The effects of *N*-glycosylation sites and the N-terminal region on the biological function of β1,3-*N*-acetylglucosaminyltransferase 2 and its secretion

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Abbreviations: AcNPV-me-GFP_{uv}-β3GnT2, AcNPV-GGT; β3GnT2, β1,3-*N*-acetylglucosaminyltransferase 2; β3GalT, β1,3-galactosyltransferase; β3GT, β1,3-glycosyltransferase; CBB, Coomassie Brilliant Blue; ER, endoplasmic reticulum; GFP_{uv}, green fluorescent protein when excited with long-wave UV light; GFP_{uv}-β3GnT2Δ25, GGTΔ25; GGT, GFP_{uv}-β3GnT2; PCR, polymerase chain reaction; WT, wild type

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Abstract

Human β1,3-*N*-acetylglucosaminyltransferase 2 (β3GnT2) is thought to be an enzyme that extends the polylactosamine acceptor chains, but its function and structure analysis are unknown. To obtain insight into the structure of β3GnT2, the effects of *N*-glycosylation on its biological function were evaluated using the addition of inhibitors, site-directed mutagenesis of potential *N*-glycosylation sites, and deletion of its N-terminal region using a fusion protein with GFP_{uv} in a baculovirus expression system. Four of five potential *N*-glycosylation sites were found to be occupied, and their biological function and secretion were inhibited with the treatment of *N*-glycosylation inhibitor, tunicamycin. The *N*-glycosylation at Asn219 was necessary for the β3GnT activity; moreover *N*-glycosylation at Asn127 and Asn219 was critical for efficient protein secretion. When Ser221 was replaced with Thr, fusion protein was expressed as a single band, indicating that the double band of the expressed fusion protein was due to the heterogeneity of the glycosylation at Asn219. The truncated protein consisting of amino acids 82-397 (GFP_{uv}-β3GnT2Δ83), which lacked both one *N*-glycosylation site at Asn79 and the stem region of glycosyltransferase, was expressed as only a small form and showed no β3GnT activity. These results suggest that the *N*-glycosylation site at Asn219, which is conserved throughout the β1,3-glycosyltransferase family, is indispensable not only with regard to its biological function, but also to its secretion. The N-terminal region, which belongs to a stem region of glycosyltransferase, might also be important to the active protein structure.

Keywords: β1,3-*N*-acetylglucosaminyltransferase 2, β1,3-glycosyltransferase, *N*-glycosylation, site-directed mutagenesis, oligosaccharide, glycoprotein

Introduction

The carbohydrate chains of glycoproteins play important roles in a variety of biological and physical properties, including the folding, secretion, solubility, and a stability of protein [1, 2]. These oligosaccharides are synthesized by the sequential action of glycosidases and glycosyltransferases localized in the endoplasmic reticulum (ER) and Golgi apparatus. The Golgi glycosyltransferases share the same structures, which consist of a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region, and a C-terminal catalytic domain. Glycosyltransferases from prokaryotes and eukaryotes can be classified into 65 families based on the similarities of their amino acid sequences and substrate specificities [3].

β1,3-*N*-acetylglucosaminyltransferase 2 (β3GnT2) belongs to the β1,3-glycosyltransferase (β3GT) family and shows 19-24% identity to the β1,3-galactosyltransferases (β3GalTs) and 40-45% identity to other β3GnTs [4-7]. One *N*-glycosylation site is strictly conserved among all β3GalTs and β3GnTs. The β3GnT2 has five potential *N*-glycosylation sites, which is the largest number in the β3GT family. β3GalT2 has only one conserved *N*-glycosylation site, and its *N*-glycosylation is crucial for biological function and trafficking to the Golgi complex from the ER [8]. Moreover, mutations of conserved domains and cysteine residues among β3GT cause β3GalT I to be inactive [9]. In the case of β3GnTs, the amino acid residues and conserved regions involved in its biological and physical properties as well as its three-dimensional structure are still not known.

In the present study, β3GnT2 was expressed extracellularly as GFP_{uv} fusion protein, and the effects of *N*-glycosylation of human β3GnT2 on its biological function and expression in the baculovirus expression system (BES) were investigated.

Materials and methods

Cell lines, media, and insect cell culture. Tn-5B1-4 cells from Trichoplusia ni were purchased from Invitrogen (San Diego, CA, USA) and were grown in 100-ml flasks with a working volume of 20 ml in specified medium. Express Five (Invitrogen) medium was used for cultivation of Tn-5B1-4 cells with 1% antibiotics-antimycotic (Invitrogen). Tunicamycin and castanospermine were added to the culture medium at 10 μg/ml and 75 μg/ml, respectively

Site-directed mutagenesis and deletion mutation. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) to investigate the glycosylation of individual *N*-glycosylation sites. The potential expected *N*-glycosylation sites were considered to be Asn79, Asn89, Asn127, Asn173, and Asn219. Six mutants named as N79Q, N89Q, N127Q, N173Q, N219Q, and S221T were constructed with mutagenic primers (Table 1) using pDEST8/GFP_{uv}-β3GnT2 as a template [10].

Deletion of β3GnT2 was performed by the PCR technique. The deletion mutants, Δ83, Δ91, Δ117, and Δ129 had amino acids 1-83, 1-91, 1-117, and 1-129 deleted from the β3GnT2 gene, respectively. These deletion mutants were amplified by PCR using mutagenic primers, with β3GnT2Δ83-F, β3GnT2Δ91-F, and β3GnT2Δ129-F being used as forward primers and β3GnT2-R as a reverse primer (Table 1), respectively.

All PCR fragments were confirmed on the basis of the dideoxynucleotide chain terminating sequence [11] using the Thermo Sequenase Cycle Sequencing kit (USB Co., Cleveland, Ohio, USA).

Construction of recombinant baculovirus. The recombinant baculovirus containing the GFP_{uv}-β3GnT2 (GGT) fusion gene fused with the melittin signal sequence, AcNPV-me-GFP_{uv}-β3GnT2 (AcNPV-me-GGT), was constructed in a previous study [10] using GATEWAY Cloning Technology and Bac-to-Bac Baculovirus Expression Systems (Invitrogen). All mutant genes were inserted into AcNPV genome as a fusion gene according to our previous report [10].

Expression and purification of GFP_{uv}-β3GnT2 fusion proteins. Suspension cultures were carried out in 100-ml flasks with a working volume of 20 ml in specified medium. Agitation and temperature were controlled at 100 rpm and 27 ^{o}C, respectively. Two million cells/ml of Tn-5B1-4 cells were infected at a multiplicity of infection (M.O.I) of 10 with a recombinant baculovirus, AcNPV-me-GGT.

After a postinfection time of 2 days, the cultures were sampled and centrifuged at 8,000 rpm for 5 min to remove the cells. Fifty milliliters of the cell culture supernatant was mixed with 0.5 ml of Ni^{2+} NTA agarose (Qiagen, Valencia, CA, USA) resin equilibrated with distilled water. The mixture was gently stirred at 4 ^{o}C for 1 hour and then loaded onto a column. The column was washed with 3 volumes of 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 40 mM imidazole. Histidine-tagged fusion protein was eluted with 3 volumes of 50 mM Tris-HCl (pH7.5) containing 150 mM NaCl and 200 mM imidazole.

Deglycosylation of GFP_{uv}-β3GnT2 fusion protein. Purified fusion protein was deglycosylated with Glycopeptidase F (PNGase F, Takara Bio., Ohtsu, Japan) according to the procedure provided by the manufacturer under either native or denatured conditions. Fusion proteins cleaved with each enzyme were confirmed by SDS-PAGE. For partial deglycosylation, PNGase F was added to the protein solution at 100, 10, 1,

and 0.1 μU.

β3GnT activity. The β3GnT activity assay was carried out as previously described [11]. One unit of enzyme activity is defined as the amount of enzyme capable of catalyzing the transfer of 1 μmol of GlcNAc per minute.

Detection and visualization of recombinant protein using fluorescence image analysis. To detect the expression of recombinant protein, cell lysates, supernatants, and partially purified preparations were subjected to SDS-PAGE [12] on either 8 or 10% polyacrylamide gel. Cell lysates were prepared with lysis buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100). To detect specific fluorescent GFP_{uv}-β3GnT2 protein on SDS-PAGE gel, samples were mixed only with sample buffer without boiling [13]. The green fluorescent bands were directly detected, and their amounts were determined by Molecular Imager FX (Bio-Rad Co., Ltd, Hercules, CA, USA). Purified protein was also detected by staining with Coomassie Brilliant blue (CBB) R-250.

Results

N-Glycosylation of β3GnT2

β3GnT2 has five potential *N*-glycosylation sites, Asn79, Asn89, Asn127, Asn173, and Asn219 (Fig. 1), and only Asn219 is strictly conserved among all β3GTs [4-7]. In a previous study [14], β3GnT2 deleted at amino acids 1-25 was fused with GFP_{uv} at its N-terminus and expressed in BES. This fusion protein is designated as GGTΔ25. To determine how many of these sites were effectively used in GGTΔ25, it was purified by Ni^{2+}-NTA agarose resin from the supernatant of Tn-5B1-4 cell culture medium. Purified fusion protein was analyzed by partial PNGase F digestion under denatured conditions

followed by SDS-PAGE (Fig. 2). Nontreated fusion protein migrated as a band of approximately 77 kDa. With 100 μU of PNGase F, the completely deglycosylated fusion protein migrated with a molecular mass of 70 kDa (0 in Fig. 2). Partially deglycosylated fusion proteins migrated at an interval of approximately 1-2 kDa between 80 and 70 kDa (1-4 in Fig. 2). These five bands demonstrated the existence of four potential *N*-glycosylation sites out of five. GFP_{uv} and the linker region have no potential *N*-glycosylation site.

N-glycosylation is often essential for enzyme activity [15, 16]. Therefore, to confirm the relationship between enzyme activity and *N*-glycosylation in β3GnT2, treatment of purified fusion protein with PNGase was performed under the native condition. The β3GnT activity decreased by 36% compared with the nontreated protein due to its partial deglycosylation [14], indicating that *N*-glycosylation is involved in the protein's enzyme activity. However, since β3GnT2 could not be completely deglycosylated under the native condition, we could not confirm the activity of nonglycosylated β3GnT2.

Tn-5B1-4 cells infected with recombinant baculovirus were treated with either 10 μg/ml of *N*-glycosylation inhibitor, tunicamycin, or 75 μg/ml of oligosaccharide trimming inhibitor, castanospermine. Secretion of fusion protein in the presence of tunicamycin was drastically inhibited (lane 2 in Fig. 3A). The intracellular fraction in the control (lane 3 in Fig. 3A) contained both high molecular mass fusion protein, with a molecular mass similar to that of the extracellular fusion protein, and the low molecular mass fusion protein. However, in the presence of tunicamycin, only the low molecular mass fusion protein was observed in the intra- and extracellular fractions (lanes 2 and 4 in Fig. 3A). These results indicate that the fusion protein with the low

molecular mass was the nonglycosylated form. When tunicamycin was added, neither intra- nor extracellular β3GnT activities were detected (lanes 2 and 4 in Fig. 3A). These results indicate that *N*-glycosylation in GGTΔ25 fusion protein is essential to its enzyme activity and trafficking in the secretory pathway.

On the other hand, when cells were treated with castanospermine, the enzyme activity of the fusion protein remained unchanged, but its mobility became low (lanes 2 and 4 in Fig. 3B), indicating that the trimming of oligosaccharides linked to the fusion protein was blocked with castanospermine treatment, but may be dispensable to the biological function of β3GnT2 (Fig. 3B).

Site-directed mutagenesis of the N-glycosylation site in β3GnT2

To analyze the function of each *N*-glycosylation in β3GnT2, each asparagine of the potential glycosylation site (^{79}NQT, ^{89}NYS, ^{127}NYS, ^{173}NQT, and ^{219}NLS) in β3GnT2Δ25 was exchanged with glutamine by site-directed mutagenesis and then expressed in BES as GFP_{uv} fusion protein. Extracellular expression of each mutant is shown in Fig. 4. GGTΔ25-N79Q and GGTΔ25-N89Q were expressed as a double-band similar to GGTΔ25; GGTΔ25-N127Q and GGTΔ25-N219Q were expressed as a single band. No band of GGTΔ25-N173Q was detected in either the medium or cell lysate (data not shown). The double-bands of GGTΔ25-N79Q and GGTΔ25-N89Q were slightly lower than that of GGTΔ25. GGTΔ25-N127Q migrated as the same molecular mass of the upper bands of GGTΔ25-N79Q and GGTΔ25-N89Q, while GGTΔ25-N219Q migrated as the same molecular weight of the lower band of GGTΔ25. It indicates that at four potential *N*-glycosylation sites, Asn79, Asn89, Asn127, and Asn219 were possibly glycosylated in β3GnT2Δ25.

All mutants except for GGTΔ25-N173Q were accumulated intracellularly to the same extent (data not shown), but secretion of GGTΔ25-N127Q and GGTΔ25-N219Q was drastically reduced to approximately 19% and 13% of GGTΔ25 (Fig. 4), respectively, suggesting that *N*-glycosylation at Asn127 and As219 is necessary for secretion of this fusion protein. Specific activity of each mutant is also shown in Fig. 4. The densitometry value of the full-length fusion protein, including a double-band on SDS-PAGE gel, was estimated as a protein concentration. Specific activity of GGTΔ25-N79Q was somewhat low compared to GGTΔ25, and specific activities of GGTΔ25-N89Q and GGTΔ25-N127Q were similar to that of GGTΔ25. On the other hand, mutation of Asn219 results in a complete loss of β3GnT activity, despite its significant secretion, thus suggesting that the oligosaccharide linked to Asn219 might be the most essential for its biological function in fusion proteins. GGTΔ25-N173Q was not expressed in either the extra- or intracellular fractions, which might be due to its aberrant conformation.

Frequency of N-glycosylation at Asn219

GGTΔ25 was expressed in BES as a double-band, whose differences in molecular mass were approximately 1-2 kDa. Migration of the lower band corresponded to that of GGTΔ25-N219Q. It may be considered that the lower band is a nonglycosylated protein at Asn219. The amino acid sequence of this glycosylation site is Asn^{219}-Leu^{220}-Ser^{221} (Fig. 1). It is known that the amino acid at the X position of an Asn-X-Ser sequon is an important determinant of *N*-glycosylation efficiency [17, 18]. The *N*-glycosylation efficiency of Asn-Leu-Ser is 35-45 %. It was hypothesized that this low efficiency causes a double-band for the fusion protein. To confirm this, the replacement of Ser^{221}

with threonine was performed because, in contrast with Asn-Leu-Ser, the *N*-glycosylation efficiency of Asn-Leu-Thr is above 90%. Extracellular expression of GGTΔ25-S221T is shown in Fig. 5. GGTΔ25-S221T was shifted to a single band that corresponded to the upper band of the wild type, indicating that the double band of the wild type was derived from the heterogeneity of usage of the *N*-glycosylation at Asn^{219}. The extracellular β3GnT activity of Tn-5B1-4 cells infected with baculovirus containing GGTΔ25-N221T gene was the same level as that of GGTΔ25, and moreover the specific activity was about twofold due to a disappearance of the lower band that was not glycosylated at Asn219.

Similarly to GGTΔ25-N219Q and GGTΔ25-S221T, GGTΔ25-N127Q was also expressed extracellularly as a single band. Based on SDS-PAGE (Fig. 4), the molecular mass of GGTΔ25-N127Q corresponded to the upper band of GGTΔ25-N79Q and GGTΔ25-N89Q, indicating that GGTΔ25-N127Q lacked only one *N*-linked oligosaccharide. GGTΔ25-N127Q would be inactive if Asn219 in GGTΔ25-N127Q was not glycosylated, but GGTΔ25-N127Q displayed a significant β3GnT activity. These results indicate that Asn219 in GGTΔ25-N127Q was glycosylated completely, and that the oligosaccharide linked to Asn127 in GGTΔ25 might affect *N*-glycosylation at Asn219.

Construction of deletion mutants of GFP_{uv}-β3GnT2

For further analysis of the role of oligosaccharides linked to Asn79, Asn89, and Asn127, deletion mutants were constructed as a fusion protein with GFP_{uv}. We generated three deletion mutants, β3GnT2Δ83 consisting of amino acids 84-397 in which Asn79 was removed; β3GnTΔ91 consisting of amino acids 92-397 in which

Asn79 and Asn89 were removed; β3GnT2Δ129 consisting of amino acids 130-397 in which Asn79, Asn89, and Asn127 were removed. Three chimeric β3GnT2s were fused with GFP_{uv} at its N-terminus, likely to GGTΔ25, and were named as GGTΔ83, GGTΔ91 and GGTΔ129, respectively. These three fusion proteins were expressed in BES. Only the low molecular mass proteins of GGTΔ83 and GGTΔ91 were observed extracellularly (Fig. 6). Full-length GGTΔ129 was expressed intracellularly (data not shown), but only low molecular mass proteins were secreted. β3GnT activity was not detected in all extra- and intracellular fractions of GGTΔ83, GGTΔ91, and GGTΔ129.

Discussion

In a previous study, β3GnT2 deleted at amino acids 1-25 was expressed in BES as a fusion protein with GFP_{uv} and purified [14]. The β3GnT activity of the purified GGTΔ25 protein was reduced to 64% after its partial deglycosylation under the native condition [14], suggesting that *N*-glycosylation is involved in its biological function. We therefore analyzed *N*-glycosylation of β3GnT2 in more detail. Deglycosylation by PNGase F under the denatured condition and site-directed mutagenesis of *N*-glycosylation sites clarified that oligosaccharides were linked to *N*-glycosylation sites in β3GnT2, probably Asn79, Asn89, Asn127, and Asn219. Because the expression of GGTΔ25-N173Q was not observed in BES, *N*-glycosylation at Asn173 could not be confirmed. However, probably *N*-glycosylation at Asn173 would not occur because practical *N*-glycosylation sites were thought to be at least four, judging from partial deglycosylation by PNGase F under the denatured condition (Fig. 2).

Elimination of the *N*-glycosylation sites at either Asn127 or Asn219 impaired the secretion of GGT fusion protein into the extracellular fraction, but the specific activity

of GGTΔ25-N127Q was similar to GGTΔ25, which was fully glycosylated. In contrast to GGTΔ25-N127Q, GGTΔ25-N219Q exhibited no activity (Fig. 4). These results suggest that oligosaccharides linked to Asn127 contributed to the secretion of the fusion protein, while oligosaccharides linked to Asn219 contributed to both its secretion and biological function. Similarly, inhibition of conserved *N*-glycosylation at Asn143 of mouse β1,3-galactosyltransferase 2 by tunicamycin treatment and site-directed mutagenesis resulted in a loss of enzyme activity and impairment of its exit from the endoplasmic reticulum (ER) [8]. Its *N*-glycosylation site corresponding to Asn219 in β3GnT2 and Asn143 in Gal-T2 is conserved among the β3GT family [4, 19]. It is likely that *N*-glycosylation at the conserved site is critical to its biological function and localization throughout the β3GT family. The oligosaccharide at Asn219 may be the most important among the four oligosaccharides linked to β3GnT2. *N*-glycosylation of other glycosyltranferases is also needed for the enzyme activity and targeting to the Golgi [20-23]. However, the enzyme activity and secretions were not abolished by the castanospermine treatment (Fig. 3), suggesting that trimming of *N*-linked oligosaccharides may not be necessary to its biological function and trafficking, unlike to Gal-T2, which was stacked in ER with castanospermine treatment.

GGTΔ25 was expressed as a double-band in BES, and GGTΔ25-N219Q (N^{219}LS→QLS) and GGTΔ25-N221T (N^{219}LS→NLT) were expressed as single bands, respectively. The molecular masses of GGTΔ25-N219Q and GGTΔ25-S221T corresponded to the lower and upper bands of the double-band of GGTΔ25, respectively (Figs. 4 and 5). In general, among Asn-X-Ser sequons, Asn-Trp-Ser, Asn-Asp-Ser, Asn-Glu-Ser, and Asn-Leu-Ser are poor oligosaccharide acceptors, while Asn-X-Thr sequons are efficiently glycosylated, even when X is Trp, Asp, Glu, or Leu [17, 18]. In

β3GnT2, the *N*-glycosylation site sequon at Asn219 is Asn-Leu-Ser. Moreover, a double-band was replaced with a single band by exchanging Ser with Thr in Asn-Leu-Ser in β3GnT2. We speculate that the double-band of GGTΔ25 might be derived from the heterogeneity in utilization of *N*-glycosylation at Asn219 in BES. In human β3GnTs, only the conserved *N*-glycosylation site of β3GnT2 is Asn-Leu-Ser, while the others are Asn-Leu-Thr [4-7]. It would be interesting to determine whether this conserved *N*-glycosylation site is completely occupied in mammalian cells and tissues.

Based on the expression patterns of GGTΔ25, GGTΔ25-N79Q, GGTΔ25-N89Q, GGTΔ25-N127Q, GGTΔ25-N173Q, and GGTΔ25-N219Q (Figs. 4 and 5), the *N*-glycosylation numbers of GGTΔ25-N127Q and GGTΔ25-N219Q may be three and two, respectively. GGTΔ25-N127Q and GGTΔ25-N219Q was found to be a single band, but GGTΔ25-N127Q had lower mobility than GGTΔ25-N219Q. The *N*-glycosylation patterns are supposed to be those of the model as shown in Fig. 7. This model hypothesizes that *N*-glycosylation at Asn127 requires an *N*-linked oligosaccharide at Asn219, but *N*-glycosylation at Asn219 does not require an *N*-linked oligosaccharide at Asn127. If this hypothesis is applied to the expression pattern of GGTΔ25, the upper band indicates that the *N*-glycosylation sites, Asn79, Asn89, and Asn127 are occupied, and the lower band indicates that the *N*-glycosylation sites, Asn79 and Asn89, are occupied. The upper band of GGTΔ25-N79Q and GGTΔ25-N89Q indicates that three *N*-glycosylation sites are occupied. However, the lower bands indicate that only either Asn79 or Asn89 is glycosylated. In this hypothesis, the N-glycosylation pattern of GGTΔ25-N219Q and GGTΔ25-S221 corresponds to the pattern of the lower and upper bands of GGTΔ25, respectively.

GGTΔ25 for which 1-25 amino acids were deleted in β3GnT2 was expressed and active in BES, but GGTΔ83 was only slightly expressed in a fragmented, inactive form (Fig. 6). GGTΔ25-N79Q was expressed and was active to the same extent as GGTΔ25 (Fig. 4), suggesting that fragmentation and inactivation of GGTΔ83 were not due to the deletion of oligosaccharides linked to Asn^{79}, but rather to a lack of 26-83 amino acids. This deleted region is in a putative stem domain, and its amino acid sequence shows no homology with other β3GnT. Therefore, regarding human β3GnT2, its stem domain is likely to be involved in the stability of β3GnT2, which is necessary for the expression of its biological function. In *Arabidopsis*, β1,2-xylosyltransferase was found to be expressed in BES but was completely inactivated after the deletion of 82 amino acids residues at its N-terminus, and only amino acids 1-36 were satisfied with its Golgi retention [23]. The stem domain of β-galactoside-α2,6-sialyltransferase could play a role in recognizing glycoprotein acceptors, and it may exert strict control onto the catalytic domain [24]. Stem domains of various glycosyltransferase have high variability in amino acid sequences and little secondary structure, but its stem domains are also important for the biological function and localization likely to *N*-glycosylation [25].

The crystallization of glycoproteins heavily linked to oligosaccharides is difficult, although the crystallization of an active glycoprotein from which several *N*-linked oligosaccharides were removed has been achieved [26]. β3GnT2Δ25-S221T, which could be expressed as a single band in BES, may be useful for the crystallization of β3GnT2. Further studies, including the determination of the minimal catalytic domain and minimal *N*-glycosylation in β3GnT2, may help its crystallization and structure analysis.

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Legend for figures

Fig. 1. Potential *N*-glycosylation sites in β3GnT2.

Fig. 2. Partial deglycosylation of purified GGTΔ25 by PNGase. Purified GGTΔ25 was analyzed by 8% SDS-PAGE and CBB stain after treatment with 0-100 μU PNGase F for overnight incubation. The fully glycosylated fusion protein migrated with a molecular mass of approximately 77kDa. The staining with CBB displayed a total of five bands, demonstrating the presence of four *N*-glycans in the native protein. Numbers on the right indicate the number of *N*-glycans present in the individual protein band. M denotes molecular marker.

Fig. 3. Effect of the addition of tunicamycin (A) and castanopermine (B) on he expression of the GGT fusion protein and β3GnT activity After the infection of the recombinant baculovirus, either 10 μg/ml tunicamycin or 75 μg/ml castanopermine was added to culture medium. Lanes 1, 2: extracellular fractions without (1) and with the addition of tunicamycin (2), respectively; Lanes 3, 4: intracellular fractions without (3) and with the addition of castanospermine (4), respectively.

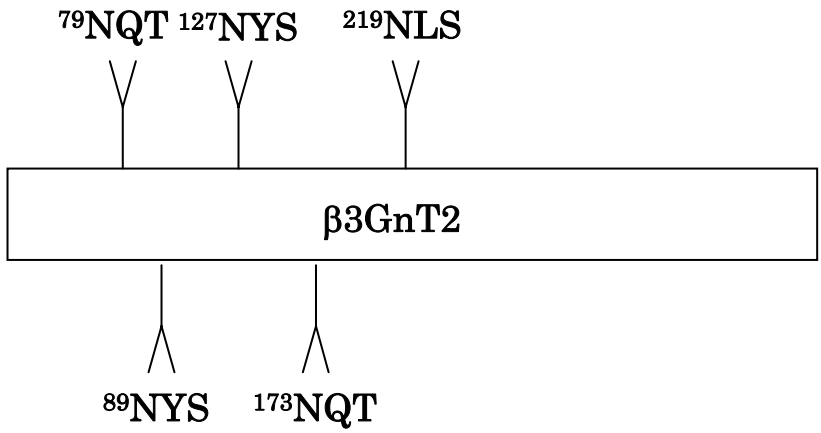
Fig. 4. Secretion and β3GnT activity of mutant fusion proteins. Tn-5B1-4 cells were infected with recombinant baculovirus which contained each mutant gene. After cultivation for 2 days, culture supernatants were analyzed by measurement of β3GnT activity and SDS-PAGE. Specific fluorescent bands were detected by Molecular Image FX (Bio-Rad). Protein concentrations are represented as the densitometric value of the total amount of the upper and lower bands. Protein concentration and the specific

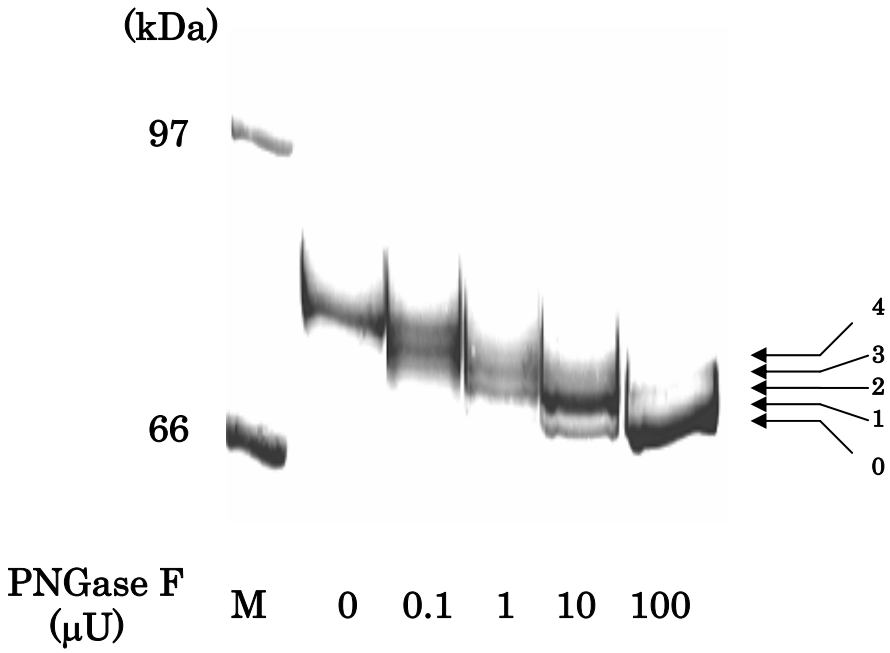
activity of wild type (WT) are used as a reference value indicating 100%. WT, N79Q, N89Q, N127Q, N173Q, and N219Q denote GGTΔ25, GGTΔ25-N79Q, GGTΔ25-N89Q, GGTΔ25-N123Q, GGTΔ25-N173Q, and GGTΔ25-N219Q, respectively.

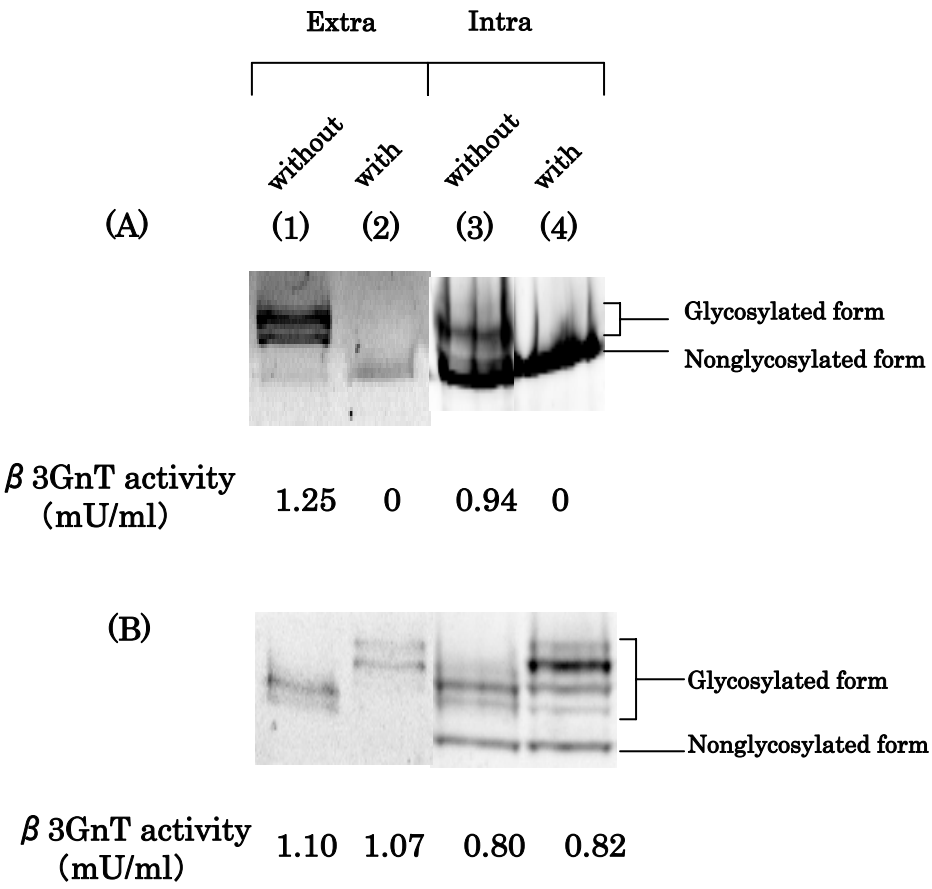
Fig. 5. Secretion and β3GnT activity of S221T. Expression of the protein concentration and the specific activity of WT are described in Fig. 4. WT and S221T denote GGTΔ25 and GGTΔ25-S221T, respectively.

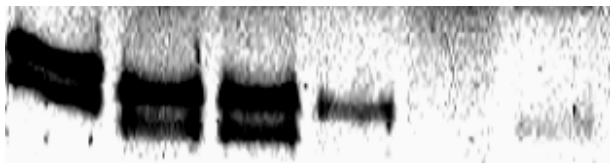
Fig. 6. Expression and secretion of deletion mutant proteins. Tn-5B1-4 cells were infected with recombinant baculovirus containing each mutant gene. After cultivation for 2 days, culture supernatants were analyzed by SDS-PAGE. Expressed bands were detected by fluorescence. Arrow indicates the full-length fusion protein of GGTΔ25. Δ25, Δ83, Δ91, and Δ129 denote GGTΔ25, GGTΔ83, GGTΔ91, and GGTΔ129, respectively.

Fig. 7. Proposed N-glycosylation patterns. Based on the molecular band patterns shown in WT, N79Q, N89Q, N127Q, N173Q and N219Q mutants, N-glycosylation sites of β3GnT2 are proposed. ●, ○, and × indicate glycosylated , unoccupied, and mutated, respectively. WT, N79Q, N89Q, N127Q, N173Q, and N219Q denote GGTΔ25, GGTΔ25-N79Q, GGTΔ25-N89Q, GGTΔ25-N123Q, GGTΔ25-N173Q, and GGTΔ25-N219Q, respectively. (U) and (L) denote the upper and lower bands, respectively.

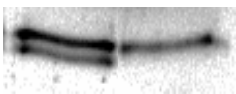

79NQT
127NYS
219NLS
β3GnT2
89NYS
173NQT

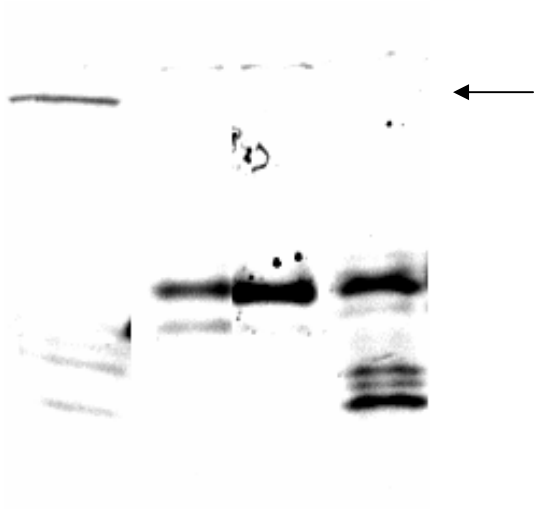

(kDa)
97
66
4
3
2
1
0
PNGase F
(μU)
M
0
0.1
1
10
100


Extra
Intra
without
with
without
with
(A)
(1)
(2)
(3)
(4)
Glycosylated form
Nonglycosylated form
β 3GnT activity (mU/ml)
1.25
0
0.94
0
(B)
Glycosylated form
Nonglycosylated form
β 3GnT activity (mU/ml)
1.10
1.07
0.80
0.82



	WT	N79Q	N89Q	N127Q	N173Q	N219Q
Extracellular fraction						
Activity (mU/ml)	1.34	0.74	0.95	0.43	0	0
Relative protein concentration (%)	100	80	84	19	0	13
Relative specific activity (%)	100	67	90	126	0	0

	WT	S221T
Extracellular fraction		
Activity (mU/ml)	1.34	1.51
Relative protein concentration (%)	100	63
Relative specific activity (%)	100	186



Δ 25 Δ 83 Δ 91 Δ 129

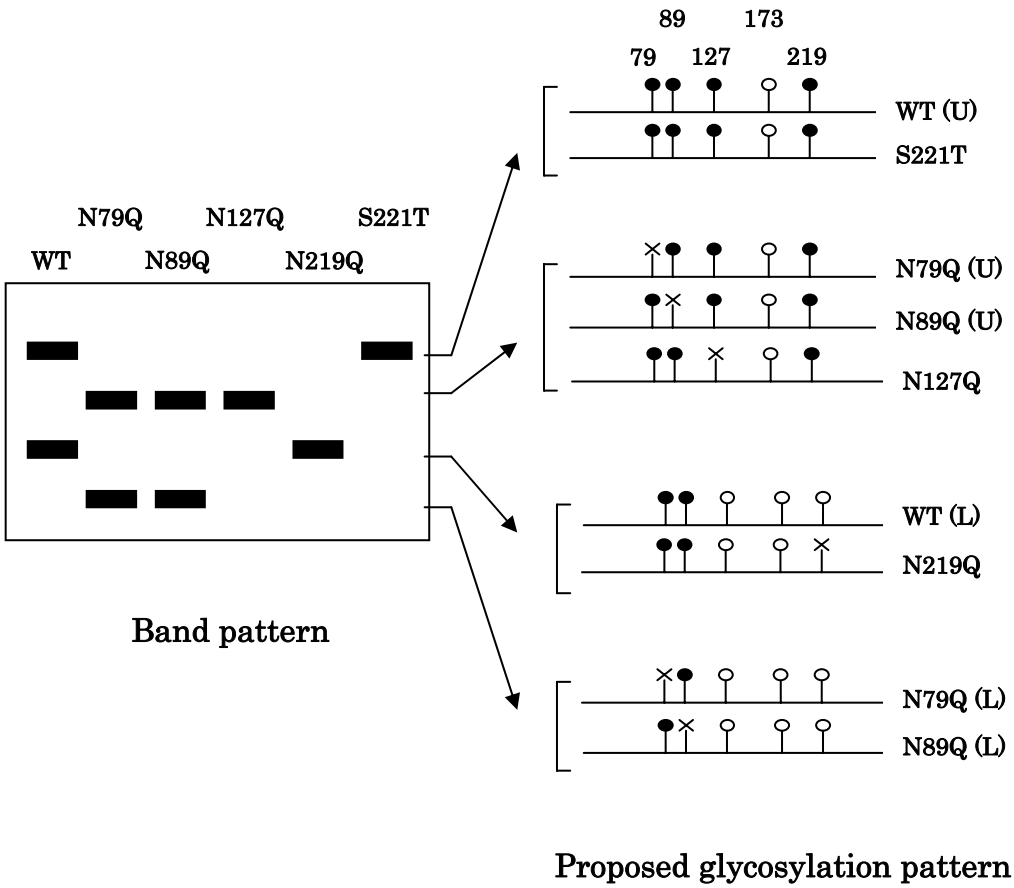

89
173
79
127
219
WT (U)
S221T
N79Q
N127Q
S221T
WT
N89Q
N219Q
N79Q (U)
N89Q (U)
N127Q
WT (L)
N219Q
N79Q (L)
N89Q (L)
Band pattern
Proposed glycosylation pattern

Table 1
Mutagenic primers

	5' - 3'
β3GnT2-N1-1	GAGCATGCTGACCCAACAGACGGGGGAG
β3GnT2-N1-2	CTCCCCCGTCTGTTGGGTCAGCATGCTC
β3GnT2-N2-1	GGCAGGCTCTCCCAAATAAGCCATCTG
β3GnT2-N2-2	CAGATGGCTTATTTGGGAGAGCCTGCC
β3GnT2-N3-1	GTATTTGAGATGCCGCCAGTATTCACTGCTTATAG
β3GnT2-N3-2	CTATAAGCAGTGAATACTGGCGGCATCTCAAATAC
β3GnT2-N4-1	GAAAGCAACGCAGGGCAACAAACGGTGGTGCG
β3GnT2-N4-2	CGCACCACCGTTTGTTGCCCTGCGTTGCTTTC
β3GnT2-N5-1	GACACTTTCTTCCAGTTGTCTCTGAAG
β3GnT2-N5-2	CTTCAGAGACAACTGGAAGAAAGTGTC
β3GnT2-S221-1	CTTTCTTCAACTTGACTCTGAAGGAAGTGC
β3GnT2-S221-2	GCACTTCCTTCAGAGTCAAGTTGAAGAAAG
β3GnT2Δ83-F	AACTGCAGCTGCGGGCAGGCTCTCCAATATAAGC
β3GnT2Δ91-F	AACTGCAGCTCATCTGAACTACTGCGAACCTGAC
β3GnT2Δ129-F	AACTGCAGCTCTGCTTATAGATCAGCCGGATAAG
β3GnT2-R	CGGAATTCTGAAGGGTTTAGAGGCCCTCAAATGGG