h. The conditioned medium was precipitated by TCA and used for Western blotting with the mAb5. The final concentration of each reagent is indicated. Leupeptin was used at 5 μg/ml. Epi-S, epithin-short.

Analysis of the genomic structure of epithin and the presence of epithin(Δ) mRNA in normal tissues

In order to examine the origin of epithin(Δ), the gene structure was analyzed. The full genomic DNA sequence for epithin was obtained from the public database (www.genome.ucsc.edu). The epithin gene is composed of 19 exons over about 42,282 bp on chromosome 11 [3] (Fig. 5A). Interestingly, the beginning and end sequences of the deleted region in epithin(Δ) were positioned in exons 15 and 16, respectively (Fig. 5A, striped box). The characteristics around the deleted region in epithin(Δ) are a 'CC' sequence in both the 5′ and 3′ sites (Figs. 5A and B, italics).

We investigated the possibility that alternative splicing contributes to the generation of epithin(Δ). In the deletion site, all possible combinations for alternative splicing

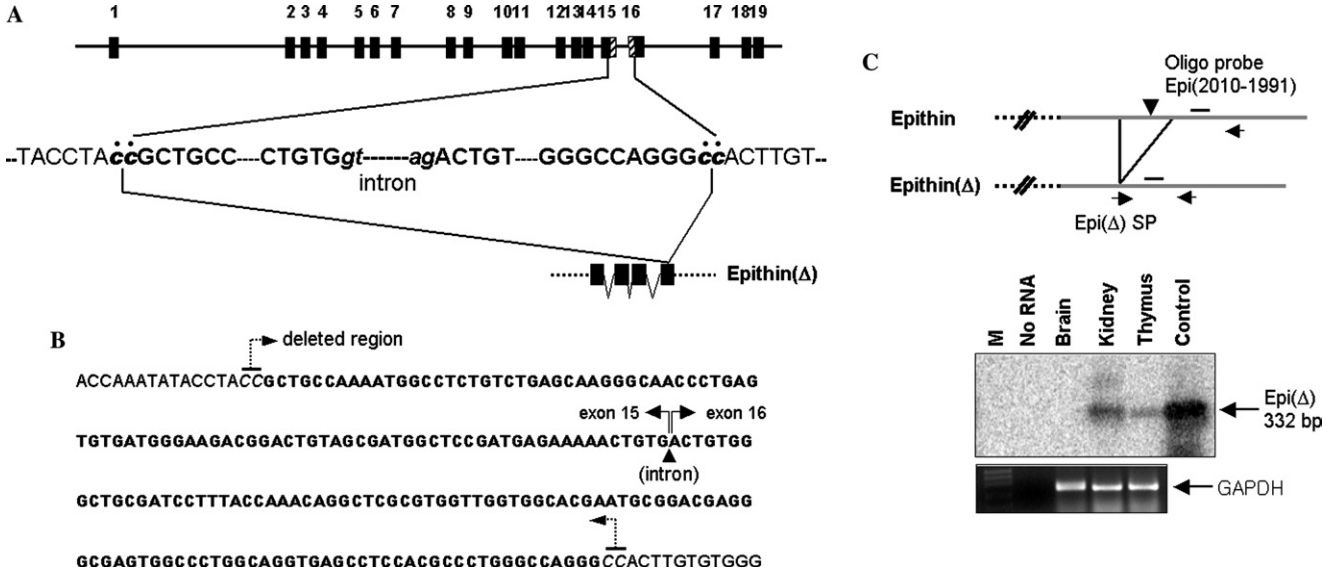

Fig. 5. Gene structure and epithin(Δ) mRNA analysis. (A) The organization of the epithin gene. The boundaries for exons and introns were determined by comparing the cDNA and full gene sequences. The overall gene structure of epithin was obtained from NCBI (http://www.ncbi.nlm.nih.gov/AceVeiw) and from the UCSC genome browser (http://genome.cse.ucsc.edu). Exons 15 and 16, which contain the start and end of the deleted sequence, are shown as partial striped boxes. The partial sequences, including the 5′ and 3′ boundaries of the deleted region from exon 15 and 16 are displayed. 'CC' sequences appearing in both deletion boundaries are indicated. (B) The cDNA sequence of the 198 bases of the deletion is shown in bold letters. These boundaries are delineated by dashed bent arrows. (C-upper panel) The diagram shows the epithin(Δ) specific primer pair for the amplification and the oligo probe Epi(2010–1991) for Southern blotting. The activation site for the protease is indicated by an arrowhead. Epi(Δ) SP, epithin(Δ) specific probe. (C-lower panel) One-step RT-PCR was done using total RNAs from brain, kidney, and thymus. After transferring the gel, Southern blotting was done using the internal oligo probe, Epi(2010–1991). The expected epithin(Δ) specific band (332 bp) is indicated by the upper arrow. As a size control, pBluscript/epithin(Δ) plasmid was used. To normalize for the amount of total RNA, the primer pair for GAPDH was also used for amplification (lower arrow).

include CC–GG, CG–GC, GC–CC, and CT–CA. None of these combinations corresponds to a conventional splicing donor/acceptor sequence. Based on the database of known splices (SpliceDB) (http://genomic.sanger.ac.uk/spldb/SpliceDB.html), only eight observed types of splice site pairs (out of 256 possible combinations) have been classified. Of these, 99.24% contain the canonical GT–AG (donor–acceptor sites), 0.69% are GC–AG, 0.05% are AT–AC, and finally 0.02% are other types of non-canonical splice sites [12,13]. In the cases of alternative splicing inside of exons, the machinery still uses the conserved splicing donor and acceptor sequences [14–16]. Therefore, it is unlikely that epithin(Δ) is generated by conventional alternative splicing. This unusual phenomenon is also different from the alternative splicing case for the rat-matriptase-2. In this case, the membrane-spanning and the secreted isoforms are generated by using alternate exons [11].

In order to exclude the possibility of a cloning artifact, we looked for the presence of the message for epithin(Δ) in tissues. First, we designed 4 types of primer sets specific for epithin(Δ) (see Materials and methods). All 4 sets of primers amplified specifically from the epithin(Δ) cDNA plasmid, but did not produce any amplifiable product from the full-length epithin cDNA (data not shown). Using the type 1 primer set, which is the most stringent epithin(Δ) specific pair, RT-PCR products was generated from total RNAs from the brain, kidney, and thymus, and probed by Southern blotting with an internal specific oligo (Fig. 5C, upper panel). An epithin(Δ) specific signal of 332 bp was clearly seen in the kidney and thymus but not in the brain (Fig. 5C, lower panel). The absence of message for epithin(Δ) in the brain was not from the lack of total RNA because a GAPDH signal was equally present (Fig. 5C, lower panel). Brain does not show any detectable epithin message in Northern blotting [17]. In addition, a ribonuclease protection assay with an epithin(Δ) specific probe and a poly(A)$^+$ RNA preparation from the thymic epithelial cell line 427.1.86 also produced the correct size product (data not shown). Therefore, we conclude that a low level of epithin(Δ) message is present in normal tissues and specific cell lines. Finally, we were able to detect a candidate Epi(Δ)-L protein in the kidney within the range of variation of glycosylation by Western blotting [17]. Unfortunately, at the current time, there is no epithin(Δ)-specific antibody to definitely test this.

There are many questions which still remain to be addressed: Is there a difference in the expression pattern between epithin and epithin(Δ)? What mechanism is involved in the production of epithin(Δ) message? How do the full-length protein and epithin(Δ) affect each other when the two proteins are expressed in the same cell? We suspect that epithin(Δ) plays a role as a negative regulator of its full-length counterpart since the two share other protein domains such as the CUBs and three LDLRAs.

Acknowledgments

We are grateful to C. Kim and C.H. Kang for their help in the Epi-S release assay with the protease inhibitors and for helpful discussion. E.G. Cho was supported by a fellowship from the BK21 program of the Korean Ministry of Education.

References

[1] J.D. Hooper, J.A. Clements, J.P. Quigley, T.M. Antalis, Type II transmembrane serine proteases: insights into an emerging class of cell surface proteolytic enzymes, J. Biol. Chem. 276 (2001) 857–860.

[2] M.G. Kim, C. Chen, F.A. Flomerfelt, R.N. Germain, R.H. Schwartz, A subtractive PCR-based cDNA library made from fetal thymic stromal cells, J. Immunol. Methods 213 (1998) 169–182.

[3] M.G. Kim, C. Chen, M.S. Lyu, E.G. Cho, D. Park, C. Kozak, R.H. Schwartz, Cloning and chromosomal mapping of a gene isolated from thymic stromal cells encoding a new mouse type II membrane serine protease, epithin, containing four LDL receptor modules and two CUB domains, Immunogenetics 49 (1999) 420–428.

[4] E.G. Cho, M.G. Kim, C. Kim, S.R. Kim, I.S. Seong, C. Chung, R.H. Schwartz, D. Park, N-terminal processing is essential for release of epithin, a mouse type II membrane serine protease, J. Biol. Chem. 276 (2001) 44581–44589.

[5] T. Takeuchi, M.A. Shuman, C.S. Craik, Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue, Proc. Natl. Acad. Sci. USA 96 (1999) 11054–11061.

[6] C.Y. Lin, J. Anders, M. Johnson, Q.A. Sang, R.B. Dickson, Molecular cloning of cDNA for matriptase, a matrix-degrading serine protease with trypsin-like activity, J. Biol. Chem. 274 (1999) 18231–18236.

[7] T. Takeuchi, J.L. Harris, W. Huang, K.W. Yan, S.R. Coughlin, C.S. Craik, Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates, J. Biol. Chem. 275 (2000) 26333–26342.

[8] K. List, C.C. Haudenschild, R. Szabo, W. Chen, S.M. Wahl, W. Swaim, L.H. Engelholm, N. Behrendt, T.H. Bugge, Matriptase/MT-SP1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis, Oncogene 21 (2002) 3765–3779.

[9] K. Yamada, T. Takabatake, K. Takeshima, Isolation and characterization of three novel serine protease genes from *Xenopus laevis*, Gene 252 (2000) 209–216.

[10] G. Velasco, S. Cal, V. Quesada, L.M. Sanchez, C. Lopez-Otin, Matriptase-2, a membrane-bound mosaic serine proteinase predominantly expressed in human liver and showing degrading activity against extracellular matrix proteins, J. Biol. Chem. 277 (2002) 37637–37646.

[11] J.D. Hooper, L. Campagnolo, G. Goodarzi, T.N. Truong, H. Stuhlmann, J.P. Quigley, Mouse matriptase-2: identification, characterization and comparative mRNA expression analysis with mouse hepsin in adult and embryonic tissues, Biochem. J. 373 (2003) 689–702.

[12] M. Burset, I.A. Seledtsov, V.V. Solovyev, Analysis of canonical and non-canonical splice sites in mammalian genomes, Nucleic Acids Res. 28 (2000) 4364–4375.
[13] M. Burset, I.A. Seledtsov, V.V. Solovyev, SpliceDB: database of canonical and non-canonical mammalian splice sites, Nucleic Acids Res. 29 (2001) 255–259.
[14] D.M. Gilligan, L. Lozovatsky, A. Silberfein, Organization of the human beta-adducin gene (ADD2), Genomics 43 (1997) 141–148.
[15] S. Ando, N.J. Sarlis, J. Krishnan, X. Feng, S. Refetoff, M.Q. Zhang, E.H. Oldfield, P.M. Yen, Aberrant alternative splicing of thyroid hormone receptor in a TSH-secreting pituitary tumor is a mechanism for hormone resistance, Mol. Endocrinol. 15 (2001) 1529–1538.
[16] U. Bodendorf, F. Fischer, D. Bodian, G. Multhaup, P. Paganetti, A splice variant of beta-secretase deficient in the amyloidogenic processing of the amyloid precursor protein, J. Biol. Chem. 276 (2001) 12019–12023.
[17] E.G. Cho, Studies on the Biochemical Properties and Release Mechanism of Epithin, a Type II Membrane Serine Protease, Ph.D. thesis, Seoul National University, 2002.