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Proteolytic cleavage of the rat heparan sulfate 6-*O*-endosulfatase SulfFP2 by furin-type proprotein convertases

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ABSTRACT

Heparan sulfate 6-*O*-endosufatases Sulf1 and Sulf2 hydrolyze the 6-*O*-sulfate of the glucosamine residues in heparin and heparan sulfate, thereby regulating multiple signaling pathways. A previous study reported that human Sulf1 and Sulf2 were proteolytically processed in a manner sensitive to a furin inhibitor. However, the exact sites of cleavage, the sequence motifs for proteolysis, and the effect of the cleavage on enzyme activity remain unknown. Here we show that the cleavage of rat Sulf2 (also called SulfFP2) occurs at two arginine residues, 543 and 570, in the hydrophilic domain. Both sites reside in the consensus sequence for the cleavage by furin-type proprotein convertases, and the consensus motifs are essential for cleavages. The cleavage at arginine 570 is sensitive to a furin inhibitor. Furthermore, the uncleavable form of SulfFP2 shows sulfatase activity comparable to the cleavable SulfFP2, indicating that the cleavage is not indispensable for activation of SulfFP2.

Introduction

Heparan sulfate (HS) regulates cell differentiation, proliferation, and migration by interacting with a wide variety of proteins, including growth factors, morphogens, and their receptors, as well as with extracellular matrix molecules [1–4]. HS is extensively modified by nonuniform epimerization and sulfation, giving rise to enormous structural heterogeneity in its chain [1–4]. In particular, the sulfation patterns of HS define the specificity and affinity of the ligand binding and play a role in the functional diversification of HS [1–4].

Sulfatases comprise a family of enzymes that hydrolyze sulfate ester bonds in a wide variety of biological compounds [5]. All the known eukaryotic sulfatases have a conserved cysteine residue in their catalytic domains that is posttranslationally converted to a C_α-formylglycine [5]. This modification carried out by sulfatase modifying factor 1 (Sumf1) is essential for enzyme activity [6,7]. Recently, a new class of sulfatases has been reported. This class comprises two orthologues in the human, mouse, rat, and quail genomes: Sulfatase 1 (Sulf1, also called Sulfatase FP1 [SulfFP1]) and Sulfatase 2 (Sulf2, also called SulfFP2) [8–13]. Sulfs comprise a putative signal sequence at the N-terminus, an enzyme catalytic domain in the N-terminal region, a hydrophilic domain in the middle portion, and a C-terminal domain [8–12; see 13 for review]. Sulfs catalyze hydrolysis of the 6-*O*-sulfate of the glucosamine residues in intact heparin and HS at neutral pH [8,9,12,14]. As a result of 6-*O*-desulfation, they regulate multiple signaling pathways positively or negatively [8,9,12–17]. Morimoto-Tomita et al. [9] reported that human Sulfs underwent proteolytic cleavage that was sensitive to a furin inhibitor. However, exactly where the cleavage occurs, what sequence motifs are present in the cleavage sites, whether all the proteolysis is mediated by furin, and whether the cleavage is necessary for the enzyme activity remain unknown.

Furin and proprotein convertases (PCs) form a family of specialized endoproteinases that cleave the multibasic motifs in proprotein substrates in the secretory pathway [18,19]. Their substrates include growth factors, hormones, neuropeptides, and extracellular matrix proteins, and this posttranslational processing transforms inactive precursors into active proteins and peptides [18,19]. The consensus sequence of the cleavage by furin and PCs is R/K-X-R/K-X-R/K-R-↓-X (R, K, X, and ↓ denote arginine, lysine, any amino acid, and the cleavage site, respectively), in which the last arginine residue is essential and at least two of the 3 R/K residues should be arginine or lysine [18]. Recently, Duckert et al. [20] developed a method to predict the cleavage sites for PCs on the basis of artificial neural networks.

In this study, we determined the cleavage sites of SulfFP2 by Edman degradation sequencing of the fragments of the secreted SulfFP2 protein. We found that there were consensus sequences for

Abbreviations: 4-MUS, 4-methylumbelliferyl sulfate; dec-RVKR-CMK, decanoyl-Arg-Val-Lys-Arg-chloromethylketone; HS, heparan sulfate; PC, proprotein convertase; Sumf1, sulfatase modifying factor 1.

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the cleavage and that these sequences were required for proteolysis. We also found that the cleavage was not indispensable for SulfFP2 activation.

Materials and methods

Expression constructs. The coding region of rat *SulfFP2* [11] tagged with a MycHis epitope at its C-terminus was subcloned into a pCEP4 vector (Invitrogen, Carlsbad, CA, USA). Mutations were introduced using PCR. Mouse *Sumf1* cDNA (UniGene ID: 2828521) was obtained using RT-PCR and inserted into a pCAGGS vector (a kind gift of J. Miyazaki, Osaka University).

Cell culture and transfection. The 293EBNA cells (Invitrogen) were transfected using LipofectAMINE 2000 (Invitrogen). After the cells were cultured in Opti-MEM I (Invitrogen) without fetal bovine serum for 3 days, the conditioned medium was analyzed. In some experiments, the cells were incubated with 10 μM decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-CMK; Bachem, Bubendorf, Switzerland) after transfection.

Protein sequencing. SulfFP2 protein in the conditioned medium of 293EBNA cells transfected with pCEP4-*SulfFP2-MycHis* was purified using a cation exchange column (HiTrap SP HP; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) on a BioLogic DuoFlow system (Bio-Rad, Hercules, CA, USA). After washing with 50 mM Hepes–NaOH buffer (pH 7.0) containing 0.1 M NaCl, eluents were obtained with a linear gradient of 0.1 M to 1.0 M NaCl. The fractions containing SulfFP2-MycHis protein, eluting at 0.64–0.82 M NaCl, were pooled and subjected to SDS–PAGE. After being blotted to an Immobilon PVDF membrane (Millipore, Billerica, MA, USA) and stained with Coomassie Brilliant Blue R-250 solution (Bio-Rad), the bands were cut and subjected to Edman degradation gas-phase protein sequencing using a protein sequencer (PPSQ-23; Shimadzu, Kyoto, Japan).

Antibody production. Two sequences in the hydrophilic domain of rat SulfFP2, amino acid residues 421–564 (designated as SulfFP2A) and 576–724 (SulfFP2B), were separately subcloned into a pBAD/His prokaryotic expression vector (Invitrogen). The recombinant SulfFP2 proteins were produced in the *Escherichia coli* and then purified using a HisTrap kit (GE Healthcare Bio-Sciences). Antibodies were raised by immunizing rabbits with the resultant proteins (TransGenic, Kumamoto, Japan).

Western blot analysis. The conditioned medium was concentrated by trichloroacetate precipitation, resolved by SDS–PAGE, and transferred to an Immobilon PVDF membrane. To remove glycosylation, the concentrated conditioned medium was incubated with 0.1 U/μl *N*-glycosidase F (Roche Diagnostics, Basel, Switzerland) at 37 °C for 16 h before SDS–PAGE. The membrane was incubated with anti-SulfFP2A (1:500), anti-SulfFP2B (1:500), or anti-Myc mouse monoclonal antibody (1:2000; Invitrogen), and then with either horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; Bio-Rad) or anti-mouse IgG (1:5000; Chemicon, Temecula, CA, USA) antibodies. After washing, chemiluminescence reaction was carried out using an ECL kit (GE Healthcare Bio-Sciences), and the membrane was exposed to X-ray films.

Bioinformatics. Amino acid sequences were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was done using ClustalW (http://www.clustal.org/). Cleavage sites for a signal peptidase and furin/PCs were predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) and the ProP 1.0 server (http://www.cbs.dtu.dk/services/ProP), respectively.

Enzyme assay. Sulfatase activity was measured using a fluorogenic substrate, 4-methylumbelliferyl sulfate (4-MUS; Sigma–Aldrich, St. Louis, MO, USA). The conditioned medium of 293EBNA cells transfected with pCEP4-*SulfFP2-MycHis* or its mutant forms in combination with pCAGGS-*Sumf1* was concentrated 30-fold using a Microcon YM-30 (Millipore). The medium was incubated with 10 mM 4-MUS, 10 mM $MgCl_2$, 150 mM NaCl, and 0.01% bovine serum albumin in 50 mM sodium acetate buffer (pH 7.4) in a total volume of 50 μl at 37 °C for 24 h. To terminate the reaction, 100 μl of 0.5 M Na_2CO_3/$NaHCO_3$ (pH 10.7) was added to a 20-μl aliquot of the reaction mixture, and the fluorescence of 4-methylumbelliferone was measured using SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA), with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Because the 293EBNA cells had endogenous sulfatase activity of unknown origin, the sulfatase activity in the conditioned medium from the *β-galactosidase*-transfected cells was subtracted.

To measure endosulfatase activity, the concentrated conditioned medium (5 μl) was incubated with 10 μg heparin, 200 mM NaCl, and 10 mM $MgCl_2$ in 10 mM Tris–HCl buffer (pH 7.5) in a total volume of 10 μl at 37 °C for 24 h. The mixture was heated at 95 °C for 2 min and then incubated with 10 μl 40 mM Hepes–NaOH (pH 7.0) containing 2 mM calcium acetate, 1 mIU heparinase I (Sigma–Aldrich), 1 mIU heparitinase I (Seikagaku, Tokyo, Japan), and 1 mIU heparitinase II (Seikagaku) at 37 °C for 24 h. After the digestion was stopped by heating at 95 °C for 2 min and the mixture cleaned using an Ultrafree-MC filter (Millipore), unsaturated disaccharides were analyzed by reversed-phase ion-pair chromatography essentially as previously reported [21]. A gradient was applied at a flow rate of 1.1 ml/min on a Senshu Pak Docosil (4.6 × 150 mm, particle size 5 μm; Senshu Scientific, Tokyo, Japan) at 55 °C using a HPLC system (Alliance UV system 2695/2487; Waters, Massachusetts, USA). The eluents were as follows: A, H_2O; B, 0.2 M NaCl; C, 10 mM tetra-*n*-butylammonium hydrogen sulfate; and D, 50% acetonitrile. The gradient of eluent B was as follows: 0–10 min, 1–4%; 10–11 min, 4–15%; 11–20 min, 15–25%; 20–22 min, 25–53%; 22–29 min, 53%. The proportions of eluent C and D were constant at 12% and 17%, respectively. The effluent was monitored spectrophotometrically (absorbance at 232 nm), and disaccharide peaks were identified and quantified by comparison with the following authentic unsaturated disaccharide markers: unsaturated HS/HEP-disaccharide mixture (H Mix; Seikagaku), ΔUA2S-GlcNAc and ΔUA2S-GlcNAc6S (Dextra Laboratories, Reading, UK). The chromatogram was analyzed using Empower software (Waters).

Results and discussion

Determination of the N-terminal sequences of the secreted SulfFP2 protein

To begin to examine the molecular mechanism by which SulfFP2 is cleaved, we first determined the cleavage site(s) by directly determining the N-terminal sequence(s) of the processed fragments. After 293EBNA cells were transfected with pCEP4-*SulfFP2-MycHis* and cultured in a serum-free medium for 3 days, SulfFP2 protein in the conditioned medium was purified using a cation exchange column. The purified protein was subjected to SDS–PAGE, blotted onto a PVDF membrane, stained with Coomassie Brilliant Blue, and analyzed.

Edman degradation of the ~90 kDa fragment yielded a mixture of two sequences of FLSYPRLKGR and DRRNIRPNII, found at the 25–34 and 38–47 residues of SulfFP2 (Fig. 1A and B), respectively. Thus, SulfFP2 was cleaved at two different sites near its N-terminus: between Ala^{24} and Phe^{25} and between Arg^{37} and Asp^{38}. The former matched the signal peptide cleavage site predicted using the SignalP 3.0 server, whereas the latter matched the consensus sequence of the cleavage site by furin family PCs [17,19]. These findings suggest that SulfFP2 underwent proteolytic cleavage at Ala^{24} by signal peptidases and at Arg^{37} by PCs.