BACE1 is a membrane-bound aspartic protease that cleaves the amyloid precursor protein (APP) at the β-secretase site, a critical step in the Alzheimer disease pathogenesis. We previously found that BACE1 also cleaved a membrane-bound sialyltransferase, ST6Gal I. By BACE1 overexpression in COS cells, the secretion of ST6Gal I markedly increased, and the amino terminus of the secreted ST6Gal I started at Glu41. Here we report that BACE1-Fc chimera protein cleaved the A-ST6Gal I fusion protein, or ST6Gal I-derivative peptide, between Leu37 and Gln38, suggesting that an initial cleavage product by BACE1 was three amino acids longer than the secreted ST6Gal I. The three amino acids, Gln38-Leu37-Gln38, were found to be truncated by exopeptidase activity, which was detected in detergent extracts of Golgi-derived membrane fraction. These results suggest that ST6Gal I is cleaved initially between Leu37 and Gln38 by BACE1, and then the three-amino acid sequence at the NH2 terminus is removed by exopeptidase(s) before secretion from the cells.

The deposition of amyloid β-peptide (Aβ) in the brain is a hallmark of the pathogenesis of Alzheimer’s disease (1). Aβ, a 39–43-amino acid peptide, is a proteolytic product derived from the amyloid precursor protein (APP). The β-secretase initially generates the NH2 terminus of Aβ, cleaving APP to produce a soluble NH2-terminal fragment (APPsβ) and a 12-kDa COOH-terminal fragment (C99) that remains membrane bound. C99 is further cleaved by γ-secretase, resulting in the production of pathogenic Aβ peptide (2, 3). As an alternative processing pathway, α-secretase cleaves within the Aβ sequence to produce a soluble NH2-terminal fragment (APPαs).

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Materials—Eight-week-old male Wistar rats, maintained in specific-pathogen free conditions, were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Tissue culture media and reagents, including Dulbecco’s modified Eagle’s medium and LipoFectin, were purchased from Invitrogen. Protein A-Sepharose Fast Flow was purchased from Amersham Biosciences. CDP-hexanolamine-Sepharose was a gift from Dr. K. N. Colley (University of Illinois at Chicago). Columns for DNA purification were obtained from Qiagen Inc. (Chatsworth, CA). PCRs were performed using LA Taq polymerase (Sigma). Protein molecular weight standards were purchased from Bio-Rad. BACE inhibitor, KTEEEEVNSTaVAEF (in which Leu was substituted with statine (S5)), for the P1 position of an APP analogue peptide, was purchased from Bachem (Bubendorf, Switzerland). A polyclonal antibody, Q38, which specifically recognizes the NH2 terminus of ST6Gal I-Q38 fragment, was prepared by immunizing a synthetic peptide, QAKEFQC, conjugated with keyhole limpet hemocyanin (23).

Expression Plasmid—For the transient transfection experiment, ST6Gal I FLAG-pSVL, protein A ST6Gal I-pcDNA, and BACE Fc-pEF were constructed as described previously (19, 24, 25). To generate ST6Gal I FLAG-pcDNA, polymerase chain reaction was performed using ST6Gal I FLAG-pSVL templates with primer 1 (5’-CGCGAATTCTAAGAAGGGGACGTACTGA-3’) and primer 2 (5’-GGGCCATCTGGAACTCTGCTGCTTGCAGTGTAAG-3’). PCR product purified with the QIAEX II Gel extraction system (Qiagen Inc.) was digested with EcoRI and XhoI and then ligated into the pcDNA EcoRI-XhoI site. ST6Gal I FLAG-pcDNA was generated from ST6Gal I FLAG-pcDNA using a QuikChange site-directed mutagenesis kit (Invitrogen) with primer 3 (5’-TTTCACTGCGAGCAGAGTCCAGAGTGCC-3’) and primer 4 (5’-GGGTCATCTGCGAGCATCTGGCTGAGTGATGAA-3’) (24).
in a 37 °C, 5% CO₂ incubator until 50–70% confluent. Cells were transfected using Lipofectin and Opti-MEM I. Expression of proteins was typically allowed to continue for 24 to 48 h.

To analyze the soluble secreted form of ST6Gal I-FLAG, COS cells were transfected with ras ST6Gal I-FLAG-pSVL. At 48 h after transfection, soluble ST6Gal I-FLAG secreted in the media was pulled down with M2-agarose (Sigma) and analyzed by immunoblotting using either the E41 (1:500), Q38 (1:1000), or anti-ST6Gal I polyclonal antibody (1:1000). Pre-absorption of the E41 antibody was performed using peptide EFQMPK (10 μg/ml) or FQMPKC (10 μg/ml). Horseradish peroxidase-anti-rabbit IgG (Cappel, 1:1000) was used as a secondary antibody, and chemiluminescent substrate (Pierce) was used for detection (19).

Rat hepatoma FTO2B cells that endogenously express ST6Gal I at a high level were transfected with human BACE1-myc-pcDNA3.1 or the control vector. At 48 h after transfection, soluble ST6Gal I secreted in the media was immunoprecipitated with anti-ST6Gal I rabbit polyclonal antibody. One-third of the immunoprecipitant was used for detection with the anti-ST6Gal I antibody, and the rest of the sample was used for detection with the E41 antibody. Each sample was treated with Laemmli sample buffer (26), subjected to 4–20% gradient SDS-PAGE, and then transferred to a nitrocellulose membrane. The membrane was incubated with either anti-ST6Gal I or the E41 polyclonal antibody.

Rat plasma (200 μl) was diluted with the buffer containing 10 mM sodium cacodylate, pH 6.5, 0.1% Triton CF-54, and 0.15 M NaCl. Protein A-Sepharose (30 μl) was added to the mixture and rotated for 30 min to remove adhesive proteins. After the beads were removed by centrifugation, 20 μl of CDP-hexanolamine-agarose was then added to pull down sialyltransferase proteins. After rotation for 16 h, beads were washed with phosphate-buffered saline. Sialyltransferase proteins immobilized to the beads were analyzed by immunoblotting with either anti-ST6Gal I or the E41 polyclonal antibody.

In Vitro BACE1 Assay Using Protein A-ST6Gal I Fusion Protein—When protein A-ST6Gal I-FLAG or protein A-ST6Gal I FLAG was used as a substrate, both BACE1-Fc and protein A-ST6Gal I fusion proteins were purified from 20 ml of culture media of COS cells that transiently expressed these proteins by absorbing them in 20 μl of protein A-Sepharose and IgG-Sepharose (50% suspension in phosphate-buffered saline), respectively. These preparations of BACE1-Fc and protein-A-ST6Gal I were mixed, resulting in a final volume of 20 μl, which comprised 50 mM sodium acetate buffer (pH 4.5) and protease inhibitors for possible contaminating proteases associated with Sepharose beads (Complete (Roche), 10 μg pepstatin, 1 μg leupeptin, 1 mg/ml pepstatin, and 2 μg aprotinin). The mixture was incubated at 37 °C for 2 h with rotation. The reaction was then terminated, and the product was analyzed by immunoblotting with anti-FLAG (M2) antibody.

In Vitro BACE1 Assay Using Synthetic Peptides—When peptides were used as substrates for assay, BACE1-Fc was purified from 50 ml of culture medium of COS cells. Each peptide (0.1 mM) was incubated for 16 h in 50 μl of the reaction mixture as described above. KTEEISENV(T)α(1-methylethyl-β-aminoethylacetyl) was used as a β-secretase inhibitor. After the incubation the reaction mixture was centrifuged to remove immobilized BACE1-Fc. The products were separated on a reversed-phase HPLC C30-UG-5 column (4.6-mm i.d. × 250 mm, No muru Chemical Co., Japan) using a Waters model 600E HPLC system, equipped with a Senshu model SSC-5200 (Tokyo, Japan) UV detector. The sample, applied to the column equilibrated in 10% acetonitrile, was then eluted with a gradient of 10–50% acetonitrile for 40 min. The elution rate was 1 ml/min. For MALDI-TOF MS analysis, peptide DYEALTLQAKEFQMPKSQE was incubated with BACE1-Fc in a 5-fold scale, and each peak, separated by HPLC, was collected manually.

NH2-terminal Amino Acid Sequence Analysis—Protein A-ST6Gal I-FLAG, prepared from 120 ml of culture medium, was cleaved by BACE1-Fc, and the soluble proteins yielded were precipitated with 75% ice-cold ethanol at −20 °C for 16 h. An aliquot of the precipitant was analyzed by SDS-PAGE and stained with silver nitrate (Daiichikagaku, Tokyo, Japan). The rest of the sample was subjected to SDS-PAGE followed by electrical blotting to an Immobilon membrane (Millipore). After staining with Coomassie Blue, the band of soluble ST6Gal I-FLAG (~49 K) was excised, and its amino-terminal amino acid sequence was determined with a Procise 492 LCSC protein sequencer (Applied Biosystems).

Exopeptidase Assay Trims NH2-terminal QAK Sequence of Q38 Form of ST6Gal I—ST6Gal I- or ST6Gal Igα3-αST6Gal I Q38 form that starts at Glu38 was prepared by cleaving protein A-ST6Gal I-FLAG or protein A-ST6Gal Igα3-FLAG by BACE1 in vitro as described above. The Q38 form protein as a substrate was mixed with a micromolar fraction (10 μg of protein) or rat Golgi membrane (10 μg of protein) pretreated with 1% Triton X-100. The mixture was incubated at 37 °C for 2 h in 20 μl of 50 mM sodium acetate buffer (pH 4.5) containing 0.1 mg/ml bovine serum albumin. Incubation was terminated by the addition of a Laemmli sample buffer (25). An aliquot of the incubation mixture was subjected to 4–20% gradient SDS-PAGE, and then the separated proteins were transferred onto a nitrocellulose membrane. The ST6Gal I-FLAG protein was detected by anti-FLAG antibody, and E41 form was detected by the E41 antibody. Several protease inhibitors such as amastatin, bestatin, 1,10-phenanthrolin, and EDTA were added to the reaction mixture to prevent the consumption of their inhibitory potency.

RESULTS

Secreted ST6Gal I from the Cells Starts at Glu38—Previously, we used ST6Gal I as a model molecule for studying how glycosyltransferases are cleaved and secreted from the cells (24). We found that BACE1 was responsible for the cleavage and secretion of ST6Gal I, i.e. overexpression of BACE1 together with rat ST6Gal I in COS cells increased the secretion of a soluble ST6Gal I, which had a Glu38→Phe38→Gln38→Met44→Pro54-Lys66 sequence at the NH2-terminus of soluble ST6Gal I (19). We prepared a polyclonal antibody, E41, that recognized the NH2-terminal sequence of soluble ST6Gal I. In the pres-
ent study, the specificity of the E41 antibody was further characterized by a pre-absorption experiment showing that the antibody was absorbed by the peptide EFQMPK but not by FQMPK (Fig. 1A). Using the E41 antibody, we also examined the secretion of endogenous ST6Gal I in rat hepatoma FTO2B cells, which express high levels of the ST6Gal I protein. Soluble ST6Gal I, immunoprecipitated with anti-ST6Gal I antibody from the media of FTO2B cells, was detected with the E41 antibody (Fig. 1B). With BACE1 overexpression in FTO2B cells, the ST6Gal I secretion markedly increased, and the increased soluble enzyme was also recognized by the E41 antibody. A similar form of soluble ST6Gal I was also present in rat plasma. The plasma enzyme was partially purified using CDP-hexanolamine-agarose resin and subjected to immunoblotting analysis. The plasma enzyme reacted with the E41 antibody as well as anti-ST6Gal I antibody (Fig. 1C). By pre-absorption of E41 antibody with peptide EFQMPK, E41 staining of the plasma enzyme was reduced (data not shown). These data suggest that in vivo cleavage and secretion of endogenous ST6Gal I are also mediated by BACE1.

BACE1 Cleaved ST6Gal I-derived Peptide between Leu37 and Gln38—We confirmed that the secreted ST6Gal I starts at Glu41 in vivo as well as in cultured cells (19), but in vitro studies by others (21, 22) on BACE1 cleavage site preference showed that Lys40 residue at the P1 position is not a preferable amino acid for BACE1 cleavage. We therefore analyzed BACE1-dependent cleavage of a peptide substrate, DYEALTLQAKEFQMPKSQE, which corresponds to Asp31/Glu49 of ST6Gal I sequence. The peptide substrate was incubated with purified BACE1-Fc chimera, and the products yielded were analyzed by reversed-phase HPLC. We detected two peptide peaks as products, the retention times of which corresponded to those of authentic peptides, DYEALTLQAKEFQMPKSQE and DYEALTL, respectively. Several other peaks (marked with an asterisk) in the HPLC chromatogram were also observed in the control reaction mixture without peptide substrate and shown by MALDI-TOF MS analysis to be non-peptide components. We did not detect peptide peaks corresponding to DYEALTLQAK and EFQMPKSQE on HPLC and MALDI-TOF MS analyses.
This result indicates that BACE1-Fc cleaves the peptide substrate exclusively between Leu and Gln.

We further characterized the cleavage of the ST6Gal I peptide, by comparison with those of APP<sub>wt</sub> (KTEEISEVKMDAEFRHDSG; APP Swedish mutant (APP-SW), KTEEISEVLDAEFRHDSG; ST6Gal I wild type substrate (ST-WT), DYEALTLQAKEFQMPKSEQE; and ST6Gal I<sub>LA</sub>, mutant (ST-LA), DYEALTLQAKEFQMPKSEQ) for 16 h. KTEEISEVN(Sta)VAEF (0.1 μM) was used as the BACE1 inhibitor. Cleaved peptides and substrate were separated by reversed-phase HPLC. The rate of BACE1-dependent cleavage was expressed as the ratio (%; mean ± S.D., n = 3) of the product to the total peptide signal by measuring their absorbance at 215 nm.

**Fig. 3.** BACE1-Fc-dependent cleavage of APP-derived or ST6Gal I-derived peptides. The BACE1-Fc protein, expressed in COS cells and then purified with protein A-Sepharose, was incubated with a series of synthetic peptides (APP wild type (APP-WT), KTEEISEVKMDAEFRHDSG; APP Swedish mutant (APP-SW), KTEEISEVLDAEFRHDSG; ST6Gal I wild type substrate (ST-WT), DYEALTLQAKEFQMPKSEQE; and ST6Gal I<sub>LA</sub>, mutant (ST-LA), DYEALTLQAKEFQMPKSEQ) for 16 h. KTEEISEVN(Sta)VAEF (0.1 μM) was used as the BACE1 inhibitor. Cleaved peptides and substrate were separated by reversed-phase HPLC. The rate of BACE1-dependent cleavage was expressed as the ratio (%; mean ± S.D., n = 3) of the product to the total peptide signal by measuring their absorbance at 215 nm.

**Fig. 4.** Purity of the protein A-ST6Gal I-FLAG cleaved by BACE1-Fc. The BACE1-Fc protein was incubated with protein A-ST6Gal I-FLAG. An aliquot of the products was analyzed by SDS-PAGE. Proteins were detected with silver nitrate.

**Fig. 5.** BACE1 did not recognize Lysα of ST6Gal I. Purified BACE1-Fc was incubated with protein A-ST6Gal I-FLAG and then analyzed by immunoblotting with anti-FLAG antibody.

**Fig. 6.** NH<sub>2</sub>-terminal QAK sequence of a ST6Gal I-Q38 form was truncated by exopeptidase activity in detergent-treated Golgi membrane fraction. A, protein A-ST6Gal I-FLAG was cleaved by BACE1-Fc. The resulting Q38 form, in which the NH<sub>2</sub>-terminal amino acid sequence starts at Gln<sup>38</sup>, was incubated with either a microsomal or cytosolic fraction prepared from COS cells. The microsomal fraction was pretreated with or without 1% Triton X-100. The product generated was analyzed by immunoblotting the anti-FLAG or E41 antibodies. B, protein A-ST6Gal I-FLAG was incubated with either the BACE-Fc or Golgi membrane fraction (10 μg of protein) or both. After incubation for 2 h, the reaction products were analyzed by immunoblotting with the E41 antibody.
BACE1 recognition. In which the NH2-terminal amino acid sequence starts at Gln 38, was protein antibody, which recognizes the ST6Gal I-Q38 form. Peptide QAKEFQ (10). As a negative control, Q38 antibody pre-absorbed with antibodies (Ab) analyzed by immunoblotting with either the Q38 or E41 polyclonal antibodies (Ab) in the three independent experiments when E41 form/total substrate was taken as 100% in the control sample.

| Inhibitor          | Concentration mN | Residual activity % |
|--------------------|------------------|---------------------|
| Amastatin          | 1.0              | 265                 |
| Bestatin           | 1.0              | 67                  |
| 1,10-Phenanthroline| 1.0              | 62                  |
| EDTA               | 1.0              | 85                  |

When we took a time course to compare the cleavage efficiency between these protein A-ST6Gal I proteins in detail, protein A-ST6Gal I40A-FLAG was cleaved at almost the same efficiency as the wild type (data not shown). These results suggest that Lys40 of ST6Gal I is not critical for same efficiency between these protein A-ST6Gal I proteins in detail, cleavage rate of wild type ST6Gal I (E41 form/total ST6Gal I-FLAG) after 2 h of incubation taken as 100%.

Our results described above suggest that BACE1 cleaves ST6Gal I between Leu37 and Gln38; the cleaved product (Q38 form) was three amino acids longer than the secreted one (E41 form). We speculated that the three-amino acid QAK sequence of the Q38 form was removed by endogenous exopeptidase(s) before secretion, and hence we set up an assay for detecting possible exopeptidase activity. As a substrate for the assay we used the Q38 form of ST6Gal I, which had been prepared by cleavage of protein A-ST6Gal I-FLAG with BACE1-Fc. When the substrate was mixed with detergent extracts of a microsomal fraction of COS cells, its NH2-terminal QAK sequence was trimmed to generate the E41 form of ST6Gal I, which was detected by the E41 antibody (Fig. 6A). This suggests that the extracts contained protease activity, as we had expected. The protease activity was not detected by adding the microsomal fraction without detergent treatment. As shown in Fig. 6B, we also detected such a protease activity in the detergent-solubilized Golgi fraction prepared from rat livers (27). These results suggest that the protease was localized mainly in the Golgi apparatus and its catalytic domain faces the luminal side. To rule out the possibility that the protease has endopeptidase activity, we added protein A-ST6Gal I-FLAG as substrate to the detergent-solubilized Golgi fraction as the enzyme fraction to see whether the ST6Gal I E41 form was produced. Because we did not detect the production of ST6Gal I E41 form, we surmised that the protease in the Golgi fraction is a kind of exopeptidase that acts on ST6Gal I-Q38 form. We also confirmed that BACE1 itself has no trimming activity, because we did not detect production of the ST6Gal I-Q38 form without detergent-solubilized Golgi fraction (Fig. 6B). Thus we demonstrate the presence of luminal exopeptidase activity that trims the NH2-terminal QAK-sequence of the Q38 form. To further characterize the trimming activity, we tested the sensitivity of the putative peptide to the various aminopeptidase inhibitors (Table II). As both EDTA and 1,10-phenanthroline significantly inhibited the peptidase activity at 1 mM, the enzyme seems to have metalloaminopeptidase-like character. Moreover, we found that bestatin, but not amastatin, significantly inhibits the activity. These results suggest that the protease that trims NH2-terminal QAK sequence of ST6Gal I-Q38 form belongs to the bestatin-sensitive metalloaminopeptidase.

**Table II**

| Inhibitor     | Concentration mN | Residual activity % |
|---------------|------------------|---------------------|
| Amastatin     | 1.0              | 265                 |
| Bestatin      | 1.0              | 67                  |
| 1,10-Phenanthroline | 1.0       | 62                  |
| EDTA          | 1.0              | 85                  |

**Fig. 7.** Soluble secreted ST6Gal I was not detected with Q38 antibody, which recognizes the ST6Gal I-Q38 form.

**Fig. 8.** Exopeptidase activity, which trims the NH2-terminal QAK sequence of ST6Gal I-Q38 form and its K40A mutant. The ST6Gal I-FLAG-Q38 form and its K40A mutant were prepared from their respective protein A-ST6Gal I proteins by BACE1 cleavage. The resulting substrates were then incubated with 10 μg of Golgi fraction solubilized with 1% Triton X-100 for 0, 0.5, 1, and 2 h. Each product was divided in half; one-half of the sample was analyzed by immunoblotting with anti-FLAG antibody to quantitate total ST6Gal I-FLAG and the other half was analyzed with E41 antibody to quantify the E41 form by Luminolimage Analyzer LAS-1000 PLUS (Fujifilm). The percentage of cleavage was an average of two independent experiments, with the cleavage rate of wild type ST6Gal I (E41 form/total ST6Gal I-FLAG) after 2 h of incubation taken as 100%.

**Fig. 9.** Proposed mechanism of ST6Gal I processing and secretion in the cell. Membrane-bound ST6Gal I protein is cleaved initially by BACE1 to produce the soluble Q38 form, which starts at Gln38. Luminal aminopeptidase(s) activity then trims its NH2-terminal Gln38, Ala39-Lys40 sequence, with the resultant E41 form secreted from the cell (21).
ST6Gal I Q38 form Was Not Secreted from the Cell—We show here that BACE1 cleaves ST6Gal I between Leu37 and Gln38. Although amino-terminal sequencing analysis showed that most of soluble secreted ST6Gal I started at Glu41, there might be small amount of soluble ST6Gal I starting at Gln38. We prepared an antibody that specifically recognizes the NH2-terminus of ST6Gal I-Q38 form. This Q38 antibody failed to detect soluble secreted ST6Gal I from COS cells, even though E41 antibody did detect the soluble ST6Gal I (Fig. 7). The results indicate that secreted ST6Gal I start mostly at Glu41.

K40A Mutation Reduced the Efficiency of Exopeptidase Activity—A simple explanation for the efficient conversion from ST6Gal I-Q38 form to E41 form is that the amount of exopeptidase activity exceeds that of the substrate. An alternative idea is that exopeptidase-dependent trimming may be a prerequisite for secretion. The latter speculation is supported by our previous cellular experiment (19) in which a ST6Gal I K40A mutant expressed in COS cells was poorly secreted (40% of wild type level), although this mutation did not affect in vitro BACE1-dependent cleavage (Fig. 5). The mutation may affect exopeptidase-dependent trimming and then the secretory process. We therefore compared the exopeptidase activity toward ST6Gal I-Q38 form with that for its K40A mutant. Exopeptidase cleavage rate of the K40A mutant was half of wild type protein over a particular time course; i.e. when we set the cleavage rate of wild type after a 2-h incubation as 100%, the cleavage rate of K40A was 46%. After a 1-h incubation, the cleavage rate of the wild type was 40% and that of K40A was 17% (Fig. 8). These results suggest that K40A mutation reduces the efficiency of the exopeptidase activity.

DISCUSSION

In the present experiment, we used ST6Gal I peptide and protein A-ST6Gal I-FLAG as substrates for BACE1, both of which were cleaved between Leu37 and Gln38. The observation fits other previous reports (20–22) describing residue preferences for substrates of BACE1; i.e. P1 site is most stringently recognized by BACE1 and only large hydrophobic residues such as Leu, Phe, Met, and Tyr are accepted at this position. Previous reports (22, 28) showing that BACE1 prefers bulky hydrophobic residues at the P3 position also correspond well with the presence of Leu at the P3 of ST6Gal I, suggesting that BACE1-Fe preferably recognizes the Leu35-Thr36-Leu37 sequence of ST6Gal I and cleaves exclusively at this site. This cleavage is also supported by our own previous cellular experiment, in which replacement of Leu37 with Ala (ST6Gal I L37A), an unfavorable substitution for BACE1 cleavage in vitro, significantly reduced the secretion from the cells (61 ± 20% of the control level, p < 0.05). Taken together, we speculate that BACE1 cleaves ST6Gal I between Leu37 and Gln38 inside the control level, although this mutation did not affect exopeptidase trimming efficiency. At present, however, we cannot exclude the possibility that an additional unidentified mechanism other than the ST6Gal I cleavage process exists to regulate the ST6Gal I secretion. The data presented here suggest that the exopeptidase has a best-sitesensitive metalloproteinase-like character. Because we used a crude Golgi membrane fraction as an enzyme source in this study, purification of this exopeptidase will be required for further understanding of the molecular mechanisms underlying the cleavage and secretion of ST6Gal I.

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Characterization of α2,6-Sialyltransferase Cleavage by Alzheimer's β-Secretase (BACE1)

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