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N-terminal Processing Is Essential for Release of Epithin, a Mouse Type II Membrane Serine Protease*

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Eun-Gyung Cho‡‖, Moon Gyo Kim¶, Chungho Kim‡‖, Seung-Ryul Kim, Ihn Sik Seong‡, Chinha Chung‡, Ronald H. Schwartz¶, and Dongeun Park‡ ‡‡**

*From the ‡School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea, ¶Laboratory of Cellular and Molecular Immunology, NIAID, National Institutes of Health, Bethesda, Maryland 20892, and the **College of Medicine, Chungbuk National University, Chungbuk 361-763, Republic of Korea*

Epithin was originally identified as a mouse type II membrane serine protease. Its human orthologue membrane type-serine protease 1 (MT-SP1)/matriptase has been reported to be localized on the plasma membrane. In addition, soluble forms of matriptase were isolated from human breast milk and breast cancer cell-conditioned medium. In this paper, we report a processing mechanism that appears to be required for the release of epithin. CHO-K1 or COS7 cells transfected with single full-length epithin cDNA generated two different-sized proteins in cell lysates, 110 and 92 kDa. The 92-kDa epithin was found to be an N-terminally truncated form of the 110-kDa epithin, and it was the only form detected in the culture medium. The 92-kDa epithin was also found on the cell surface, where it was anchored by the N-terminal fragment. The results of *in vivo* cell labeling experiments indicate that the 110-kDa epithin is rapidly processed to the 92-kDa epithin. Using site-directed mutagenesis experiments, we identified Gly^{149} of the GSVIA sequence in epithin as required for the processing and release of the protein. These results suggest that N-terminal processing of epithin at Gly^{149} is a necessary prerequisite step for release of the protein.

Membrane type serine proteases play important roles in cell migration and tumor cell metastasis (1). Previously, we reported the cloning of a mouse type II membrane serine protease, epithin, from a polymerase chain reaction-based subtractive cDNA library of isolated fetal thymic stromal cells (2). Northern analysis revealed that epithin mRNA was expressed in intestine, kidney, lung, thymus, and spleen, and enriched in the severe combined immunodeficiency (SCID)[1] thymus. In the thymus, the message is present in the stromal compartment not in the thymocytes. The gene was mapped at 17 centimorgan from the centromere on the mouse chromosome 9. Epithin has a multidomain structure containing a putative N-terminal transmembrane region, two CUB domains, four LDLRA repeats, and a C-terminal serine protease domain (Fig. 1). The human orthologues of epithin, membrane type-serine protease 1 (MT-SP1) and its N-terminal truncated form, matriptase, have also been reported (Refs. 3 and 4 and Fig. 1). MT-SP1 cDNA was cloned from the PC-3 human prostate cancer cell line and shares 81% amino acid identity with epithin. The purified serine protease domain of MT-SP1 expressed in *Escherichia coli* as a His-tagged fusion protein was found to be autoactivated during the purification process. Ecotin and variants of ecotin, which inhibit the differentiation of rat prostate and the growth of human PC-3 prostate cancer tumors, inhibited the activated protease domain of MT-SP1 at subnanomolar concentrations. These findings suggested a possible role for MT-SP1 in prostate differentiation and the growth of prostatic carcinomas (3). Using a positional scanning synthetic combinatorial library and substrate phage technique, protease-activated receptor 2 (PAR2) and single-chain urokinase-type plasminogen activator (sc-uPA) were identified as macromolecular substrates of MT-SP1 (5). The identification of these molecules as putative *in vivo* substrates suggests that MT-SP1 regulates the functions mediated by PAR2, such as the inflammatory response or cell adhesion, and by uPA, such as tumor cell invasion and metastasis.

Matriptase was initially identified as an 80-kDa matrix-degrading protease from conditioned medium of human breast cancer cells (6), and later it was purified from human breast milk as a complex with a kunitz-type serine protease inhibitor (7). The isolated cDNA for matriptase appears to be a truncated form of MT-SP1, lacking the N-terminal 172 amino acids of the coding sequence (4). Using active matriptase isolated from human milk, it has been demonstrated that the protein can convert hepatocyte growth factor/scattering factor to its active form in Madin-Darby canine kidney epithelial cells, and can activate c-Met tyrosine phosphorylation in A549 human lung carcinoma cells (8). Studies with MT-SP1/matriptase suggest that epithin and its human orthologue may play important roles in cell migration as well as cancer invasion and metastasis.

The presence of an N-terminal transmembrane region in epithin/MT-SP1 led us to predict that these molecules would be localized in the membrane (2). Later it was shown that MT-SP1 is in fact on the plasma membrane by immunofluorescent mi-

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‡‡ To whom correspondence should be addressed: School of Biological Sciences, Seoul National University, Kwanak-gu, Shilim-dong, Seoul, 151–742, Republic of Korea. Tel.: 82-2-880-5753; Fax: 82-2-872-1993; E-mail: depark@snu.ac.kr.

[1] The abbreviations used are: SCID, severe combined immunodeficiency; MT-SP, membrane-type serine protease; CUB, complement subcomponents C1r/C1s, urchin embryonic growth factor, and bone morphogenic protein; LDLRA, low density lipoprotein receptor class A; sc-uPA, single-chain urokinase-type plasminogen activator; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; NTA, Ni-nitrilo-tri-acetic acid; TLCK, tosyl lysyl chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; AMC, 7-amido-4-methylcoumarin; EpiPD, epithin protease domain; Epi-S, epithin-short; Epi-L, epithin-long; MuEpithin, mutant epithin; *t*-Boc, *t*-butoxycarbonyl.

croscopy and a cell surface biotinylation technique (5). Interestingly, matriptase, which lacked the N-terminal transmembrane region in the initial protein preparation, was also found on the plasma membrane (6). This suggests either that there is an additional signal peptide other than the N-terminal transmembrane domain, or that matriptase noncovalently associates with other proteins in the membrane. Finally, the purification of matriptase from human breast cancer cell-conditioned medium (6) or from human breast milk (4, 7) indicates the existence of soluble forms of epithin/MT-SP1, although the mechanism for producing such forms and their exact nature are not yet understood.

In the present study, we first characterized the mouse epithin protease domain by showing its activation with trypsin-like serine proteases, including itself, and demonstrating its substrate specificity. We then presented evidence for both N-terminal processing of epithin during protein synthesis and subsequent release of the processed form from the cell membrane into the culture medium. Site-directed mutagenesis identified Gly^{149} in the N-terminal region as essential for both processing and release. These results suggest that translational processing is important for subsequent release of the protein to carry out its functions in the extracellular environment.

MATERIALS AND METHODS

Cell Lines and Culture Condition—COS7 cells, 427.1.86 cells, and 1308 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) with 10% fetal bovine serum (FBS, Life Technologies, Inc.) in a 10% CO_2 incubator. CHO-K1 cells were cultured in F-12 nutrient mixture medium (Life Technologies, Inc.) with 10% FBS in a 5% CO_2 incubator. To obtain the conditioned medium from COS7, 427.1.86, and 1308 cells, they were each cultured in medium supplemented with insulin-transferrin-sodium selenite (Roche Molecular Biochemicals) without FBS. The S2 cell line was grown in a flow hood in Complete DES™ Expression medium with L-glutamine (Invitrogen) containing 10% heat-inactivated FBS, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate (Life Technologies, Inc.) at 23 °C.

Vector Constructions—The vector constructions follow. 1) pGEX-5X-1/epithin: the cDNA (nucleotides 1,287–3,106) containing the CUB2, LDLRA1–4, and serine protease domains was ligated into the pGEX-5X-1 vector (Pharmacia Biotech). 2) pcDNA3/epithin: the full-length epithin cDNA was ligated into the pcDNA3 vector (Invitrogen) under the control of a cytomegalovirus promoter. 3) pcDNA3.1/HisB/epithin protease domain (EpiPD): the cDNA (nucleotides 1,754–3,067) was amplified by the polymerase chain reaction and cloned into the pcDNA3.1/His B vector (Invitrogen). The construct was designed to be in-frame with an N-terminal His tag and expressed the His-tagged LDLRA4 repeat along with the serine protease domain, spanning amino acids 565–855 of epithin. 4) pMT/BiP/V5-HisB/EpiPD: the cDNA (nucleotides 1,754–2,614) was amplified by polymerase chain reaction and ligated into 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 promoter, and could be secreted into the culture medium when the Drosophila S2 cells were induced with $CuSO_4$.

Antibody Preparation—A glutathione *S*-transferase (GST)-epithin fusion protein encoded by pGEX-5X-1/epithin was expressed in bacterial cells and purified by affinity chromatography. Polyclonal antibodies against the purified GST/epithin (anti-epithin antiserum) and against the N-terminal peptide, ^{12}GSQDFGAGLKYNSR^{25} (anti-N antiserum), were produced in rabbits. The purified GST-epithin was also injected into BALB/c mice for the production of a monoclonal antibody, mAb5. Hybridomas were prepared by fusing spleen cells with cells of the myeloma line SP2/O-Ag14. Enzyme-linked immunosorbent assay and Western blotting were performed to ensure that mAb5 reacted with epithin. For the isotype mapping of mAb5 (IgG2a), mouse monoclonal antibody isotyping reagents (Sigma) were used on ascitic fluid (1–1000 dilution in phosphate buffered saline (PBS)). The prepared mAb5 recognized the C-terminal end of epithin. This was demonstrated by Western blotting of cell lysates transfected with two different constructs, pcDNA3.1/HisB/EpiPD, which has the full C-terminal end of epithin, and pMT/BiP/V5-HisB/EpiPD, which does not have the C-terminal 4 amino acids because they have been replaced with a His tag.

In Vitro Processing of the Epithin Protease Domain by Trypsin—pcDNA3.1/HisB/EpiPD was *in vitro* translated as described below. Eighty μl of Ni-nitrilo-tri-acetic acid (NTA)-agarose (Qiagen) was added to 50 μl of the *in vitro* translation mixture and 870 μl of PBS and then incubated on a rotator for 1 h at room temperature. The Ni-NTA and protein complexes were washed three times with lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40), and a final wash was done with a trypsin assay buffer (50 mM Tris-HCl, pH 7.6, 5 mM $CaCl_2$). After adding 200 μl of fresh trypsin assay buffer to the bead complexes, the mixture was divided into aliquots and various concentrations of either trypsin (Sigma), with or without 100 μM tosyl lysyl chloromethyl ketone (TLCK), or 15 nM chymotrypsin (Sigma) was added. The final 36 μl of reaction mixtures were incubated for 30 min at 37 °C and stopped with 2× Laemmli sodium dodecyl sulfate-reducing sample buffer. After SDS-polyacrylamide gel electrophoresis (PAGE), the gel was dried and exposed to Fuji x-ray film for 1 week.

The Expression of the Epithin Protease Domain in Drosophila S2 Cells—The pMT/BiP/V5-HisB/EpiPD construct was 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 Molecular Biochemicals) for 3–4 weeks. For the purification of the protease domain, the stable S2 cells were cultured in 500 ml 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 harvested 2 days after induction. After centrifugation at 15,000 rpm for 15 min at 4 °C, the medium 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, 10 mM imidazole) and eluted with 1 ml elution buffer (2 mM Tris-HCl, pH 7.9, 50 mM NaCl, 100 mM imidazole). Elution was repeated five 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). Protein quantification was done by a dot-METRIC 1 μl of PROTEIN ASSAY (Chemicon).

The Assay for Protease Activity—The activity of the purified protease domain was assayed using the fluorogenic peptide substratesisopropyl-1-thio-β-D-galactopyranoside *N-t*-Boc-QAR-7-amido-4-methylcoumarin (AMC), *N-t*-Boc-AGPR-AMC, *N-t*-Boc-EKK-AMC, *N*-succinyl-AAPF-AMC, *N*-succinyl-LT-AMC, and *N*-succinyl-LLVY-AMC (Sigma). 10 ng of purified enzyme was added to 0.1 mM of each 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) configured with the excitation wavelength at 380 nm and the emission wavelength at 440 nm. For the determination of enzyme kinetics, 1 ng of the protease was used, and the measurements were plotted using Microcal Origin software version 3.5 (Microcal Software, Inc.). For the inhibitor assay, 1 ng of enzyme was preincubated with each inhibitor for 20 min, and then the protease activity was assayed using 0.1 mM *N-t*-Boc-QAR-AMC for 20 min as described above.

In Vitro Translation, Co-translational Modification, and Deglycosylation of Epithin from Cell Extracts—*In vitro* translation of the pBlueScript/epithin under the T3 promoter was performed using the transcription/translation-coupled reticulocyte lysate system (Promega) in the presence of [^{35}S]Met. For the co-translational modification, 2.5 μl of canine pancreatic microsomal membrane (Promega) were added to the 25 μl of reaction mixture. For deglycosylation, stably transfected CHO-K1 cells on a 100-mm dish were lysed with radioimmunoprecipitation assay buffer (RIPA; 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100, 100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 mM EDTA). Lysates were clarified by centrifugation at 15,000 rpm for 10 min. Clarified lysates were incubated with anti-epithin antiserum for 4 h, followed by an additional incubation with protein A-Sepharose (Pharmacia Biotech) for 2 h. The immunoprecipitates were washed three times with the same lysis buffer, mixed with denaturing buffer (0.5% SDS and 1% β-mercaptoethanol), boiled at 100 °C for 10 min, and divided into two aliquots. One aliquot was digested with 100 units of endoglycosidase H_f (New England Biolabs) in 50 mM sodium citrate, pH 5.5, for 10 min, and the other was used as a control without added enzyme. Finally, the reaction mixture was diluted with an SDS sample buffer, boiled at 100 °C for 3 min, and analyzed by SDS-PAGE and Western blotting with mAb5.

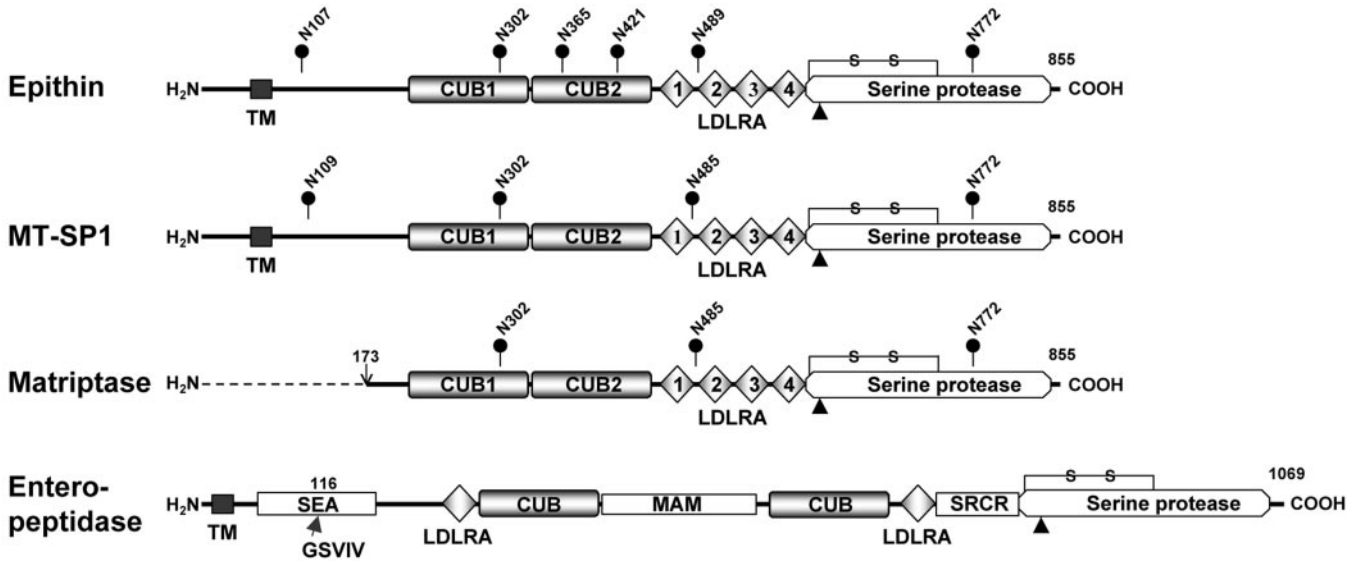

FIG. 1. **Domain structures of epithin and related proteins.** The domain structure of mouse epithin is compared with those of human MT-SP1/matriptase and enteropeptidase. Each structure was predicted at the PFAM data base configured to a 1.0 E-value cut-off level and fragment search (pfam.wustl.edu). The autoactivation site between the non-catalytic and catalytic domains (*arrowhead*) and the disulfide bond connecting them (labeled as S-S) are shown. The N-terminal cleavage site of enteropeptidase (G ↓ SVIV) is indicated by an *arrow*. The potential *N*-linked glycosylation sites of epithin and its human orthologue are marked by a "lollipop" symbol with amino acid numbers. Epithin and MT-SP1 share identical numbers of amino acids and structural domains; however, epithin has two more glycosylation addition sites. *TM*, transmembrane region; *SEA*, sea urchin sperm protein, enterokinase, agrin, a module associated with *O*-glycosylation; *MAM*, a domain homologous to members of a family defined by motifs in the mammalian metalloprotease meprin, the *Xenopus laevis* neuronal protein *A*5, and the protein tyrosine phosphatase μ; *SRCR*, scavenger receptor cysteine-rich domain.

Transient or Stable Transfection of Epithin cDNA—The pcDNA3/epithin constructs were transfected into COS7 cells for transient overexpression or into CHO-K1 cells for both transient and stable expression by using LipofectAMINE Plus reagent (Life Technologies, Inc.). 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 or CHO-K1 cells in a 35-mm dish. At 48 h posttransfection, the cell lysate and conditioned medium of COS7 or CHO-K1 cells were harvested and used for Western blot or immunoprecipitation. For the selection and maintenance of CHO-K1 cells stably expressing epithin (CHO-K1/epithin cells), 700 μg/ml of geneticin 418 (Life Technologies, Inc.) was added to the culture medium.

Surface Biotinylation—An enhanced chemiluminescence (ECL) protein biotinylation module (Amersham Life Science) was used to label the surface proteins of CHO-K1 cells following the manufacturer's protocol. The cells in a 35-mm dish were rinsed twice with ice-cold PBS and labeled with 20 μl of biotinylation reagent in 1 ml of bicarbonate buffer, pH 8.6, on a rocking plate for 30 min. After incubation, the cells were washed twice with PBS and lysed with 1 ml of RIPA buffer on the rocking plate for 20 min, and the lysate was centrifuged at 15,000 rpm for 15 min. The supernatant was used for immunoprecipitation with anti-epithin antiserum and protein A-Sepharose. After SDS-PAGE, the bands were visualized using the ECL detection system (Amersham Life Science).

Immunoprecipitation of the N-terminal Fragment of Epithin-Short (Epi-S)—CHO-K1/epithin cells in two 100-mm dishes were washed twice with ice-cold PBS, scraped, and centrifuged at 13,000 rpm for 5 min at 4 °C. The cell pellet was lysed with RIPA buffer for 1 h at 4 °C and then centrifuged at 15,000 rpm for 15 min at 4 °C. Separate aliquots of supernatant were used for immunoprecipitation by either anti-N antiserum or anti-epithin antiserum. The samples were analyzed in a gradient SDS-PAGE gel (6–15%) and transferred to a nitrocellulose membrane (Gelman). The top and bottom halves of the membranes were separately immunoblotted with anti-epithin antiserum or anti-N antiserum, respectively.

In Vivo Cell Labeling—CHO-K1/epithin cells in a 35-mm dish were washed twice with Met- and Cys-free DMEM (Life Technologies, Inc.) and incubated for 20 min at 37 °C in pre-warmed Met- and Cys-free DMEM to deplete the intracellular pools of sulfur-containing amino acids. After the medium was removed, the cells were incubated in DMEM containing 100 μCi of [^{35}S]Met/[^{35}S]Cys (PerkinElmer Life Sciences, EXPRE ^{35}S^{35}S) for 5 min (pulse) at 37 °C. The cells were then rapidly washed twice with PBS and incubated for 0, 5, 10, 20, 40, or 80 min (chase) at 37 °C in fresh F-12 medium containing FBS. After washing twice with ice cold PBS, cell-free extracts were prepared in lysis buffer (25 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% Triton X-100, 100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 mM EDTA) and immunoprecipitated with anti-epithin antiserum. After SDS-PAGE, the gel was soaked in destaining solution (10% acetic acid, 30% methanol in distilled water) for 1 h and dried. The dried gel was exposed to x-ray film (Fujifilm) for 24 h.

Site-directed Mutagenesis—Site-directed mutagenesis of the putative N-terminal processing site was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene). The following mutagenic primers were used: primer 1, 5′-CTGCCTTCAGTGAGaaCAGTGTCATCGCCTAC-3′, and primer 2, 5′-GTAGGCGATGACACTGttCTCACTGAAGGCAG-3′. Mismatches are indicated by lowercase letters. The mutant construct was verified by DNA sequencing and then transfected into COS7 cells. At 48 h posttransfection, the cells and conditioned medium were harvested for Western blotting or immunoprecipitation.

Immunoprecipitation of Lysates and Conditioned Medium from 427.1.86 Cells and 1308 Cells—Cells in 100-mm dishes were washed twice with ice-cold PBS, scraped, and centrifuged at 13,000 rpm for 5 min at 4 °C. The cell pellet was lysed with 1 ml RIPA buffer for 1 h at 4 °C and then centrifuged at 15,000 rpm for 15 min at 4 °C. The conditioned medium (8 ml) from about 90% confluent cells was harvested and centrifuged at 15,000 rpm for 15 min at 4 °C. The lysate and the conditioned medium (1 ml) were used for immunoprecipitation with anti-epithin antiserum and protein A-Sepharose. After SDS-PAGE, Western blotting was done with mAb5, and the bands were visualized using the ECL detection system.

RESULTS

In Vitro Processing of the Epithin Protease Domain by Trypsin—The protease domain of epithin contains the sequence RVVGG (Fig. 1, *arrowhead*). Cleavage at such a site (on the C-terminal side of Arg) by trypsin-like serine proteases is responsible for the activation of most zymogen serine proteases. Autoactivation of the human orthologue of epithin, MT-SP1 by cleavage at this site has been previously reported (3). To test whether mouse epithin could be the specific substrate of trypsin-like serine proteases, we generated the construct pcDNA3.1/HisB/EpiPD (Fig. 2*A*), which can be *in vitro* translated. The translation product was purified by metal-chelate affinity chromatography. The resulting 36-kDa (327 amino acids) protein was composed of three regions: 1) 36 amino acids

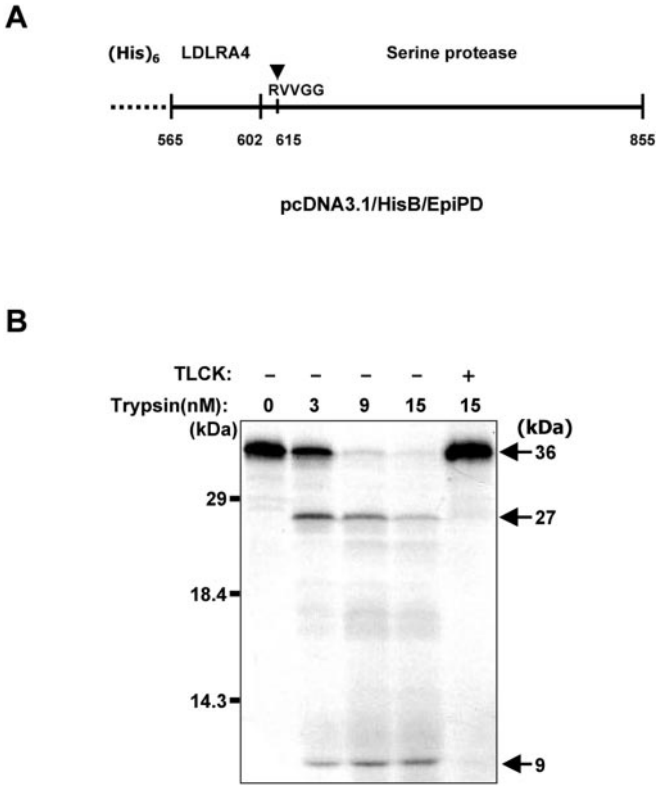

FIG. 2. ***In vitro* processing of the epithin protease domain by trypsin.** *A,* the diagram of the construct, pcDNA3.1/HisB/EpiPD, which was used for *in vitro* translation, is shown. The potential cleavage site by trypsin is indicated with an *arrowhead*. *B,* the *in vitro* translated [^{35}S]Met-labeled protease domain of epithin was incubated with various concentrations of trypsin in the absence or presence of the serine protease inhibitor, TLCK. The bands were visualized by autoradiography following SDS-PAGE.

containing a metal binding site, 2) the LDLRA4 repeat (38 amino acids), and 3) the serine protease domain (253 amino acids), including the RVVGG sequence. When the *in vitro* translated protein was incubated with a low concentration of trypsin, two major fragments (27 and 9 kDa) were generated (Fig. 2*B*). The larger fragment corresponds in size to the mature enzyme of 241 amino acids and the smaller fragment to a prodomain composed of the metal binding site, the LDLRA4 repeat, and 12 amino acids from the N terminus of the protease domain (Fig. 2*A*). This cleavage was blocked by TLCK, a trypsin-like serine protease inhibitor (Fig. 2*B*, *right-most lane*) and was not observed with 15 nM chymotrypsin (data not shown). These results indicate that the protease domain of epithin can be efficiently cleaved by trypsin family serine proteases.

Autoactivation, Kinetics, and Inhibitor Profile of the Epithin Protease Domain—To study epithin protease activity, we expressed the protease domain construct pMT/BiP/V5-HisB/EpiPD using a Drosophila expression system and purified the protein using metal-chelate affinity chromatography. The size of the expressed protein on reducing SDS-PAGE gels was 39 kDa. When the eluted fractions were pooled, concentrated, and resuspended in trypsin assay buffer, the single-chain zymogen protease domain was autocatalytically converted to its two-chain active form (data not shown) as previously reported for the MT-SP1 protease domain (3). The catalytic activity of the purified protease domain was tested by using AMC-tagged, synthetic peptide substrates that contained various amino acids at the P1 position (P1-Arg, Lys, Phe, Thr, or Tyr). The only substrates on which the enzyme showed prominent activity were those with P1-Lys or -Arg (Table I). The best of the screened substrates was *N-t*-Boc-QAR-AMC. Interestingly, the

TABLE I
Activity of the epithin protease domain on various peptide substrates

Substrate	Released AMC
	$pM \times 10^4$
N-t-Boc-QAR-AMC	1548.3
N-t-Boc-AGPR-AMC	134.3
N-t-Boc-EKK-AMC	131.4
N-succinyl-AAPF-AMC	0.4
N-succinyl-LT-AMC	0.3
N-succinyl-LLVY-AMC	0

TABLE II
Effects of various inhibitors on the activity of the epithin protease domain

Inhibitor	Concentration	Inhibition
		%
Leupeptin	10 μM	88
Ecotin	500 nM	85
DFP	1 mM	47
PMSF	1 mM	41
TLCK	500 μM	41
TPCK	500 μM	17
NEM	1 mM	0
Idoacetamide	1 mM	0
EDTA	20 mM	0

sequence of the predicted cleavage site in epithin for protease activation is QAR↓VVGG. Thus, the relative specificity of the enzyme strongly suggests that the QAR↓VVGG sequence is the mouse epithin autoactivation site. This is in agreement with N-terminal sequencing result on activated protease from MT-SP1 (3).

The activity profile of the protease domain was further characterized using the N-t-Boc-QAR-AMC substrate. These experiments showed concentration- and time-dependent increases in activity (data not shown), yielding K_m = 257 μM, K_{cat} = 1.76 s^{-1}, and $K_{cat}/K_m = 6.85 \times 10^3$ m^{-1} s^{-1}. The effect of inhibitors on the protease activity of epithin is summarized in Table II. The fact that leupeptin is one of the strongest inhibitors of epithin agrees with the results obtained with matriptase (9). Ecotin, which has been used for the identification of MT-SP1 (3), was also a potent inhibitor for mouse epithin.

Two Forms of the Epithin Protein—In our previous study, epithin was predicted to be a type II membrane serine protease with six *N*-linked glycosylation sites (Ref. 1 and Fig. 1). The molecular mass of the predicted protein is about 95 kDa. In *in vitro* translation experiments, before microsomal modification, epithin cDNA encoded predominantly a 95-kDa protein under reducing conditions (Fig. 3*A*, *lane 1*). Thus, the size of the translated protein matched that of the calculated molecular mass of epithin without glycosylation. After co-translational modification using microsomal membrane preparations, which can cleave signal sequences and glycosylate the target protein *in vitro*, the size of the translated protein increased to 110 kDa (Fig. 3*A*, *lane 2*). This is consistent with the glycosylation of all six predicted *N*-linked carbohydrate addition sites in the epithin protein. In contrast to these *in vitro* translation studies, the stable expression of epithin in CHO-K1 cells revealed predominantly a 92-kDa protein (Epi-S), although a small amount of the 110-kDa protein (Epi-L) was also detected (Fig. 3*B*, *lane 1*). The size of the Epi-L protein matches the fully modified epithin protein in the *in vitro* translation experiments. When the two epithin proteins from CHO-K1 extracts were treated with endoglycosidase H_f, which removes high-mannose oligosaccharides, the sizes of both forms were reduced by about 15 kDa (Fig. 3*B*, *lane 2*). This indicates that both Epi-L and Epi-S are high-mannose type glycoproteins containing a similar degree of glycosylation.

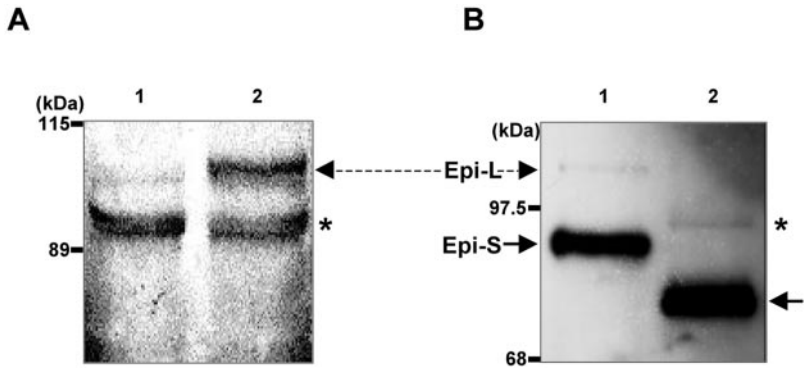

FIG. 3. ***In vitro* co-translational modification and deglycosylation of epithin from cell extracts.** *A, in vitro* translation of epithin cDNA was done in the absence (*lane 1*) or presence (*lane 2*) of canine microsomal membranes. The *asterisk* and the *arrowhead* indicate the unmodified and modified forms of epithin, respectively. *B,* deglycosylation of the immunoprecipitated epithin was done in the absence (*lane 1*) or presence (*lane 2*) of endoglycosidase H_f. The *arrowhead* indicates Epi-L, the fully glycosylated precursor, and the *arrow* indicates Epi-S, the smaller processed form of epithin. In *lane 2*, the *asterisk* indicates the deglycosylated form of Epi-L, and the *arrow* the deglycosylated Epi-S. *Panel A* is an autoradiogram of [^{35}S]Met-labeled *in vitro* translated products, and *panel B* is a Western blot using the mAb5 after immunoprecipitation from a CHO-K1/epithin cell extract with anti-epithin antiserum.

To examine the membrane localization of Epi-S and Epi-L, a surface biotinylation experiment was performed in CHO-K1/epithin cells. Both Epi-S and Epi-L were biotinylated and specifically immunoprecipitated with an anti-epithin antiserum (Fig. 4*A*, *left panel*). The recognition of the same proteins by a mAb5 directed against the carboxyl-terminal region of epithin confirmed that the immunoprecipitated proteins were both epithin (Fig. 4*A*, *right panel*). Because biotinylation is restricted to the cell surface, this result indicates that both Epi-S and Epi-L are located on the surface of CHO-K1/epithin cells. To examine the relationship between Epi-L and Epi-S, we employed antibodies that recognize different parts of the epithin protein. The mAb5 against the C terminus recognized both Epi-L and Epi-S in a Western blot of CHO-K1/epithin cell lysates (Fig. 4*B*, *left panel*). Interestingly, however, an anti-N antiserum, directed against the N-terminal peptide, recognized only Epi-L in the same blot (Fig. 4*B*, *right panel*). This observation indicates that Epi-S lacks the N terminus of epithin. The apparent molecular mass of 92 kDa in SDS-PAGE and the lack of immunoreactivity with anti-N antiserum suggested that Epi-S might be generated from Epi-L by N-terminal truncation of an 18-kDa polypeptide, which would include the transmembrane domain. To investigate how Epi-S could localize to the plasma membrane without an N-terminal-containing transmembrane region, immunoprecipitation experiments were carried out using anti-N and anti-epithin antisera on CHO-K1/epithin cell lysates. All of Epi-L (110 kDa), Epi-S (92 kDa), and the N-terminal fragment (18 kDa) were co-immunoprecipitated by either the anti-N or the anti-epithin serum (Fig. 4*C*). These results suggest that Epi-S and the N-terminal fragment are associated with each other on the CHO-K1/epithin cell surface. The interaction seems to be non-covalent because the migration of Epi-S was not changed under reducing *versus* non-reducing conditions (data not shown).

To test whether Epi-S is a processed product of Epi-L, *in vivo* cell labeling experiments were carried out using a pulse-chase procedure (Fig. 5). Both Epi-L and Epi-S were already substantially labeled at the end of a 5-min pulse. Surprisingly, the 110-kDa Epi-L persisted for at least 20 min before it slowly disappeared over the next hour. The amount of 92-kDa Epi-S increased during this latter time period. An experiment with pulse labeling for 10 min gave similar results (data not shown). The results are consistent with Epi-L being very rapidly processed to Epi-S following synthesis. The persistence of a significant cohort of Epi-L molecules, which may have escaped the early rapid processing, is potentially why Epi-L is still detected on the CHO-K1/epithin cell surface. The persistence of Epi-L and then the slow conversion to Epi-S suggest that processing can also occur in later compartments, such as the Golgi, although it seems less efficient there than at earlier stages.

Epi-S Is Released into the Culture Medium by COS7 Cells—To examine whether exogenously expressed epithin could be released from the cell surface, we first looked for the protein by immunoprecipitation in concentrated CHO-K1/epithin cell culture supernatants. We failed to detect any protein in supernatants from four independently selected clones. Transient expression in CHO-K1 cells without selection, however, revealed a small amount of epithin released into the medium (data not shown). To see if we could increase this amount for study, epithin was transiently expressed in the COS7 cell line. Here, Epi-S was readily detected in the culture medium by immunoprecipitation with an anti-epithin antiserum (Fig. 6*A*, *left panel*, *lane CM*). Note that in contrast to CHO-K1/epithin clones, most of protein found in the lysates of COS7 cells was Epi-L and very little was Epi-S (Fig. 6*A*, *left panel, lane Lysate*). This difference probably arises from insufficient processing of epithin in COS7 cells due to the high level of expression in transient transfection as well as the release of Epi-S into the medium. The release difference between stable CHO-K1 clones and COS7 cells suggests that there might be a shedding mechanism for epithin in COS7 cells that is absent or inactive in the selected CHO-K1/epithin clones. Finally, note that a 95-kDa protein that migrates slower than the 92-kDa Epi-S was also detected in the COS7 cell lysates. This protein is likely to be the unglycosylated form of Epi-L because it is recognized by the anti-N antiserum (Fig. 6*A*, *right panel*) and because its size is the same as the unglycosylated epithin produced in the *in vitro* translation experiments (Fig. 3*A*, *lane 1*).

Cleavage at Gly^{149} Is Essential for the Release of Epithin—The release of Epi-S, but not Epi-L, into the culture medium from epithin-expressing COS7 cells suggests that the processing step is required for release. Enteropeptidase is also a type II membrane serine protease, which has a similar modular structure to that of epithin and a highly homologous protease domain (Fig. 1). Purified enteropeptidase is composed of three different polypeptides, a heavy, a light, and a mini-chain. The N-terminal amino acid sequence of the heavy chain was shown to start with SVIV (Ref. 10 and Fig. 1). This suggests that the C-terminal side of glycine in the sequence GSVIV in enteropeptidase is cleaved by some unknown protease. Interestingly, the sequence ^{149}GSVI(A) is present in epithin in the region of potential processing predicted from the size of Epi-S. There-

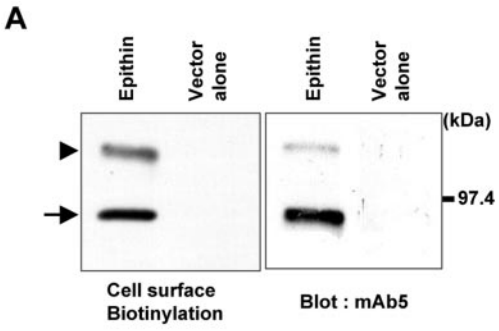

FIG. 4. **Expression of epithin in CHO-K1 cells.** *A,* biotinylation of cell surface proteins in CHO-K1/epithin cells. The biotinylated proteins were visualized using an ECL detection system after immunoprecipitation with anti-epithin antiserum (*left panel*). The same membrane was used for Western blotting with the mAb5 (*right panel*). *B,* 30 μg of CHO-K1/epithin cell extracts were used for a Western blot with mAb5 (*left panel*) or with anti-N antiserum (*right panel*). *C,* association of the N-terminal fragment with Epi-S. CHO-K1/epithin cell extracts (*left two lanes*) or CHO-K1/vector cell extracts (*right two lanes*) were used for the immunoprecipitation by anti-N or anti-epithin antisera. The upper half of the figure was blotted with anti-epithin antiserum and the lower half with anti-N antiserum. The *arrowheads* indicate Epi-L, and the *arrows* indicate Epi-S. The *asterisk* indicates the 18-kDa N-terminal fragment of processed epithin.

fore, we hypothesized that Gly^{149} in epithin might be the processing site to produce Epi-S from Epi-L. To test this hypothesis, a mutant epithin construct, which substituted Gly^{149} with Asn, was prepared by site-directed mutagenesis. When the mutant epithin (MuEpithin) was transiently expressed in COS7 cells, Epi-S was not detected in the culture medium, and the amount of Epi-L protein in cell lysates was increased (Fig. 6*B*). When the gel was exposed for a longer time, the 92-kDa Epi-S form was observed in the lysates of epithin but not of MuEpithin-transfected cells (data not shown). These results suggest that Gly^{149} is required for the cleavage and release of Epi-S from COS7 cells.

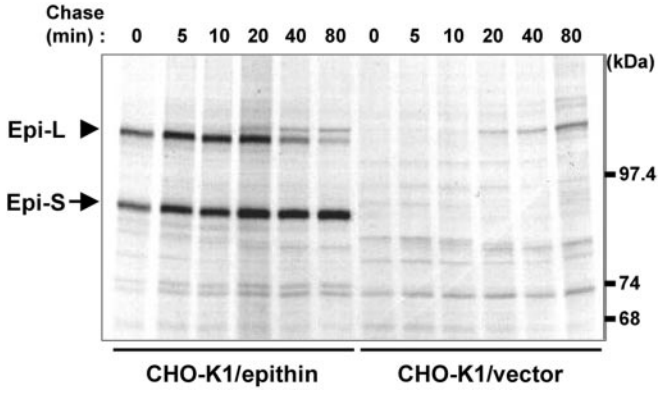

FIG. 5. ***In vivo* cell labeling by the pulse-chase method using [^{35}S]Met/[^{35}S]Cys in CHO-K1/epithin cells.** Pulse labeling was done for 5 min, followed by two washes with PBS and a chase with fresh medium for 0, 5, 10, 20, 40, and 80 min. The labeled epithin was immunoprecipitated at each time point using anti-epithin antiserum. Immunoprecipitated samples were analyzed on an 8% SDS-PAGE gel, and an autoradiogram of a 24-h exposure is shown.

Endogenous Epi-S Is Released into the Medium from a Thymic Epithelial Cell Line—To determine whether epithin release is a normal physiological process, we examined a thymic epithelial cell line called 427.1.86, which in our previous experiments was shown to express epithin mRNA (2). As a control, we used another epithelial cell line, 1308, which did not express epithin mRNA. Protein lysates from both lines were immunoprecipitated with anti-epithin antiserum and Western blotted with the anti-C-terminal mAb5. Only 427.1.86 cells showed a single specific band of about 95 kDa (Epi-S′ in Fig. 7, *left panel, Lysate lanes*). This band was also immunoprecipitated from the culture medium (*CM* lanes). The band did not react with anti-N antiserum (data not shown), suggesting that it is not a full-length Epi-L but rather an N-terminal-truncated form similar to Epi-S. The size appears to be slightly larger than that of Epi-S found in CHO-K1/epithin cells (Fig. 7, *right panel*); however, when the cells were treated with tunicamycin, which blocks carbohydrate modification at an early stage, both bands migrated with the same molecular weight (data not shown). Thus, the size difference between Epi-S and Epi-S′ from the two types of cells appears to be only a difference in posttranslational carbohydrate modification. The fact that only Epi-S and not Epi-L is detected in the thymic epithelial cell lysates and the medium (as well as on the cell surface, data not shown) strongly suggests that epithin is normally processed very quickly inside the cell, placed on the cell surface exclusively as the processed form, and then released into the medium.

DISCUSSION

Epithin and its human orthologue MT-SP1 are both predicted to be 95-kDa proteins without glycosylation. *In vitro* translation of the full-length epithin cDNA resulted in a protein band of 95 kDa, which agrees with the predicted size. The increase in molecular mass of the *in vitro* translated epithin protein by about 15 kDa after posttranslational modification is consistent with the existence of six predicted glycosylation sites in the epithin protein sequence if the average mass of the high-mannose type glycosylation per site is assumed to be about 2.5 kDa. It has been reported that PC3 cells express an MT-SP1 protein with an apparent size of 87 kDa in a reducing gel (5). There is strong sequence conservation between epithin and the human orthologue MT-SP1. The N-terminal region from Ser^{141} to Pro^{162}, which encompasses the putative processing site at Gly^{149}, is the longest segment of 100% identity outside of the protease domain. If MT-SP1 is subject to similar N-terminal processing as described for epithin in this report,

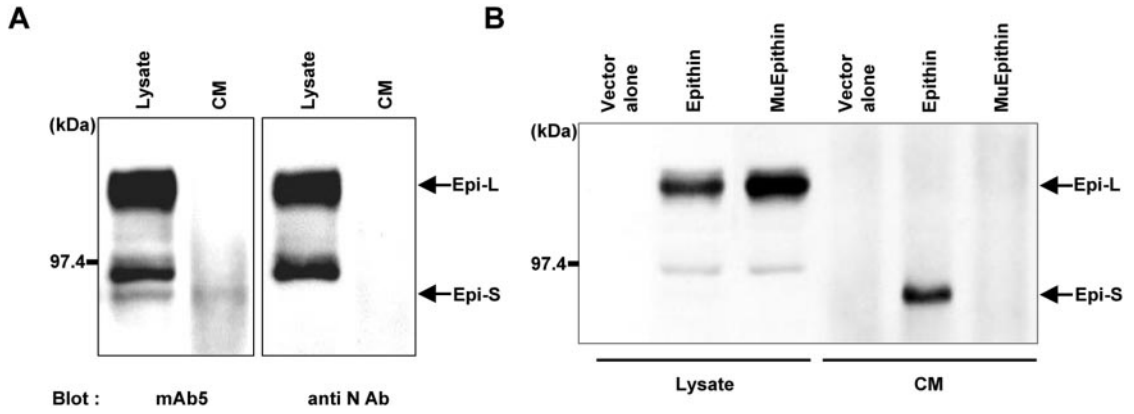

FIG. 6. **Epi-S is released from transiently transfected COS7 cells, and N-terminal cleavage is necessary for the release.** *A,* cell lysates and conditioned medium (*CM*) of transiently transfected COS7 cells were used for Western blotting with mAb5 (*left panel*) and with anti-N antiserum (*right panel*). *B,* cDNA of wild type epithin or a point-mutated epithin (*MuEpithin*) was transiently transfected into COS7 cells. In MuEpithin, Gly of ^{149}GSVIV was changed to Asn. Vector alone is pcDNA3-transfected, epithin is pcDNA3/epithin-transfected, and MuEpithin is pcDNA3/MuEpithin-transfected.

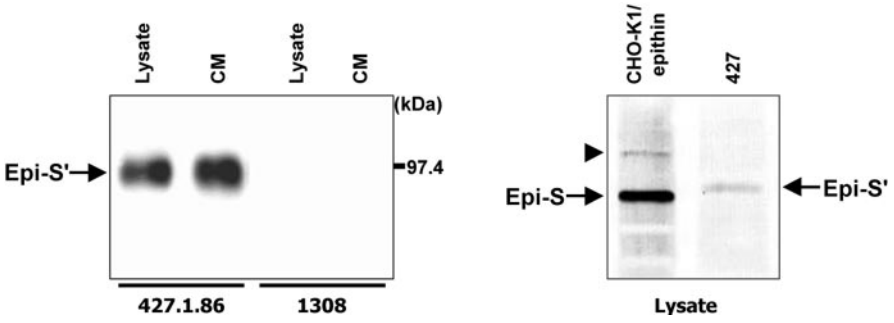

FIG. 7. **Epi-S is normally released into the medium from 427.1.86 cells.** The lysates and the conditioned medium (*CM*) from 427.1.86 cells and 1308 cells (*left panel*) and the lysates from CHO-K1/ epithin cells and 427 cells (*right panel*) were immunoprecipitated with anti-epithin antiserum followed by Western blotting with mAb5. The *arrowhead* indicates the Epi-L form. The *arrows* indicate either the Epi-S form from CHO-K1 cells or the slightly larger Epi-S′ form from 427.1.86 cells.

then the observed 87-kDa MT-SP1 protein in PC3 cells might correspond to our 92-kDa Epi-S protein if one takes into account the fact that MT-SP1 has two fewer glycosylation sites (Fig. 1).

Two forms of the epithin protein, Epi-L and Epi-S, were detected in CHO-K1 and COS7 cells that were transfected with epithin cDNA. Epi-L appears to be the unprocessed epithin protein, possibly persisting by escaping from N-terminal processing due to the high level of expression from exogenous cDNA under a strong promoter. Under physiological conditions, as seen with the thymic epithelial cell line, all of the endogenously expressed epithin is completely subject to N-terminal processing and expressed as only the Epi-S form in the lysates and medium (Fig. 7) as well as on the cell surface (data not shown). Results of *in vivo* cell labeling experiments in which not only Epi-L but also Epi-S was labeled in less than 5–10 min (Fig. 5) support the idea that Epi-L is normally rapidly processed into Epi-S. Detection of a single form of MT-SP1 in PC3 cells, which may express the protease endogenously (5), is also consistent with this interpretation. In contrast, some of the Epi-L form found in CHO-K1 cells still makes it to the cell surface, as detected by cell-surface biotinylation; however, none of this form is ever released into the medium. Similar observations were made for COS7 cells. In the latter case, only Epi-S was found to be released into the medium, suggesting that Epi-L must to be processed in order for the protease to be released. Rapid N-terminal cleavage of epithin during protein synthesis suggests that the processing is occurring in the endoplasmic reticulum, possibly co-translationally (Fig. 8).

Cell-surface biotinylation of Epi-S, which lacks a transmembrane region, was somewhat unexpected. In our experiments, cell-surface localization of Epi-S seems to be mediated by association with the N-terminal fragment that contains a transmembrane region. This observation suggests that the N-terminal fragment, which results from the processing of Epi-L, remains in the cell membrane and may serve as an anchoring site for Epi-S. According to this model, epithin in the cell membrane would be present as a two-chain complex (Fig. 8). The nature of the association between the N-terminal fragment and Epi-S is not known but does not involve a Cys bridge. Also, we have not excluded other mechanisms, such as the association of Epi-S with another membrane protein. It has been reported that the expression in COS cells of MT-SP1, in which the transmembrane region was deleted, resulted in some MT-SP1 remaining bound to the cell surface (5). Furthermore, matriptase, which is the N-terminal-truncated form of MT-SP1, was also localized to the cell-surface (6). In both cases, the mechanism for anchoring the protein is not known. It has been suggested that extracellular domains of MT-SP1 other than the transmembrane region are involved in these cell-surface interactions.

Although epithin and MT-SP1 were originally predicted to be type II transmembrane proteins, the presence of a soluble form of MT-SP1/matriptase in breast milk suggests a more complex story. In particular, the 70-kDa active matriptase form was shown to be composed of two major and one minor cleaved products of the membrane-bound form. N-terminal amino acid sequencing of the 70-kDa matriptase revealed two cleavage sites for the major bands at Lys^{189}-Ser^{190} and Lys^{204}-Thr^{205} (8). In our experiments, Epi-S, which is the N-terminal-processed product of Epi-L, was mostly released into the medium as is when transiently expressed in CHO-K1 and COS7 cells, while it remained associated with the cell membrane when stably expressed in CHO-K1 clones specifically selected for high surface expression. These observations suggest that Epi-S is initially bound to the cell surface and then released into the

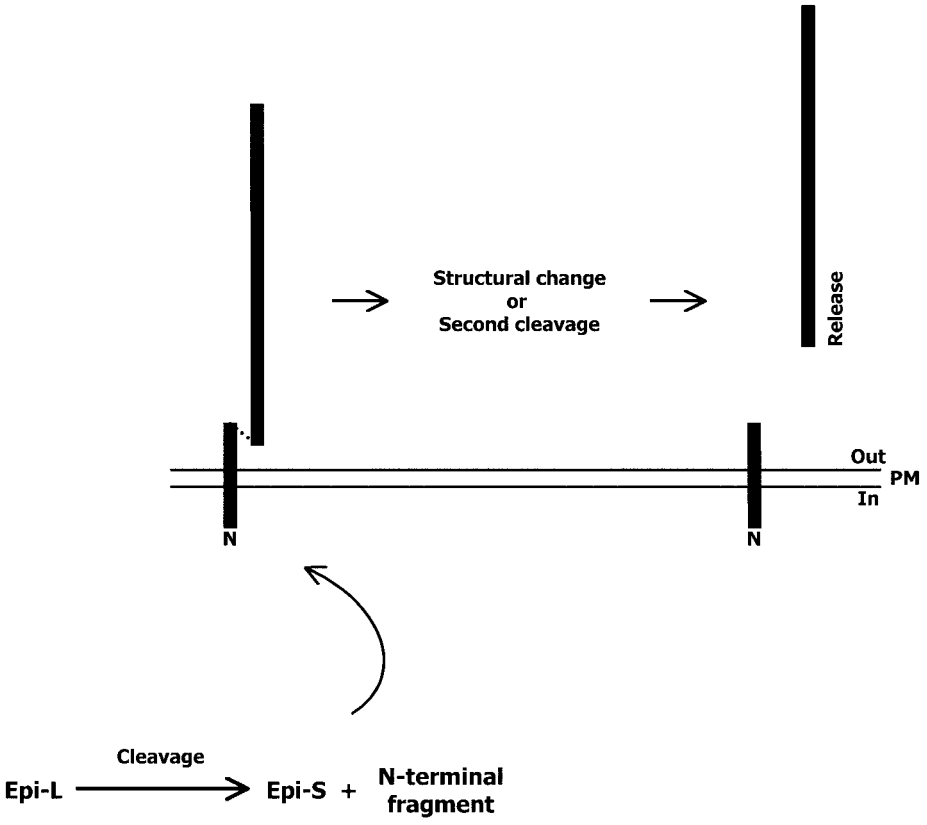

FIG. 8. **Model of epithin processing.** Epithin is synthesized as the 110-kDa Epi-L form and then cleaved in the N-terminal region by an unknown protease located in an intracellular compartment. This process produces Epi-S and an N-terminal fragment, which remain associated with each other by non-covalent forces. After movement to the cell surface, Epi-S can be released by further structural change or cleavage following some triggering event. *PM*, plasma membrane.

medium following some secondary triggering event, *i.e.* the N-terminal ectodomain cleavage itself is not sufficient for release of the Epi-S protein (Fig. 8). The nature of this triggering mechanism(s) remains to be investigated. However, one possible mechanism is the activation of the cell surface protease domain by extrinsic factors or autoactivation. Recently, Benaud *et al.* (11) reported that serum-dependent activation of matriptase on a cultured epithelial cell surface is followed by ectodomain release of matriptase. Their results suggest that the activation of the protease domain in MT-SP1/matriptase is required for the release of the protein from the cell surface. Although this observation is consistent with our hypothesis that there is a triggering mechanism, in addition to N-terminal processing, required for release of the protein from the cell surface, in our experiments the stable CHO-K1/epithin cells grown in serum-containing medium did not release detectable amounts of epithin into the culture medium. Thus, the reported serum-dependent activation of the protease might act through an indirect mechanism, which is probably absent or inactive in the CHO-K1/epithin clones.

Although it is not clear at present whether the N-terminal fragment is essential for the function of epithin, it is not degraded and remains in the cell membrane. In CHO-K1/epithin cells, the N-terminal fragment seems to play the role of a linker between the cell membrane and Epi-S (Fig. 4, *panel C*). Among other type II membrane serine proteases, hepsin has also been suggested to be cleaved in an ectodomain near the transmembrane region and released as a soluble form (12). The nature of the protease(s) responsible for N-terminal processing of either hepsin or epithin is not known. One possible candidate is an elastase-like serine protease whose specificity is directed toward small uncharged side chains as found in the GSVIA processing site of epithin. Another possibility is one of the members of the ADAM (A Disintegrin And Metalloproteinase) family, which has been implicated in the processing of many membrane proteins.

Most of the serine proteases are synthesized as a zymogen, and they exhibit proteolytic activity only after cleavage at an activation site. Since we could not detect protease activity of the expressed epithin, Epi-L and Epi-S, in CHO-K1 or COS7 cell lysates (data not shown), N-terminal ectodomain processing itself does not seem to be sufficient for activation. This is in contrast to the case of tumor necrosis factor-α converting enzyme, which is a membrane-anchored ADAM family protein involved in the release of tumor necrosis factor-α. Here, the ectodomain processing itself is the maturation process for catalytic activity (13). In contrast, in order for epithin to become an active protease, it has to be cleaved at its activation site by another trypsin-like protease or by autocatalysis. In this sense, the cleavage of the epithin protease domain by trypsin (Fig. 2) may have some biological relevance because epithin is highly expressed in the intestine where trypsin could play a physiological role in epithin activation.

There are many studies that document autoactivation of serine proteases for the initiation of the intrinsic (factor VII (14, 15)) and extrinsic (factor XII (16–18)) blood coagulation cascades, the initiation of the classical pathway of the complement cascade (C1 (19, 20)), and the initiation of the fibrinolytic cascade uPA (21). Very convincing evidence has been reported for hepsin, which is capable of concentration-dependent autoactivation in the mouse embryo as early as the two-cell stage, where there has not been any evidence to demonstrate the presence of other proteases (12). The same may be true of the recently identified *Xenopus* homologue of epithin, XMT-SP1 (22), because its expression has been detected in *Xenopus* embryos as early as the two-cell stage. The demonstration that epithin/MT-SP1 can be autoactivated and that one of its substrates is sc-uPA, raises the possibility that this protease could be an initiator of the biologically important fibrinolytic (or other) cascade mediated by activated uPA.

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