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Mutational Analysis of the Golgi Retention Signal of Bovine β-1,4-Galactosyltransferase*

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To examine the role of the NH_2-terminal region of the 402-residue-long β-1,4-galactosyltransferase (β-1,4-GT), a series of mutants and chimeric cDNA were constructed by polymerase chain reaction and transiently expressed in COS-7 cells, the enzyme activities were measured, and the protein was localized in the cells by subcellular fractionation or indirect immunofluorescence microscopy. We showed earlier that the deletion of the amino-terminal cytoplasmic tail and transmembrane domain from GT abolishes the stable expression of this protein in mammalian cells (Masibay, A. S., Boeggeman, E., and Qasba, P. K. (1992) *Mol. Biol. Rep.* 16, 99–104). Further deletion analyses of the amino-terminal region show that the first 21 amino acids of β-1,4-GT are not essential for the stable production of the protein and are consistently localized in the Golgi apparatus. In addition, analysis of hybrid constructs showed that residues 1–25 of α-1,3-galactosyltransferase can functionally replace the β-1,4-GT amino-terminal domain (residues 1–43). This fusion protein also showed Golgi localization. On the other hand, the α-2,6-sialyltransferase/β-1,4-GT fusion protein (α-2,6-ST/β-1,4-GT) needed additional COOH-terminal sequences flanking the transmembrane domain of the α-2,6-ST for stability and Golgi localization. Substitution of Arg-24, Leu-25, Leu-26, and His-33 of the β-1,4-GT transmembrane by Ile (pLFM) or substitution of Tyr by Ile at positions 40 and 41 coupled with the insertion of 4 Ile residues at position 43 (pLB) released the mutant proteins from the Golgi and was detected on the cell surface. Our results show that (*a*) the transmembrane domains of β-1,4-GT, α-1,3-galactosyltransferase, and α-2,6-ST, along with its stem region, all play a role in Golgi targeting and participate in a common mechanism that allows the protein to be processed properly and not be degraded *in vivo*; (*b*) increasing the length of the transmembrane domain overrides the Golgi retention signal and directs the enzyme to the plasma membrane; and (*c*) the length of the hydrophobic region of the transmembrane domain of β-1,4-GT is an important parameter but is not sufficient by itself for Golgi retention.

The carbohydrate moieties of glycoproteins serve a broad range of biological roles from cell adhesion and targeting of lysosomal hydrolases (1–7) to morphological differentiation and metastases (8–12). This variety is believed to be governed by the differential expression of the many glycosyltransferase genes (1, 2, 9, 13, 14). Much is known about the biosynthetic pathways of these carbohydrates (15), but it is only recently that we are beginning to get a better understanding of the molecular regulation, subcellular organization, and targeting of the glycosyltransferases.

Several of the glycoslytransferases have been cloned to date (for a review, see Ref. 9), and they all share a common structural topology: a 6–24-residue-long NH_2-terminal cytoplasmic tail, a 16–20-amino acid signal/anchor region, an extended stem structure, and a large COOH-terminal catalytic domain. It has been postulated that the Golgi retention signal for the glycosyltransferases would reside in the cytoplasmic or membrane-spanning region (16), but sequence comparison of the glycosyltransferases cloned to date show no obvious putative Golgi retention signal in their transmembrane domain. Each glycosyltransferase is grouped according to the enzyme's donor substrate and the formation of a specific linkage; for the β-1,4-galactosyltransferase (β-1,4-GT),[1] UDP-galactose is the donor substrate that is linked to *N*-acetylglucosamine (GlcNAc) to produce *N*-acetyllactosamine with a β-1,4-glycosidic linkage (9, 14, 15). The β-1,4-GT has been localized by immunoelectron microscopy to the *trans*-cisternae of the Golgi (17, 18), but there have also been a number of reports on the cell surface expression of β-1,4-GT (19–25). In addition to the membrane-associated forms of β-1,4-GT, active soluble forms of the enzyme exist in several body fluids such as milk, colostrum, and serum (26).

The intracellular transport and targeting of proteins from their site of synthesis to their correct destination is a process instrumental to the maintenance of cellular integrity. According to the current bulk flow model (27, 28), both plasma membrane and secreted proteins, which have a signal or leader sequence, follow a similar transport pathway through the cell (29, 30). The proteins are retained at points along the way via a retention signal (18, 31–36). The bovine β-1,4-GT full-length cDNA has been expressed (37, 38) and found to code for a fully functional enzyme. We recently showed that the transmembrane segment of β-1,4-GT may play a role in the stable expression of the protein (39). Since β-1,4-GT is detected both in the Golgi and on the cell surface, we wanted to determine which parameters play a role in the sorting/retention machinery. We have constructed amino-terminal deletion mutants, produced chimeric proteins, and used site-directed mutagenesis to demonstrate that (*a*) the presence of

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[1] The abbreviations used are: β-1,4-GT, β-1,4-galactosyltransferase; α-1,3-GT, α-1,3-galactosyltransferase; α-2,6-ST, α-2,6-sialyltransferase; GlcNAc, *N*-acetylglucosamine; ATPase, ouabain-sensitive Na^+K^+-ATPase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

the transmembrane domain is essential for the stable expression of glycosyltransferases in mammalian cells, (*b*) transmembrane domains of glycosyltransferases are exchangeable, and (*c*) increasing the length of the hydrophobic membrane-spanning region of β-1,4-GT releases the protein from the Golgi and is transported onto the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials

Reagents such as restriction endonucleases, T4 DNA ligase, T4 polymerase, and *Taq* polymerase were purchased from Boehringer Mannheim and Perkin-Elmer Cetus Instruments and were used according to the manufacturers' directions. Oligonucleotide primers were synthesized by Genosys, The Woodlands, TX. [^{32}P]dCTP (3000 Ci/mmol) and UDP-[^{3}H]galactose (20 Ci/mmol) were obtained from Amersham Corp.. Cell culture reagents were obtained from either Whittaker Bioproducts or GIBCO. Rhodamine-conjugated and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG were purchased from Organon Teknika Corp., West Chester, PA.

Methods

Cell Culture and Transfections—Cell lines were maintained in exponential growth as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamycin (500 μg/ml). COS-7 cells were seeded at 5 × 10^5 cells/100-mm dishes, and after overnight incubation at 37 °C in a 5% CO_2, 95% air atmosphere, were transfected with 15 μg of DNA (1 μg/μl) using the calcium phosphate transfection protocol of Chen and Okayama (40). After 48 h, the cells were washed three times with cold isotonic phosphate-buffered saline (PBS), harvested by scraping the cells from each plate in 1 ml of PBS and centrifuged at 7000 × *g* for 10 min, and the pellet was stored at −70 °C.

General DNA Recombinant Techniques and Construction of Mutants—Plasmid preparation, restriction endonuclease digestions, ligations, kinase reactions, phosphatase treatment, end-labeling of DNA fragments, screening of bacterial colonies, and Northern blot analysis were carried out as described by Ausubel *et al.* (41). The following primers were synthesized by Genosys. 1) NH_2-terminal deletion mutants: GT1 (GGACTAGTCCACCATGAAGTTTCGGG AGCCGCTCCTG; 1M14, GGACTAGTATGCCGGGCGCGTCCCT GCAGCGG; 1M22, GGACTAGTCCACCATGGCCTGCCGCCTCC TCGTGGCT; 1M43, GGACTAGTATGGCCGGCCGAGACCTAAG ACGC; CL1, TCCAAGGCCACTTTAGCCACCAGCGGCCGC; and CL2, TTACTTGGATTAGGAGTCTCCATCCTTAAGCC. 2) α-1,3-GT: BR1-3A, GGACTAGTATGAATGTCA; BL1-3B, TCATACTT ACAGTTTCCTTTTCACTAAGACAGTTACG; BR1-3C, ATTCTG TCAATGCTGGTTGTCTCAACTGTCATTGTTGTGTTTT; and BL1-3, TAACAACACAAAACCCTTATATAGCGGCCGGCTT. 3) α-2,6-ST: RR2-6A, GGACTAGTATGATTCATACCAACT; RL2-6B, CTAAGTATGGTTGAACTTCTTTTTTCAAGTCGGAGAAGT AGGAC; RR2-6C, CTCTTCATCCTGGTCTTTCTCCTGTTCGCA GTCATCTGTGTTT; and RL2-6, CAGTAGACACAAACCTTCTT TCCGCCGGCTT. 2-6D,TCAGTAGACACAAACCTTCTTTCCGT CGCTGATACTC; 2-6STSA, GAAGCGACTATCAGGCCCTTACA CTGCAAGCCAAGGAA; and 2-6E, GAATGTGACGTTCGGTTCC TTAAGGTCTACCCGCCGGCTCTC. 4) β-1,4-GT transmembrane mutants: GT1B, CTCGGCGAGGACCCGCCGTCACGGCGCTACG GCCCGCGCAGGGACGTCGCCCGGACG; GT1CIle, CAGCGGGC CTGCatcatcatcGTGGCTGTCTGCGCCCTGatcCTTGGCGTCACC CTCGTC; GT1D, CAGTGGGAGCAGATAATGGACCGGCCGGC TC;GT1C,CAGCGGGCCTGCCGCCTCCTCGTGGCTGTCTGCGC CCTGCATCTTGGCGTCACCCTCGTC; and GT1DIle, CAGTGG GAGCAGtagtagGACtagtagtagtagCGGCCGGCTC.

Primers GT1, 1M14, 1M22, and 1M43, which have a *Spe*I site (*underlined*) at the 5′ end, correspond to nucleotides 423–446, 462–485, 486–506, and 549–569 of pLsGT (37), respectively. The primer CL1 is complementary to nucleotides 1593–1614 of pLbGT-1 near the *Eco*RI site, whereas CL2 is complementary to nucleotides 835–855 (42). Primer CL1 was used with either primers GT1, 1M14, 1M22, or 1M43 to amplify the sequences from pLsGT (37) using Perkin-Elmer Cetus Instruments' PCR reagent kit and DNA Thermal Cycler. For clone pLbGT14Δ6, 1M14 and CL2 primers were used. Each PCR product was digested with *Spe*I and *Eco*RI and then subcloned into the *Spe*I/*Eco*RI site of pL2M (39), which was generated from pLbGT-1 and pLsGT. The pL2M construct previously reported is the same construct as pLbGTd43 reported in this study (Fig. 1). The nomenclature was changed to make it consistent with the new constructs generated from this parental construct.

Overlapping oligomers were synthesized that covered the entire region of interest of α-1,3-GT (BR1–3A, BL1–3, BR1-C, and Bl1–3) and α-2,6-ST (RR2–6A, RL2–6B, RR2–6C, RL2–6, 2–6D, 2–6STSA, and 2–6E). After allowing the overlapping sequences to hybridize, the DNA was filled in. This double-stranded DNA was used as a template for PCR amplification using the extreme left primers (BR1–3A for α-1,3-GT and RR2–6A for α-2,6-ST) with *Spe*I restriction site (*underlined*) and right primers (BL1–3 for α-1,3-GT and RL2–6 or 2–6E for α-2,6-ST) with the *Eag*I site (*double underlined*). After digestion with *Spe*I and *Eag*I, the PCR product was subcloned into the *Spe*I/*Eag*I site of pLbGTd43. The same approach was used for construction of transmembrane mutants pLFM (GT1, GT1B, GT1CIle, and GT1D), pLB (GT1, GT1B, GT1C, and GT1DIle) and pLFMB (GT1, GT1B, GT1CIle, and GT1DIle) of β-1,4-GT. Lower case letters in the above sequences indicate the mutated nucleotides, as compared with the wild-type sequence, which is indicated by upper case letters. Each 5′-synthetic primer was ^{32}P-end-labeled and used as a probe to pick up positive clones, and the fidelity of the union of the PCR products at the unique *Spe*I site was confirmed by primary sequence analysis through the junction. This also confirmed that the correct reading frame was maintained.

Fractionation of Membranes from pLGTF Sucrose Gradient Centrifugation—The protocol of Barbosa and Wettstein (43) was used to fractionate COS-7 cell membranes. Briefly, cells from eight 100-mm dishes (approximately 16–24 × 10^6 cells) were scraped in PBS and pelleted (500 × *g*, 10 min, 4 °C). The pellet was resuspended in 2 ml of homogenizing buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, proteinase inhibitors) and homogenized using a tight-fitting Dounce homogenizer (30 strokes). The homogenate was centrifuged at 500 × *g* for 10 min to remove unbroken cells and debris; the supernatant (crude cell extract) contained total membranes. Total cellular membranes were obtained by further centrifugation (145,000 × *g*, 1 h) of crude extract. The resulting pellet was resuspended in 1 ml of homogenizing buffer and layered over a continuous sucrose gradient ranging from 1.3 M (bottom) to 0.73 M (top), having a 1-ml 2 M sucrose cushion in an SW41 rotor. After overnight centrifugation (107,000 × *g*) at 4 °C, 0.5-ml fractions were collected and pelleted. The pellet was resuspended and divided for protein determinations and enzyme activity measurements as described below.

Preparation of Cell Extracts for Protein Determination and β-1,4-Galactosyltransferase and Ouabain-sensitive Na^+K^+-ATPase Assays—Each cell pellet was resuspended in 100 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 1% Triton X-100). The suspension was kept on ice for 15–20 min and gently homogenized using a hand-held pellet pestle (Kontes). The suspension was centrifuged at 7000 × *g* at 4 °C for 20 min, and the supernatant was assayed. Protein concentration of the extracts from transfected cells was determined using the Bio-Rad protein assay kit. UDP-galactose-*N*-acetylglucosamine galactosyltransferase activity was assayed by a radiochemical procedure (44, 45). Cell extracts were assayed at 30 °C for 30 min in a 100-μl incubation mixture containing 0.5 μCi of UDP-[^{3}H]galactose (20 Ci/mmol), 5 nM UDP-galactose, 400 nM $MnCl_2$, and 1 μl of Triton X-100. Assays were performed in duplicates with and without acceptor molecule (2 μM *N*-acetylglucosamine) to assess the extent of hydrolysis by the nucleotide pyrophosphatases. The reaction was terminated by adding 200 μl of ice-cold H_2O, and the unincorporated UDP-[^{3}H] galactose was separated from ^{3}H-labeled products on an anion-exchange column (AG 1-X8, Bio-Rad) as described (45). Ouabain-sensitive Na^+K^+-ATPase (ATPase) activity was measured according to Lernmark *et al.* (46). Briefly, ATPase activities were assayed in duplicate in 310 μl of total volume of 30 mM histidine buffer, pH 7.4, containing 1.0 mM $MgCl_2$, 0.1 mM EDTA, 130 mM NaCl, 20 mM KCl, 1 mM ATP (sodium salt), as well as trace amounts of [γ-^{32}P]adenosine-5′-triphosphate. Incubations were carried out with or without 1 mM ouabain (Sigma) at 37 °C for 60 min. The reaction was stopped by the addition of 1 ml of 0.1 M HCl containing 4% (w/v) activated charcoal (Sigma), 0.2 mg/ml bovine serum albumin, 1 mM sodium phosphate, and 1 mM sodium pyrophosphate. All samples were vortexed and allowed to stand on ice for 30 min to adsorb unreacted ATP before being centrifuged at 4 °C for 10 min at 5,000 × *g*. The supernatant (1 ml) was pipetted into 10 ml of distilled water, and the Cerenkov radiation of $^{32}P_i$ was counted in a Beckman liquid scintillation counter (Beckman LS-3801). ATPase activity was estimated from the difference between the activities obtained with and without

ouabain. Activity was expressed as counts per microgram of protein.

Immunoblotting—The cell pellet from two 100-mm dishes (4–6 × 10^6 cells) was resuspended in 100 μl of lysis buffer. The crude extract was centrifuged at low speed to get rid of the nucleus and cell debris. After determining protein concentrations, equivalent amounts of protein (20 μg) were resolved in a 12% SDS-polyacrylamide gel electrophoresis gel (47). The separated proteins were electrophoretically transferred onto an Immobilin-P (Millipore) membrane at 17 V for 2 h using the Novex® mini-gel apparatus. After transfer, the membrane filter was probed with a monospecific antibody against bovine GT (37) following standard protocols (41). The Ag·Ab complex was visualized using the horseradish peroxidase conjugate substrate kit from Bio-Rad.

Immunofluorescence Microscopy—Transfection of COS-7 cells were carried out as described before (37) with minor modifications. Briefly, sterile coverslips were placed in 100-mm dishes and seeded at 5 × 10^5 cells/dish. The transfection proceeded as previously described above. After 43 h, the cells were washed with PBS, and were subsequently fixed with 3% paraformaldehyde for 15 min and then treated with PBS containing 10% goat serum for nonpermeabilized samples. Parallel cultures were permeabilized with 0.2% Triton X-100 in PBS. After several washings with PBS, the cells were incubated in PBS containing 10% goat serum. All of the cells were incubated with anti-GT antiserum at 4 °C overnight. After washing with PBS, cells were stained with either fluorescein isothiocyanate- or rhodamine-labeled goat anti-rabbit IgG and examined by immunofluorescence microscopy.

Computational Methods and Calculation of Hydropathic Indices—Using the Kyte and Doolittle hydropathic scale (48) for each amino acid residue, we calculated and generated hydropathic profiles for the transmembrane domains of various Golgi and plasma membrane proteins, including mutant proteins generated in this study and elsewhere. The total sum of hydropathic values of 7 contiguous residues was placed at position 4. This window of 7 residues was moved along the polypeptide chain, generating a hydropathic indices profile for a given segment of the chain. The transmembrane domain with additional residues flanking each side was used as input data to generate a hydropathic profile. The length of the transmembrane domain was determined by counting the number of residues within the longest contiguous stretch of positive hydropathic values that indicates the hydrophobic segment.

RESULTS

The Transmembrane Domain of β-1,4-GT Is Necessary for Protein Production in COS-7 Cells—To determine whether the cytoplasmic or the transmembrane domain is important for production of β-1,4-GT in COS-7 cells and to investigate what effect these domains have on Golgi localization of β-1,4-GT, a series of deletion mutants were constructed. Progressive deletion of amino acids from the NH_2-terminal portion of bovine β-1,4-GT was achieved by synthesizing primers corresponding to the appropriate regions of interest. These primers were paired up with a COOH-terminal primer (CL1 or CL2) to generate the appropriate clones (see "Experimental Procedures") that were used for transfection of COS-7 cells. Fig. 1*A* shows a schematic diagram of the corresponding protein that should be produced from each deletion construct. Measurement of enzyme activity showed that the first 21 amino acid residues of the cytoplasmic region of β-1,4-GT is not essential for its production in COS-7 cells (Fig. 2). The disruption of the transmembrane domain causes a significant reduction in enzyme activity (Fig. 2, pLbGTd43). DNA sequence analysis of the deletion clones showed that the loss of activity is not due to any disruption of the reading frame or any other sequence artifacts (data not shown). The protein coded by the mutants should correspond to the ones schematically shown in Fig. 1*A*. The results indicate that the stable expression of this protein is sensitive to the deletions in the transmembrane region, since removal of the first 5 amino acids of the transmembrane segment also abolishes the observed activity, as compared with the wild-type protein (data not shown). Deletion of the transmembrane region may have resulted in the protein not entering the secretory pathway due to the lack of an intact signal anchor.

To determine if these proteins are being produced, Western blot analysis was performed. Cell extracts from transfected COS-7 cells were loaded onto SDS-polyacrylamide gel electrophoresis, blotted, and probed with a monospecific antibody against β-1,4-GT. Fig. 3 shows the result of such an analysis. Consistent with the observed enzyme activity, protein is detected from clones with intact transmembrane region (*lanes 2–4*). The 8-residue difference in protein sizes between the NH_2-terminal mutants, pLbGTd14 and pLbGTd22 (*lanes 3* and *4*), is not great enough to be resolved by the gel. Barely detectable amounts of β-1,4-GT are observed when the transmembrane segment is deleted (Fig. 3, *lane 5*). These results suggest that the transmembrane region of bovine β-1,4-GT is important for the stable accumulation of the protein when produced in COS-7 cells. A construct of bovine β-1,4-GT that had portions of the COOH-terminal sequence deleted (pLGT14Δ6) showed no enzyme activity (Fig. 2). The protein from this mutant accumulates in large quantities (Fig. 3, *lane 6*), which is, as expected, smaller in size than the wild-type, since 43 amino acids are missing from the COOH-terminal domain of the protein.

The Bovine α-1,3-GT Transmembrane Region Can Replace Bovine β-1,4-GT, Whereas the Rat α-2,6-ST Transmembrane Domain Requires Flanking Sequences to Rescue pLbGTd43—Since β-1,4-GT is but one member of a group of glycosyltransferases that exhibit conserved topological structures (9), our hypothesis was that the corresponding transmembrane regions of other glycosyltransferases may directly substitute for the transmembrane segment of bovine β-1,4-GT. Assembled DNA segments coding for the cytoplasmic and transmembrane regions of α-1,3-GT or α-2,6-ST that would replace the corresponding transmembrane segment of β-1,4-GT were used for the generation of chimeric constructs pL1,3-bGTd43, pL2,6-bGTd43, and pL2,6S-bGTd43 to give rise to fusion proteins (Fig. 1*B*). The DNA segment coding for the cytoplasmic and transmembrane regions of α-1,3-GT can rescue the production of protein from the construct pLbGTd43, which does not contain the coding sequence for the β-1,4-GT transmembrane domain. From this construct, pL1,3-bGTd43, there is a stable accumulation of the protein in transfected COS-7 cells (Fig. 2). On the other hand, the α-2,6-ST cytoplasmic and transmembrane domain (amino acids 1–29) substituting for the β-1,4-GT transmembrane domain does not produce a stable protein (pL2,6-bGTd43). Major segments of the construct pL2,6-bGTd43 were sequenced to confirm that no deletions or mutations have taken place, which would terminate or alter the expression of this protein (data not shown). Furthermore, all of our constructs were shown to be transcribed, as determined by Northern blot analysis (data not shown). However, if the flanking sequences that code for a part of the neck or stalk region of α-2,6-ST (amino acids 30–44; Fig. 1*B*) are added to the construct, a stable active protein is produced again (Fig. 2, pL2,6S-bGTd43).

Localization of Wild-type, Deletion Mutants, and Hybrid Proteins of Bovine β-1,4-GT—β-1,4-GT has traditionally been located within the Golgi compartment (17, 18) but has also been detected on the plasma membrane of many cell types (19–25). Recently, it has been suggested that the 402-residue-long full-length protein and the short form of β-1,4-GT (residues 1–13 deleted) are targeted to different subcellular compartments (49). To better understand the mechanisms underlying the sorting of this glycosyltransferase, subcellular fractionation and immunofluorescence experiments were performed to see where the overexpressed proteins of wild-

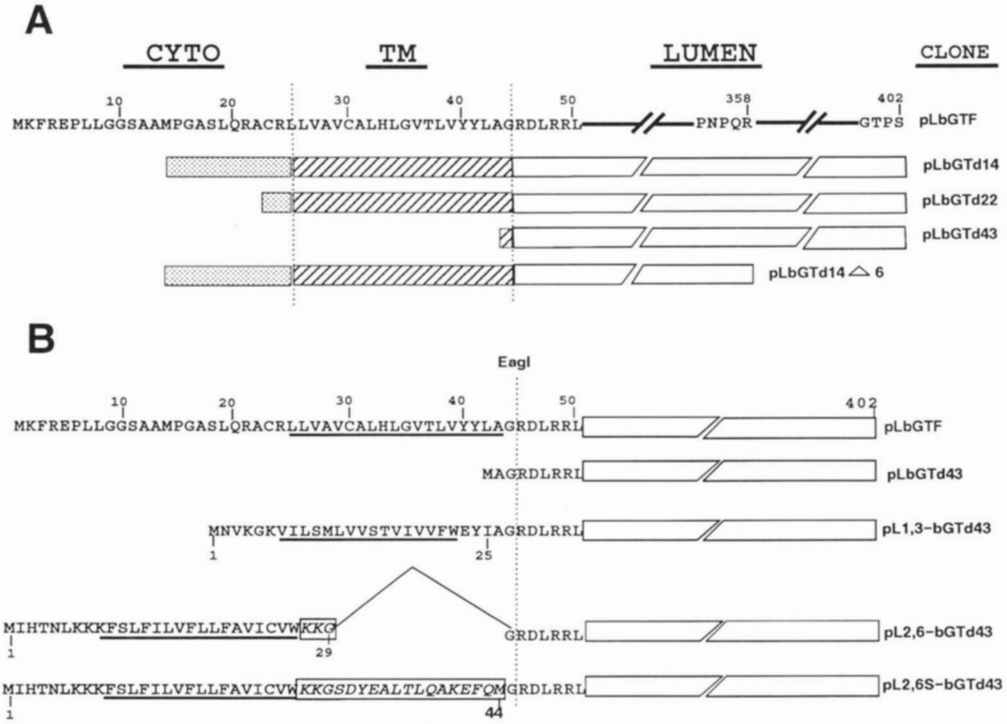

FIG. 1. **Construction of mutants.** *A*, NH_2-terminal protein sequence of β-1,4-GT and schematic representation of wild-type 402-residue-long bovine β-1,4-GT, deletion, and hybrid mutants. *pLbGTF*, amino acids 1–402; *pLbGTd14*, 14–402; *pLbGTd22*, 22–402; *pLbGTd43*, 43–402; *pLbGTd14Δ6*, 1–358; *CYTO*, cytoplasmic side; *TM*, transmembrane; *LUMEN*, luminal side; *stippled box*, cytoplasmic domain of β-1,4-GT; *hatched box*, transmembrane segment; *open box*, neck and COOH-terminal region. *B*, schematic illustration of resulting fusion proteins from the chimeric constructs and their comparison with the full-length wild-type protein from pLbGTF and the protein from pLbGTd43 that is missing the transmembrane domain. *Numbers above* the protein sequence refer to bovine β-1,4-GT protein residues, whereas *numbers below* refer to either bovine α-1,3-GT (pL1,3-bGTd43) or rat α-2,6-ST (pL2,6-bGTd43) amino acids. The *boxed residues* in pL2,6S-bGTd43 is the portion of the stem region; the *open box* is the rest of the β-1,4-GT protein sequence; the membrane-spanning region is *underlined*.

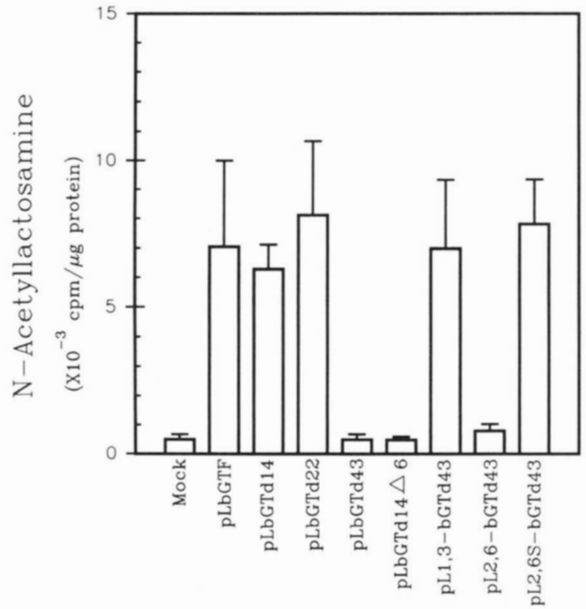

FIG. 2. **β-1,4-GT activities of the deletion and hybrid mutants.** The cell extracts from transfected COS-7 cells were assayed in the presence of GlcNAc. The value obtained from the extract assayed in the absence of GlcNAc was subtracted from each corresponding sample and expressed as activity/μg of protein. Values are the mean (± S.E.) of four or seven (hybrid or deletion mutants, respectively) independent experiments as calculated by SigmaPlot®.

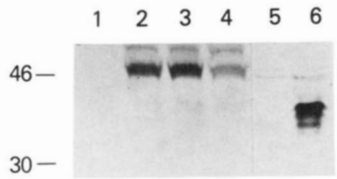

FIG. 3. **Detection of β-1,4-GT protein by immunoblot.** Equivalent amounts of cell extracts from transfected COS-7 cells were subjected to SDS-polyacrylamide gel electrophoresis on a 12% glycine mini-gel, blotted onto a filter, and probed with a monospecific antibody against bovine β-1,4-GT. *Lanes 1–6*, mock, pLbGTF, pLbGTd14, pLbGTd22, pLbGTd43, and pLbGTd14Δ6, respectively.

type and deletion mutants of β-1,4-GT are targeted. Since β-1,4-GT has been classically used as a Golgi marker, we initially characterized the profile of β-1,4-GT activity as it fractionates on a sucrose gradient. Fig. 4*A* shows that when cell extract of pLbGTF-transfected COS-7 cells was sedimented on a sucrose gradient, the majority of the activity is observed between fractions 8–12. The top fractions, 18–22, represent plasma membranes as marked by the ouabain-sensitive Na^+K^+-ATPase activity. This clearly shows that the Golgi fraction can be separated from the plasma membrane component in COS-7 cells as has also been observed by others (43). Comparison of the subcellular fractionation profile of the pLbGTF with pLbGTd14-transfected cells show no significant difference (Fig. 4*B*), indicating that both full-length and short forms of β-1,4-GT are targeted mostly to the Golgi fraction.

A series of immunofluorescence experiments were performed on the amino-terminal deletion mutants to assure that the overproduced β-1,4-GT is indeed mostly targeted to the

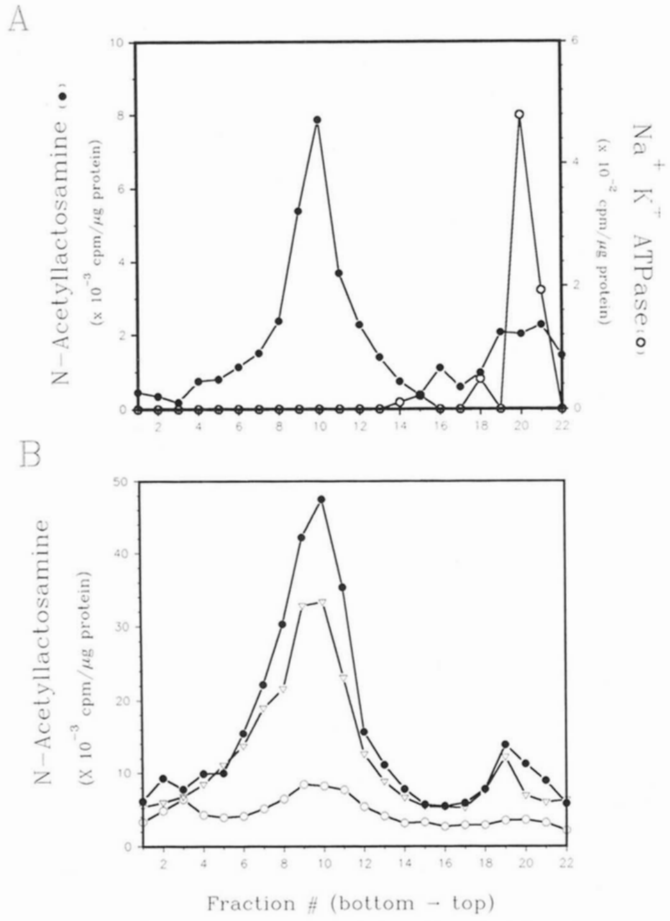

FIG. 4. **Sucrose gradient centrifugation of COS-7 cell extract.** A, fractionation of Golgi *versus* plasma membranes from pLbGTF-transfected COS-7 cells. Golgi and membrane components of transfected COS-7 cells were separated by continuous sucrose gradient centrifugation. Galactosyltransferase enzyme activity (*closed circles*) was used to monitor Golgi fraction, whereas ouabain-sensitive Na^+K^+-ATPase activity (*open circles*) was utilized as a marker for plasma membranes. *B*, subcellular distribution of the long form (pLbGTF) and the short form (pLbGTd14) of bovine β-1,4-GT. COS-7 cells were transfected with either construct pLbGTF (first Met, *closed circles*) or pLbGTd14 (start from second Met, *open triangles*). The cell extract from these cells were fractionated and galactosyltransferase activity was determined for each fraction, as described under "Experimental Procedures." *Open circles*, mock-infected.

Golgi. The immunofluorescence data show that most of the overexpressed protein is present in the Golgi and not on the cell surface (Fig. 5). This is consistent with what was already observed in the fractionation experiments, where no shift was observed in the fractionation profile of the wild-type pLbGTF *versus* the amino-terminal 14- or 22-residue deletion mutants, pLbGTd14 and pLbGTd22, respectively. The localization of the fusion proteins produced from the hybrid constructs, pL1,3-bGTd43 and pL2,6S-bGTd43, were also determined by indirect immunofluorescence. The results show that the majority of the protein is directed to the Golgi compartment (Fig. 5).

Effect of Mutations on Hydrophobicity of the Golgi Retention Signal Domain and on Cellular Localization of Bovine β-1,4-GT—The amino acid sequence of the β-1,4-GT transmembrane region has neither any homology with the transmembrane region of various glycosyltransferases cloned so far nor with the transmembrane domains of integral membrane proteins. However, all these transmembrane domains exhibit the classical hydrophobic property associated with a membrane-spanning domain. When the membrane-spanning region of β-

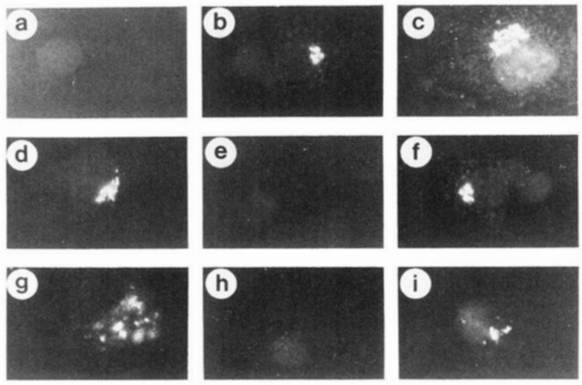

FIG. 5. **Localization of the full-length (*b*), deletion mutants (*c–f*), and hybrid constructs (*g–i*) of β-1,4-GT in COS-7 cells by indirect immunofluoresence.** Transfected cells grown on coverslips were fixed with paraformaldehyde and permeabilized with Triton X-100. After incubation with anti-galactosyltransferase, a secondary antibody conjugated to fluoroisothiocyanate or rhodamine was used to visualize the Ag-Ab complex under a fluorescence scope (Zeiss). *a*, mock; *b*, pLbGTF; *c*, pLbGTd14; *d*, pLbGTd22; *e*, pLbGTd43; *f*, pLbGTd14Δ6; *g*, pL1,3-bGTd43; *h*, pL2,6-bGTd43; and *i*, pL2,6S-bGTd43. Magnification, × 630.

1,4-GT was modeled into an α-helix, using InsightII from Biosym Inc. as a molecular modeling package, we noticed that the polar residues line up on one side of the α-helix. We began examining membrane-spanning domains of other proteins that were targeted to the cell surface and observed that a majority of these proteins had stretches of hydrophobic residues uninterrupted by any charged residues on one side of the α-helix. In contrast, proteins targeted to other compartments, such as the Golgi or endoplasmic reticulum have this hydrophobic stretch interrupted by a charged or polar residue(s). To calculate the hydropathic index of each residue within the transmembrane domain of these various proteins, we used the Kyte and Doolittle scale (48) and a window of 7 amino acids. The first positive number of this stretch was then considered residue number 4 for comparative plotting purposes. The adjusted residue number *versus* the hydropathic index was plotted as shown in Fig. 6. The length of the transmembrane was determined by counting the number of residues within this positive hydropathic stretch. The length of the transmembrane domain based on the number of residues with a positive hydropathic index fall into two basic categories: one with a broad and the other with a short hydrophobic region. The proteins targeted to the plasma membrane tend to have a broader hydrophobic region, and the hydrophobicity values are greater, as compared with proteins targeted to the Golgi, suggesting a possible relationship between the length of the transmembrane domain and their localization (Fig. 6). Although the average length of the transmembrane domain of Golgi and plasma membrane proteins show a difference, there is, however, an overlap (Fig. 6, compare *D versus G* or *J*). Based on these analyses, we investigated if by increasing the hydropathic index of the signal anchor domain of the Golgi-associated β-1,4-GT, one could target the enzyme to the cell surface. Fig. 7*A* shows that these mutations were accomplished by substitution or a combination of substitution and insertion of Ile in the membrane-anchor domain. The mutant pLFM had Ile substitutions at positions 24–26 and 33, whereas the pLB mutant had Ile substitution at positions 40 and 41 and insertion of 4 Ile residues after position 42. The construct pLFMB has mutations of both pLFM and pLB mutants. Fig. 7*B* shows the hydropathic profile of wild-type β-1,4-GT (pLbGTF) and transmembrane mutants, pLFM, pLB, and pLFMB. Comparison of the hydrophobic profiles

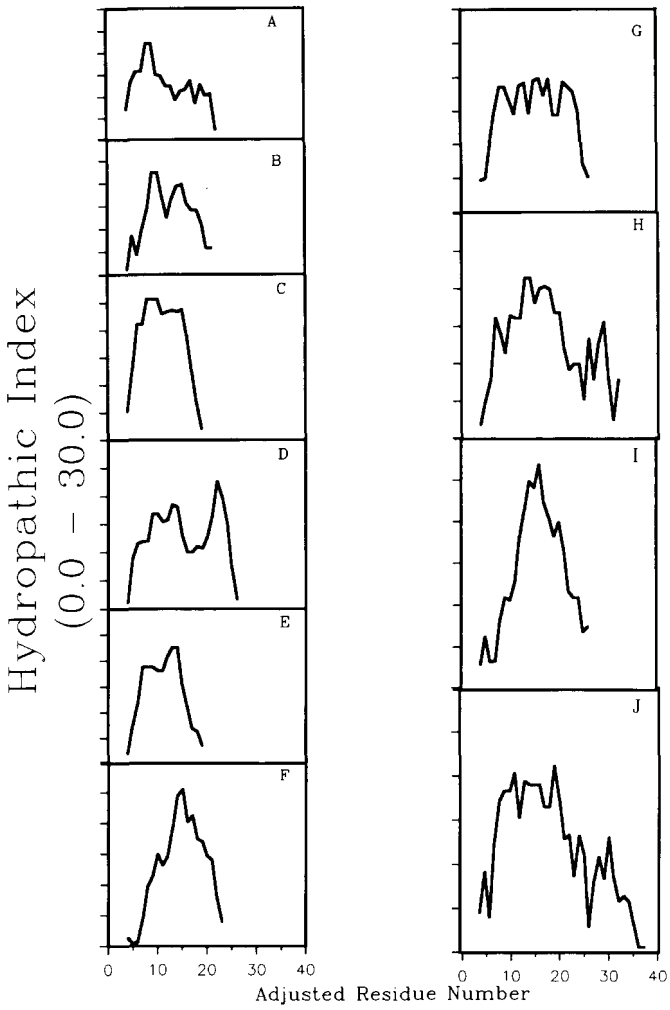

FIG. 6. **Comparison of the hydropathic profile of the membrane region of Golgi proteins *versus* plasma membrane proteins.** Using the scale of Kyte and Doolittle (48) and a window of 7 residues, the hydropathic profile for each protein was calculated, and the longest contiguous hydrophobic stretch was determined. The first value above 0 (hydrophobic) of this stretch for all proteins was assigned residue 4, and the rest of the values were plotted accordingly (see "Methods"). *A–F*, Golgi-associated proteins (*A*, β-1,4-GT; *B*, α-1,3-GT; *C*, α-2,6-ST; *D*, *N*-acetylglucosaminyltransferase I; *E*, infectious bronchitis virus E1 glycoprotein; *F*, α-mannosidase II) and *G–J*, plasma membrane proteins (*G*, dipeptidylpeptidase IV; *H*, neuraminidase; *I*, transferrin receptor; *J*, chicken hepatic lectin). Each *hash mark* on the *vertical axis* represents 5 units.

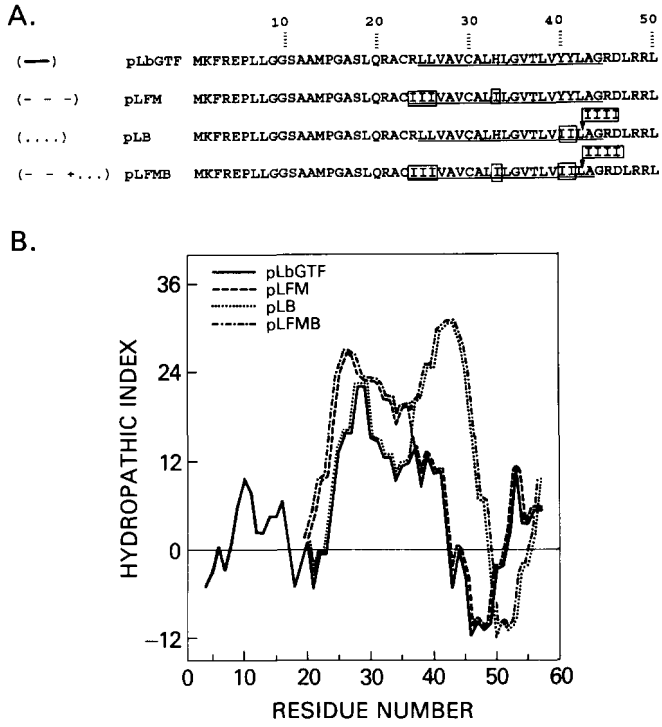

FIG. 7. **Substitution or insertion of Ile changes the hydropathic profile of the transmembrane domain.** *A*, amino-terminal sequence of the wild-type β-1,4-GT (pLbGTF) and the mutant transmembrane regions (pLFM, pLB, and pLFMB). The first 50 residues of the wild-type protein are shown and compared with the sequence of site-directed transmembrane mutants. Substitution and/or insertion of Ile is shown in *boxes*. The signal anchor domain is *underlined*. *B*, hydropathic profile of the transmembrane domain of wild-type and mutant β-1,4-GT proteins. Using the Kyte and Doolittle scale (48) and a window of 7 amino acids, the hydropathic index of each residue in the transmembrane region of pLbGTF (*solid line*), pLFM (*dashed line*), pLB (*dotted line*) and pLFMB (*dashed* plus *dotted lines*) was plotted. The *peak* above the value of 0 indicates the hydrophobic region.

indicate that these mutations affect the hydrophobic characteristic of the signal anchor region. The hydropathic index of the pLFM mutant (Fig. 7*B*, *dashed line*) shows a slight increase in the left shoulder, whereas the pLB mutant (Fig. 7*B*, *dotted line*) shows a drastic shift in height, as well as width, of hydrophobicity in the right shoulder. Mutant pLFMB shows the greatest increase in hydrophobic width relative to wild type and the other two mutants, since it incorporates all of the mutations (*dashed* and *dotted lines*).

We determined the effect of these mutations on the localization of β-1,4-GT by fractionation of the cell extracts on sucrose gradients, whereby the Golgi fraction is separated from plasma membranes, or by indirect immunofluorescence of transfected COS-7 cells. The enzyme activity from these mutant-transfected cells show that these site-directed mutageneses did not inactivate the protein produced. The enzyme activity of the fractionated cell extracts shows that there is more of GT activity present in the plasma membrane fractions of mutant pLFM, pLB (Fig. 8), and pLFMB (data not shown) as compared with the wild-type protein, pLbGTF. The presence of β-1,4-GT on the cell surface upon transfection with the membrane mutants was verified using indirect immunofluorescence of permeabilized (Fig. 9, *a-e*) and nonpermeabilized (Fig. 9, *f-j*) COS-7 cells. Fig. 9 shows that there is an increase in β-1,4-GT expression on the cell surface of pLFM (*h*), pLB (*i*), and pLFMB (*j*) transfectants, indicating that the Golgi retention signal can be disrupted by increasing the hydrophobic character of the transmembrane domain. Additionally, by keeping intact the critical residues for Golgi retention, Cys-30 and His-33 (52), and increasing the hydrophobic length of the membrane-spanning region (pLB membrane mutant), the mutant protein is now released from the Golgi and detected on the plasma membrane.

DISCUSSION

β-1,4-GT is anchored in the lipid bilayer of a cell through its amino-terminal region with an orientation reverse to that of class I membrane proteins that are anchored by the carboxyl terminus. It has been localized in the *trans*-Golgi (17, 18), but there are reports that also indicate its presence on the cell surface (19–25). In the present study, site-directed mutagenesis was used to ascertain the functional role of the membrane-spanning region and to identify the requirements needed for this function. To this end, wild-type and mutant

forms of β-1,4-GT were transiently expressed in COS-7 cells, and the proteins were localized by indirect immunofluorescence microscopy and subcellular fractionation. We analyzed the function(s) of the membrane-spanning domain either by sequential deletion of the amino-terminal portion of the molecule or by replacing the amino-terminal segment by corresponding regions of other glycosyltransferases. Comparative protein sequence analysis of the transmembrane domain of various glycosyltransferases show little homology, but the domains are always hydrophobic. This suggests that not only are the specific residues important for its function(s), but so is the hydrophobicity of the region. To alter the hydropathic character of the transmembrane region, we introduced hydrophobic residues either by substitution or by insertion and substitution at the amino-terminal and the carboxyl-terminal ends of the membrane-spanning domain, respectively. Three conclusions have been derived from this study: 1) the membrane-spanning region is essential for normal processing of the protein leading to its stable accumulation, 2) to produce the stable protein, the cytoplasmic and signal anchor domain of β-1,4-GT can be substituted by an equivalent domain of α-1,3-GT, whereas the cytoplasmic and transmembrane segment of α-2,6-ST needs extra sequences, and 3) to target glycosyltransferases to various cellular compartments, the hydrophobic length of the signal anchor domain, in addition to specific residues, may contribute significantly to the intracellular transport (sorting/retention signal) machinery in the cell.

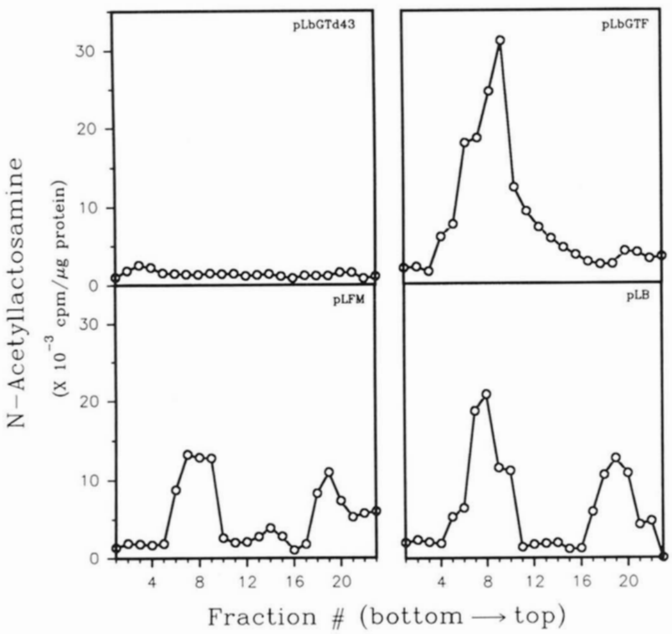

FIG. 8. **Subcellular fractionation of β-1,4-GT activity in COS-7 cells transfected with site-directed mutants.** Extracts from transfected COS-7 cells were subjected to a sucrose gradient centrifugation. Fractions collected from the gradient were assayed for galactosyltransferase activity as previously described.

Although there is ample evidence to show that the *trans*-membrane region of β-1,4-GT contains a Golgi retention signal (50–52), it is still not clear what the requirements and the exact mechanism(s) needed for the retention machinery are. Our previous (39) and present studies indicate that not only is the membrane-spanning region of β-1,4-GT important for Golgi localization, but it is also essential for stable accumulation in COS-7 cells. As shown by Western blot analysis (Fig. 3) and indirect immunofluorescence experiments (Fig. 5), the entire transmembrane domain must be intact for proper processing of the protein leading to its accumulation within the Golgi. Disruption of the first 5 amino acids of the membrane-spanning domain causes the recognition machinery either to shunt the protein somewhere else or to retain it and then initiate its degradation, *i.e.* without a proper signal anchor, the protein does not enter the secretory pathway. This hypothesis is supported by the fact that the membrane-spanning region of another transferase, α-1,3-GT, can replace the corresponding region of β-1,4-GT and produce a stable protein. It is also possible, as speculated earlier (39), that attachment of a signal peptide to pLbGTd43 would have produced a secreted protein similar to α-2,6-ST (16).

The construct, pL2,6-bGTd43, which contains the membrane-spanning region of α-2,6-ST, but lacks its flanking neck region, did not produce any stable protein. Once the flanking neck region is restored (pL2, 6S-bGTd43), the protein accumulates in the Golgi (Fig. 5). Our results regarding the importance of the neck region of α-2,6-ST in protein stability and in the targeting of the protein to the Golgi are consistent with the observations made by others (53–55). It, however, remains to be determined why the fusion protein from the chimeric construct does not accumulate in the absence of the neck region. The sorting/retention machinery may distinguish the signals between α-2,6-ST and β-1,4-GT transmembrane region. Instead of receiving a distinct signal, the membrane-spanning region of α-2,6-ST without its neck region may cause the protein to be tagged as an unfolded molecule or may induce a conformational change that accounts for the instability of the pL2,6-bGTd43 fusion protein. The difference in the requirements of the transmembrane region of galactosyltransferase and sialyltranferase for Golgi retention, observed by us and by others (53–55), may reflect already known

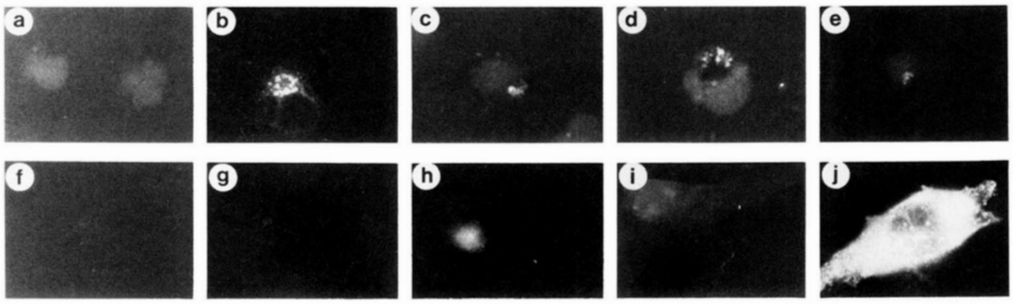

FIG. 9. **Cellular localization of transmembrane mutant proteins in permeabilized (*a–e*) or nonpermeabilized (*f–j*) transfected COS-7 cells by indirect immunofluorescence.** COS-7 cells after transfection were fixed and permeabilized (*a–e*) with Triton X-100 in PBS and stained by indirect immunofluorescence with the monospecific anti-β-1,4-GT antibody. Staining of nonpermeabilized transfected cells (*f–j*) followed the same procedure as above, except for the permeabilization step. *a* and *f*, pLbGTd43; *b* and *g*, pLbGTF; *c* and *h*, pLFM; *d* and *i*, pLB; *e* and *j*, pLFMB. Magnification × 630.

differences in the subcellular localization of these enzymes in the *trans*-Golgi. It is possible that there may be a set of graded retention signals involved in *trans*-Golgi retention. Further studies involving immunoelectron microscopy will delineate whether such graded sets of retention signals actually exist within the *trans*-Golgi subcompartment(s).

The possibility that the hydrophobic nature of the transmembrane domain of glycosyltransferases may influence the retention machinery that is operating *in vivo* prompted us to analyze the hydropathic nature of the transmembrane domain of several integral membrane proteins. These analyses suggest that the hydrophobic length of the transmembrane is one of the contributing factors for sorting of these proteins. Comparison of the hydropathic profile of the membrane-spanning domain of plasma membrane proteins with those of Golgi proteins show that the former has, on the average, a hydrophobic length longer than the latter (Fig. 6, *G–J versus A–F*). Increasing the hydrophobic length of the transmembrane domain by substituting and/or inserting Ile in this region causes a shift in the localization of Golgi β-1,4-GT mutants toward the cell surface (Figs. 8 and 9), indicating that it was sufficient to override the retention signal, thus releasing the protein from the Golgi. Golgi retention was partially lost in the mutant pLB, where the amino-terminal half of the membrane-spanning domain (which has within it Cys-29 and His-33) was kept intact while mutating the carboxyl-terminal end of the transmembrane domain. Although Cys-29 and His-33 have been shown to be critical for Golgi retention (52), there may be other parameters that influence the retention/sorting machinery. The carboxyl-terminal half of the transmembrane domain of human GT (residues 38–47), which does not contain Cys and His, has been shown to be sufficient for Golgi targeting (50). This would be consistent with the observation that simply substituting a Cys and a His residue in the corresponding positions of the transmembrane domain of the transferrin receptor, a plasma membrane protein, does not cause the cell surface-bound protein to be retained in the Golgi (52). It has been found that a chimeric protein between lysozyme, a secretory protein, and the α-2,6-ST transmembrane domain can be targeted to the Golgi by replacing 16 residues of this transmembrane region with 17 Leu residues (53). Increasing the hydrophobic length of this transmembrane region by adding 6 more Leu residues resulted in the protein being targeted from the Golgi to the plasma membrane. A similar strategy with reverse consequences has been reported earlier (56), whereby the influenza virus neuraminidase, a plasma membrane protein, accumulated in the Golgi and RER when the hydrophobic length of the transmembrane region was reduced. This was accomplished by replacing the hydrophobic residue with a charged residue. The increased hydrophobicity of the transmembrane domain may allow it to hydrophobically interact with other proteins and lipids and possibly form vesicle scaffolding that may be a part of the sorting machinery that transports plasma membrane-bound proteins. By increasing the hydrophobic character of the transmembrane domain of β-1,4-GT or α-2,6-ST, one also increases their ability to oligomerize, which may help ensure their transport to the plasma membrane instead of the Golgi. The transmembrane region of β-1,4-GT or other glycosyltransferases may contain signals that specifically direct the movement of the protein along the exocytotic pathway and allow it to be retained in their respective locations in different subcompartments of the Golgi apparatus, and this signal can be overcome by increasing the hydrophobic character of the transmembrane domain. Local alterations of the transmembrane structure might also disable this signal, thereby preventing transport of the complete molecule to its normal destination. The importance of chain length and hydrophobicity of transmembrane domains in protein-membrane interaction is definitely something that needs to be addressed in the cellular targeting of glycosyltransferases. There are indications that the lipid bilayer may not have a constant thickness and that differences in the fatty acid composition of the lipid bilayer contribute to the varying thickness of the membrane in different parts of the cell or organelle structures (57, 58). One can envision that the interaction between a thicker lipid bilayer and the transmembrane domain of a protein will be greater when the latter has a longer and more hydrophobic character. This will facilitate the movement of the protein toward the cell surface via the bulk flow mechanism. On the other hand, if Golgi or endoplasmic reticulum membranes are thinner, the transmembrane region of a protein that is of a shorter hydrophobic length than that of plasma membrane proteins may result in more favorable interactions. The specific residues may subsequently be critical for the retention process.

The question still remains as to how the cell targets the wild-type β-1,4-GT to the cell surface without mutation. Our results, along with others (50–52) rule out the possibility that the 13 residues before the second Met are responsible for the differential targeting, as has been suggested by some (49). We favor the hypothesis that the sorting signal is within the transmembrane domain itself. The presence of β-1,4-GT on the cell surface has been associated with certain cell states such as cell growth and differentiation (10, 11, 19–25), in which the cell responds to a stimulus and undergoes drastic changes. It is quite possible that during one of these changes a post-translational event occurs that increases the hydrophobicity of the transmembrane region that overcomes the Golgi retention signal of β-1,4-GT. Fatty acylation is a recently discovered post-translational modification and is suspected to have some involvement in membrane targeting (59, 60). Palmitate is usually attached to Cys residues via a thioester bond, and the β-1,4-GT has a Cys within the transmembrane domain and another one very close to the NH_2-terminal side of this region. Palmitylation of integral membrane proteins has been proposed to be involved in protein-protein and/or protein-lipid interactions within membranes, possibly by influencing protein folding or protein orientation (61, 62). In this light, it is interesting to point out that the α-2,6-ST also has a Cys within the transmembrane domain, and this glycosyltransferase has been detected on the cell surface of stimulated B cells (4).

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