Regulation of Heparan Sulfate 6-O-Sulfation by β-Secretase Activity

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The enzymes involved in glycosaminoglycan chain biosynthesis are mostly Golgi resident proteins, but some are secreted extracellularly. For example, the activities of heparan sulfate 6-O-sulfotransferase (HS6ST) and heparan sulfate 3-O-sulfotransferase are detected in the serum as well in the medium of cell lines. However, the biological significance of this is largely unknown. Here we have investigated by means of monitoring green fluorescent protein (GFP) fluorescence how C-terminally GFP-tagged HS6STs that are stably expressed in CHO-K1 cell lines are secreted/shed. Brefeldin A and monensin treatments revealed that the cell-permeable β-secretase inhibitor N-benzyloxy carbonyl-Val-Leu-Leucinal (Z-VLL-CHO) specifically inhibits HS6ST secretion, although this effect was specific for HS6ST3 but not for HS6ST1 and HS6ST2. However, Z-VLL-CHO treatment did not increase the molecular size of the HS6ST3-GFP that accumulated in the cell. Z-VLL-CHO treatment also induced the intracellular accumulation of SP-HS6ST3(-TMD)-GFP, a modified secretory form of HS6ST3 that has the preprotrypsin leader sequence as its N-terminal hydrophobic domain. Diminishment of β-secretase activity by coexpressing the amyloid precursor protein of a Swedish mutant, a potent β-secretase substrate, also induced intracellular HS6ST3-GFP accumulation. Moreover, Z-VLL-CHO treatment increased the 6-O-sulfate (6S) levels of HS, especially in the disaccharide unit of hexuronic acid-GlcNS (6S). Thus, the HS6ST3 enzyme in the Golgi apparatus and therefore the 6-O sulfation of heparan sulfates in the cell are at least partly regulated by β-secretase via an indirect mechanism.

Heparan sulfate (HS) proteoglycans play important roles in various biological processes by acting as co-receptors, by serving as reservoir molecules in morphogen gradient formation etc. (1–5). The sulfation domains (S domains) of HS are the binding sites for the growth factors and morphogens. Analyses of fruit fly, zebrafish, and mice mutants have revealed that the specific patterns of the sulfations determine the bioactivities of the HS proteoglycans (6–10). In particular, 6-O-sulfation of HS has been shown to be required for the fibroblast growth factor (FGF) and Wnt signaling pathways in Drosophila and zebrafish (11, 12). With regard to the link between 6-O-sulfation of HS and the FGF signaling pathway, heparan sulfate 6-O-sulfotransferase (HS6ST) RNA interference experiments in fruit fly have demonstrated that the interfered phenotype closely resembles those of mutants that are defective in FGF signaling components (11). In addition, by subjecting a sulfated octasaccharide library to an affinity chromatographic assay, Ashikari-Hada et al. (13) have shown that the 6-O-sulfation of HS is important for binding activities of FGF-10, -4, and -7. With regard to the link between HS 6-O-sulfation and the Wnt signaling pathway, the knockdown of HS6ST in zebrafish with morpholin antisense oligonucleotides results in perturbed muscle differentiation that is associated with higher expression of the Wnt target genes myoD and eng2. Q5ulf1, the avian heparan sulfate 6-O-endsulfatase, is required for the activation of MyoD, which is a Wnt-induced regulator of muscle specification (14). Thus, the 6-O sulfation of HS plays important roles in regulating HS-binding growth factor signaling and morphogen gradient formation.

Three isoforms of HS6STs have been identified in mice and humans (15–18). The expression patterns of these isoforms are regulated in spatially and temporally different manners. Their substrate specificities also differ (16, 17), since HS6ST1 preferentially catalyzes the sulfation of the L-iduronic acid (IdoA)-GlcNS disaccharide unit, whereas HS6ST2 prefers the D-glucuronic acid (GlcA)-GlcNS and IdoA(2S)-GlcNS disaccharides, and HS6ST3 has intermediate substrate specificity between

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HS6ST1 and HS6ST2. Thus, different patterns of HS 6-O-sulfation could be produced by three HS6ST isoforms with different substrate specificities. Since every HS-modifying enzyme is thought to be a Golgi resident protein, but HS6STs are rapidly secreted into the culture medium (19), the degree of 6-O-sulfation occurring in the cell may also be regulated by the amount of the enzyme protein in the Golgi apparatus.

In this study, we have investigated the mechanism by which HS6STs are secreted. We have focused the analysis on HS6ST3 for several reasons. First, it is secreted at higher levels than the other two HS6STs (Fig. 1B). Second, the HS6ST1 gene has another ATG codon 30 bp upstream from the translation initiation codon, as reported previously (16). This results in a 10-amino acid longer form that can be also translated in HS6ST1-green fluorescent protein (GFP)-transfected CHO-K1 cells. This may confuse analyses that seek to determine whether the protein has been processed or not. Third, the HS6ST2 gene also has another ATG codon, albeit far upstream from the other translation initiation codon. Transfection of CHO-K1 cells with this longer HS6ST2-GFP expression vector revealed poor translation initiation codon. Transfection of CHO-K1 cells with this longer HS6ST2-GFP expression vector revealed poor translation initiation codon, as reported previously (16). This results in a 10-amino acid longer form that can be also translated in HS6ST1-GFP, HS6ST2-GFP, and HS6ST3-GFP is described in the next section and may thus play important roles in modulating 6-O-sulfation (Fig. 5). Therefore, it is likely that β-site APP-cleaving enzyme (BACE1)-like enzymes that have β-secretase activity may be involved in HS6ST3 secretion and may thus play important roles in modulating 6-O-sulfated HS structures.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The construction of vectors expressing HS6ST1-GFP, HS6ST2-GFP, and HS6ST3-GFP is described in a previous paper (20). The vectors for HS6ST1(A)-GFP, HS6ST1(Q)-GFP, HS6ST1(AA)-GFP, and HS6ST3-2ST-GFP were constructed by PCR-based mutagenesis as described previously (20) using the following primers: HS6ST1(A)-GFP forward (5′-GTCGAACCGATCGAGCAACAT-3′) and reverse (5′-ATAGATTCATCCTGAGGAG-3′); HS6ST1(Q)-GFP forward (5′-ACGACGAGCAACTGAGCAGA-3′) and reverse (5′-ATAGATTCATCCTGAGGAG-3′); HS6ST1(HA)-GFP forward (5′-ACGACGAGCAACTGAGCAGA-3′); FLAG-HS6ST3(AGT-AGC)-GFP forward (5′-ATGGATGAAAGGTTCAACAAG-3′) and reverse (5′-GATCGATGATTCGGCCGCGC-3′); HS6ST3-2ST-GFP forward (5′-GAGAACCAGATCGAGCAAGCTG-3′) and reverse (5′-GTCCCCGGATGATCCTGAC-3′); the SP-HS6ST3(-TMD)-GFP expression plasmid was constructed by subcloning the SacI fragment of pHS6ST3-GFP into the SacI site of pFLAG-CMV3-HS6ST3. The SP-GFP expression vector was constructed by PCR using forward primer 5′-ATGGTGAGGAAGGCGAGGAGGAG-3′ and reverse primer 5′-CCCGGCAGCAAGTCTGAC-3′ from SP-HS6ST3(-TMD)-GFP. The cDNAs encoding the N termini of HS6ST1Long-GFP and HS6ST2Long-GFP were cloned by reverse transcription-PCR using mouse embryonic day 15 total RNA as the template.

**Chemicals**—The protease inhibitors used in this study (KTEIESEVN-Stat-VAEF, Z-VAL-CHO) were purchased from Calbiochem, Brefeldin A (BFA), monensin sodium salt, aprotinin, EDTA, iodoacetic acid, leupeptin, N-ethylmaleimide, pepstatin A, phenylmethylsulfonl fluoride, Nα-tosyl-L-lysine chloromethyl ketone hydrochloride, and N-p-tosyl-L-phenylalanine chloromethyl ketone were from Sigma. Radioisotopes were from PerkinElmer Life Sciences.

**Cell Culture, Transfection, and Western Blotting**—CHO-K1 and its transfectants were maintained in Dulbecco's modified Eagle's medium/F-12 medium (Sigma) supplemented with 10% fetal calf serum (Cell Culture Technologies, Lugano, Switzerland) except that when the medium fraction was to be subjected to Western blotting, the medium was supplemented with 1% fetal calf serum. The cells were transfected with the APPsw or BACE1 expression plasmids (21) by using FuGENE6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. In some cases, cells were treated with BFA (10 μg/ml) or monensin sodium salt (5 μM) for 4 h. Cell lysates were prepared as described previously (20). Medium aliquots were centrifuged at 1,000 × g for 5 min to remove cell debris. The resulting cell lysates and medium aliquots were processed for Western blotting according to standard procedures and subjected to electrophoresis in 8% polyacrylamide gel. The proteins in the gels were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) and reacted with rabbit polyclonal anti-GFP antibody (diluted 1:1000). To detect the antibody, a peroxidase-conjugated secondary antibody (CAPPEL, Irvine, CA) was used, and immunocomplexes were revealed by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

**Triton X-114 Phase Separation**—Cell lysates were prepared by using Triton X-114 as a detergent. Cell lysates were incubated at 37 °C for 3 min and centrifuged at 1700 × g for 5 min at room temperature. An upper detergent-poor phase and a lower detergent-rich phase were collected separately and subjected to Western blotting with an anti-GFP or anti-calnexin antiserum (Stressgen Bioreagents, Ann Arbor, MI).

**Cell Staining**—Mouse monoclonal anti-GM130 was obtained from BD Transduction Laboratory (Lexington, KY), mouse monoclonal anti-Myc antibody (9B11) was from Cell Signaling Technology (Danvers, MA), and Alexa Fluor-conjugated goat anti-mouse IgG antibodies were from Invitrogen Japan (Tokyo, Japan). Cells were fixed and stained as described previously (20). Immunofluorescence was detected under an
We speculated that these residues might be a common cleavage or secretion signal. Indeed, we found that Tyr25 and Gln24 are conserved in all of the HS6ST isoforms (HS6ST1, -2, and -3) that have been identified to date in mice, humans, rats, dogs, chicken, and zebrafish (Fig. 1A). This indicates that these residues do not after all participate in HS6ST cleavage or secretion.

To further analyze the cleavage and secretion of the HS6STs, we cultured CHO-K1 cell lines that stably express HS6STs tagged with GFP at their C termini and then measured the GFP fluorescence that had been secreted into the culture medium (Fig. 1B). Fluorescence in the culture medium and cell lysate was then detected by Hitachi F-3010 fluorescence spectrophotometry with an excitation wavelength of 388 nm and an emission wavelength of 407 nm. HS6ST secretion was expressed as a ratio of the fluorescence intensity of the culture medium to that of the cell lysate. For comparison, the secretion of the stable transfectant expressing HS6ST1-GFP was set to 100%.

The results of the disaccharide analysis are shown in Table 1. The disaccharides found in the conditioned medium of CHO-K1 cells expressing HS6ST1-GFP, HS6ST2-GFP, and HS6ST3-GFP showed a similarity to those found in CHO-K1 conditioned medium (15). *, amino acids that are identical in the HS6STs. TMD, transmembrane domain.

**RESULTS**

We previously showed that 6-O-sulfotransferase activity could be detected in the conditioned medium of cultured CHO-K1 cells (19). Analysis of the amino acid sequence of HS6ST1 in the culture medium revealed that its N-terminal amino acid started from tyrosine 25. Subsequent cDNA cloning showed that Tyr25 and Gln24 are conserved in all of the HS6ST isoforms (HS6ST1, -2, and -3) that have been identified to date in mice, humans, rats, dogs, chicken, and zebrafish (Fig. 1A).
steps of the intracellular transport process, and examined the resulting molecular form of HS6ST3-GFP by Western blot analysis employing anti-GFP antiserum. If HS6ST3-GFP processing occurs after it reaches the trans-Golgi, both its full-sized and processed forms would be present in BFA-treated cells. This is because BFA treatment disassembles the early Golgi complex and its fusion with the endoplasmic reticulum, and thus the uncleaved product would accumulate in the cell. Monensin reversibly slows the intracellular transport rate of newly synthesized proteins, in particular by interfering with the transfer across Golgi compartments; thus, it compromises secretion from the trans-Golgi (23). If HS6ST3-GFP is cleaved only after reaching the cell surface, both monensin and BFA treatment would result in the accumulation of the uncleaved HS6ST3-GFP product. As shown in Fig. 2A, HS6ST3-GFP secretion was almost completely blocked when the cells were treated with either inhibitor. Western blot analysis was then performed to determine the molecular sizes of the intracellular HS6ST3-GFP synthesized by the untreated and BFA- and monensin-treated cells. For this analysis, the cell lysates were first incubated with PNGase F to remove N-glycans that could simplify the analysis. The HS6ST3-GFP molecules in the BFA/monensin-treated cells were the same size as those in the untreated controls even when electrophoresed for a long time to intensify the size difference (Fig. 2B, top). This suggests that HS6ST3-GFP is processed early in the secretory pathway, namely in either the endoplasmic reticulum or the cis/medial-Golgi. To confirm this possibility, we performed the Triton X-114 phase separation assay. Border (24) has shown that transmembrane proteins can be specifically and quantitatively recovered in the detergent-rich phase. Heating of aqueous solution containing Triton X-114 above the temperature called the cloud point leads to the formation of large micelles that sediment rapidly during low speed centrifugation. An upper detergent-poor phase and a lower detergent-rich phase are formed, with the latter phase containing membrane lipids and transmembrane proteins. HS6ST3-GFP was extracted into the aqueous phase after centrifugation regardless of the BFA and monensin treatment, suggesting that the N-terminal hydrophobic domain was cleaved off early in the secretory pathway (Fig. 2B, bottom).

Since the N-terminal cytoplasmic sequence of HS6ST3 is relatively short, we hypothesized that the N-terminal cytoplasmic and hydrophobic domain might have the signal peptide activity of a secretory protein. Signal peptides of secretory proteins, which are cleaved by the signal peptidase, are known to consist of three contiguous regions; a 1–5-amino acid region of positively charged residues, a 7–15-hydrophobic amino acid region, and a 5–7-amino acid region containing residues with small side chain at the −1- and −3-positions (25–28). If the N-terminal hydrophobic sequence of HS6ST3 is cleaved by signal peptidase, adding several hydrophilic amino acids to its N terminus would compromise its recognition by the signal peptidase. To test this possibility, we added FLAG-peptide to the N terminus of HS6ST3-GFP and substituted the ATG codon of HS6ST3 with ACG (FLAG-HS6ST3(ACG)-GFP), Cell lysates and the medium of CHO-K1 cells stably expressing HS6ST3-GFP or FLAG-HS6ST3(ACG)-GFP were subjected to Western blotting analysis using anti-GFP antiserum. As shown in Fig. 2D, HS6ST3-GFP was detected both in the cell lysate and in the conditioned media. However, FLAG-HS6ST3(ACG)-
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ACG)-GFP was exclusively found in the cell lysate. Next, we substituted the N-terminal hydrophobic domain of HS2ST with that of HS6ST3, resulting in a HS2ST chimeric protein (HS6ST3-2ST-GFP). HS2ST-GFP was not detected in the culture medium (Fig. 2D). If the N-terminal hydrophobic domain of HS6ST3 was involved in signal peptidase recognition, substituting the N-terminal hydrophobic domain of HS2ST with that of HS6ST3 would result in a secretory protein. Western blotting was performed on the cell lysates and the medium of CHO-K1 cells stably expressing HS2ST-GFP or HS6ST3-2ST-GFP. HS6ST3-2ST-GFP, but not HS2ST-GFP, was detected in the conditioned medium (Fig. 2D). Thus, the N-terminal cytoplasmic and hydrophobic domains of HS6ST3 are sufficient for secretion. Next, we performed the Triton X-114 phase separation experiment for these mutants. As shown in Fig. 2C, SP-GFP (secretory form of GFP), HS6ST3-GFP, and HS6ST3-2ST-GFP were extracted into the aqueous phase after Triton X-114 phase separation. HS2ST-GFP, FLAG-HS6ST3(ATG>ACG)-GFP, and endoplasmic reticulum membrane protein calnexin were found both in the aqueous and detergent-rich phase. Thus, the N-terminal cytoplasmic and hydrophobic domains of HS6ST3 and HS6ST3-2ST-GFP would be cleaved off before secretion to become soluble protein. On the other hand, FLAG-HS6ST3(ATG>ACG)-GFP was not cleaved, since the addition of FLAG tag sequence would compromise the recognition by signal peptidase.

These results support our hypothesis that the N-terminal cytoplasmic and hydrophobic domains of HS6ST3-GFP behave as a signal peptide sequence of secretory protein.

In the course of the experiment to identify the protease that is responsible for the cleavage of HS6ST3-GFP, we treated the cells with various protease inhibitors (aprotinin, EDTA, iodoacetic acid, leupeptin, N-ethylmaleimide, pepstatin A, phenylmethylsulfonyl fluoride, N\(^{-}\)tosyl-L-lysine chloromethyl ketone hydrochloride, N\(^{-}\)p-tosyl-L-phenylalanine chloromethyl ketone, KTEEISEVN-Stat-VAEF, N-benzzyloxyacrylonyl-Leu-leucinal, Z-VLL-CHO, and 1,3-di-(N-carboxybenzoyl-Leu-Leu)-amino acetone). If a particular inhibitor was involved in blocking HS6ST3-GFP secretion, the extracellular GFP fluorescence would decrease, whereas the intracellular fluorescence would increase. We found no significant effect when the following protease inhibitors were used: aprotinin, EDTA, iodoacetic acid, leupeptin, N-ethylmaleimide, pepstatin A, phenylmethylsulfonyl fluoride, N\(^{-}\)tosyl-L-lysine chloromethyl ketone hydrochloride, N\(^{-}\)p-tosyl-L-phenylalanine chloromethyl ketone, KTEEISEVN-Stat-VAEF, N-benzzyloxyacrylonyl-Leu-leucinal, Z-VLL-CHO, and 1,3-di-(N-carboxybenzoyl-Leu-Leu)-amino acetone. If a particular inhibitor was involved in blocking HS6ST3-GFP secretion, the extracellular GFP fluorescence would decrease, whereas the intracellular fluorescence would increase. We found no significant effect when the following protease inhibitors were used: aprotinin, EDTA, iodoacetic acid, leupeptin, N-ethylmaleimide, pepstatin A, phenylmethylsulfonyl fluoride, N\(^{-}\)tosyl-L-lysine chloromethyl ketone hydrochloride, N\(^{-}\)p-tosyl-L-phenylalanine chloromethyl ketone, KTEEISEVN-Stat-VAEF, N-benzzyloxyacrylonyl-Leu-leucinal, Z-VLL-CHO, and 1,3-di-(N-carboxybenzoyl-Leu-Leu)-amino acetone (data not shown).

However, the potential effect was observed with the cell-permeable β-secretase inhibitor Z-VLL-CHO (Fig. 3A), since it decreased extracellular fluorescence intensity by 30–50% of the intensity of untreated cells (Fig. 3A). Concomitantly, it increased the intracellular fluorescence intensity by 200–300% of the intensity of untreated cells (Fig. 3A). We used two lines of CHO-K1 cells stably expressing different levels of HS6ST-GFP and found that both cell lines respond to Z-VLL-CHO similarly (data not shown). Other inhibitors did not show any significant effect. Western blotting of the cell lysate and the medium with anti-GFP antibody showed that Z-VLL-CHO treatment did not appreciably change the overall amount of HS6ST3-GFP (Fig. 3A, bottom left). The cell-impermeable β-secretase inhibitor KTEEISEVN-Stat-VAEF had no effect. To ensure that the observed effect of Z-VLL-CHO did not arise from a block to general cellular transport, we treated CHO-K1 cells expressing the secretory form of GFP (SP-GFP) with Z-VLL-CHO or vehicle for 8 h and measured the fluorescent intensity of the culture medium and the cell lysates (Fig. 3A). Z-VLL-CHO treatment decreased the fluorescent intensity of the culture medium to 75%. This may be due to Z-VLL-CHO toxicity. However, the fluorescence of the cell layer was nevertheless increased by Z-VLL-CHO treatment. Therefore, the observed effect of Z-VLL-CHO does not result from nonspecific inhibition of cellular transport.

Although Z-VLL-CHO is widely used as a specific inhibitor of β-secretase, there may be other proteases that can also be inhibited by this inhibitor. To examine the specificity of the inhibitory effect of Z-VLL-CHO on HS6ST3-GFP secretion, the accumulation of HS6ST3-GFP in the treated cells was observed up to 12 h by fluorescence microscopy. In the untreated cells, HS6ST3-GFP co-localized with the Golgi marker GM130 (Fig. 3B), as shown in our previous paper (20). However, when the cells were treated with Z-VLL-CHO, HS6ST3-GFP accumulated in the cell, initially in the Golgi apparatus (Fig. 3B). Longer exposure of the cells to the inhibitor resulted in the leaking of HS6ST3-GFP from Golgi apparatus, since HS6ST3-GFP fluorescence showed wider distribution than the GM130 fluorescence labeled with Alexa594.

To investigate whether β-secretase is directly responsible for the secretion of HS6ST3-GFP, we first examined if transiently expressing human APP with a Swedish mutation (APP\(^{sw}\)), a well known and potent β-secretase substrate (29), would alter HS6ST3-GFP secretion (data not shown). We also investigated the effect of transiently transfecting the vector expressing BACE1, which has β-secretase activity (30, 31) (data not shown). The effects of expressing APP\(^{sw}\) or BACE1 were quite small; this is because the efficiency of transient transfection was very low (about 15%) even under the best possible conditions. The competition of β-secretase activity by APP\(^{sw}\) expression increased the GFP fluorescence in the cell, whereas conversely, BACE1 expression enhanced HS6ST3 secretion (data not shown). These observations suggest that HS6ST3 secretion is dependent on β-secretase activity. Since the difference in fluorescence measured above was quite small, we confirmed the enhanced secretion of HS6ST3-GFP following BACE1 transfection by fluorescence microscopy. HS6ST3-GFP-expressing cells were transiently transfected with the Myc-tagged BACE1 expression vector and treated with or without BFA to visualize HS6ST3-GFP expression. Cells were stained with anti-Myc antibody followed by Alexa594-conjugated anti-mouse antibody and counted the number of BACE1-positive cells with or without HS6ST3-GFP expression. Before BFA treatment, the number of BACE1- and HS6ST3-GFP-double-positive cells/BACE1-positive cells was 38/136. After BFA treatment, the number of BACE1- and HS6ST3-GFP-double-positive cells/
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**FIGURE 3.** Effect of enzyme inhibitors or transiently expressing APP<sup>sw</sup> or BACE1 on HS6ST3-GFP secretion and effect of Z-VLL-CHO on the secretion of the longer HS6ST1 and HS6ST2 isoforms. A, cells stably expressing HS6ST3-GFP were treated with the cell-permeable β-secretase inhibitor Z-VLL-CHO or the nonpermeable β-secretase inhibitor KTEESEYN-Stat-VAEF for 12 h. Upper left, HS6ST3-GFP secretion was determined by fluorescence spectrophotometry of the culture medium. The fluorescence intensity of the conditioned medium produced by untreated cells was set at 100%. Only the cell-permeable β-secretase inhibitor reduced the extracellular GFP fluorescence while concomitantly elevating the intracellular GFP fluorescence. Upper right, CHO-K1 cells expressing SP-GFP was treated with Z-VLL-CHO or vehicle. The fluorescence intensities of the conditioned medium and cell lysate of untreated cells were set to 100%, respectively. Treatment with Z-VLL-CHO had no effect on the secretion of SP-GFP. Lower left, the Z-VLL-CHO-mediated inhibition of HS6ST3-GFP secretion was verified by Western blotting analysis of the lysates of the cells treated with or without Z-VLL-CHO for 8 h. For this, equal amounts (30 μg) of cell lysates were subjected to Western blotting using anti-GFP antiserum after treatment with PNGase F. The HS6ST3-GFP molecules in the cell were similarly sized regardless of the treatment with Z-VLL-CHO. The overall amounts of HS6ST3-GFP (cell lysate and medium) did not change after Z-VLL-CHO treatment. Equal amounts (30 μg) of cell lysates were subjected to Western blotting using anti-GFP antiserum after PNGase F treatment. Lower right, Western blotting of Z-VLL-CHO-treated or nontreated cell lysates after Triton X-114 phase separation without PNGase F treatment. The aqueous (A) and the detergent-rich (D) phase were subjected to Western blotting with anti-GFP or anti-calnexin antiserum. B, time-dependent increase of intracellular HS6ST3-GFP levels in Z-VLL-CHO-treated cells that stably express HS6ST3-GFP. The cells were fixed at the indicated time after Z-VLL-CHO treatment, permeabilized, and stained with mouse monoclonal antibody followed by Alexa Fluor 568 anti-mouse IgG. C, CHO-K1 cells expressing HS6ST3-GFP were transiently transfected with the BACE1 expression vector, and half of the transfected cells were treated with BFA to block the secretion. Cells were stained with anti-Myc (9B11) antibody followed by Alexa594-conjugated goat anti-mouse IgG antibody. D, cells stably expressing the isoforms of HS6ST1 and HS6ST2 that have longer N termini by 10 and 146 amino acids, respectively, were cultured. Left, the GFP-derived fluorescence in the conditioned medium and cell lysates was analyzed by fluorescence spectrophotometry. The secretion efficiency was calculated as a ratio of the fluorescence intensity of the culture medium divided by that of the total GFP fluorescence. The representative photographs were shown in Fig. 3C. BACE1-positive cells was 79/129. The relative number of BACE1- and HS6ST3-GFP-double-positive cells increased significantly by BFA treatment (p < 0.01; Fisher’s exact probability test). The representative photographs were shown in Fig. 3C. Cells expressing BACE1 were less GFP-fluorescent compared with BACE1-nonexpressing cells (no treatment). BFA treatment increased the relative number of doubly positive cells (BFA). The relative number of HS6ST3-GFP-expressing cells/total cells (no treatment, 127/146; BFA, 109/137; p > 0.05; Fisher’s exact probability test) was not significantly increased by BFA treatment. The cells transiently expressing APP<sup>sw</sup> had an abnormal morphology (data not shown), which may be caused by the toxic effect of Aβ40 and Aβ42 produced by BACE (32–34). To determine whether β-secretase directly processes HS6ST3-GFP, we analyzed the molecular weight of HS6ST3-GFP within cells after the treatment with Z-VLL-CHO or vehicle by Western blotting. Z-VLL-CHO treatment increased the overall cellular HS6ST3-GFP levels (Fig. 3A, bottom), which is consistent with the Z-VLL-CHO-induced elevation in intracellular fluorescence intensity shown in the top of Fig. 3A. However, the molecular weight of the intracellular HS6ST3-GFP did
not increase (Fig. 3A, lower left). By Triton X-114 phase separation, HS6ST3-GFP was extracted into the aqueous phase after centrifugation regardless of the Z-VLL-CHO treatment (Fig. 3A, lower right). Thus, it appears that β-secretes regulates the level of intracellular HS6ST3 that received the cleavage. This is further supported by our observation that cleavage products were not detected when BACE1-Fc (21) was incubated in vitro with the HS6ST3-protein A fusion protein.

By analyzing the DNA database of the full-length cDNA project, the HS6ST1 gene was revealed to have another ATG codon 30 bp upstream from the translation initiation codon as reported previously (16). This results in a 10-amino-acids longer form that can be also translated in HS6ST1-GFP-transfected CHO-K1 cells. We also discovered that the N termini HS6ST2 can be 146 amino acids longer, respectively, than those we previously reported (16), due to the presence of an additional initiation codon upstream from our previously published initiation codon. To investigate whether these longer HS6ST1 and HS6ST2 are also secreted into the medium like their shorter isoforms, we cloned them by reverse transcription-PCR and subcloned them into the GFP expression vector such that the GFP tag was on their C termini. Stable cell lines transfected with these vectors secreted HS6ST1Long-GFP but not HS6ST2Long-GFP into the culture medium (Fig. 3D). Interestingly, treatment of HS6ST1-GFP- and HS6ST2Long-GFP-expressing cells with Z-VLL-CHO had no effect of their secretion patterns (Fig. 3D), which indicates that the β-secretes-dependent mechanism that controls the intracellular pool of HS6ST is specific to HS6ST3.

The above data suggested that the N-terminal domain of HS6ST3-GFP is cleaved off to generate the secreted form, whose secretion is subsequently regulated in β-secretase-dependent manner. To test this hypothesis further, we asked whether Z-VLL-CHO treatment could also cause the secreted form of HS6ST3-GFP to accumulate in the cell. To do this, we stably expressed in CHO-K1 cells a modified form of HS6ST3-GFP in which the N-terminal hydrophobic domain has been replaced with the signal peptide of human pro-trypsin; this generated the secretory HS6ST3-GFP fusion protein SP-HS6ST3(-TMD)-GFP (Fig. 4). We then assessed the effect of Z-VLL-CHO treatment on the secreted GFP fluorescence. As shown in Fig. 4, the secreted fluorescence intensity in the medium of Z-VLL-CHO-treated cells was lower than that of untreated controls. Thus, the cleaved form of HS6ST3 is retained intracellularly if β-secretase activity is blocked, which further supports the notion that the mechanism behind HS6ST3 secretion is dependent on β-secretase.

The intracellular accumulation of HS6ST3-GFP induced by inhibiting β-secretase activity would affect the 6-O sulfation of newly synthesized HS. We labeled HS6ST3-GFP-expressing cells with [35S]H2SO4 and examined the sulfation pattern in the presence or absence of the inhibitor by analyzing their disaccharide compositions. HS isolated from Z-VLL-CHO-treated cells had more 6-O-sulfated structures (the 6-O sulfation per total sulfation was 36.9 ± 0.8%) than the untreated cells (21.8 ± 3.3%) (Fig. 5). These values were calculated as follows: (HexA-GlcnAc(6S) count + (HexA-GlcnNS(6S)) count)/2 + (HexA(2S)-GlcNS(6S) count)/total count. Significantly, a specific increase was observed in the HexA-GlcnNS(6S) (untreated versus treated: 4.9 ± 0.4% versus 16.9 ± 0.6%), HexA-GlcnAc(6S) (16.9 ± 30.0% versus 23.6 ± 15.5%), and HexA(2S)-GlcNS(6S) (7.2 ± 2.6% versus 14.6 ± 2.8%) disaccharide units. We calculated as follows: in the case of HexA-GlcnNS(6S), (count of HexA-GlcnNS(6S) area/2 per total count, taking into account the number of sulfation of each disaccharide. These observations together indicate that when β-secretase inhibition induces the intracellular accumulation of HS6ST3-GFP, the 6-O sulfation levels of the cells are increased.

D I S C U S S I O N

That HS sulfation is important for the functionality of many HS-binding growth factors and morphogens has been revealed by analyzing mutant animals in which the activity of various HS biosynthetic enzymes has been disrupted. For example, the embryos of Drosophila sugarless and sulfatless, which lack the UDP-N-glucose dehydrogenase and heparan sulfate N-deacetylase/N-sulfotransferase enzymes, respectively, have similar phenotypes to embryos lacking the functions of fibroblast growth factors (35). RNA interference-mediated knockdown of HS6ST in Drosophila also resulted in defective fibroblast growth factor signaling (11). Thus, the regulation of HS sulfation may well be a key mechanism that controls the bioactivity of many HS-interacting proteins. That the sulfotransferases themselves may participate in regulating HS sulfation is suggested by the fact that the HS6ST isoforms have inherently

3 S. Kitazume, unpublished observation.
different substrate specificities and generate a variety of sulfation patterns. Although their substrate specificities partially overlap, each individual isoform has a characteristic preference for the uronic acid residue neighboring the N-sulfoglucosamine residue. HS6ST1 predominantly sulfates the IdoA-GlcNS unit, whereas HS6ST2 preferentially sulfates the GlcA-GlcNS and IdoA(2S)-GlcNS units to generate trisulfated disaccharide units in HS, and HS6ST3 has the intermediate preference between HS6ST1 and HS6ST2. The degree of sulfation may also play an important role in regulating the bioactivity of HS-binding proteins. So far, little is known about the mechanism regulating the level of sulfation. In this study, we showed for the first time that cells are able to regulate the degree of 6-O-sulfation by changing their intracellular levels of HS6ST3, which is one of the three enzymes that 6-O-sulfate HS. Moreover, we showed that \( \beta \)-secretase activity may be partly responsible for regulating the intracellular localization of HS6ST3.

Of the three HS sulfotransferases, HS6ST and heparan sulfate 3-O-sulfotransferase, but not HS2ST, can be purified from the culture medium, which indicates that these enzymes are secretory proteins (15). We previously showed that 6-O-sulfotransferase activity could be detected in the conditioned medium of cultured CHO-K1 cells (19). Analysis of the amino acid sequence of HS6ST1 in the culture medium revealed that its N-terminal amino acid was Tyr\(^{39}\). Since Tyr\(^{39}\) and Gln\(^{24}\) are conserved in all HS6ST isoforms (HS6ST1, -2, and -3), we speculated that HS6ST3 may be cleaved at this position. When the conserved QY residues were altered to AY, QA, and AA, the secretion of these mutant proteins was not affected or was even enhanced (Fig. 1B), which indicates that these residues did not participate in HS6ST3 cleavage. Alternatively, the mutations may have resulted in cleavage at a different position (see below).

Although we showed previously that HS6STs are Golgi-resident type II transmembrane proteins (20), there were no experimental data about the cellular compartment in which HS6ST is cleaved. To investigate this, we treated the HS6ST3-GFP-expressing cells with BFA and monensin (Fig. 2B). Both inhibitors almost completely abrogated HS6ST3-GFP secretion but, unexpectedly, had no effect on HS6ST3-GFP cleavage, as determined by 8% polyacrylamide gel electrophoresis of the cell lysates and conditioned medium after removing the N-linked sugar chain with the PNGase treatment (Fig. 2B). Although it remains possible that the extracellular form of HS6ST3-GFP may have been modified in some way that compensates for the loss of molecular weight arising from the cleavage of the N-terminal hydrophobic domain, the above data nevertheless suggest that HS6ST3-GFP is cleaved in the early phase of the secretory pathway, before reaching the trans-Golgi.

This was further confirmed by the Triton X-114 phase separation assay. As shown in Fig. 2B, HS6ST3-GFP was extracted into the aqueous phase after centrifugation regardless of the BFA and monensin treatment. These results suggest that the N-terminal hydrophobic domain of HS6ST3-GFP was cleaved to become soluble protein early in the secretory pathway.

We hypothesized that the N-terminal cytoplasmic and hydrophobic sequences of HS6ST3 would behave like a signal peptide of a secretory protein for several reasons. First, the predicted cytoplasmic domain of HS6ST3 is short (7 amino acids) and has two positively charged amino acids followed by a hydrophobic domain and some small amino acids. This domain was predicted to be a signal peptide by the SOSUI signal analysis (available on the World Wide Web at bp.nuap.nagoya-u.ac.jp/sosui/sosui signal/sosui signal submit.html). Second, the phase partitioning assay (Fig. 2C) demonstrated that HS6ST3-GFP was soluble, suggesting the cleavage of its hydrophobic transmembrane domain. The molecular form of HS6ST3-GFP in the cell was the same as that of the extracellular form, even after treating the cells with BFA (Fig. 2B). Third, when several hydrophilic residues were added to its N terminus, HS6ST3-GFP was retained in the cell (Fig. 2D) as a membrane protein (Fig. 2C). Fourth, substituting the N-terminal hydrophobic domain of HS2ST for that of HS6ST3 rendered the chimeric protein soluble (Fig. 2C) and secretory (Fig. 2D). Although we could not show directly that a signal peptide cleaves HS6ST3-GFP, these experiments strongly suggest that the N terminus of HS6ST3-GFP behaves like a signal peptide of a secretory protein. A series of mutants with substitutions in the conserved QY residues (AY, QA, AA) were also predicted to have signal peptide activity according to SOSUI signal analysis. It is not an easy task to determine the N-terminal amino acid of the cleavage products of the HS6ST3-GFP and its QY mutants, because limiting amounts are available. At the moment, we cannot state categorically whether the N-terminal hydrophobic sequences of HS6ST1 and HS6ST2 behave as signal peptides of secretory protein or not.

We treated the transfected CHO-K1 cells with a series of protease inhibitors and found that the secretion of HS6ST3 was only inhibited by the cell-permeable \( \beta \)-secretase inhibitor.
Z-VLL-CHO (Fig. 3A). We used two lines of CHO-K1 cells stably expressing HS6ST3-GFP for the protease inhibitor assays. In these cell lines, HS6ST activity in the cell layer was 4.67- and 9.84-fold higher than that of the parental CHO-K1 cells. On the other hand, HS6ST activity in the medium was 4.31- and 9.35-fold higher, respectively. Importantly, both cell lines respond to Z-VLL-CHO as well. We could not completely exclude the possibility that the responsiveness of HS6ST3-GFP to Z-VLL-CHO is merely caused by overexpression. However, we assumed that HS6ST3 actually responded to Z-VLL-CHO, since two cell lines with different expression levels responded to this inhibitor. CHO-K1 cell lines overexpressing proteins other than HS6ST3-GFP (SP-GFP, HS6ST1Long-GFP, and HS6ST2Long-GFP) did not respond to Z-VLL-CHO. It may also be important to investigate the effect of the inhibitor on cells that endogenously express HS6ST3. Unfortunately, no method is available at the moment to measure the HS6ST3-specific activity in HS6ST1-, -2-, and -3-expressing cells.

BACE1 is a type I membrane-associated aspartyl protease with β-secretase activity that cleaves APP (30, 31). The few other BACE1 substrates that have been identified include the APP homologues amyloid precursor-like protein APLP1 and -2 (36), P-selectin glycoprotein ligand-1, membrane-bound α2,6-sialyltransferase (21, 37), and low density lipoprotein-receptor related protein (38). Like HS6STs, ST6Gal I is a Golgi-resident enzyme that has a type II transmembrane topology. After the cleavage of the luminal domain of ST6Gal I by BACE1, the enzyme is secreted extracellularly. To investigate whether BACE1 is involved in HS6ST3 secretion, we transiently transfected HS6ST3-GFP-expressing CHO-K1 cells with a BACE1 expression vector and investigated whether this would increase HS6ST3 secretion. Cells treated with or without BFA were counted the number of BACE1-positive cells with or without HS6ST3-GFP expression (Fig. 3C). The relative number of BACE1 and HS6ST3-GFP double positive cells increased by BFA treatment, suggesting that BACE1-positive cells having HS6ST3-GFP lost the intracellular enzyme pool if the secretion was allowed. The molecular weight of the intracellular HS6ST3-GFP did not increase upon the treatment with Z-VLL-CHO (Fig. 3A, bottom). In addition, when the recombinant BACE1-Fc protein was incubated with the HS6ST3-protein A fusion protein, the HS6ST3 protein was not cleaved. Thus, it appears that β-secretase activity indirectly regulates the intracellular levels of HS6ST3. It may be possible that β-secretase cleaves an unidentified protein that resides in the Golgi and with which HS6ST3 interacts; alternatively, β-secretase may affect the secretory pathway in which HS6ST3 is transported. Recently, the Drosophila homologues of human BACE1, namely, DASp1 and DASp2, have been cloned, and it has been shown that these proteases can induce Drosophila HS6ST secretion upon co-transfection in COS7 cells (39). However, it is unclear whether DASp1 and DASp2 cleave Drosophila HS6ST directly or only indirectly up-regulate its secretion. To confirm that even the secretory form of HS6ST3-GFP can accumulate in the Golgi upon Z-VLL-CHO treatment, we stably expressed SP-HS6ST3(-TMD)-GFP in CHO-K1 cells, which is a modified secretory form of HS6ST3 whose N-terminal hydrophobic domain has been replaced with the signal peptide of human preprotrypsin. We found that Z-VLL-CHO treatment also blocked the secretion of this form, since it accumulated in the cell (Fig. 4). This supports the hypothesis that the cleaved form of HS6ST3 is retained in the Golgi apparatus when β-secretase activity is blocked and that the secretion mechanism is β-secretase-dependent. Notably, with regard to the inhibitory effect of Z-VLL-CHO, this inhibitor had little, if any, effect on the secretion of HS6ST1-GFP and HS6ST2Long-GFP. This suggests that the β-secretase-dependent regulation of secretion is specific to HS6ST3 and not to the other HS6STs. Since the C-terminal domains of HS6ST1 and HS6ST3 do not resemble each other, this domain may play an important role in β-secretase-dependent regulation. The reason why the HS6ST3-specific regulation exists is currently unknown. Since HS6ST3 has intermediate substrate specificity between HS6ST1 and HS6ST2, HS6ST3 may specifically participate in the regulation of sulfation level. Alternatively, HS6ST1 and HS6ST2 also regulate the sulfation level by different mechanisms.

We also showed that the inhibition of β-secretase activity increased HS 6-O-sulfation (Fig. 5). The HexA-GlcNS(6S) levels were significantly more increased than the HexA-GlcNAc(6S) and HexA-GlcNS(6S) units. Since the HexA-GlcNS unit is abundant in the untreated cells, it is conceivable that this marked increase in HexA-GlcNS(6S) levels simply reflects the greater levels of the pre-6-O-sulfated substrate in the cell. Notably, we also analyzed the disaccharide composition of BACE1-overexpressing cells and found that the HexA-GlcNAc(6S) and HexA-GlcNS(6S) levels were lower than in the control mock-transfected cells (data not shown). HS proteoglycans colocalize with amyloid β-peptide (Aβ) (40, 41) and promote its aggregation (42). Moreover, HS and heparin bind fibrillar amyloid protein and enhance fibril formation and stability (43). Lindahl et al. (44) also reported that the minimal binding site for the Aβ fibril occurs in N-sulfated hexasaccharide domains and contains critical 2-O-sulfated iduronic acid residues, whereas the binding of Aβ monomers requires 6-O-sulfated groups on glucosamine residues in addition to these residues. Since HS6ST3 has been shown to be expressed in brain, although at a lower level than HS6ST1 and HS6ST2 (16), it will be interesting to determine whether the up-regulation of BACE1 activity not only augments Aβ production by increasing APP cleavage but also facilitates Aβ fibril formation by lowering the levels of HexA-GlcNS(6S) and HexA(2S)-GlcNS(6S) on HS.

REFERENCES
1. Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G., and Yayon, A. (1994) Cell 79, 1005–1013
2. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777
3. Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D., and Vlodavsky, I. (1988) Annu. Rev. Pathol. 130, 393–400
4. Kijlstra, L., and Lindahl, U. (1991) Annu. Rev. Biochem. 60, 443–475
5. Rapraeger, A. C. (1993) Curr. Opin. Cell Biol. 5, 844–853
6. Bullock, S. L., Fletcher, J. M., Beddington, R. S., and Wilson, V. A. (1998) Genes Dev. 12, 1894–1906
7. Fan, G., Xiao, L., Cheng, L., Wang, X., Sun, B., and Hu, G. (2000) FEBS Lett. 467, 7–11
8. Ringvall, M., Ledin, J., Holmborn, K., van Kuppevelt, T., Elin, F., Eriksson, I., Olofsson, A. M., Kjellen, L., and Forsberg, E. (2000) J. Biol. Chem. 275,
29. Haass, C., Lemere, C. A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., and Selkoe, D. J. (1995) *Nat. Med.* 1, 1291–1296
30. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, A., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Bieri, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Trenor, J., Rogers, G., and Citron, M. (1999) *Science* 286, 735–741
31. Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Hewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S. M., Wang, S., Walker, D., Zhao, J., McConlogue, L., and John, V. (1999) *Nature* 402, 537–540
32. Zhang, Y., McLaughlin, R., Goodyer, C., and LeBlanc, A. (2002) *J. Cell Biol.* 156, 519–529
33. Canevari, L., Abramov, A. Y., and Duchen, M. R. (2004) *Neurochem. Res.* 29, 637–650
34. Cuello, A. C. (2005) *Brain Pathol.* 15, 66–71
35. Lin, X., and Perrimon, N. (1999) *Nature* 400, 281–284
36. Li, Q., and Sudhof, T. C. (2004) *J. Biol. Chem.* 279, 10542–10550
37. Kitazume, S., Tachida, Y., Oka, R., Kotani, N., Ogawa, K., Suzuki, M., Dohmae, N., Takio, K., Saido, T. C., and Hashimoto, Y. (2003) *J. Biol. Chem.* 278, 14865–14871
38. von Arnim, C. A., Kinoshita, A., Peltan, I. D., Tangredi, M. M., Herl, L., Lee, B. M., Spoelgen, R., Hshieh, T. T., Ranganathan, S., Battey, F. D., Liu, C. X., Bacskai, B. J., Sever, S., Iruzarry, M. C., Strickland, D. K., and Hyman, B. T. (2005) *J. Biol. Chem.* 280, 17777–17785
39. Kotani, N., Kitazume, S., Kamimura, K., Takeo, S., Aigaki, T., Nakato, H., and Hashimoto, Y. (2005) *J. Biol. Chem.* 280, 17777–17785
40. Snow, A. D., Mar, H., Nochlin, D., Sekiguchi, R. T., Kimata, K., Koike, Y., Lee, B. M., Spoelgen, R., Zhao, J., McConlogue, L., and John, V. (1999) *Nature* 402, 537–540
41. Su, J. H., Cummings, B. J., and Cotman, C. W. (1992) *Neuroscience* 51, 801–813
42. McLaurin, J., Franklin, T., Kuhns, W. J., and Fraser, P. E. (1999) *Annu. Rev. Neurosci.* 23, 233–243
43. Castillo, G. M., Ngo, C., Cummings, J., Wight, T. N., and Snow, A. D. (1999) *J. Neurochem.* 69, 2452–2465
44. Lindahl, B., Westling, C., Gimenez-Gallego, G., Lindahl, U., and Salmivirta, M. (1999) *J. Biol. Chem.* 274, 30631–30635