Alzheimer's β-secretase, β-site amyloid precursor protein-cleaving enzyme, is responsible for cleavage secretion of a Golgi-resident sialyltransferase

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The deposition of amyloid β-peptide (Aβ) in the brain is closely associated with the development of Alzheimer's disease. Aβ is generated from the amyloid precursor protein (APP) by sequential action of β-secretase (BACE1) and γ-secretase. Although BACE1 is distributed among various other tissues, its physiological substrates other than APP have yet to be identified. ST6Gal I is a sialyltransferase that produces a sialylα2,6galactose residue, and the enzyme is secreted out of the cell after proteolytic cleavage. We report here that BACE1 is involved in the proteolytic cleavage of ST6Gal I, on the basis of the following observations. ST6Gal I was colocalized with BACE1 in the Golgi apparatus by immunofluorescence microscopy, suggesting that BACE1 acts on ST6Gal I within the same intracellular compartment. When BACE1 was overexpressed with ST6Gal I in COS cells, the secretion of ST6Gal I markedly increased. When APP_{SW} (Swedish familial Alzheimer's disease mutation), a preferable substrate for BACE1, was coexpressed with ST6Gal I in COS cells, the secretion of ST6Gal I significantly decreased, suggesting that that the β-cleavage of overexpressed APP_{SW} competes with ST6Gal I processing. In addition, BACE1-Fc (Fc, the hinge and constant region of IgG) chimera cleaved protein A-ST6Gal I fusion protein *in vitro*. Thus, we conclude that BACE1 is responsible for the cleavage and secretion of ST6Gal I.

Alzheimer's disease is a widespread neurodegenerative dementia-inducing disorder. Generation of amyloid β-peptide (Aβ) is regarded as a crucial process for the pathogenesis of Alzheimer's disease (1, 2). For initiation of Aβ formation, amyloid precursor protein (APP) is cleaved by β-secretase to generate a soluble NH_2-terminal fragment (APPsβ) and a 12-kDa COOH-terminal fragment (C99), which remains membrane bound. C99 is further cleaved by γ-secretase, resulting in production of pathogenic Aβ (3, 4). In an alternate pathway, APP is cleaved by α-secretase within an Aβ sequence to generate a soluble NH_2-terminal fragment (APPsα) and 10-kDa membrane-bound COOH-terminal fragment (C83) (5, 6). C83 is also cleaved by γ-secretase, resulting in production of nonpathogenic p3 peptide. Because β- and γ-secretases play a critical role for Aβ formation, inhibitors of these proteases are promising therapeutics for Alzheimer's disease (7, 8). β-site APP-cleaving enzyme (BACE)1, β-amyloid-converting enzyme 1, has been recently identified as a membrane-bound aspartic protease (9–11) and is now considered to carry the major β-secretase activity *in vivo* (12, 13). Northern blot analysis showed that BACE1 mRNA is expressed in most peripheral tissues, including those of the brain, but its physiological substrates other than APP have yet to be identified.

Many glycosyltransferases are type II membrane proteins and are retained in the Golgi apparatus for oligosaccharide biosynthesis (14). Some of these enzymes are then cleaved by an endogenous protease, or proteases, and secreted out of the cell. Indeed, many glycosyltransferases have been found as extracellular soluble forms in bodily fluids such as serum, colostrum, and milk (15–19). It has been well documented that the proteolytic cleavage and secretion of glycosyltransferases into bodily fluids are affected by various pathological conditions such as malignant transformation and inflammation, but the endogenous protease responsible for the cleavage and secretion has not yet been identified (20). In the present study, we demonstrate that BACE1 is involved in the proteolytic cleavage of a Golgi-resident sialyltransferase, ST6Gal I, that produces a sialylα2,6galactose residue.

Materials and Methods

Materials. Tissue culture media and reagents, including DMEM and Lipofectin, were purchased from Invitrogen. Protein A Sepharose Fast Flow was purchased from Amersham Pharmacia Biotech. Columns for DNA purification were obtained from Qiagen (Chatsworth, CA.). Protein molecular weight standards were purchased from Bio-Rad. [^{35}S]Express protein-labeling mix was purchased from DuPont/NEN. BACE inhibitor, KTEEISEVN(Sta)VAEF, was purchased from Bachem. A polyclonal antibody, E41, which specifically recognizes the N terminus of soluble ST6Gal I, was prepared, by immunizing a synthetic peptide, EFQMPKC, which was conjugated with keyhole limpet hemocyanin (21).

Expression Plasmid. For transient transfection experiment, ST6Gal I-pSVL and ST6Gal I FLAG-pSVL were constructed as described previously (22). For a stable expression experiment, ST6Gal I FLAG-pcDNA3.1 was constructed by excision of ST6Gal I FLAG from ST6Gal I-pBluescript with *Bam*HI and *Xho*I, followed by ligation into the *Bam*HI and *Xho*I sites of pcDNA3.1 (Invitrogen). APP_{SW}-pcDNA was generated by ligating the full length APP_{SW} cDNA into the *Xba*I and *Apa*I sites of pcDNA3.1 (23). BACE-Fc chimera protein was generated by inserting human BACE1 amino acids 1–460 into the *Cla*I and *Bam*HI sites of PEF-Fc encoding the Fc region of human IgG1 in frame. The protein A-ST6Gal I chimera protein was generated by inserting ST6Gal I amino acids 27–103 into *Eco*RI and *Xho*I sites of pCDSA encoding the signal peptide plus IgG-binding domain of protein A (24).

Cell Culture and Transfection. COS-7 or human embryonic kidney (HEK)293 cells maintained in DMEM/10% FBS were plated

Abbreviations: Aβ, amyloid β-peptide; APP, amyloid precursor protein; APP_{SW}, Swedish familial Alzheimer's disease mutation of APP; BACE, β-site APP-cleaving enzyme; HEK, human embryonic kidney; Fc, the hinge and constant region of IgG; SNA, *Sambucus nigra*.

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on 100-mm tissue culture dishes and grown in a 37°C, 5% CO_2 incubator until 50–70% confluent. Cells were transfected by using the Lipofectin method and Opti-MEM I (25). Expression of transfected proteins was typically allowed to continue for 16–36 h. To obtain rat ST6Gal I or myc-tagged human BACE1 transfectants, HEK293 cells were transfected with either ST6Gal I FLAG-pcDNA3.1 or BACE1 myc-pcDNA3.1. After selection in culture medium supplemented in G418, a high-expressor clone of ST6Gal I or BACE1 was obtained by limiting dilution.

Immunofluorescence Microscopy. HEK293 cells that stably expressed ST6Gal I FLAG were transfected with cDNA of BACE1-myc. At 24 h after transfection, cells were rinsed with PBS and fixed with ice-cold methanol for 10 min and then incubated in PBS with 5% goat serum as a blocking step. For indirect immunofluorescence experiments, cells were incubated for 45 min with anti-ST6Gal I rabbit polyclonal antibody plus anti-myc mAb (Invitrogen) at 1:100 in blocking buffer (25). Cells were washed with PBS then incubated with Alexa Fluor 488 goat anti-rabbit IgG plus Alexa Fluor 594 goat anti-mouse IgG antibodies (Molecular Probes) at 1:100 in blocking buffer for 45 min. After PBS washes, cells were mounted in 30% glycerol and observed by using an Axiovert 100 M inverted microscope (Zeiss LSM510) by using C-Apochromat (×63, 1.4 n.a.) oil immersion objective. Appropriate excitation and barrier filters were used to observe fluorescence.

Pulse–Chase Analysis and Immunoprecipitation of Transiently Expressed Proteins. Metabolic labeling of cells and immunoprecipitation of expressed proteins were performed essentially as previously described (22, 25). COS cells were cotransfected with rat ST6Gal I and either human BACE1 cDNA or vector alone. Cells were then labeled by using [^{35}S]Express protein labeling mix (100 μCi/ml) in methionine- and cysteine-free DMEM for 1 h and chased in 4 ml of DMEM/10% FBS for 3 h. Cells were lysed in immunoprecipitation buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/5 mM EDTA/0.5% Nonidet P-40/0.1% SDS). Immunoprecipitations of expressed proteins were processed as described previously (25). Immunoprecipitated proteins were denatured in Laemmli sample buffer with 5% β-mercaptoethanol by boiling for 5 min. Immunoprecipitated proteins were analyzed by using 5–20% gradient SDS–polyacrylamide gels (26), and radiolabeled proteins were visualized by using the BAS 2000 radio image analyzer (Fuji). The percentage of secretion, taken as an average of three independent experiments, was defined as the ratio of the radioactivity of soluble ST6Gal I in the medium to the total ST6Gal I radioactivity (medium + cells).

For competition experiments, COS cells were cotransfected with ST6Gal I-pSVL plus either APP_{SW}, C99, or control pcDNA3.1 vector, and then labeled for 1 h and chased for 6 h. ST6Gal I immunoprecipitates from both cell lysates and media were analyzed as described above.

Western Blotting. For detection of APP and APPsβ, cell lysates, and media of COS cells transiently expressing APP were collected 24 h after transfection. APPsβ was pulled down by using heparin–agarose (Pierce) from the media. APPsβ and cell lysates (20 μg of protein) for APP detection were taken in Laemmli sample buffer (26), subjected to 4–20% gradient SDS/PAGE, and then transferred to nitrocellulose membrane. The blotted membrane was incubated with antibodies 6E10 (1:500) and βNL (1:1,000) for APP and APPsβ, respectively, followed by incubation with appropriate second antibody, horseradish peroxidase–goat anti-mouse or rabbit IgG (Cappel), and then visualized by using chemiluminescent substrate (Pierce).

For analysis of soluble secreted ST6Gal I, COS cells were cotransfected with rat ST6Gal I FLAG-pSVL and with either human BACE-pcDNA3.1 cDNA or control pcDNA vector alone. At 24 h after transfection, soluble secreted ST6Gal

Fig. 1. Cleavage of ST6Gal I for secretion is sequence specific. (*A*) Schematic structure of rat ST6Gal I and its soluble secreted form. (*B*) Site-directed mutagenesis was carried out with primers incorporating the desired mutations with the QuickChange system (Stratagene). COS cells transiently expressing wild-type or mutant ST6Gal I in the pSVL expression vector, were pulse labeled and chased for 6 h for further immunoprecipitation analysis by using anti-ST6Gal I antibody (C, cell lysates; M, media.). (*C*) The percentage of secretion (mean ± SD, $n = 3$), was defined as the ratio of the radioactivity of soluble ST6Gal I in the medium to the total ST6Gal I radioactivity (medium + cells). Significant difference is indicated with an asterisk ($P < 0.05$).

I-FLAG in the media was pulled down with M2-agarose (Sigma) and analyzed by immunoblotting by using E41 polyclonal antibody (1:500).

***Sambucus nigra* (SNA) Lectin Blotting.** Microsome fractions were prepared from HEK293 cells that stably express BACE1 or from their parent cells, and equivalent amounts of proteins (10 or 40 μg for SNA lectin staining) were subjected to 4–20% gradient SDS/PAGE and transferred to nitrocellulose membrane. The blotted membrane was incubated with 1% BSA in 10 mM sodium phosphate buffer (pH 7.2), incubated with 10 μg/ml of SNA-horseradish peroxidase (EY Laboratories), and then visualized by using chemiluminescent substrate. Twenty micrograms of each microsome fraction were also subjected to 4–20% gradient SDS/PAGE and then stained with Coomassie (Wako Biochemicals, Osaka).

***In Vitro* BACE Assay.** Both BACE-Fc and protein A-ST6Gal I proteins were purified from 20 ml of culture media of COS cells that transiently expressed these proteins by absorbing them to 20 μl of protein A-Sepharose and IgG-Sepharose (50% suspension in PBS), respectively. Reaction mixture contained 50 mM sodium-acetate buffer (pH 4.5), 1 μl of BACE-Fc and protein A-ST6Gal I preparation, and protease inhibitors for possible contaminating proteases that associate with Sepharose beads [Complete (Roche)], 10 μM pepstatin/1 μM Leupeptin/1 mg/ml of pepstatin/2 μM Amastatin] with or without 30 or 100 nM β-secretase inhibitor (Bachem). The mixture was incubated at 37°C for 0 or 30 min with rotation. Reaction was quenched and the product was analyzed by immunoblotting with anti-ST6Gal I antibody.

Results

We have studied ST6Gal I as a model protein for understanding the molecular mechanisms of the cleavage secretion of Golgi glycosyltransferases. Because our previous studies showed that soluble secreted ST6Gal I from the cells starts at Glu-41 (Fig. 1*A*; ref. 22), we analyzed the effects of amino acid substitutions in the region from Leu-37 to Glu-41 on ST6Gal I secretion. COS cells were transiently transfected with a series of mutant ST6Gal I cDNA, metabolically labeled, and chased. Immunoprecipitated ST6Gal I proteins from both cell lysates and media were then analyzed (Fig. 1 *B* and *C*). Substitution of Lys-40 to Ala or Leu led to a marked decrease of ST6Gal I secretion. The level of ST_{K40A} secretion was lower than that of ST_{K40L}, in which substituted amino acid residue was hydrophobic and bulky. Replacement of Leu-37 with Ala also decreased ST6Gal I secretion. Substitutions at Gln-38 or Glu-41 showed subtle effects on ST6Gal I secretion. Thus the single amino acid substitutions led to substantial decrease of ST6Gal I secretion, suggesting that the protease, or proteases, responsible for the cleavage and secretion are sequence specific. BACE1 is a sequence-specific protease (27) that expressed in the Golgi apparatus of various types of cells (10). Site-directed mutagenesis of cleavage site of APP revealed that a minimum recognition region of BACE1 is from P3 to P2′ position, which contains hydrophobic amino acid at P1 position and acidic one at P1′ position (27). Although ST6Gal I does not contain a hydrophobic amino acid at the putative P1 position, it does contain an acidic one, glutamic acid, at P1′ position. BACE1 cleaves APP between Met-671 and Glu-672, i.e., 28 amino acids distant from the transmembrane domain, whereas ST6Gal I is cleaved between Lys-40 and Glu-41, i.e., 14 amino acids distant from the transmembrane domain. The distance of the latter is much shorter than that of the former, but BACE1 cleavage tolerates changes of distance from the membrane, as previously reported (27). Therefore, we asked whether BACE1 is responsible for the cleavage of ST6Gal I.

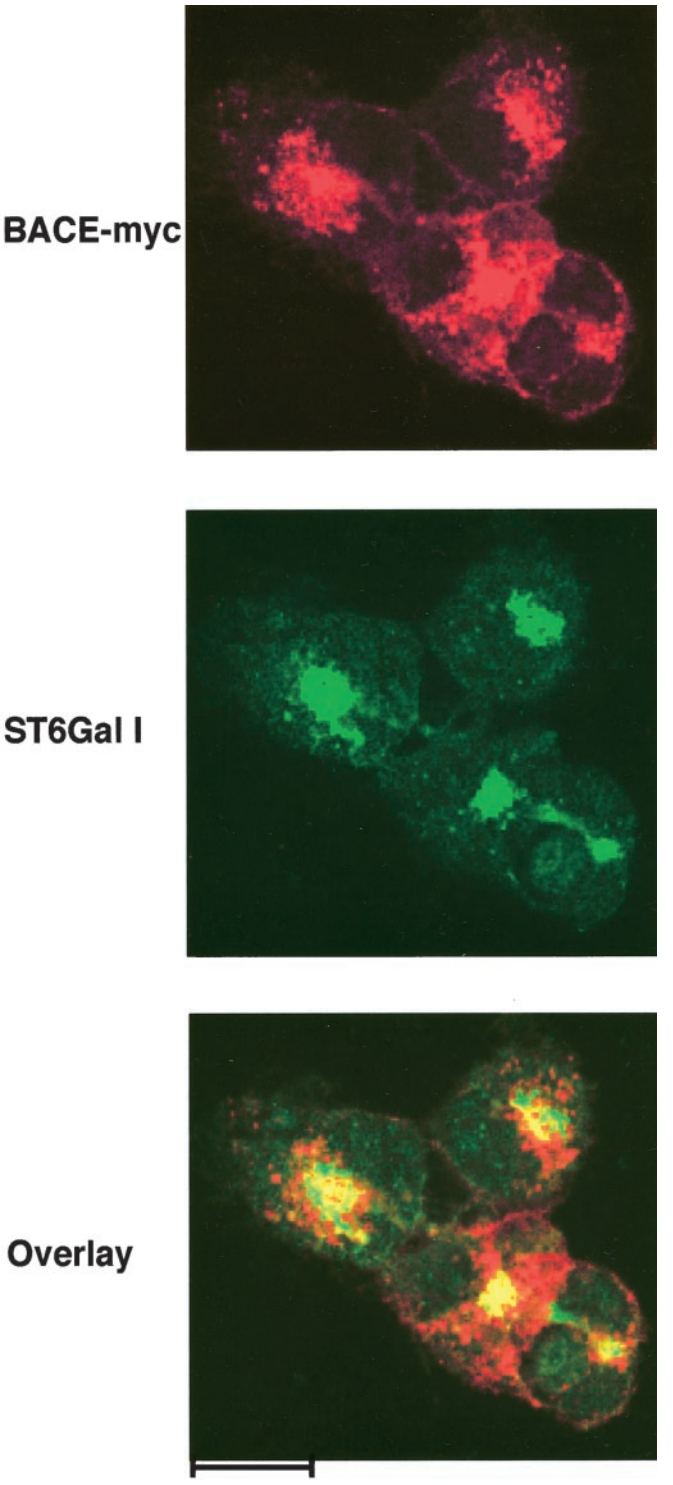

Fig. 2. Intracellular colocalization of ST6Gal I and BACE1-myc in HEK293 cells. HEK293 cells that stably expressed ST6Gal I were transfected with cDNA of BACE1-myc. Cells were stained with anti-myc and anti-ST6Gal I antibodies and then subjected to confocal fluorescence microscopy. (*Top*) Anti-myc immunostaining (red). (*Middle*) Anti-ST6Gal I immunostaining (green). (*Bottom*) Overlay of myc and ST6Gal I immunostaining. Their colocalization staining appears yellow. (Bar = 20 μm.)

First, we examined whether ST6Gal I exhibits any intracellular colocalization with BACE1. HEK 293 cells that stably expressed ST6Gal were transiently transfected with cDNA of

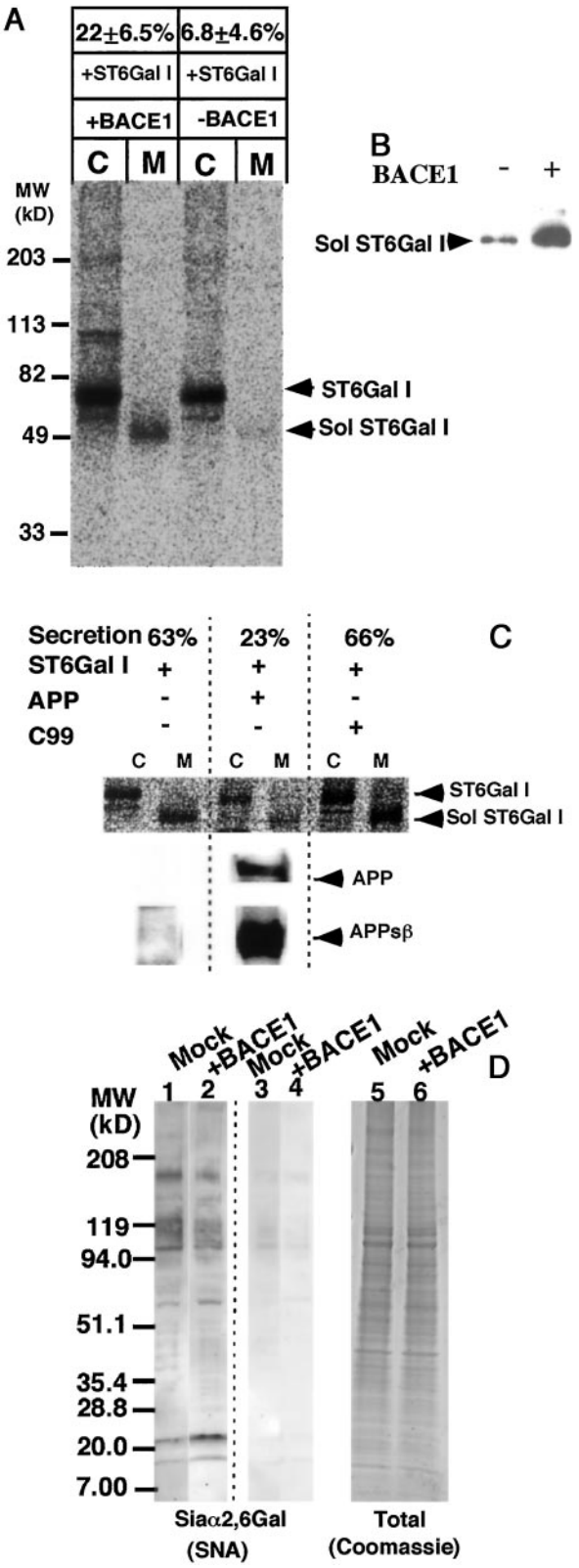

Fig. 3. Involvement of BACE1 in ST6Gal I secretion. (*A*) COS cells expressing rat ST6Gal I in the pSVL vector and either human BACE1 or vector were pulse labeled and chased for 3 h for further immunoprecipitation analysis by using anti-ST6Gal I antibody (C, cell lysates; M, media.) The percentage of secretion (mean ± SD, $n = 3$) was defined as the ratio of the radioactivity of soluble ST6Gal I in the medium to the total ST6Gal I radioactivity (medium + cells). (*B*) COS cells were transiently cotransfected with ST6Gal I FLAG-pSVL and either human BACE1 or vector. After 24 h of expression, soluble ST6Gal I FLAG in the media were pulled down with M2-agarose and then detected with an E41 antibody. (*C*) Cells expressing ST6Gal I plus either APP_{SW}, C99 or control pcDNA3.1 vector, were pulse labeled and chased for 6 h, and analyzed as shown in *A*. The percentage of secretion, taken as an average of two independent experiments, was defined as the ratio of the radioactivity of soluble ST6Gal I in the medium to the total ST6Gal I radioactivity (medium + cells). APP and APPsβ were detected with 6E10 and βNL antibodies, respectively. (*D*) Microsome fractions were prepared from HEK293 cells that stably express BACE1 or from their parent cells, and equivalent amounts of proteins were used for staining with SNA lectin that recognizes Siaα2,6Gal oligosaccharide structure. (For SNA lectin staining, 40 μg of protein was used for lanes 1 and 2, 10 μg for lanes 3 and 4, and 20 μg for Coomassie staining.)

BACE1 tagged with myc epitope, which was fused to the COOH terminus of BACE1 protein (BACE1-myc). The cells were stained with anti-myc epitope and anti-ST6Gal I antibodies, then subjected to confocal fluorescence microscopy. ST6Gal I was localized in the Golgi apparatus as reported previously (25) (Fig. 2). BACE1-myc was colocalized with ST6Gal I in the Golgi apparatus, suggesting that BACE1 could act on the ST6Gal I within the shared intracellular compartment. Additional BACE1-myc staining appeared to be in the endosome (10).

We then analyzed the effect of overexpression of BACE1 on secretion of ST6Gal I. When BACE1 was transiently expressed in COS cells together with ST6Gal I, secretion of ST6Gal I was significantly increased (22% secretion) as compared with the control experiment (6.8% secretion) (Fig. 3*A*). The result indicates that the BACE1 overexpression induces secretion of ST6Gal I. A polyclonal antibody, E41, which specifically recognizes the N terminus of soluble ST6Gal I, was prepared by immunizing a synthetic peptide, EFQMPKC, which was conjugated with keyhole limpet hemocyanin. When BACE1 was coexpressed with ST6Gal I in COS cells, soluble ST6Gal I that is detected with the antibody E41 significantly increased, suggesting that BACE1 cleaves at exactly the same position as that of the endogenous protease (Fig. 3*B*) (21). Next we examined whether APP_{SW}, which is a preferable substrate for BACE1, competes with ST6Gal I with regard to the secretion by the endogenous protease. Cotransfection of APP_{SW} cDNA with ST6Gal I diminished the ST6Gal I secretion (23%; see Fig. 3*C*) along with the generation of Aβ, whereas cotransfection with C99 cDNA, or a vector, did not affect the secretion of ST6Gal I at all (66% for C99 and 63% for a vector). This result indicates that the β-cleavage of overexpressed APP_{SW} competes with ST6Gal I processing. Thus we conclude that BACE1 is responsible for the cleavage and secretion of ST6Gal I in the cells.

To demonstrate that ST6Gal I is a BACE1 substrate *in vitro*, we developed an assay system in which purified BACE1-Fc (10) was incubated with purified ST6Gal I that lacked a transmembrane domain, instead containing a signal peptide plus protein A, protein A-ST6Gal I (24). As shown in Fig. 4 *A* and *B*, purified BACE1-Fc cleaved protein A-ST6Gal I to remove the protein A part, resulting in the soluble ST6Gal I, which was observed at 49 kDa, the same as that of the soluble secreted form *in vivo* (28). BACE inhibitor, KTEEISEVN-(Sta)VAEF, in which Leu was substituted with statine for P1 position of a APP analogue peptide, was inhibitory for the ST6Gal I cleavage. The statine peptide showed dose-dependent inhibition; 20 and 50% inhibition was observed at the concentration of 30 and 100 nM, respectively. Because BACE1 cleaves ST6Gal I to secrete from the cell, BACE1 overexpression could reduce not only the level of cellular ST6Gal I but also α2,6-sialylation in the cell. Indeed, HEK293 cells, which stably overexpress BACE1, had lower levels of sialylα2,6galactose residue as compared with their parent cells, judging from the blot stained with SNA lectin that specifically

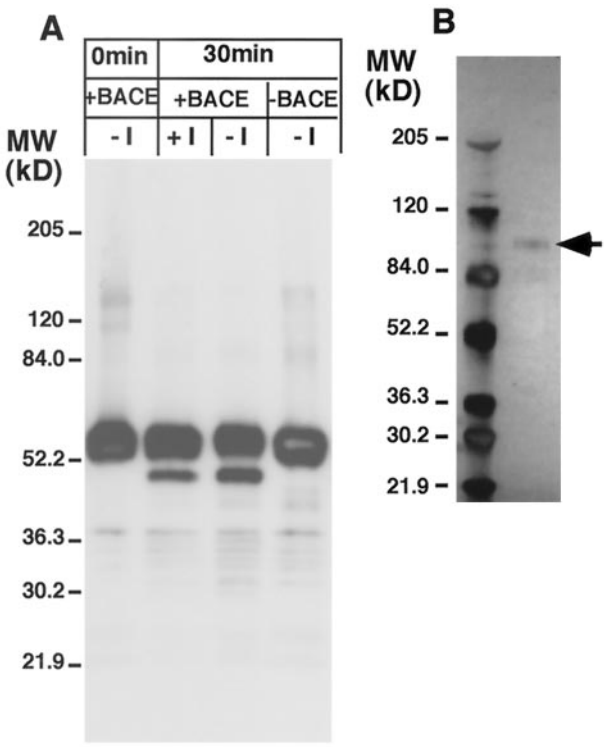

Fig. 4. Purified BACE1-Fc cleaves protein A-ST6Gal I *in vitro*. (*A*) Both BACE-Fc and protein A-ST6Gal I proteins, respectively, were purified from culture media of COS cells expressing those proteins by absorbing them to protein A-Sepharose and IgG-Sepharose. Reaction mixture, containing BACE-Fc and protein A-ST6Gal I and protease inhibitors for possible contaminating proteases that associate with Sepharose beads, was incubated at 37°C in the presence or absence of 100 nM BACE inhibitor (I) for 0 or 30 min with rotation. Reaction was quenched and analyzed by immunoblotting with anti-ST6Gal I antibody. The experiment was repeated four times, and the representative result is shown in *A*. (*B*) Silver-stained gel of purified BACE-Fc.

recognizes this carbohydrate structure (Fig. 3*D*). Taken together, we concluded that BACE1 cleaves ST6Gal I *in vivo* as well as *in vitro* and affects the cellular level of α2,6-sialylation.

Discussion

The interaction of a sialylα2,6galactose residue, which is synthesized solely by ST6Gal I, with a B cell-specific lectin, CD22/Siglec-2, is important for B cell function (29–31), and mice deficient in ST6Gal I showed reduced levels of serum IgM (32). Our results raise the possibility that BACE1 deficiency may cause abnormalities in ST6Gal I metabolism and α2,6-sialylation. Indeed, BACE1 is expressed in various tissues including liver and leukocytes, in which ST6Gal I is abundantly expressed (10). It is interesting that mice deficient in BACE1 appeared to exhibit abnormal levels of serum globulin (see supplementary data for ref. 12 at http://neurosci.nature.com/web_specials), supporting the notion that BACE1 deficiency may cause an abnormality in B cells through aberrant ST6Gal I metabolism. BACE1 may be also responsible for the secretion of other glycosyltransferases. Indeed, we found that overexpression of BACE1 in mammalian cells induced significant changes in glycoconjugate metabolism other than α2,6-sialylation (S.K., N. Kotani, Y.T., R.O., and Y.H., unpublished observations). Mice deficient in BACE1, however, appeared to be normal at the age of 3$\approx$4 months (12, 13). It is notable that mice deficient in some glycosylation enzymes that appear to grow normally show subtle neurological abnormalities with increasing age; glycosphingolipid-deficient mice show lethal audiogenic seizures induced by a sound stimulus (33) or deficits in motor behavior associated with either dysmyelination or demyelination depending on defect that advance with age (34), and mice deficient in the polysialyltransferase show impaired long-term potentiation and long-term depression of hippocampal neurons with increasing age (35). Therefore, BACE1 deficiency or inhibition may lead to rather modest defects, not manifested under normal conditions, through dysmetabolism of glycosyltransferases and glycoconjugates.

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