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# Involvement of proteases in glycosyltransferase secretion: Alzheimer's $\beta$-secretase-dependent cleavage and a following processing by an aminopeptidase

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**Alzheimer's beta-secretase (BACE1) cleaves amyloid precursor protein to produce amyloid beta-peptide, which is a crucial initiation process of the pathogenesis of Alzheimer's disease. We previously found that BACE1 also cleaves a membrane-bound sialyltransferase (ST6Gal I). Here we report that, when the protein A-ST6Gal I fusion protein, or ST6Gal I-derived peptide, was used as an *in vitro* substrate for BACE1, it cleaved the substrates between Leu$^{37}$and Gln$^{38}$. However, a soluble form of ST6Gal I secreted from COS cells started from Glu$^{41}$, which was three amino acids shorter than the *in vitro* product. The results suggested that the BACE1 product was truncated by an aminopeptidase(s) before secretion. The aminopeptidase activity was successfully detected in detergent extracts of Golgi-membrane fraction. Taken together, we concluded that BACE1 initially cleaved ST6Gal I between Leu$^{37}$ and Gln$^{38}$, and the $NH_2$-terminal three amino acids of the yielded product was further trimmed by the aminopeptidase.**
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## Introduction

Glycosyltransferases catalyze glycan biosynthesis in the Golgi apparatus or endoplasmic reticulum, and play critical roles for glycan expression. The majority of glycosyltransferases are type II membrane proteins, and their structures are characterized by a luminal catalytic domain, followed by a transmembrane domain and a small cytoplasmic tail. They have a "stem region," which is often cleaved by endogenous protease(s) to generate a soluble form of catalytic domain [1–4]. The soluble form was secreted from the cells. Indeed, soluble forms of glycosyltransferases are detected in body fluids such as serum, urine, and milk. Some of the soluble forms in serum are utilized as diagnostic markers, because their levels are correlated with inflammation or malignant transformation [5–7]. In order to understand the molecular mechanism underlying the cleavage and secretion of glycosyltransferases, we need to identify the endogenous protease(s) that is responsible for the cleavage. Alzheimer's $\beta$-secretase (beta-site amyloid precursor protein-cleaving enzyme 1: BACE1) has been recently identified as a protease that cleaves $\alpha$2,6-sialyltransferase (ST6Gal I) [8–10]. We discuss the cleavage of ST6Gal I by BACE1 and a following processing of the cleaved ST6Gal I by an aminopeptidase.

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## Cleavage of $\alpha$2,6-sialyltransferase by BACE1 protease

We have studied on the mechanism of glycosyltransferase secretion using $\alpha$2,6-sialyltransferase (ST6Gal I) as a model. In 2001, we found that ST6Gal I was cleaved by Alzheimer's $\beta$-secretase (BACE1) (Figure 1). BACE1 was originally identified as a protease that cleaved amyloid precursor protein (APP) to initiate the production of beta-amyloid (A$\beta$) peptide [11–13]. The production and deposition of the neurotoxic A$\beta$-peptide in the brain is a hallmark of the pathology of Alzheimer's disease [14]. Substrate specificity of BACE1 has been extensively analyzed by a series of studies [15,16]. When an APP-derived

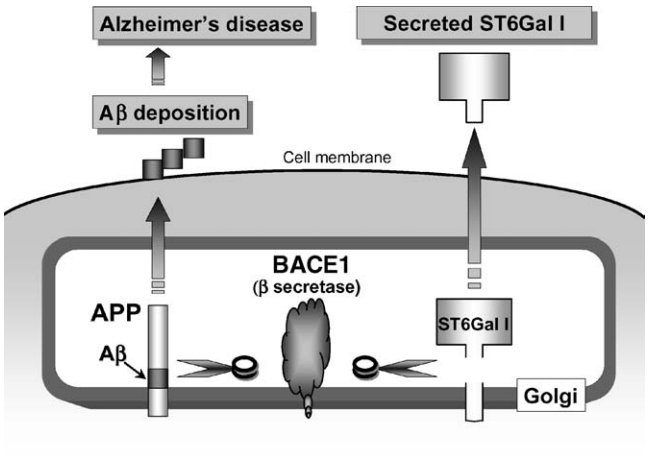


**Figure 1.** BACE1 cleaves ST6Gal I as well as APP.

peptide containing Glu-Val-Lys-Met-Asp-Ala-Glu-Phe sequence was used as a substrate, BACE1 cleaved the substrate between methionine and aspartic acid. In newly generated $NH_2$-terminal portion (P subsite), Glu, Val, Lys, and Met were called P4, P3, P2, and P1, amino acid, respectively, whereas, in newly generated COOH-terminal portion (P′ subsite), Asp, Ala, Glu, and Phe were called P′1, P′2, P′3, and P′4, amino acid, respectively (Figure 2). Amino acid substitution analysis revealed that P1 position of the cleavage site was the most stringent, preferring residues in the order of Leu > Phe > Met > Tyr ≫ others [15]. In general, the substitutions of P subsites (P1–P4) are more stringent than those of P′ subsites (P′1–P′4). The preference for leucine at P1 position accounts for the rapid cleavage of a mutant APP, Swedish-type mutant (APPsw); *i.e.*, the mutant contains Asn-Leu instead of Lys-Met as P2-P1 sequence, and it is cleaved 6–7-fold faster than wild-type APP. The early onset of dementia of the patient with Swedish-type mutation is attributed to the rapid cleavage of APPsw [14].

We examined *in vitro* cleavage of an ST6Gal I-derived peptide (DYEALTLQAKEFQMPKSQE: corresponding to amino acid residue 31–49 of rat ST6Gal I sequence) [9]. BACE1 cleaved the substrate and produced two peptide fragments, structures of which were identified to be DYEALTL and QAKEFQMPKSQE by reversed-phase HPLC, followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Figure 3). The result indicated that BACE1 cleaved the peptide substrate between 7th($L^{37}$) and 8th($Q^{38}$) amino acid residue. Replacement of the leucine at P1 position with alanine, as an unfavorable substitution, significantly reduced the velocity of cleavage (less than 40% of wild type), which was consistent with the subsite specificity described above. We also examined cleavage of protein A-ST6Gal I fusion protein, which had sialyltransferase activity and appeared to be correctly folded as a native enzyme. The fusion protein was cleaved by BACE1, and $NH_2$-terminal sequence newly generated was $Q^{38}$AKEFQMPK- - -, as was the case of the peptide substrate. The soluble form of ST6Gal I thus produced *in vitro* was designated as Q38 form.

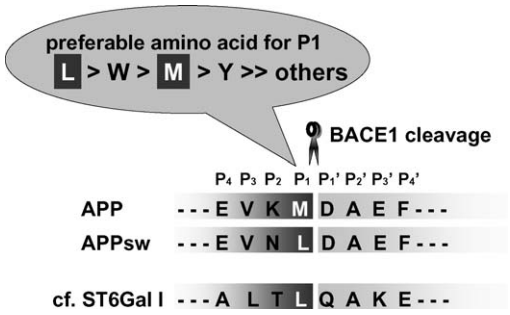


**Figure 2.** BACE1 prefers hydrophobic or bulky amino acid at P1 position.

## Q38 form of ST6Gal I was truncated by aminopeptidase before secretion

When ST6Gal I was overexpressed together with BACE1 in COS cells, secretion of the transferase was markedly enhanced [8,10]. Sequence analysis revealed that the $NH_2$-terminus of secreted ST6Gal I started from $Glu^{41}$ ($E^{41}$FQMPKSQE- - -), indicating that the secreted form (E41 form) was three amino acids shorter than Q38 form, which was produced by *in vitro* experiment. We therefore speculated that the $NH_2$-terminal three amino acids, $Q^{38}A^{39}K^{40}$, of Q38 form were trimmed by aminopeptidase(s) before secretion. For the detection of the aminopeptidase activity, Q38 form was prepared as a substrate and it was incubated with detergent-treated Golgi fraction. E41 form as a product was detected with antibody that specifically reacted with $NH_2$-terminal $E^{41}$FQMPK sequence [9] (Figure 4), suggesting that the aminopeptidase was present in the Golgi fraction. It was noted that incubation of Q38 form with intact Golgi fraction, without detergent treatment, did not generate E41 form at all, suggesting that the catalytic domain of the aminopeptidase faces the luminal side of the Golgi apparatus. To rule out the possibility that the protease has endopeptidase activity, the detergent-treated microsomal fraction was incubated with an intact fusion protein, protein A-ST6Gal I. No production of E41 form was detected. Taken together, we concluded that a membrane-bound form of ST6Gal I is initially cleaved by BACE1 to produce Q38 form, and then converted to E41 form by a Golgi-luminal aminopeptidase before secretion.

The aminopeptidase was further characterized by the use of several inhibitors [9]. Bestatin, EDTA, and 1,10-pheananthroline significantly inhibited the activity, suggesting that the protease is bestatin-sensitive metalloaminopeptidase. Alanine substitution experiment revealed that replacement of $Lys^{40}$ with Ala diminished the trimming velocity (44% of wild type), suggesting that $Lys^{40}$ is recognized by the aminopeptidase. In contrast to the effect on the aminopeptidase activity, K40A mutation did not affect BACE1-dependent cleavage, which was consistent with subsite specificity reported for BACE1.

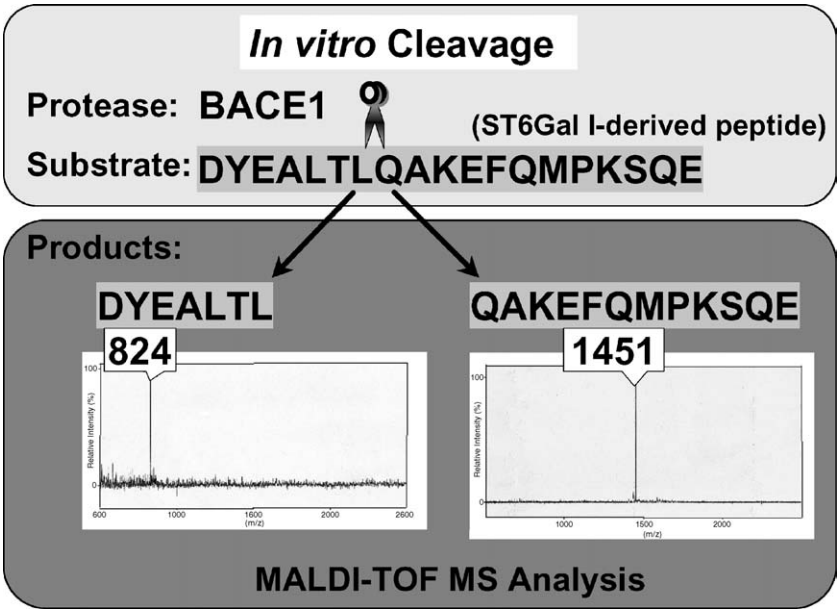


**Figure 3.** *In vitro* cleavage of ST6Gal I-derived peptide by BACE1. BACE1 cleaved an ST6Gal I-derived peptide (DYEALTLQAKEFQMPKSQE) *in vitro*. The reaction products are identified to be DYEALTL and QAKEFQMPKSQE by MALDI-TOF MS analysis.

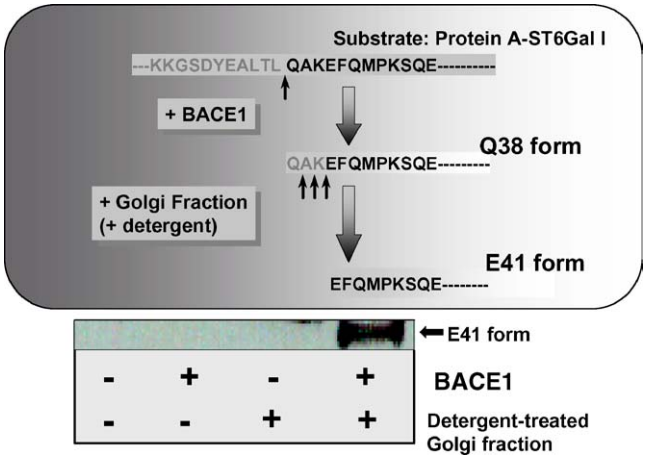


**Figure 4.** Aminopeptide trimms Q38 form to produce E41 form. Protein A-ST6Gal I fusion protein is cleaved by BACE1 to produce Q38 form. The yielded Q38 form is trimmed by an aminopeptidase in the detergent extracts of the Golgi membrane fraction. The generated E41 form is detected with antibody that specifically reacts with EFQMPK sequence at $NH_2$-terminus.

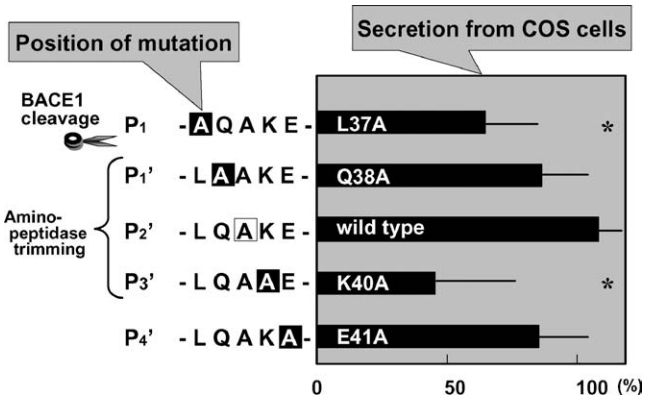


**Figure 5.** Secretion of alanine mutants from COS cells. Alanine mutants around the cleavage site ($Leu^{37}$–$Glu^{41}$) are overexpressed in COS cells. Note that the secretion of L37A and K40A mutants is significantly reduced. Significant difference is indicated with asterisk ($p < 0.05$).

### Effect of alanine substitution on ST6Gal I secretion from COS cells

It was noted that the main portion of ST6Gal I secreted from COS cells was E41 form, and Q38 form was hardly detected in the culture medium. A possible explanation for lacking the secretion of Q38 form is that the amount of aminopeptidase activity is high enough to digest all of the endogenous Q38 substrate; *i.e.*, Q38 form produced by BACE1 is entirely converted to E41 form before secretion. Alternatively, the trimming by the aminopeptidase may be prerequisite for the secretion from the cells. To address the issue, we prepared a series of alanine mutants around the cleavage site (amino acid residue 37–41), and they were overexpressed in COS cells [8,10]. The replacement of $Leu^{37}$ with Ala, unfavorable P1 substitution for BACE1, significantly diminished the secretion (60% of the control level), suggesting that BACE1-dependent cleavage is critical for the initiation of ST6Gal I secretion (Figure 5). Most significant reduction of the secretion was observed with the replacement of $Lys^{40}$, unfavorable replacement for aminopeptidase, (40% of the control level), suggesting that the aminopeptidase-dependent trimming is required for the efficient secretion of ST6Gal I.

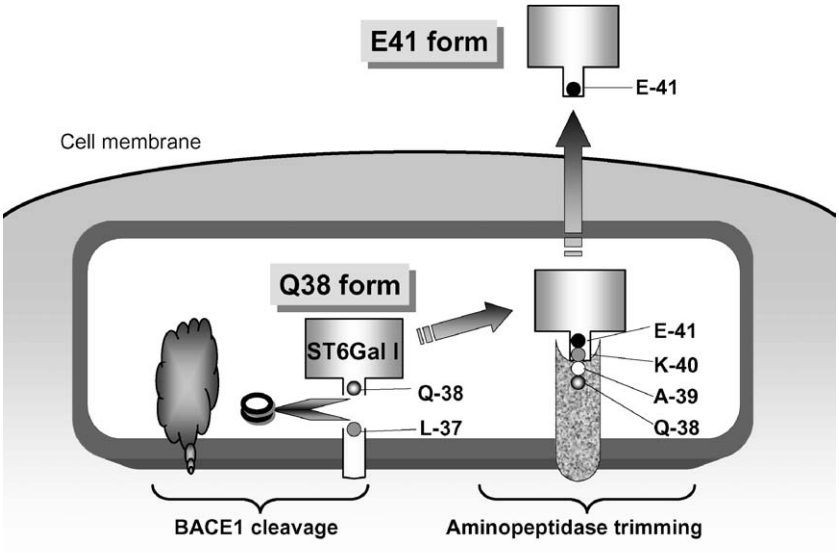


**Figure 6.** Possible mechanism of ST6Gal I processing and secretion. BACE1 cleaves ST6Gal I between $Leu^{37}$ and $Gln^{38}$ to generate Q38 form. Q38 form is converted to E41 form by a Golgi-luminal aminopeptidase before secretion.

The secretion machinery for ST6Gal I appears to comprise at least two steps: one is BACE1-dependent cleavage to generate Q38 form and the other is trimming of the N-terminal QAK sequence of Q38 form by the aminopeptidase (Figure 6). It is tempting to hypothesize that these two steps are tightly associated, *e.g.*, forming a functional complex in a particular compartment in the Golgi apparatus. To support the hypothesis, we need to purify and characterize the aminopeptidase in the future.

## Concluding remarks

We have analyzed whether BACE1cleaved other glycosyltransferases as well, and found that only a few transferases could be BACE1 substrates, although majority of them were secreted from cells upon overexpression. The result suggests that some other proteases are responsible for the processing of the latter transferases. Identfication of the proteases will be required for further understanding of the processing of glycosyltransferases.

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