N-Glycosylation is requisite for the enzyme activity and Golgi retention of N-acetylglucosaminytransferase III

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UDP-N-acetylglucosamine: β-d-mannoside β-1,4N-acetylglucosaminytransferase III (GnT-III, EC 2.4.1.144) is a glycoprotein involved in the biosynthesis of N-linked oligosaccharides. Rat GnT-III contains three potential N-glycosylation sites, which have been predicted to be Asn243, Asn261, and Asn399. To study the roles of N-glycosylation in the GnT-III function, rat GnT-III was expressed in COS-1 cells under tunicamycin or castanospermine treatment. The tunicamycin-treated GnT-III, which was not N-glycosylated, had almost no activity. The castanospermine-treated GnT-III was not localized in the Golgi, but glucosylation did not affect its activity. To clarify the role of individual N-glycosylations, we obtained a series of mutant cDNAs in which some or all of the potential glycosylation sites were eliminated by site-directed mutagenesis, and expressed them in COS-1 cells. All the mutants exhibited lower enzyme activity than the wild-type, but deglycosylation at individual sites had different effects on the enzyme activity. The deglycosylation at Asn243 or Asn261 resulted in slightly lower activity, corresponding to the case of tunicamycin-treated wild-type GnT-III. Kinetic analysis revealed that the deglycosylation at Asn243 or Asn261 resulted in slightly lower affinity for the donor substrate, but the other mutation did not significantly change the Km value for either the donor or acceptor. None of the mutant GnT-III showed perinuclear localization or Golgi retention, that was observed for the wild-type protein. This is the first demonstration that the glycosyltransferase localized in the Golgi apparatus requires N-glycosylation for its activity and retention.

Key words: N-acetylglucosaminytransferase III/N-glycosylation/Golgi apparatus/glycoprotein/protein folding

Introduction

Oligosaccharides of glycoproteins play crucial roles in a variety of biological and physical properties, such as folding, secretion, solubility, stability, and clearance from the blood stream, of glycoproteins (for reviews, see Rademacher et al., 1988; Varki, 1993). These sugar chains, and those of glycolipids, are synthesized by glycosidases and glycosyltransferases localized in the ER and Golgi apparatus (Field and Wainwright, 1995). All known glycosyltransferases except for β-1,2-N-acetylglucosaminytransferase I (Kumar et al., 1990; Sarkar et al., 1991) have potential N-glycosylation sites. However, the roles of these N-linked oligosaccharides have not been evaluated, except in a few cases, such as α-2,6-sialyltransferase and β-1,4-N-acetylglactosaminytransferase (Fast et al., 1993; Haraguchi et al., 1995).

UDP-N-Acetylglucosamine: β-d-mannoside β-1,4N-acetylglucosaminytransferase III (GnT-III, EC 2.4.1.144) catalyzes the attachment of a GlcNAc residue to β-1-4 mannose in the core region of N-glycans and forms bisecting GlcNAc (Figure 1). GnT-III is presumed to be involved in pathological conditions, because increased expression is accompanied by malignant transformation or oncofetal changes (Koenderman et al., 1987; Koenderman et al., 1989; Miyoshi et al., 1993; Yoshimura et al., 1995). Rat GnT-III is a type II membrane glycoprotein composed of 536 amino acids and has a domain structure typical of glycosyltransferases (Nishikawa et al., 1992). The domain structure comprises a short amino terminal cytoplasmic tail, transmembrane and neck regions, and a long carboxyl terminal catalytic domain protruding into the Golgi lumen. The catalytic domain has three potential N-glycosylation sites at Asn243, Asn261, and Asn399. The recombinant GnT-III expressed in E.coli is almost inactive, and the activity is not always correlated with the mRNA levels in various tissues (Yoshimura et al., 1995). These findings suggest that GnT-III activity is regulated by posttranslational processes, such as phosphorylation and glycosylation. N-linked oligosaccharide structures are sequentially synthesized through the removal and addition of specific sugar residues by specific glycosidases and glycosyltransferases. It must, therefore, be important to clarify the mechanisms by which these processing enzymes, including GnT-III, are retained in the Golgi apparatus as well as their activation.

In the present study, a full-length rat GnT-III was expressed in COS-1 cells. The roles of core glycosylation and subsequent glucose trimming in the activity and Golgi retention of GnT-III were examined by using processing inhibitors, tunicamycin and castanospermine, and a series of mutants that lack potential N-glycosylation sites. We confirmed that all three N-glycosylation sites are necessary for Golgi retention and full expression of the enzyme activity.

Results

Characterization of the anti-rat GnT-III antibody

To check the immunoreactivity of antibody against the active GnT-III expressed in COS-1 cells, the lysate of transfected COS-1 cells was incubated with increasing volumes of anti-GnT-III antiserum. And then, the immune complex was precipitated with protein-G Sepharose. The residual GnT-III ac-
Fig. 1. The reaction catalyzed by GnT-III. GlcNAc and Man denote N-acetylglucosamine and mannose, respectively. R was GlcNAcβ1-4GlcNAc2-aminopyridine in the assay system used in the present study.

Activity in the supernatant was reduced in a dose-dependent manner (Figure 2).

Effects of tunicamycin and castanospermine on GnT-III in COS-1 cells

The Asn residues at 243, 261, and 399 of GnT-III are potential N-glycosylation sites (Asn-X-Thr) (Figure 3a). Tunicamycin blocks core glycosylation, and castanospermine, an inhibitor of glucosidase I and II, blocks the initial step of N-glycan processing. The Western blotting pattern of GnT-III with or without treatment is presented in Figure 4. The wild-type GnT-III migrated on SDS-PAGE to 68.1 kDa. Compared with intact GnT-III, the tunicamycin-treated enzyme migrated faster, to 60.3 kDa, due to the lack of N-glycans, and the castanospermine-treated form migrated slowly, to 69.7 kDa, because of the remaining glucose residues. The enzyme activities of these structurally modified GnT-IIs were measured and are presented in Figure 5. GnT-III activity was completely abolished by tunicamycin, whereas it was not affected by castanospermine. Intracellular localization was then analyzed by fluorescence microscopy. As shown in Figure 6, wild-type GnT-III displayed a Golgi staining pattern similar to Gal-T and WGA. In contrast, the inhibitor-treated enzymes were not localized in the Golgi apparatus.

These results, taken together, indicated that the normal enzyme activity requires core glycosylation but not glucose trimming, although these steps are necessary for GnT-III to be retained in the Golgi apparatus.

Enzyme activity of mutant GnT-III

From the wild-type GnT-III cDNA constructed in an expression vector, pSVK3, we generated seven types of GnT-III mutant by site-directed mutagenesis, replacing potential N-glycosylation site Asn residues with Gln (Figure 3b). Mutants 1-3 (MT1-3) were missing one of the N-glycosylation sites, mutants 1, 2, 1, 3, and 2, 3 (MT1,2, MT1,3, and MT2,3) lacked two sites, and mutant 1, 2, 3 (MT1,2,3) had no N-glycosylation sites. As shown in Figure 7, the mutant GnT-IIs migrated differently from one another on SDS-PAGE, according to the number of oligosaccharide chains attached. A 68.1 kDa protein...
was generated from wild-type GnT-III, whereas carbohydrate removal by site-directed mutagenesis at a single site resulted in a decrease in molecular mass of 2.0–3.0 kDa, a value corresponding to a single sugar chain. The molecular mass of the triple mutant, MT1,2,3, was almost the same as that of tunicamycin-treated GnT-III (Figures 4, 7). The GnT-III activities of these mutants are shown in Figure 5. MT1 or MT2 transfectants exhibited 40–50% of the activity of wild-type transfectants, while MT3 transfectants showed a decrease of 90%. MT1,2 transfectants expressed only a little less activity compared with MT1 and MT2. MT1,2,3 transfectants exhibited almost no activity, corresponding to the case of tunicamycin-treated GnT-III described above. These results suggested that glycosylation at Asn243 and Asn261 is important for the enzyme to express GnT-III activity, while that at Asn99 is not essential.

**Intracellular localization of mutant GnT-IIIIs**

The distributions of the wild-type and mutant GnT-IIIIs in transfected COS-1 cells were analyzed by the immunohistochemical technique (Figure 8). Anti-rat GnT-III antibody revealed perinuclear staining for wild-type GnT-III, and the stained compartment turned out to be the Golgi apparatus, as judged by the WGA lectin binding. On the other hand, all mutants were diffusely distributed in the cells, indicating that N-glycosylation all the three potential sites are indispensable for GnT-III to be retained in the Golgi apparatus.

**Kinetics of mutant GnT-IIIIs**

In order to examine the enzymological effect of the mutations in detail, kinetic analysis of GnT-III as to the donor and acceptor was performed. As shown in Figure 9, the $V_{\text{max}}$ values were different for the wild-type and three mutant (MT1, MT2, and MT3) transfectants. On the other hand, the apparent $K_m$ values for the wild-type and MT3 transfectants were similar to each other for either the donor or acceptor, while MT1 and MT2 exhibited a little higher $K_m$ value for the donor. These findings indicated that the MT3 mutation scarcely affected the affinity to the donor or acceptor, whereas the MT1 and MT2 mutations decreased the affinity to the donor but not that to the acceptor. The $K_m$ values for the wild-type transfectant in the present study were similar to those in rat tissue reported by Nishikawa et al. (1990).

**Discussion**

In eukaryotes, the sugar moieties of glycoproteins are synthesized by specific glycosidases and glycosyltransferases, and most of the glycosyltransferases with known structures are themselves glycoproteins (Field and Wainwright, 1995). However, the significance of N-glycosylation in these glycosyltransferases has been examined in only a few studies so far (Fast et al., 1993; Haraguchi et al., 1995). In the present study, we examined whether or not three potential sites on GnT-III are actually glycosylated. We found that all three potential sites are N-glycosylated in COS-1 cells.

To study the role of N-glycosylation in GnT-III, we completely blocked the N-glycosylation of GnT-III with tunicamycin. The GnT-III activity was almost completely lost by tunicamycin-treatment, as was expected from the recombinant GnT-III expressed in E.coli (our unpublished data).

Subsequently, we used castanospermine, an inhibitor of glucosidase I and II. Castanospermine-treated GnT-III migrated on SDS-PAGE to 69.7 kDa, the size corresponding to that of glucosylated GnT-III (Figure 4). Although the activity of castanospermine-treated GnT-III was almost the same as that of the wild-type, the transfected enzyme was not retained in the Golgi. In some glycoproteins, the inhibition of glucosidase activity was found to block secretion or cell surface expression in a line of studies (Gross et al., 1983; Lodish and Kong, 1984; Schlesinger et al., 1984; Moore and Sprio, 1993). These results suggested that the glucose on N-glycans function as specific signals for glycoprotein retention and/or degradation in the ER. Conversely, in the case of other glycoproteins, a glucosidase inhibitor was found not to inhibit the secretion or cell surface expression in another line of studies (Collet and Fielding, 1991; Enns et al., 1991). They suggested that glucose trimming is involved in the protein folding of particular glycoproteins. Indeed, in a soybean lecithin, the high-mannose sugar chain acquires the protein folding function on glucose trimming (Nagai et al., 1993). In the present study, however, castanospermine-treated GnT-III exhibited sufficient activity suggesting that GnT-III was completely folded into an active form. This suggests a possibility that the completely folded form recognized by cells is not the same as the completely active form.

Although removal of any one of the three N-linked oligosaccharides resulted in a significant, but not marked, decrease in the enzyme activity, deglycosylation at individual sites showed different effects on the enzyme activity. While the MT3 mutation resulted in a −30% decrease, the MT1 and MT2 ones showed 50–60% less activity than that of the wild-type. The double mutation, MT1,2, showed enzyme activity reduced to −10% of the wild-type level, but the MT1,3 and MT2,3 mutations showed only a little less activity than single ones, MT1 and MT2. Complete deglycosylation of GnT-III resulted in negligible activity, corresponding to the loss of activity of

Fig. 5. GnT-III activity in the COS-1 cells treated with inhibitors or overexpressing wild-type and mutant GnT-IIIIs. GnT-III activities in the COS-1 cell lysates were measured using a fluorescence-labeled oligosaccharide acceptor and a donor UDP-GlcNAc as substrates. TM and CS denote the wild-type GnT-III treated with tunicamycin and castanospermine, respectively. MT1–MT1,2,3 are GnT-III mutants. Normalization of the GnT-III activity is based on the cotransfected β-galactosidase activity. The mean values from three experiments are presented.
tunicamycin-treated GnT-III. These results indicate that N-glycosylation at any of the three potential sites significantly contributes to the expression of GnT-III activity, but the glycosylations at Asn243 and Asn261 are more important than that at Asn399. To determine the mechanism by which the removal of single or multiple N-glycosylations in GnT-III resulted in an additive decrease in enzyme activity, kinetic analysis was then performed. Though the $V_{\text{max}}$ values apparently varied between the wild-type and mutant transfectants, glycosylations at Asn243 and Asn261 affect the $K_{m}$ value for the donor but not for the acceptor sugar. On the other hand, glycosylation at Asn399 does not contribute to the affinity for either the donor or acceptor sugar. These results suggested that glycosylations at Asn243 and Asn261 participate in the binding to donor, and the glycosylation at Asn399 is not directly involved in the interaction of the enzyme with donor or acceptor. The glycosylation at the Asn399 may contribute to the stability of the enzyme structure and is thus required for the full enzyme activity.

During the process of biosynthesis of N-linked oligosaccharides in the ER and Golgi, certain sugar residues are removed and others are added through a defined order of reactions cata-

Fig. 6. The effects of tunicamycin and castanospermine on the subcellular localization of GnT-III in COS-1 cells. (a) Transfected COS-1 cells were stained with anti-rat GnT-III polyclonal antibody (wild-type, TM, and CS). Typical ER staining or Golgi staining pattern was shown by anti-calreticulin antibody (ER) or anti-Gal-T serum (Gal-T), respectively. The Golgi staining pattern of Gal-T and wild-type was verified by double staining with FITC-labeled WGA (Golgi) (panels on the left). TM, tunicamycin; CS, castanospermine. (b) The relative proportions of staining patterns, ER versus Golgi, in the transfected cells. The number of cells counted is given in parentheses.
lyzed by specific enzymes. Therefore, the glycosylating enzymes (glycosyltransferases and glycosidases) including GnT-III should have a targeting mechanism by which precise glycosylation steps should be organized and completed. GnT-III is one of the medial Golgi enzymes. Although the mechanism underlying Golgi retention has not been resolved, two hypothetical models have been reported. One is that the length of the transmembrane domain determines the behavior of proteins in the secretary pathway (Munro, 1991, 1995), and the other is the "kin recognition" model (Nilsson et al., 1994). On the basis of the latter hypothesis, a protein that should be localized in the medial Golgi is retained in the proper compartment through the formation of "hetero-oligomers," which are too large to enter forward-moving transported vesicles, in situ, with other medial Golgi proteins. Elimination of any one of the three N-glycosylation sites might result in failure to form such "hetero-oligomers" and thus in loss of retention in the Golgi. Although the activity of MT3 was 70-80% of the wild-type level, MT3 was not localized in the Golgi (Figure 8). This finding, as well as the localization of castanospermine-treated GnT-III, suggests that the native folding of the domains, which contain these glycosylation sites but are not so important for enzyme activity, is required for Golgi retention. Otherwise, glycosylation and glucose trimming of GnT-III may participate in the "kin recognition."  

In conclusion, this study demonstrated that all three potential sites are glycosylated in GnT-III, and that they are required for Golgi retention and for full expression of the catalytic activity of this enzyme. This study also suggested that the active form in vitro is not always the correctly folded one. However, further studies are required to determine the precise roles of individual N-linked carbohydrate chains, and the mechanism by which they affect enzyme activity and Golgi retention.

Figure 7. SDS-PAGE and Western blotting analysis of GnT-III mutants. Lysates of the COS-1 cells transfected with mock, wild-type enzyme, or various mutants were separated on an 8% polyacrylamide gel under reducing conditions. The GnT-III bands were detected with rabbit polyclonal antibody.

Materials and methods

Materials

Materials were obtained from the following sources: pSVK3, protein-G-Sepharose (Pharmacia, Uppsala, Sweden), pET3d and pCH110 (Novagen), tunicamycin, α-fucosidase, UDP-GlcNAc and bovine γ-globulin (Sigma Chemical Co.), castanospermine (Wako Chem. Co., Osaka, Japan), β-galactosidase (Seikagaku-Kogyo, Tokyo, Japan), ODS-80TM column (Tosoh, Tokyo, Japan), Lab-Tek chamber slide (Nunc), rhodamine-conjugated anti-rabbit immunoglobulin (Tago), FITC-labeled wheat germ agglutinin (Honen, Tokyo, Japan), Vectashield mounting medium (Vector Lab.), FITC-labeled anti-goat immunoglobulins and horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Dako, Glostrup, Denmark), ECL chemiluminescence detection kit (Amersham), goat anti-calciumcitulin serum and rabbit anti-galactosyltransferase serum were gifts from Dr. Maclennan at University of Toronto and Dr. Shaper at Johns Hopkins University, respectively.

Preparation of antiserum against rat GnT-III

A GnT-III protein lacking the N-terminal 22 amino acid residues was produced in E.coli by using a pET3d expression vector. The recombinant GnT-III isolated on SDS-PAGE, and recovered directly from the gel after staining, was used to raise antibody in rabbits. One of the rabbits produced an antiserum that reacted strongly with the GnT-III expressed in COS-1 cells as judged by Ouchterlony methods and immunoblotting.

Construction of a rat GnT-III expression vector and site-directed mutagenesis

The construction of plasmid pSVK3-(rat GnT-III) was described previously (Nishikawa et al., 1992). Site-directed mutagenesis was carried out using synthetic oligonucleotide primers according to the method of Kunkel (1985). The uracil-substituted single-stranded DNA was prepared from an E.coli strain C1236 transformed with the plasmid. The uracil template was used together with oligonucleotide primers to generate mutant sequences. The synthetic oligonucleotide primers used to replace Asn by Gin were as follows: 5'-CTCCCCGTAAACCCGTTGAACCTGGATCCCGAG3' for N243Q; 5'-CTCGAAGGTACCGTGGATGCACTG3' for N261Q; and 5'-GATGGGCGGTGAGCTGTCATTGCG3' for N399Q. The mutations in the pSVK3-(GnT-IIIIs) were verified by sequence analysis.

Transient expression of rat GnT-III in COS-1 cells and tunicamycin and castanospermine treatments

Transfection of pSVK3-(rat GnT-III) into COS-1 cells was performed by electroporation as described previously (Nishikawa et al., 1992). A β-galactosidase expression vector pCH110 was co-transfected in order to normalize the transfection efficiency (Kang et al., 1996). Tunicamycin or castanospermine was added to the cells at a concentration of 1 µg/ml or 50 µg/ml, respectively, 6 h after transfection. Two days later, the total 5 x 10⁶ cells were harvested and lysed by sonication in 0.5 ml of phosphate-buffered saline (PBS).

Immunoadsorption of GnT-III activity with anti-GnT-III serum

Lysates (~0.1 mg protein) of the transfected COS-1 cells were incubated in 50 µl PBS with increasing volumes (0-10 µl) of anti-GnT-III serum or pre-immune rabbit serum at 4°C for 3 h with gentle agitation. The solution was then incubated with 10 µl of protein-G-Sepharose Fast Flow for 30 min, and the immune complex was precipitated by centrifugation at 2000 x g for 5 min. GnT-III activity in the supernatant was measured.

Measurement of GnT-III activity

A fluorescencelabeled acceptor sugar chain, GlcNAcβ1-2Manα1-6GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc2-aminopyridine, was prepared from bovine γ-globulin. Briefly, a pronase digest of γ-globulin was subjected to hydrazinolysis and N-acetylation, and the resulting oligosaccharides were pyridylaminated according to the method of Hase et al. (1984). After consecutive digestions with β-galactosidase and α-fucosidase, the acceptor sugar chain was purified using a reverse-phase ODS-80TM column. The standard GnT-III assay was carried out in a 50 µl solution containing 40 µM of the fluorescencelabeled acceptor sugar chain, 20 mM of the donor substrate UDP-GlcNAc, and 10 µl of cell lysate at 37°C for 1 h (Nishikawa et
Fig. 8. Subcellular localization of mutant GnT-IIIIs in the transfected COS-1 cells. (a) The COS-1 cells transfected with wild-type, or mutant, enzymes were stained with anti-rat GnT-III polyclonal antibody. (b) Stained cells were counted as described in Figure 6.
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Abbreviations

UDP-GlcNac, uridine 5'-diphospho-N-acetylglucosamine; GnT-III, UDP-GlcNac: β-N-acetylglucosamine 1-phosphate uridylyltransferase III; Gal-T, UDP-galactose: GlcNAc β-1,4 galactosyltransferase; Man, mannose; FTTC, GlcNAc: P-D-mannoside β 1,4 N-acetylglucosaminyltransferase HI; Gal-T, UDP-GlcNAc, undine 5'-diphospho-N-acetylglucosamine; GnT-I, UDP-N-acetylglucosamine: β-N-acetylglucosaminyltransferase I; GnT-II, UDP-N-acetylglucosamine: β-N-acetylglucosaminyltransferase II; GnT-III, UDP-N-acetylglucosamine: β-N-acetylglucosaminyltransferase III.

References


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