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

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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.
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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

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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