

**Proteolytic cleavage of the rat heparan sulfate 6-*O*-endosulfatase SulFP2 by furin-type proprotein convertases**

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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.

## **Abstract**

Heparan sulfate 6-*O*-endosulfatases 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 2 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.

Keywords: heparan sulfate, heparan sulfate 6-*O*-endosulfatase, furin, proprotein convertase, cleavage, sulfatase

## 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<sub>α</sub>-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 2 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 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 endoproteases 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 2 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 SulffFP2 by Edman degradation sequencing of the fragments of the secreted SulffFP2 protein. We found that there were consensus sequences for the cleavage and that these sequences were required for proteolysis. We also found that the cleavage was not indispensable for SulffFP2 activation.

## **Materials and methods**

*Expression constructs.* The coding region of rat *SulffFP2* [11] tagged with a Myc-His 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  $\mu$ M decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-CMK; Bachem, Bubendorf, Switzerland) after transfection.

*Protein sequencing.* SulffFP2 protein in the conditioned medium of 293EBNA cells transfected with pCEP4-*SulffFP2-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 SulffFP2-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 SulffFP2, amino acid residues 421-564 (designated as SulffFP2A) and 576-724 (SulffFP2B), were

separately subcloned into a pBAD/His prokaryotic expression vector (Invitrogen). The recombinant SulFP2 proteins were produced in the *E. 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/ $\mu$ l *N*-glycosidase F (Roche Diagnostics, Basel, Switzerland) at 37°C for 16 h before SDS-PAGE. The membrane was incubated with anti-SulFP2A (1:500), anti-SulFP2B (1:500), or anti-Myc mouse monoclonal antibody (1:2,000; Invitrogen), and then with either horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000; Bio-Rad) or anti-mouse IgG (1:5,000; 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<sub>2</sub>, 150 mM NaCl, and 0.01% bovine serum albumin in 50 mM sodium acetate buffer (pH 7.4) in a total volume of 50  $\mu$ l at 37°C for 24 h. To terminate the reaction, 100  $\mu$ l of 0.5 M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> (pH 10.7) was added to a 20- $\mu$ l 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  *$\beta$ -galactosidase*-transfected cells was subtracted.

To measure endosulfatase activity, the concentrated conditioned medium (5  $\mu$ l) was incubated with 10  $\mu$ g heparin, 200 mM NaCl, and 10 mM MgCl<sub>2</sub> in 10 mM Tris-HCl buffer (pH 7.5) in a total volume of 10  $\mu$ l at 37°C for 24 h. The mixture was heated at 95°C for 2 min and then incubated with 10  $\mu$ l 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 x 150 mm, particle size 5  $\mu$ m; 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<sub>2</sub>O; 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),  $\Delta$ UA2S-GlcNAc and  $\Delta$ UA2S-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 SulffFP2 protein*

To begin to examine the molecular mechanism by which SulffFP2 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-*SulffFP2-MycHis* and cultured in a serum-free medium for 3 days, SulffFP2 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 2 sequences of FLSYPRLKGR and DRRNIRPNII, found at the 25-34 and 38-47 residues of SulffFP2

(Fig. 1A and B), respectively. Thus, SulffFP2 was cleaved at 2 different sites near its N terminus: between Ala<sup>24</sup> and Phe<sup>25</sup> and between Arg<sup>37</sup> and Asp<sup>38</sup>. 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 SulffFP2 underwent proteolytic cleavage at Ala<sup>24</sup> by signal peptidases and at Arg<sup>37</sup> by PCs.

Next, Edman degradation of ~51 kDa, ~48 kDa, and ~43 kDa yielded the same sequence of SVAIEVDGEV, found at 544-553 residues of SulffFP2, indicating that all these fragments were generated by the cleavage between Arg<sup>543</sup> and Ser<sup>544</sup>. In addition, minor products of Edman degradation in the 48-kDa fragment indicated the existence of a cleavage between Arg<sup>570</sup> and His<sup>571</sup>. The amino acid sequences preceding these 2 cleavage sites (RNRSIR and RNLTKR) matched the consensus sequence of the cleavage by furin-type PCs (Fig. 1C) predicted by the ProP program [20]. Thus, SulffFP2 protein underwent cleavage at 2 sites in the hydrophilic domain. Moreover, high conservation of the consensus sequences between many vertebrate species suggests that the cleavage mechanism is evolutionarily preserved.

#### *Proteolytic processing of SulffFP2 protein*

We next examined SulffFP2 processing by western blot analysis. First, we examined SulffFP2 protein in the cell lysate and conditioned medium using anti-Myc antibody, which recognized the C terminus of SulffFP2-MycHis protein. This antibody detected a band of ~130-kDa in the cell lysate (fragments F1+F2; see Fig. 2B for the

names of the SulFP2 fragments), whereas a very faint band of similar size was detected in the conditioned medium after long exposure to X-ray films (Fig. 2A; data not shown). The sizes of both bands were reduced to 102 kDa after treatment with *N*-glycosidase F (data not shown). Because the calculated molecular mass of SulFP2-MycHis after signal peptide cleavage (100.1 kDa) was close to the observed size of the *N*-glycosidase-treated protein (102-kDa), the 130-kDa bands appeared to represent the full-length protein. In addition, processed fragments of 51 kDa and 48 kDa (fragments C1 and C2) were detected in both the cell lysate and the conditioned medium (Fig. 2A). *N*-glycosidase F treatment reduced their sizes to 41 kDa and 38 kDa, respectively (data not shown; see also Fig. 2A), indicating that these C-terminal fragments were *N*-glycosylated. Furthermore, a weak band of ~20-kDa (fragment C3) was detected in the conditioned medium (Fig. 2A); the size of this band was reduced to less than 10 kDa after *N*-glycosidase F treatment (data not shown). These findings indicated that some additional processing occurred near the C terminus.

To examine how SulFP2 protein was processed, we generated polyclonal antibodies that specifically recognized the N-terminal and C-terminal fragments of SulFP2. We selected 2 sequences in the hydrophilic domain of SulFP2: the N-terminal half (designated as SulFP2A) containing residues 421-564 and the C-terminal half (SulFP2B) containing residues 576-724 (Fig. 2B). We produced the recombinant proteins in *E. coli* and immunized rabbits. Western blotting of the conditioned medium with anti-SulFP2A antibody detected multiple bands of 85-98 kDa (fragments N1-N4), whilst anti-SulFP2B antibody detected multiple bands of 39-51 kDa (fragments C1-C2

and M1-M2; Fig. 2A). The sum of the apparent molecular masses of the fragments detected by anti-SulfFP2A and anti-SulfFP2B antibody was close to the size of the full-length protein (~130 kDa), indicating that SulfFP2 protein was largely cleaved into the N- and C-terminal fragments. Anti- SulfFP2A antibody also detected faint bands of 51 kDa and 43 kDa, indicating that a fraction of the polyclonal antibody reacted with the C-terminal fragments (C1 and M1 fragments). These data were compatible with previous findings that western blotting detected the N-terminal (72-75 kDa) or C-terminal (40-50 kDa) fragments of human Sulf1 or Sulf2 in the conditioned media of Chinese Hamster Ovary cells, 293 cells, or HT1080 cells transfected with *Sulf1* or *Sulf2* [9, 16-17, 22] in the conditioned medium of some pancreas tumor cells expressing endogenous Sulf2 [23] or in the cartilage of patients with osteoarthritis [24].

Interestingly, anti-SulfFP2B antibody detected more bands than anti-Myc antibody did: both anti-SulfFP2B and anti-Myc antibody detected bands of 51 kDa and 48 kDa (fragments C1 and C2), whereas anti-SulfFP2B but not anti-Myc antibody detected smaller bands of 43 kDa and 39 kDa (fragments M1 and M2; Fig. 2A). These data indicated that the 43/39-kDa fragments lacked the C-terminal portion recognized by anti-Myc. It was also notable that the 20-kDa band (fragment C3) detected by anti-Myc was not detected by anti-SulfFP2B and that the size difference between 51/48 kDa and 43/39 kDa was close to the size of the C3 fragment after *N*-glycosidase F treatment. Taken together, these findings indicate that SulfFP2 was cleaved into the N- and C-terminal fragments in the hydrophilic domain, and that the C-terminal fragments were further cleaved at an unidentified site near the C terminus (Fig. 2B).

### *Cleavage of SulFP2 by furin-like PCs*

Given that SulFP2 was cleaved at 2 sites (Arg<sup>543</sup> and Arg<sup>570</sup>) that matched the consensus sequence for the cleavage by furin-type PCs, SulFP2 likely underwent cleavage by these endopeptidases. To examine this, we tested whether introduction of a point mutation in the cleavage motifs or treatment with a furin inhibitor blocked the cleavage. In these experiments, we performed western blotting of SulFP2 after *N*-glycosidase F treatment because it was easier to identify the detected bands by referring to the calculated molecular masses of the putative SulFP2 fragments.

We first tested a mutant SulFP2 protein in which arginine at 543 was converted to glycine (termed SulFP2 (R543G)). Western blot analysis by anti-Myc antibody detected 2 bands of 41 kDa and 38 kDa (fragments C1 and C2) in *SulFP2*-transfected cells, whereas in *SulFP2* (R543G)-transfected cells, the 41-kDa band disappeared, the 38-kDa band remained unchanged, and a 102-kDa band appeared (Fig. 3A). Similarly, in a mutant in which arginine at 570 was converted to glycine (termed SulFP2 (R570G)), the 38-kDa band disappeared whilst the 41-kDa band remained unchanged (data not shown). These results demonstrated that the 41-kDa and 38-kDa fragments derived from the cleavage at Arg<sup>543</sup> and Arg<sup>570</sup>, respectively, and that the processing required the furin/PC cleavage motifs.

We next examined the effect of a furin/PC inhibitor, dec-RVKR-CMK. It specifically inactivates PCs by irreversibly modifying the active sites of the enzymes [25]. Treatment with this inhibitor resulted in selective loss of the 38-kDa band and

appearance of the 102-kDa band (Fig. 3B), indicating that the cleavage at Arg<sup>570</sup> was sensitive to dec-RVKR-CMK, whereas the cleavage at Arg<sup>543</sup> was relatively resistant. The combination of the R543G mutation and the inhibitor treatment abolished all the processed fragments (Fig. 3B), indicating that major cleavage of SulffFP2 occurred at these 2 sites. We also performed western blotting of the same samples with a mixture of anti-SulffFP2A and anti-SulffFP2B antibodies. This revealed that 2 bands of 33 kDa and 31 kDa (fragments M1 and M2) were detected in addition to the N-terminal (55-65 kDa) and the C-terminal (38 kDa and 41 kDa) fragments (Fig. 3B). The 33-kDa band disappeared upon introduction of the R543G mutation, whereas the 31-kDa bands disappeared upon treatment with dec-RVKR-CMK (Fig. 3B), indicating that they derived from the cleavage at Arg<sup>543</sup> and Arg<sup>570</sup>, respectively.

#### *Sulfatase activity of the uncleavable SulffFP2*

Finally, we tested whether the proteolytic cleavage was necessary for activating SulffFP2. To avoid the possibility of a furin inhibitor influencing sulfatase activity by acting on unknown targets, we decided not to use the inhibitor and instead to use SulffFP2 mutants SulffFP2 (R543G) and SulffFP2 (R570G) and a double mutant SulffFP2 (R543G/R570G). We co-transfected 293EBNA cells with *Sumf1* because Sumf1 increased the SulffFP2 activity (data not shown). We confirmed that cleavages at both Arg<sup>543</sup> and Arg<sup>570</sup> were abolished in SulffFP2 (R543G/R570G) (data not shown). When sulfatase activity in the conditioned medium was measured using a fluorogenic substrate 4-MUS at pH 7.4, all SulffFP2 mutants showed sulfatase activity comparable to that of

wild-type SulffFP2 (Fig. 4A). Next, we measured endosulfatase activity using heparin as a substrate. After heparin was incubated with the conditioned medium, it was completely digested by a mixture of heparinases, and the resultant unsaturated disaccharides were analyzed by reversed-phase ion-pair chromatography. SulffFP2 led to the decrease of  $\Delta$ UA2S-GlcNS6S with a concomitant increase of  $\Delta$ UA2S-GlcNS (Fig. 4B), indicating that SulffFP2 hydrolyzed the 6-*O*-sulfate in the trisulfated disaccharide units in heparin, as reported previously [9, 12, 14]. SulffFP2 (R543G), SulffFP2 (R570G), and SulffFP2 (R543G/R570G) showed endosulfatase activity comparable to that of wild-type SulffFP2. Thus, proteolytic cleavage was not indispensable for activation of SulffFP2.

The hydrophilic domain of Sulfs is a unique structure that is absent in other sulfatases [13]. Deletion studies showed that this domain in quail Sulff1 was required to anchor Sulf protein on the cell surface [8]. In addition, this domain, which carries many basic amino acids, bound to negatively-charged heparin/HS and was required for sulfatase activity [12, 22, 26]. We found that proteolytic processing of SulffFP2 occurred in its hydrophilic domain. Therefore, although the uncleavable SulffFP2 mutants showed endosulfatase activity comparable to wild-type SulffFP2, proteolytic cleavage may influence the functions of SulffFP2 in physiological environments. Future studies are required to elucidate the role of Sulf processing in vivo.

## **Conclusion**

In this study, we demonstrated that SulffFP2 protein was proteolytically cleaved at

Arg<sup>543</sup> and Arg<sup>570</sup> in the hydrophilic domain. Both sites resided in the consensus sequence for the cleavage by furin-type PCs. Cleavages at Arg<sup>570</sup> and Arg<sup>543</sup> were sensitive and relatively resistant to dec-RVKR-CMK, respectively. Uncleavable SulfFP2 showed sulfatase activity, suggesting that proteolytic cleavage was not indispensable for activation of SulfFP2.

Note added in proof. During the preparation of this manuscript, similar results for human Sulf1 and Sulf2 were reported [26].

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## Figure legends

**Figure 1.** Cleavage sites of SulfFP2 protein. (A) Schematic structure of rat SulfFP2. The black boxes at the N- and C-termini indicate the signal sequence (SS) and a Myc-His tag, respectively. The gray box indicates the hydrophilic domain. Arrows indicate proteolytic cleavage sites. Underlines indicate the consensus sequence for cleavage by furin-type PCs. (B-C) Amino acid sequence alignment of vertebrate Sulf2. N-terminal sequences (B; 1-40 of rat SulfFP2) and the sequences in the hydrophilic domain (C; 536-574 of rat SulfFP2) are shown. Shading indicates arginine (R) and lysine (K) residues conserved among species. Arrows indicate proteolytic cleavage sites determined by Edman degradation sequencing.

**Figure 2.** Proteolytic cleavage of SulfFP2 protein. (A) Western blot of SulfFP2 protein. The cell lysate and the conditioned medium of mock- or pCEP4-*SulfFP2-MycHis*-transfected 293EBNA cells were analyzed by western blotting with anti-Myc, anti-SulfFP2A, or anti-SulfFP2B antibody. Sizes of the markers (kDa) are shown on the left. The names of fragments shown on the right are indicated in (B). (B) Schematic structures of the putative SulfFP2 fragments. The sizes of the bands (kDa) detected by western blotting (WB) with or without *N*-glycosidase F (NGase) treatment and calculated molecular masses (Mr) for each fragment are shown on the right. The gray regions indicate the sequences used to make anti-SulfFP2A or anti-SulfFP2B antibodies. Arrows indicate the cleavage site determined by Edman degradation sequencing, whereas the dotted arrow indicates the putative cleavage site. Dots over boxes indicate

putative *N*-glycosylation sites.

**Figure 3.** Effects of a furin inhibitor and a point mutation in the cleavage motif on SulffFP2 processing. 293EBNA cells were transfected with pCEP4-*SulffFP2-MycHis* or pCEP4-*SulffFP2 (R543G)-MycHis* and cultured with or without 10  $\mu$ M dec-RVKR-CMK. The conditioned media were concentrated, treated with *N*-glycosidase F, and subjected to western blotting with anti-Myc antibody (A) or with anti-SulffFP2A and anti-SulffFP2B antibodies (B). Sizes of the markers (kDa) are shown on the left. Names of the fragments shown on the right are indicated in Figure 2B.

**Figure 4** Sulfatase activity of SulffFP2 and its mutants. (A) Sulfatase activity measured using 4-MUS. (B) Endosulfatase activity measured using heparin as a substrate. Heparin was incubated with SulffFP2 or its mutants and then subjected to disaccharide analysis. Molar percentages of 8 unsaturated disaccharides are shown. The conditioned medium from the  $\beta$ -galactosidase-transfected cells was used as a negative control. Averages from 3 independent experiments are shown. The bars indicate standard deviation.

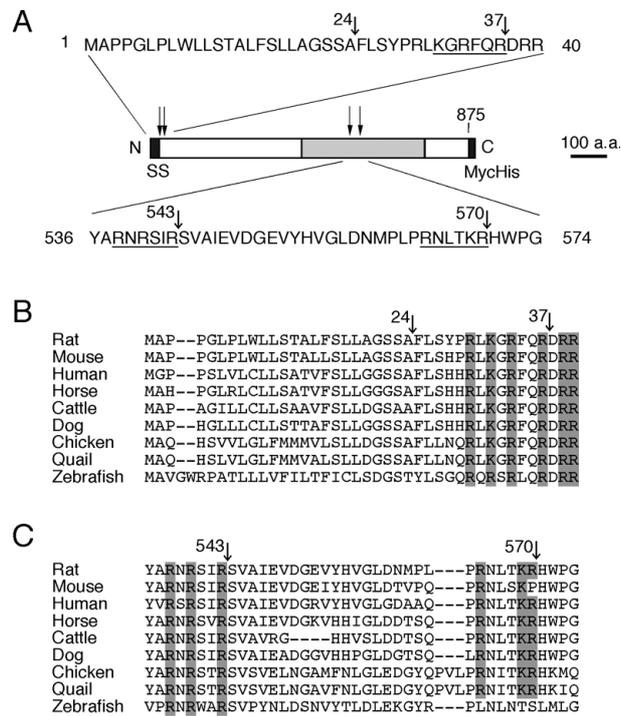


Fig. 1

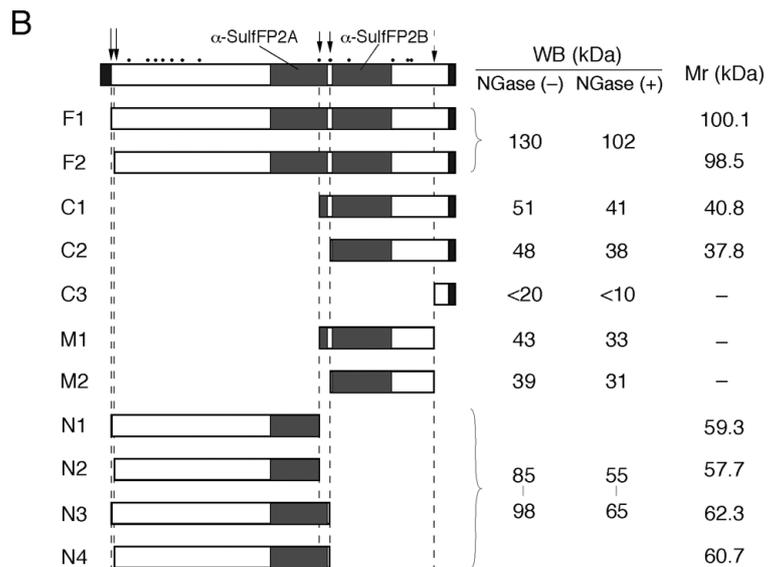
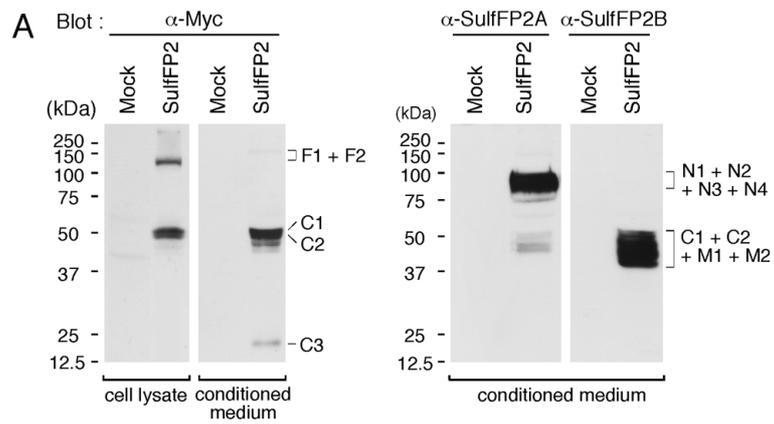


Fig. 2

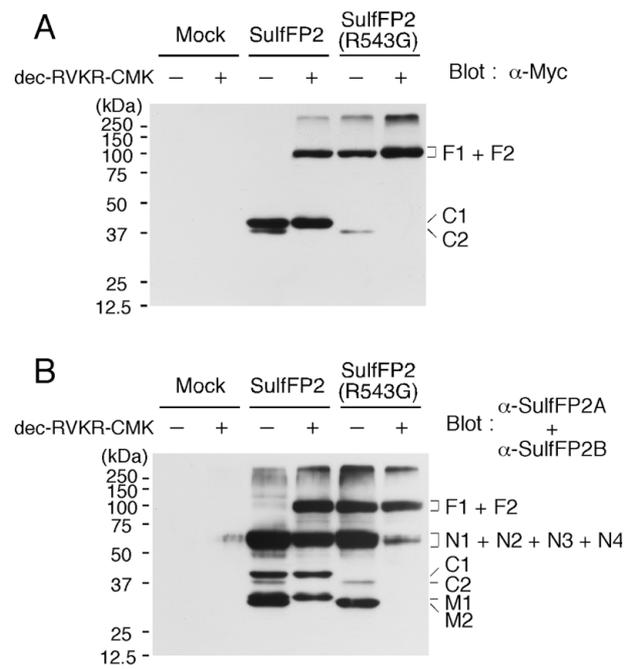


Fig. 3

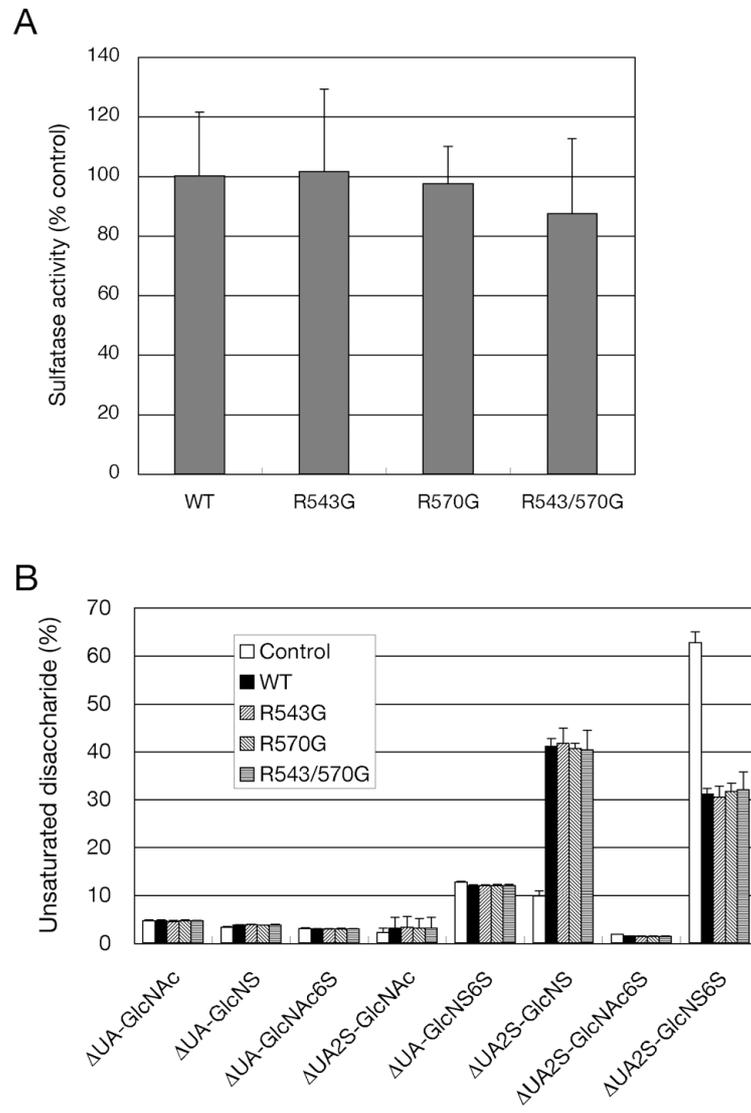


Fig. 4