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The Critical Role of the Stem Region as a Functional Domain Responsible for the Oligomerization and Golgi Localization of *N*-Acetylglucosaminyltransferase V

THE INVOLVEMENT OF A DOMAIN HOMOPHILIC INTERACTION*

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We demonstrated that a region in the stem of *N*-acetylglucosaminyltransferase V (GnT-V), a Golgi resident protein, is not required for enzyme activity but serves as functional domain, responsible for intracellular localization. Deletion of the domain led to complete retention of the kinetic properties but resulted in the cell surface localization of the enzyme as well as its efficient secretion into the medium. The lack of this domain concomitantly abolished the disulfide-mediated oligomerization of GnT-V, which appears to confer the Golgi retention. When the domain was inserted into the stem region of a cell surface-localized type II membrane protein, the resulting chimeric protein was substantially oligomerized and predominantly localized in the intracellular organelle. Furthermore, it was found that the presence of this domain is exclusively responsible for homo-oligomer formation. This homophilic interaction appears to involve a hydrophobic cluster of residues in the α-helix of the domain, as indicated by secondary structure predictions. These findings suggest that the domain specifically participates in the Golgi retention of GnT-V, probably via inducing homo-oligomer formation, and would also provide a possible mechanism for the oligomerization, which is critical for localization in the Golgi.

β1,6-*N*-Acetylglucosaminyltransferase V (EC 2.4.1.155, GnT-V),[1] a type II membrane protein that is localized in the Golgi apparatus, is involved in the biosynthesis of Asn-linked oligosaccharides. The enzyme catalyzes the transfer of a GlcNAc residue from UDP-GlcNAc to an α1,6-linked mannose moiety of Asn-linked oligosaccharides via a β1,6-linkage (1, 2). It is known that the levels of products of GnT-V, which are β1,6-branched *N*-glycans, are frequently increased in many malignant tumors and that this is closely correlated with tumor progression (3–5). Several studies have suggested that the β1,6-branched *N*-glycans directly contribute to the tumor growth and metastasis (6–8). The GnT-V protein appears to consist of an N-terminal cytoplasmic tail, a transmembrane region, a stem region, and a large C-terminal catalytic domain, as suggested by the primary structure (9, 10), and thus shares a domain structure that is common to many glycosyltransferases (11).

Oligosaccharides of glycoconjugates such as glycoproteins and glycolipids are synthesized and processed via catalysis by a variety of glycosyltransferases in the endoplasmic reticulum and the Golgi apparatus. Such enzymes, which are involved in oligosaccharide biosyntheses, must be localized to the appropriate intracellular destination to permit a properly ordered processing of the oligosaccharides. It is generally thought that many glycosyltransferases contain a structural region that is responsible for localization in the Golgi. Such a portion is often referred to as a Golgi retention signal. However, a common motif for serving as such a retention signal has not been found to date, probably because of the lack of homologous sequences among the Golgi-localized glycosyltransferases (11). It is believed that GnT-V is localized to the Golgi apparatus and participates in *N*-linked oligosaccharide processing therein. Nevertheless, the mechanism of the retention of the enzyme in the Golgi apparatus has not been explored, and a structural region responsible for this localization has not been identified.

Two models, the bilayer thickness model and the kin recognition model, have been proposed to date for the mechanism of the Golgi retention of glycosyltransferases (11). The bilayer thickness model, also known as the lipid sorting model, postulates that the length of the transmembrane domain of glycosyltransferases mediates Golgi retention (12, 13). Alternatively, in the case of the kin recognition model, it is proposed that a homo-/hetero-oligomerization of glycosyltransferases prevents the delivery of the enzymes to secretary vesicles and ongoing transport to the plasma membrane, and, as a result, this process functions as a Golgi retention signal (14, 15). However, except for a few exceptions, a clear and discrete structural factor that plays a primary role in the Golgi retention has not yet been defined in glycosyltransferases.

To better understand the mechanism of Golgi retention, additional experimental data will be required and further studies on a variety of Golgi resident proteins, including many glycosyltransferases, will be highly desirable. The mechanism that

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[1] The abbreviations used are: GnT-V, β1,6-*N*-acetylglucosaminyltransferase V; GnT-III, β1,4-*N*-acetylglucosaminyltransferase III; GGT, γ-glutamyl transpeptidase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; GST, glutathione S-transferase; MES, 4-morpholineethanesulfonic acid; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; HIV-1, human immunodeficiency virus type 1.

allows proteins to be localized in the Golgi have been substantially established by many studies using glycosyltransferases, and in fact it appears that glycosyltransferases provide a typical model for the Golgi retention of type II membrane proteins. In the present study, we expressed various deletion mutants of GnT-V and its chimeric proteins in COS cells and analyzed their intracellular localization by immunofluorescence microscopy. As a result, a region in the stem of GnT-V was identified as a functional domain that is specifically responsible for intracellular localization and that is not associated with the activity of the enzyme. Functional characterization of the domain and related studies suggests that homo-oligomer formation of GnT-V takes place and that this process is mediated by the domain that is responsible for intracellular localization. These conclusions were further confirmed by conversion of a cell surface-localized protein into an intracellularly localized form by insertion of the domain.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA-modifying enzymes were purchased from Takara, Toyobo, or New England Biolabs. UDP-GlcNAc and GlcNAc were obtained from Sigma. Oligonucleotide primers were synthesized by Greiner Japan. Antibodies were obtained from the following sources: anti-human GnT-V monoclonal antibody (a gift from FUJIREBIO Inc.); anti-FLAG epitope antibody (M5, Sigma); anti-His tag antibody (Tetra-His antibody, Qiagen); anti-GST antibody (Amersham Pharmacia Biotech); horseradish peroxidase-conjugated anti-mouse IgG antibody (Promega); horseradish peroxidase-conjugated anti-goat IgG (DAKO); and rhodamine isothiocyanate-labeled anti-mouse IgG antibody and rhodamine isothiocyanate-labeled anti-goat IgG antibody (Santa Cruz Biotechnology). Other common chemicals were obtained from Wako pure chemicals, Nacalai Tesque, and Sigma.

Expression Plasmids for a Human GnT-III and Human GnT-V—An *Eco*RI fragment, which contained the entire coding sequence of human GnT-III (hGnT-III) (16), and a *Kpn*I-*Xba*I fragment, which encodes human GnT-V (hGnT-V) (10), were subcloned into an SV40-based expression vector, pSVK3 (Amersham Pharmacia Biotech), and were used for the expression of their wild type proteins.

Construction of Chimeric GnT-V Proteins—In this study, three types of the chimeric proteins, GnVd73, GnVd187, and GnVd242, were used in which the GnT-III N-terminal sequences including the cytoplasmic tail, transmembrane domain, and portions of the stem region were fused to the luminal regions of GnT-V. For the preparation of the GnVd73 protein, a *Bsp*EI site was created by a site-directed mutagenesis according to the method of Kunkel (17), as described previously (18), to fuse the hGnT-V sequence (10) following Ala-74 to the N-terminal 62 residues of rat GnT-III (rGnT-III) (19). An oligonucleotide primer (5′-GTGGTGGATGGTCCGGACGCTGGAGTC-3′) was used for this mutation with a uracil-substituted single stranded template of the hGnT-V sequence, which was prepared using *Escherichia coli* CJ236 (*dut*$^-$, *ung*$^-$). The 2.0-kilobase fragment that encodes Ala-74 to Leu-741 (the C-terminal residue) of hGnT-V was obtained by digestion with *Bsp*EI and *Xba*I and was inserted into the *Eco*RI and *Xba*I sites of the pSVK3 along with an *Eco*RI-*Bsp*EI fragment for Met-1–Pro-62 of rGnT-III (19). When the other two chimeric proteins were constructed, the nucleotide sequences corresponding to Tyr-188–Leu-741 and Arg-243–Leu-741 were amplified by the polymerase chain reaction (PCR) to yield the fragments flanked by *Kpn*I and *Xba*I sites for 5′ and 3′ ends, respectively. A primer set used for the amplification was a combination of 5′-CAAGGTACCGGCGAATGGCTG-3′ for Tyr-188–Leu-741 or 5′-CCTGGTACCTCAGTGAGGTTG-3′ for Arg-243–Leu741 and a common antisense primer 5′-GAGCTCTAGAGGCAGTCTTTGC-3′. The amplified DNA fragments were digested by *Kpn*I and *Xba*I and were used to replace the hGnT-III nucleotide sequence encompassing Tyr-149 to the C terminus in the hGnT-III/pSVK3.

Deletion Mutant Constructs—A DNA fragment encoding Met-1-Ser-44 of hGnT-V was amplified by PCR using the following primers: 5′-CAGGTACCATGGCTCTCTTCAC-3′ and 5′-CGAGGTACCCGGAGCTGCTTTCAG-3′. A 0.13-kilobase *Kpn*I fragment resulting from the digestion of the amplified DNA was inserted, in an appropriate orientation, into the *Kpn*I site in the plasmid in which the 1.7-kilobase *Kpn*I-*Xba*I fragment for Tyr-188–Leu-741 had been ligated, thereby leading to the hGnT-V sequence lacking Met-45–Ile-187. This deletion mutant was designated GnVΔ1. Another deletion mutant, GnVΔ2, was prepared by the removal of the region covering Asp-136–Ser-435 from the expression plasmid carrying the wild type hGnT-V by *Eco*RV digestion followed by self-ligation.

FLAG Epitope-tagged Constructs for the Wild Type and the Deletion Mutants of GnT-V—To add the FLAG epitope at the N terminus, *Eco*RI-*Xba*I fragments of the complete sequences for the wild type and GnVΔ1 and GnVΔ2 mutants, excised from their respective expression plasmids constructed using the pSVK3, were ligated into a pME18FLAG vector (20), a generous gift from Dr. Takaomi Ishida (Institute of Medical Science, University of Tokyo). These FLAG-tagged constructs were designated FWT for the wild type, FΔ1 for GnVΔ1, and FΔ2 for GnVΔ2. The FN235 mutant was also prepared by ligating the PCR-amplified fragment encoding Met-1–Phe-235, in which both ends were flanked by *Eco*RI, into the *Eco*RI site of the pME18FLAG. For the construction of the FCStem mutant, in which Met-45–Ser-184 was fused to C terminus of FΔ1 mutant, two DNA fragments were amplified by PCR using the following primer sets: 5′-CAGAATTCATGGCTCTCTTCACTC-3′ and 5′-CCAACTCGAGTAGGCAGTCTTTGCAG-3′ for a full length of FΔ1 mutant and 5′-ACATCTCGAGATGCTGCGCGAGCAGATC-3′ and 5′-TATCTCGAGCTAAGAGCAGGTGGATCC-3′ for Met-45–Ser-184. The amplified DNA fragments were ligated into *Eco*RI and *Xho*I sites of a pME18FLAG vector.

Insertion of the GnT-V Sequence of Glu-41–Ser-184 into γ-Glutamyl Transpeptidase—The DNA fragment corresponding to the Glu-41–Ser-184 of hGnT-V was amplified by a PCR with primers, 5′-TTGGCCGCGGAAAGCAGCTCCATGCT-3′ and 5′-TGTCCGCGGCAAAAGAGCAGGTGGA-3′ and then digested by *Sac*II. The resulting fragment was inserted into the *Sac*II site in the human GGT/pSVK (21), whose position corresponds to amino acid residues 44–46, which is located adjacent to the membrane anchoring domain in the luminal region of GGT.

Cell Culture—COS-1 cells and CHO-K1 cells were grown and maintained at 37 °C in Dulbecco's modified Eagle medium and Ham's F-12 medium, respectively, supplemented with 10% fetal calf serum, 50 units/ml penicillin G, and 50 mg/ml streptomycin under a humidified atmosphere of 95% air and 5% CO_2.

DNA Transfection—Expression plasmids were transfected into cells by electroporation (22) using a Gene Pulser (Bio-Rad), as described previously (21). The cells were washed with Hepes-buffered saline and resuspended. Plasmids (20 μg), purified by CsCl gradient ultracentrifugation, were added to the cell suspension (1×10^7), followed by electrification. The transfected cells were subjected to biochemical and histological analyses, 48 h after transfection.

Subcellular Fractionation—Cells were homogenized in phosphate-buffered saline (PBS) and centrifugation at 10,000 × *g* for 10 min at 4 °C to remove cellular debris and nuclei. The resulting supernatants were further centrifuged at 100,000 × *g* for 1 h at 4 °C. The pellets were resuspended in a volume of PBS containing 1% Triton X-100, which was equal to that of the supernatants.

SDS-PAGE, Immunoblotting, and Lectin Blot Analysis—SDS-PAGE was carried out according to Laemmli (23). The separated proteins were electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell, PROTORAN), followed by blocking with 5% skim milk or 2% bovine serum albumin. The resulting membrane was incubated with the first antibody or biotinylated lectin. After washing, the membrane was reacted with an appropriate second antibody that was conjugated to horseradish peroxidase for immunoblotting or with an avidin-biotin complex with the peroxidase for the lectin blot. The reactive (glyco)protein bands were visualized by chemiluminescence using an ECL system (Amersham Pharmacia Biotech).

GnT-V Activity Assay and Kinetic Analysis—The GnT-V activity was assayed using a fluorescence-labeled oligosaccharide acceptor, as described previously (24). Cell homogenates (5–20 μg of proteins) were incubated at 37 °C for 2 h with 20 μM pyridylaminated agalacto bi-antennary oligosaccharide as an acceptor and 40 mM UDP-GlcNAc as a donor in 125 mM MES-NaOH (pH 6.25) containing 200 mM GlcNAc, 0.5% Triton X-100, and 10 mM EDTA. The reaction was terminated by heating the mixture at 100 °C for 3 min, and the sample was then centrifuged at 15,000 rpm for 5 min in a microcentrifuge. The resulting supernatant was analyzed by reversed phase high performance liquid chromatography (Shimazu) using a TSKgel ODS-80TM (4.6 × 150, TOSOH). The solvent used was a 20 mM, pH 4.0, ammonium acetate buffer, and the substrate and the product were isocratically separated. Fluorescence was detected with a fluorescence detector (RF-10AXL, Shimazu) at excitation and emission wavelengths of 320 and 400 nm, respectively. In the kinetic analysis, the activity was assayed with various concentrations of UDP-GlcNAc in the presence of 20 μM acceptor to determine the apparent parameters for the donor. To obtain the parameters for the acceptor, various concentrations of the acceptor were

used with a fixed concentration of the donor, namely, 40 mM UDP-GlcNAc. Other assay conditions were identical to those described above.

Immunofluorescence Microscopy—The transfected COS-1 cells (1×10^4) were seeded into individual wells of an 8-well chamber slide (Lab-Tek) and grown for 48 h, as described for the cell cultures. The cells were fixed with 3.7% formaldehyde in PBS and then permeabilized by treatment with 0.2% Triton X-100 in PBS for 15 min. The resulting cells were blocked by 2% bovine serum albumin in PBS for 60 min at room temperature and then incubated sequentially with a primary antibody for 60 min and an appropriate rhodamine isothiocyanate-conjugated second antibody for 20 min. The stained cells were then examined by fluorescence microscopy (Olympus) and photographed.

Assay for Protein Interaction by an Yeast Two-hybrid System—A DNA fragment encoding Met-45–Ser-184 was amplified by PCR using GnT-V cDNA as a template and a primer set of 5′-CATGAATTCAT-GCTGCGCGAGCAGATC-3′ and 5′-TATCTCGAGCTAAGAGCAGGTG-GATCC-3′ to introduce *Eco*RI and *Xho*I sites at the 5′ and 3′ ends, respectively. A 1.6-kilobase *Eco*RI-*Xho*I fragment that encodes the region of Glu-234–Leu-741 was excised from the human GnT-V/pSVK plasmid. To perform a yeast two-hybrid assay, a LexA two-hybrid system (CLONTECH) was employed in this study, and the assay was carried out according to the manufacturer's recommended protocol. The DNA fragments were subcloned into the *Eco*RI and *Xho*I sites of pLexA-B.D. and pB42-A.D. plasmids, and the resulting respective plasmids enabled the expression of LexA and B42 fusion proteins with these GnT-V sequences. *Saccharomyces cerevisiae* EGY48[p8op-lacZ] was used as host strain. This strain had been transformed with a reporter plasmid, p8op-lacZ, conferring LEU2 and lacZ expression under the control of LexA operators. The host cells were transformed with the LexA and B42 plasmids and grown at 30 °C for 3 days on the SD plates, which contain 2% dextrose and an amino acid mixture that lacks histidine, tryptophan, and uracil. The colonies, when grown, were then transferred to the X-gal plates, which contain 2% galactose instead of dextrose, 1% raffinose, a mixture of amino acids that lacks in leucine as well as histidine, tryptophan, and uracil. These colonies were allowed to grow at 30 °C for a further 3 days.

Glutathione S-Transferase Pull-down Assay—The GnT-V *Eco*RI-*Xho*I fragments, prepared for the yeast two-hybrid assay, were ligated into pGex4T1 (Amersham Pharmacia Biotech) and pET32a (Invitrogen) to express GST fusion proteins and polyhistidine-tagged thioredoxin fusion proteins, respectively, in *E. coli*. In the cells transformed with these plasmids, expression of both proteins was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside. GST was also expressed similarly in the cells transformed with an empty pGex4T1. The cells were lyzed by sonication, and the proteins were extracted. The GST fusion protein and nonfused GST were immobilized on glutathione Sepharose beads (Amersham Pharmacia Biotech) and were then incubated with the thioredoxin fusion protein at 4 °C for 3 h. After washing three times with PBS, the precipitated samples were subjected to SDS-PAGE followed by immunoblot analysis using anti-GST and anti-polyhistidine antibodies.

Protein Determination—Protein concentrations were determined according to the method of Bradford (25) using bovine serum albumin as a standard.

RESULTS

It is known that the GnT-V molecule contains a larger number of amino acids than many other glycosyltransferases, and, as a result, it is a reasonable assumption that the protein contains several functionally separable domains, each of which contributes to the overall function of the glycosyltransferase, but in a different manner (for example, catalytic activity *versus* the Golgi retention). The present study was initiated to identify a functional domain that is different from the catalytic domain and to investigate its specific role. Such a study would potentially lead to elucidation of the "functional domain architecture" in GnT-V and possibly in other glycosyltransferases, and the detailed functional characterization of the specific domain might provide a clue for the issue of, for example, the mechanism of Golgi retention.

To identify the structural region that is not associated with enzyme activity, chimeric GnT-V proteins, which contain the GnT-V sequences truncated using several restriction enzyme sites, were expressed in COS cells, and the enzyme activities of these products were examined. These chimeric proteins were

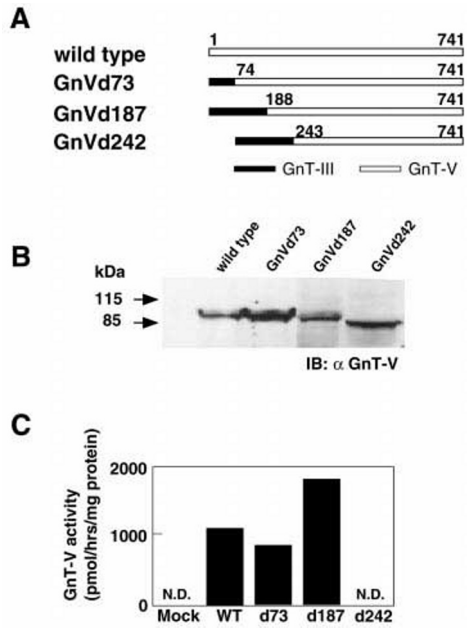

FIG. 1. **Enzyme activity of the wild type GnT-V and chimeric proteins expressed in COS-1 cells.** *A*, schematic representation of the chimeric proteins used in this study. Regions indicated by *filled* and *open boxes* were derived from GnT-III and GnT-V, respectively. *B*, detection of chimeric proteins by immunoblotting using anti-GnT-V antibody. *C*, specific activities in cell homogenates. *N.D.*, not detected; *WT*, wild type; *IB*, immunoblotting.

prepared by fusion of the GnT-V regions of Ala-74–Leu-741, Tyr-188–Leu-741, and Arg-243–Leu-741 to the GnT-III N-terminal 62 residues for Ala-74–Leu-741 or 149 residues for the others, and were designated as GnVd73, GnVd187, and GnVd242, respectively (Fig. 1*A*). An assay of GnT-V activity using the homogenates of the transfected COS-1 cells indicated that GnVd73 and GnVd187 were enzymatically active, whereas GnVd242 exhibited no detectable activity, even though its protein expression level was comparable with other chimeric enzymes, as shown in Fig. 1 (*B* and *C*). Thus, it can be concluded that the N-terminal 187 amino acid residues are not required for GnT-V activity but that the loss of additional 55 residues abolishes all activity.

To further confirm that the region corresponding to residues 45–187 is not associated with enzyme activity, deletion mutants of the GnT-V, in which residues 45–187 and residues 136–435 were deleted, were also prepared and designated as GnVΔ1 and GnVΔ2, respectively (Fig. 2*A*). Consistent with the results obtained for the chimeric proteins, the GnVΔ1 mutant was found to be active, whereas the mutation made in GnVΔ2 led to a complete loss of activity (Fig. 2, *B* and *C*). The kinetic properties of the GnVΔ1 mutant was nearly indistinguishable from those of the wild type, as revealed by kinetic analysis using cell homogenates; the apparent K_m for the donor and acceptor were 13 mM and 77 μM, respectively, in the wild type, and the respective values for the mutant were 9.1 mM and 67 μM. These results clearly indicate that the region corresponding to residues 45–187 is not involved in enzyme activity.

Despite the fact that no difference in enzymatic properties was observed, when the enzyme activity in the culture medium was assayed and compared with the cell-associated activity, it was found that the GnVΔ1 mutant is much more efficiently released from the cells, compared with the wild type (Fig. 3*A*). Furthermore, even in the cell-associated activity of the mutant, a major fraction (≈ 50%) was found in the soluble fraction, as evidenced by subcellular fractionation by ultracentrifugation (Fig. 3*B*). In the case of the wild type, on the other hand, the

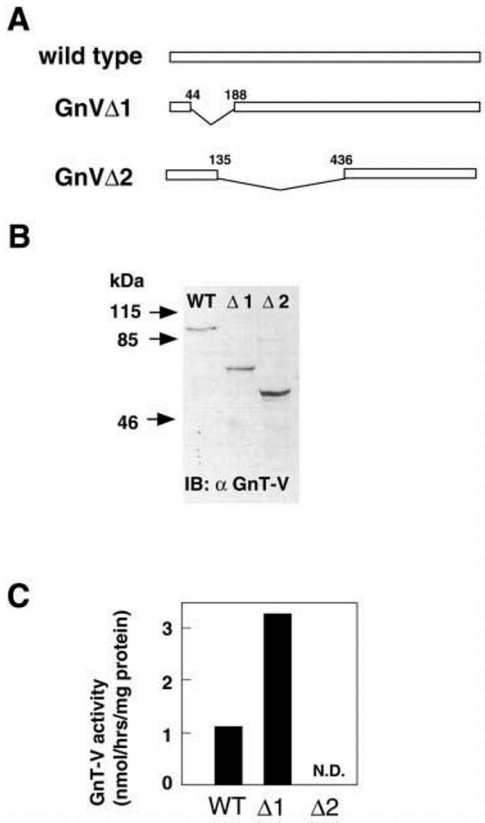

FIG. 2. **GnT-V activity of the deletion mutants.** *A*, schematic representation of the GnT-V deletion mutants, GnVΔ1 and GnVΔ2. *B*, immunoblot analysis of the expression of the deletion mutants. *C*, specific activity of the deletion mutants in the transfected COS-1 cells. *N.D.*, not detected; *WT*, wild type; *IB*, immunoblotting.

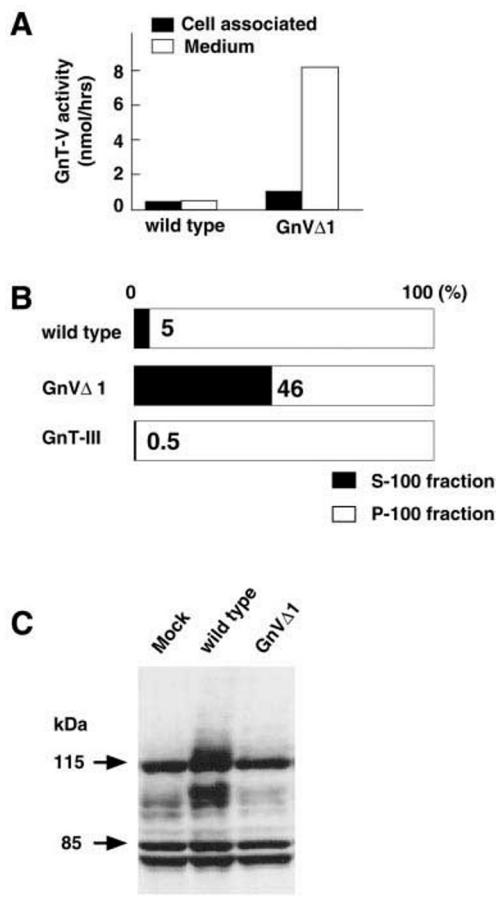

FIG. 3. **Characterization of GnVΔ1 mutant.** *A*, comparison of secreted (*open bars*) and cell-associated activities (*filled bar*) in the wild type and the mutant. *B*, subcellular distribution of the enzyme activities. The activity in the S-100 (*filled bar*) and P-100 (*open bar*) fractions are shown. *C*, lectin blot analysis of the CHO-K1 cells transfected with the GnVΔ1 mutant. Detailed experimental conditions are described under "Experimental Procedures."

enzyme was nearly exclusively located in the membrane fraction, which is consistent with the fact that many glycosyltransferases are generally anchored to the Golgi membrane. This distribution profile for the wild type is nearly the same as that of another glycosyltransferase, GnT-III, which is also known to be localized in the Golgi apparatus (26). These results show that the loss of the region corresponding to residues 45–187 causes a dramatic alteration in the cellular distribution of GnT-V.

Although the significant activity of the GnVΔ1 mutant remained associated with the membrane, it appeared that β1,6-branched sugar chains, which are formed by the action of GnT-V, were not increased in the case of the GnVΔ1-transfected CHO-K1 cells, as shown by lectin blot analysis using leukoagglutinating phytohaemagglutinin, which preferentially binds to a β1,6-branched oligosaccharide (Fig. 3*C*). In this experiment, CHO-K1 cells were used instead of COS-1 cells because the reactivity of the lectin is not substantially increased, even by the overexpression of the wild type GnT-V in COS-1 cells, probably the result of relatively high background signals. The deletion of region 45–187 resulted in the inability of the protein to modify cellular oligosaccharides even though this deletion mutant is sufficiently enzymatically active. It thus appears that the mutant is not properly localized within the cells. This suggests that the region that had been deleted could serve as a functional domain that could play a role in appropriate intracellular localization.

To investigate the intracellular localization of the wild type and GnVΔ1 mutant of GnT-V in more detail, the cellular distribution of these proteins were analyzed by immunofluorescence microscopy. Because the anti-GnT-V antibody used in the immunoblot analyses did not appear to react with the nondenatured proteins, N-terminally FLAG-tagged forms of the wild type and the mutants were constructed (Fig. 4*A*) and probed with an anti-FLAG epitope antibody. An FN235 mutant, an N-terminal 235-amino acid fragment that contains the stem region but lacks the majority of the large catalytic domain was also prepared to explore the requirement of this domain in the intracellular localization of GnT-V. As shown in Fig. 4*B*, the wild type displayed a typical Golgi-staining pattern, as reported (27), whereas the FΔ1 mutant was not localized in the Golgi apparatus but, rather, was diffusely distributed throughout the cells, probably the result of being expressed on the cell surface. These results indicate that the absence of the region corresponding to residues 45–187 (which is tentatively referred to as the "stem domain" to discriminate from "stem region") perturbs the normal Golgi localization of the GnT-V, suggesting that this domain is involved in the retention of the enzyme in Golgi. Because other mutants, FΔ2 and FN235, are also localized in the Golgi apparatus, as was the full-length wild type protein (Fig. 4*B*), the localization of GnT-V in Golgi apparatus appears to be primarily conferred by the stem domain, particularly the region encompassed by residues 45–135. As shown by the analysis of an additional mutant (Fig. 4*A*, FC-Stem), however, relocation of the stem domain to the C terminus led to different intracellular localization (Fig. 4*B*). This result suggests that the position of the domain is important, possibly because of collaboration with the transmembrane domain in the vicinity.

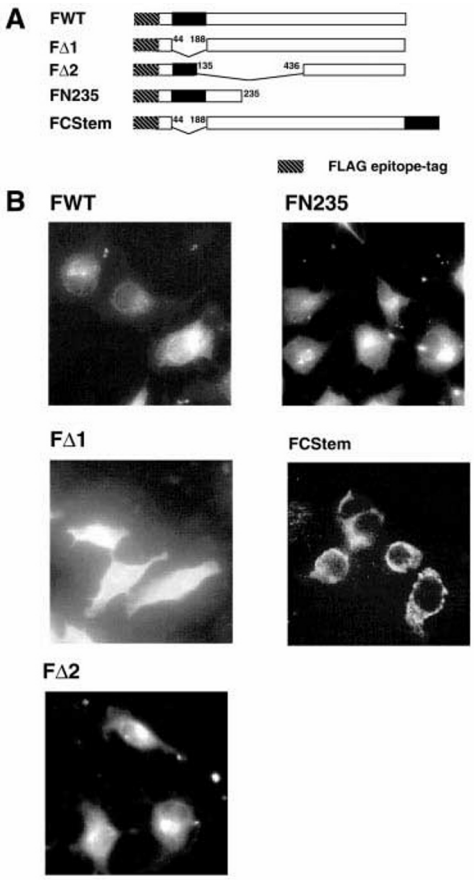

FIG. 4. **Subcellular localization of the wild type GnT-V and its deletion mutants.** *A*, schematic representation of FLAG epitope-tagged constructs of the wild type (*FWT*) and mutants (*FΔ1*, *FΔ2*, *FN235*, and *FCStem*). FLAG epitope tag and the region of Met-45–Ser-184 are shown by *hatched* and *filled boxes*, respectively. *B*, localization of FLAG epitope-tagged GnT-V proteins, as probed by an anti-FLAG antibody.

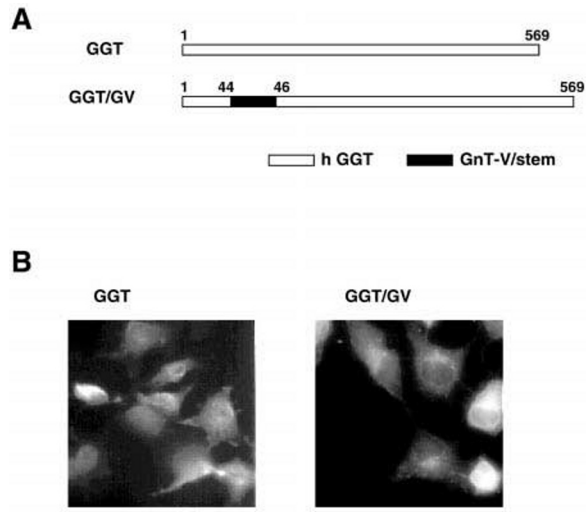

FIG. 5. **Subcellular localization of GGT and its chimeric protein.** *A*, a construct of chimeric GGT protein, in which the region of Glu-41–Ser-184 of hGnT-V was inserted. A GnT-V portion is indicated by a *filled box*. *B*, localization of the nonchimeric GGT and the chimeric GGT/GV proteins in the transfected COS-1 cells.

If the function of the stem domain is exclusively as a "Golgi retention signal" for GnT-V and is independent of other structural regions such as the transmembrane domain and the catalytic domain, it would be expected that the insertion of the domain would be sufficient to convert a plasma membrane-localizing type II membrane protein, which is unrelated to GnT-V, into a Golgi localized form. γ-Glutamyl transpeptidase (GGT) represents a typical type II membrane glycoprotein that is of critical importance to glutathione metabolism and is known to be localized on the cell surface (28, 29). Hence, this protein was used as a model protein to examine the effect of the domain on the localization of such a type II membrane protein. An amino acid sequence nearly identical to the domain comprised by residues 41–184 was inserted into almost the equivalent position in GGT (Fig. 5*A*), and the resulting chimeric protein was expressed in COS cells. As revealed by immunofluorescence microscopic analysis using anti-GGT antibody (30), the insertion of the domain had a dramatic effect on the localization of the protein: the chimeric protein was localized in the intracellular organelles, predominantly the Golgi apparatus, in contrast to the cell surface expression of the nonchimeric GGT (Fig. 5*B*). This altered intracellular distribution did not appear to be precisely identical to that of GnT-V, and thus this slight difference could be due to the subtle interference of the GGT transmembrane region, the length and amino acid composition of which are distinct from those of glycosyltransferases. However, such a dramatic conversion into an intracellularly localized form clearly indicates that the stem domain from GnT-V is fully capable of altering the localization of the cell surface-localized type II membrane protein. It thus appears likely that the mechanism of the intracellular localization of GnT-V is dependent, almost exclusively, on the nature of the stem domain but does not necessarily involve its intrinsic transmembrane domain nor any other structural regions. It also appears that the intracellular localization of GnT-V does not essentially depend on lipid sorting, a process for which the transmembrane domain plays a critical role.

When the homogenates from cells that express GnT-V were analyzed by immunoblot analysis following SDS-PAGE, performed under nonreducing conditions, it was found that the wild type GnT-V is highly oligomerized via disulfide linkages (Fig. 6*A*). This feature appears to be frequently observed in the glycosyltransferases whose intracellular localization is determined by the oligomerization-based or kin recognition mechanisms (14, 31, 32). The FΔ2 mutant, which was found to be localized in the Golgi (Fig. 4*B*), was also significantly oligomerized via disulfide linkages, as was the wild type. On the other hand, oligomer formation was substantially decreased in the cell-associated forms of the FΔ1 mutant, intracellular localization of which is impaired because of the lack of the stem domain (Fig. 6*A*). Moreover, essentially no oligomer formation was observed in the secreted enzyme, even the wild type, as well as the GnVΔ1 mutant (Fig. 6*B*). These secreted GnT-Vs, both wild type and Δ1 mutant, were smaller by about 5 kDa than those of intracellular forms, as estimated by SDS-PAGE. Thus, the secreted species of the wild type appeared to still contain the stem domain, but both secreted proteins appeared to lack the cytoplasmic tail and transmembrane domain. It seemed that their secretion results from proteolytic cleavage at the site(s) immediately close to the transmembrane domain. The disulfide bridge-mediated oligomerization appeared to fail in some fractions of the translated GnT-V protein. Considering the lack of disulfide linkages in the GnVΔ1, it is conceivable that the consequence of this failure may be the result of the release of the enzyme from the cells even in the case of the wild type. Therefore, it is likely that a GnT-V fraction that remained in the nonoligomeric state because of absence of disulfide linkages could no longer be retained in the Golgi and might subsequently be released. Oligomer formation such as this was also observed in the case of a significant fraction of the chimeric GGT, in which the stem domain of GnT-V was inserted into

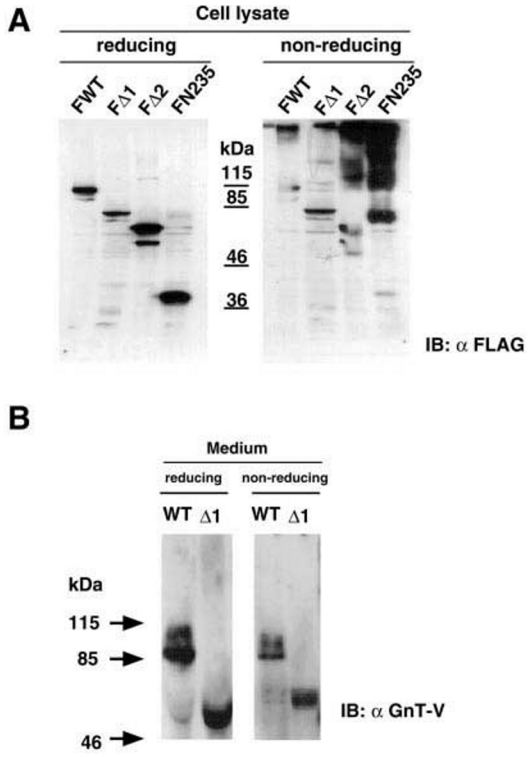

FIG. 6. **Disulfide-linked oligomerization of the wild type GnT-V and the deletion mutants.** *A*, the FLAG epitope-tagged wild type and mutants expressed in COS-1 cells were analyzed by immunoblotting using an anti-FLAG antibody, under both reducing and nonreducing conditions. *B*, the GnT-V proteins secreted from the COS-1 cells transfected with wild type GnT-V and GnVΔ1 were analyzed by immunoblotting using an anti-GnT-V antibody, under both reducing and nonreducing conditions. *WT*, wild type.

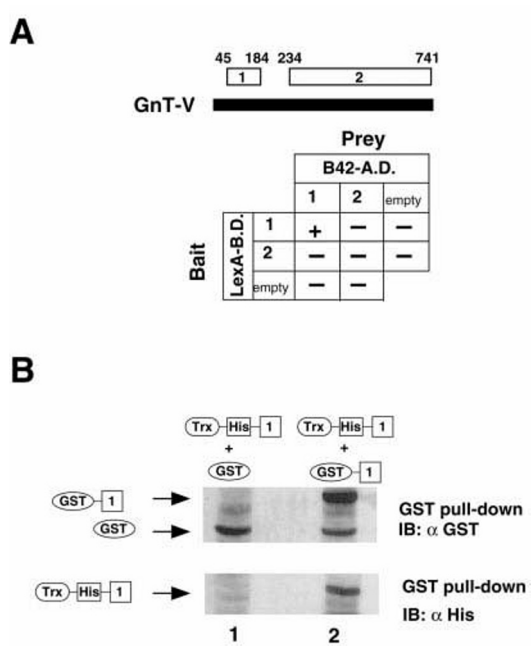

FIG. 7. **Homophilic interaction of the region of Met-45-Ser-184 in GnT-V.** Detection of the homophilic interaction by a yeast two-hybrid system (*A*) and GST pull-down assay (*B*). Detailed assay conditions are described under "Experimental Procedures." *IB*, immunoblotting.

GGT, whereas the nonchimeric GGT, which is localized on the cell surface, failed to form any detectable disulfide-linked oligomer (data not shown). The results showing that the absence of disulfide-linked oligomer formation is concomitant with the release of protein from the cells and impaired localization are consistent with the conclusion that the domain identified in this study serves to localize GnT-V in the Golgi apparatus by facilitating the formation of oligomers. Because the FN235 mutant oligomerized via disulfide bridges to a lesser extent, the formation of the covalent linkages in GnT-V may involve not only the stem domain but also other regions.

As shown in Fig. 6*A*, immunoblot analysis of the FN235 mutant gave a series of the immunoreactive bands corresponding to dimer, trimer, tetramer, and higher oligomer, suggesting homo-oligomer formation. This result and the substantial dependence of the oligomer formation of GnT-V on the stem domain strongly suggest that the domain is exclusively capable of forming a homo-oligomer via intrinsic homophilic interactions. Thus, such a homophilic interaction was examined using a yeast two-hybrid system, and, as the result, specific homophilic binding of the domain was observed, but interaction with the C-terminal catalytic domain was not (Fig. 7*A*). This homophilic interaction was also confirmed by a GST pull-down assay. The His-tagged thioredoxin fusion protein bound to the GST fusion protein but not to nonfused GST (Fig. 7*B*). In both of these two assays, interaction or binding was detected only in the combination of fragments that contained residues 45–184 but not in any other combination. These results suggest that the domains mutually interact in a homophilic manner. Thus, it is more likely that oligomerization of GnT-V is primarily mediated via the homophilic interaction of the domain, thus further supporting the critical role of the stem domain in the Golgi localization of GnT-V, which appears to be accomplished by an oligomerization-based mechanism.

DISCUSSION

In this study, we report the identification of the region comprised by ~45–184 amino acid residues in the stem region as the functional domain that is responsible for the Golgi localization but is not associated with enzymatic activity, thus demonstrating that GnT-V consists of the separable functional domains that confer either catalytic activity, intracellular localization, or membrane anchoring. The absence of the domain identified in the stem region of GnT-V led to a loss of disulfide linkage-mediated oligomer formation in conjunction with a considerably diminished Golgi retention, whereas the enzymatic properties remained nearly completely intact. Furthermore, it was found that even if the protein contains the domain, *i.e.* a full-length wild type GnT-V, a failure in forming this covalently linked oligomer may also lead to an impaired retention of protein in the Golgi, leading to its subsequent release from the cells. It was also shown that the domain that was identified as being essential for retention is capable of forming its homo-oligomer by the homophilic interaction. These findings suggest that the intracellular localization of GnT-V is due, nearly exclusively, to this domain and that the mechanism for the intracellular localization involves homo-oligomer formation, triggered by the domain, but not substantially affected by the other regions that are intrinsic to GnT-V, such as the transmembrane domain and the catalytic domain.

The mechanism for the retention of GnT-V in Golgi is consistent with kin recognition or a similar mechanism and is most probably based on homo-oligomerization (11). Formation of the disulfide-linked homo-oligomerization would represent a critical step for the retention of this glycosyltransferase. Intermolecular covalent linkages appear to be efficiently formed, not only in the stem domain, in which 4 cysteine residues are present, but also in the other regions, probably because the entire luminal region of GnT-V contains as many as 20 cysteine residues (10). In fact, although FΔ2 lacks those 4 cysteine

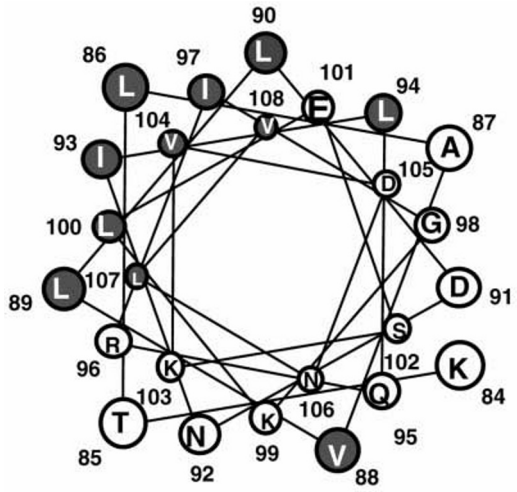

FIG. 8. **A prediction of secondary structure of the region Lys-84–Val-108 of GnT-V.** A helical wheel model based on the method of Chou and Fasman (35). Hydrophobic amino acids (*I*, *L*, and *V*) are highlighted.

residues in the stem domain, this mutant forms the disulfide-linked oligomer and is localized in the Golgi apparatus. The initial event leading to such covalent homo-oligomer formation would involve a clustering of newly biosynthesized GnT-V molecules, in which the covalent linkages have not yet been formed, and, subsequently, the clustered proteins might be covalently linked by enzymatic or nonenzymatic oxidation in the Golgi or more proximal subcellular compartments. Because the domain is absolutely required but not necessarily sufficient for the formation of covalent linkages, as suggested by the absence of intermolecular disulfides in the secreted form of the wild type GnT-V, the primary function of the domain would involve triggering the initial clustering via its homophilic interactions. The domain might also be able to facilitate intermolecular disulfide formation by increasing the probability of intermolecular contact, although a possible noncovalently associated homo-oligomer, formed only via homophilic interactions of this domain, is unstable to retain the GnT-V in the Golgi apparatus.

All secondary structural predictions, which are based on the methods of Garnier and co-workers (33), Eisenberg and co-workers (34), and Chou and Fasman (35), suggest an α-helical structure for the region encompassed by residues Lys-84–Val-108 in the stem domain, as shown in Fig. 8. The helical wheel model of the region reveals the presence of a one-sided hydrophobic amino acid cluster in the helix. It is known that such a structural feature is frequently involved in an intermolecular or intermolecular hydrophobic contact formed by multiple helices, as found, *e.g.* in gp41, a transmembrane subunit of HIV-1 envelope glycoprotein complex, gp120-gp41 complex (36, 37). The putative gp41 amphipathic α-helix, Leu-550–Leu-582, was demonstrated to be essential for hetero-oligomeric formation with gp120, indicating that hydrophobic contacts contribute to this oligomeric structure (37, 38). Therefore, it may also be possible that such a hydrophobic contact could enable the stem domain to display a homophilic interaction, which plays a role in the formation of the initial clustering of the GnT-V protein.

In the kin recognition via hetero-oligomeric interaction or homo-oligomer-based mechanism for the retention of various proteins in Golgi, it is generally thought that the formation of a sufficiently large molecule prevents the delivery of proteins from the Golgi to more distal compartments, such as the plasma membrane (11). Although oligomerization is a critical event that is common to these Golgi resident proteins, as suggested by many studies, a causal process seems to be different and divergent among individual proteins. The elucidation of the mechanistic and structural bases for the formation of an oligomer would be desired, to better understand the mechanism for the retention of glycosyltransferases and other Golgi proteins. The present study has clearly identified and characterized the domain responsible for the retention of GnT-V and thereby provides a possible mechanism for conferring Golgi retention, namely, the formation of oligomers.

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