β1,4-Galactosyltransferase: A Short NH₂-terminal Fragment That Includes the Cytoplasmic and Transmembrane Domain Is Sufficient for Golgi Retention

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β1,4-galactosyltransferase (β1,4-GT) is a Golgi-resident, type II membrane-bound glycoprotein that functions in the coordinate biosynthesis of complex oligosaccharides. Additionally, β1,4-GT has been localized to the cell surface of a variety of cell types and tissues where it is proposed to function in intercellular recognition and/or adhesion. Thus β1,4-GT is an appropriate molecule to be used in analyzing the molecular basis for retention of a membrane-bound enzyme in the Golgi complex and its subsequent or alternative transport to the cell surface.

Previously we have shown that the gene for bovine and murine β1,4-GT is unusual in that it specifies a short (SGT) and long (LGT) form of the enzyme (Russo, R. N., Shaper, N. L., and Shaper, J. H. (1990) J. Biol. Chem. 265, 3324-3331). The only difference between the two related forms is in the primary structure of the cytoplasmic domains, where LGT has an NH₂-terminal extension of 13 amino acids. In this study, we have tested the hypothesis that LGT and SGT are differentially retained in the Golgi or directed to the cell surface. LGT, SGT or chimeric proteins, containing the NH₂-terminal cytoplasmic and transmembrane domain of SGT and LGT fused to the cytoplasmic protein pyruvate kinase, were each stably expressed in Chinese hamster ovary cells. Proteins expressed from each construct were localized by immunofluorescence staining exclusively to a perinuclear region, identified as the Golgi by co-localization with wheat germ agglutinin. Furthermore, the subcellular distribution of both SGT and LGT was restricted to the trans-Golgi compartment as assessed by EM immunoelectron microscopy. These data suggest that both forms of β1,4-GT are resident trans-Golgi proteins and that an NH₂-terminal segment containing the cytoplasmic and transmembrane domains of SGT (39 amino acids) or LGT (52 amino acids) is sufficient for Golgi retention.

The Golgi apparatus serves a central role in directing incoming soluble and membrane-bound proteins from the endoplasmic reticulum to their different subcellular compartments or alternatively, export from the cell. Golgi resident enzymes carry out a variety of post-translational modifications of proteins ranging from the completion of N-linked oligosaccharide side chains to sulfation. Specific post-translational modifications have been functionally linked to the routing of proteins to their final destination. Significant progress has been made in establishing the molecular basis for the targeting and retention of both soluble and membrane-bound proteins in a number of subcellular compartments including the nucleus (Silver, 1991), ER (Pelham, 1989), lysosome (Kornfeld and Mellman, 1989), mitochondria (Pfanner and Neupert, 1990), and the yeast vacuole (Vallis et al., 1990). The Golgi apparatus, however, is conspicuous for the lack of detailed information on the retention of resident proteins. An additional complication is that within the Golgi complex there is further subcompartmentalization. The enzymes that function sequentially in the completion of N-linked carbohydrate side chains illustrate this point. Representative enzymes involved in trimming (mannosidase II), elongation (GlNAC transferase I), and terminal glycosylation (β1,4-galactosyltransferase, α2,6-sialyltransferase) are located in the cis, medial, and trans-Golgi, respectively (reviewed by Farquhar and Palade (1981) and Roth and Berger (1982)).

Work from a number of investigators has resulted in the isolation of full-length cDNA clones for a subset of Golgi-resident glycosyltransferases (reviewed by Paulson and Colley (1989)) (see Sarkar et al. (1991) and references therein). A comparison of the primary structures of the cloned glycosyltransferases has revealed several interesting patterns. First, each enzyme is oriented as a type II membrane-bound protein with a characteristic architecture consisting of a large, potentially glycosylated COOH-terminal luminal domain containing the catalytic center, a single transmembrane domain, and a short NH₂-terminal cytoplasmic domain. Second, with the exception of the blood group A and B allelic glycosyltransferases (Yamamoto et al., 1990) and α1,3-galactosyltransferase (Joziasse et al., 1989; Larsen et al., 1989), which appear to have evolved from a common ancestor, and two structurally related fucosyltransferases, α1,3/4-fucosyltransferase (Goelz et al., 1990; Kukowska-Latallo et al., 1990) and α1,3-fucosyltransferase (Lowe et al., 1991), there is essentially no sequence similarity between the different enzymes. Consequently, by comparing the primary structure of these Golgi resident proteins, it has not been possible to identify a sequence motif in common that could potentially function as a targeting or retention signal.

With the long range goal of determining the molecular basis for retention and subcompartmentalization of Golgi-resident proteins, we have initiated studies with the trans-Golgi en-

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zyme β1,4-galactosyltransferase (β1,4- GT).

β1,4-GT functions in the coordinate biosynthesis of complex oligosaccharides by catalyzing the transfer of galactose (Gal) from UDP-Gal to N-acetylglucosamine (GlcNAc), forming the Galβ1,4GlcNAc linkage found in glycoproteins, glycolipids and proteoglycans (Beyer and Hill, 1982).

β1,4-GT has also been localized to the surface of many cell types by immunocytochemistry (Shaper et al., 1985; Roth et al., 1985; Lopez et al., 1985; Bayna et al., 1988; Penno et al., 1989), where it may have a dual function in intercellular recognition and/or adhesion.

Previously, we demonstrated that the gene for murine β1,4-GT (Russo et al., 1990) each specify two sets of mRNA transcripts of different lengths, presumably by promoter switching (Kozak, 1988).

Translation of each set of mRNAs results in the biosynthesis of two related forms of the protein, short β1,4-GT (SGT) and long β1,4-GT (LGT), which differ only in the length of their NH2-terminal, cytoplasmic domains. Based on the observation that β1,4-GT is found both in the Golgi and at the cell surface, we have speculated that the functional significance of SGT and LGT might be related to their subcellular localization. By extrapolation, one would predict that the information specifying either Golgi retention or export to the cell surface would be located in the NH2-terminal region of the protein. In order to test the hypotheses that SGT and LGT are differentially retained in the Golgi or directed to the cell surface and that signals for subcellular destination are located in the NH2-terminal region of each polypeptide, SGT, LGT, and chimeric proteins containing the NH2-terminal cytoplasmic/transmembrane domain of SGT and LGT fused to the -galactosyltransferase protein prynvate kinase were each stably expressed in CHO cells. Proteins expressed from each construct were localized by immunofluorescence staining exclusively to a perinuclear region, identified as the Golgi by co-localization with WGA. Subcellular localization at the level of the electron microscope demonstrated that both SGT and LGT were restricted to the trans-Golgi. These data suggest that both forms of β1,4-GT are resident trans-Golgi proteins and neither form is differentially directed to the cell surface.

Furthermore the NH2-terminal segment containing the cytoplasmic and transmembrane domains of SGT (39 amino acids) or LGT (52 amino acids) is sufficient for Golgi retention of these type II membrane-bound proteins. A preliminary report of this work has been presented (Shaper et al., 1990).

**EXPERIMENTAL PROCEDURES**

**Materials—**BamHI linkers were obtained from New England Bio-labs; the mammalian expression vector pSVL and protein A were obtained from Pharmacia LKB Biotechnology. Tissue culture media and the antibiotic G418 were purchased from GIBCO. Lipofectin reagent was from Bethesda Research Laboratories. Methanol-free formaldehyde was obtained from Polysciences, and rabbit serum albumin was from Sigma. Nitrocellulose and Nytran membranes were from Schleicher & Schuell. The Texas Red-conjugated WGA was from E-Y Labs.

The FITC-conjugated secondary antibodies and FITC, fluorescein isothiocyanate; kb, kilobase(s); TMD, transmembrane domain; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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The FITC-conjugated secondary antibodies and FITC, fluorescein isothiocyanate; kb, kilobase(s); TMD, transmembrane domain; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Recent studies have demonstrated that each of these mAbs can recognize, by Western blot analysis, the recombinant bovine β,1,4-GT polypeptide (catalytic domain) expressed in Escherichia coli, indicating that the two different epitopes are contained within the primary structure (Russo, 1990).

Transfected cells were plated onto nitric acid-cleaned, ethanol-sterilized glass coverslips in 35-mm plastic dishes at approximately 1 × 10⁶ cells/dish. Medium was replaced after 48 h. After 4 days, the cells were rinsed with Ca²⁺/Mg²⁺-free PBS, fixed for 15 min at room temperature with 4% (v/v) methanol-free formaldehyde in PBS, and rinsed in PBS. Fixed cells were either used directly (for cell surface localization) or, alternatively, permeabilized for 5 min at ~20°C in methanol (for intracellular localization), and were again rinsed in PBS. All subsequent incubations were carried out for 30 min at 37°C and were followed by a PBS rinse. The lectin and antibody reagents were diluted in PBS containing 2 mg/ml rabbit serum albumin. Cells transfected with β,1,4-GT expression vectors were first stained with Texas Red-conjugated WGA diluted to 1 pg/ml, then with the appropriate mAb (H5 or H162) diluted to 50 µg/ml, and finally with FITC-conjugated sheep anti-mouse IgG antibody diluted to 50 µg/ml. Cells transfected with PK expression vectors were first stained with WGA as described above, followed by staining with a rabbit anti-chicken muscle PK polyclonal antibody (kindly provided by Dr. Bruce Roberts) diluted to 1:50, and finally with a FITC-conjugated goat anti-rabbit F(ab)² fraction diluted to 80 µg/ml. Coverslips were mounted on slides in PBS/glycerol (1:1) and sealed with nail polish. Slides were viewed with a Zeiss-Universal microscope equipped with epifluorescent illumination and appropriate filters for fluorescein and Texas Red fluorescence. Fluorescence was recorded on Kodak Ektachrome EES film (PS00 ASA).

Preparation of Cells for Immunoelectron Microscopy—Subconfluent transfected cells were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in PBS for 30 min at 22°C, washed twice with PBS, and subsequently incubated with 0.05 M NH₄Cl in PBS for 20 min. The fixed cells were washed 2 times with PBS and then were scraped from the dishes, pelleted, and encapsulated in 6% gelatin (from porcine skin, 300 bloom) in PBS. Small pieces of gelatin containing cells were cryoprotected by immersion into 2.3 M sucrose overnight at 4°C. The next day, gelatin pieces were frozen by plunging into liquid nitrogen and then stored in cryovials in liquid nitrogen until sectioning.

For the immunolocalization of β,1,4-GT at the electron microscopic level, ultrathin melted cryosections were processed with the protein A-gold technique essentially according to Slot et al. (1988). Briefly, ultrathin sections mounted on parlodion/carbon-coated nickel grids were floated on drops of PBS containing 0.1% BSA and 0.4% gelatin (PBS/BSA/Gel) for three times, 5 min each, followed by transfer to an alkaline phosphate mixture composed of mAbs H5 and H162 (final concentrations 50 µg/ml each) for 30 min at room temperature. After rinses with PBS/BSA/Gel (3 × 5 min), the grids were incubated on drops of affinity-purified rabbit anti-mouse IgG (10 µg/ml, diluted with PBS/BSA/Gel) for 30 min. Following rinses as above, the grids were floated for 3 min on drops of protein A-gold (10-nm particles; diluted with PBS/BSA/Gel to yield an A₂₆⁰₅₀nm = 0.2). Finally, the sections were contrasted and embedded in a methyl cellulose/uranyl acetate mixture according to Tokuyasu (1980).

Demonstration of galactose residues at the electron microscopic level was performed by using R. communis lectin I followed by an asialofetuin-gold complex as previously described (Taattjes et al., 1990).

RESULTS

Expression of the Short and Long Forms of Bovine β,1,4-Galactosyltransferase in Stably Transfected CHO Cells—The observed mRNA size for mammalian β,1,4-GT (including CHO cells) is characteristically ~4.1 kb. This transcript size is large relative to the required coding region of ~1.2 kb; it is due to the presence of an unusually long 3' untranslated region (Shaper et al., 1988). In order to individually express SGT or LGT, the respective coding regions (see Fig. 1A) were subcloned into pSVL, a mammalian expression vector that utilizes the SV40 late promoter. These constructs lack about 2 kb of the 3'-untranslated sequence. Consequently the predicted size of the bovine specific β,1,4-GT transcript is 2.1 kb and therefore is easily distinguishable from the endogenous CHO β,1,4-GT transcript of 4.1 kb.

![Diagram](image1.png)

**FIG. 1.** Panel A, diagrammatic representation of two related forms of β,1,4-GT which are encoded by pRRSGT and pRRLGT. SGT and LGT refer to short and long β,1,4-GT, respectively. The predicted size of each protein in amino acid residues (A.A.) is indicated. The thin horizontal line represents primary amino acid sequence, and the transmembrane domain is shown by the dark rectangle. Consensus sites for N-linked oligosaccharide addition (Y) are indicated. Methionines (M) in the NH₂-terminal domain are indicated. Panel B, diagrammatic representation of two related β,1,4-GT/PK chimeric proteins encoded by pRRSPK and pRRLPK. SPK and LKP refer to chimeric proteins in which the NH₂-terminal and transmembrane domains from either SGT or LGT have been fused to PK. The predicted size of each protein in amino acid residues (A.A.) is indicated. The thin horizontal line represents primary amino acid sequence, with the transmembrane domain indicated by the dark rectangle. The open box represents PK primary amino acid sequence.

![Diagram](image2.png)

**FIG. 2.** RNA blot analysis of bovine β,1,4-GT transcripts expressed in stably transfected CHO cells. Ten µg of total RNA from cells transfected with pSVL (lane 1), pRRSGT (clone 4B; lane 2), or pRRLGT (clone 6E; lane 3) were electrophoresed on a 1% agarose-formaldehyde gel. After transfer to nitrocellulose the blot was hybridized with 32P-labeled cDNA clone 7A. Sizes of RNA standards (in kb) are shown to the left.

The resulting constructs, pRRSGT and pRRLGT, were co-transfected with pSV₂-neo into CHO cells. After selection for 10–14 days in the presence of the antibiotic G418, RNA was isolated from the drug-resistant cells, and examined by Northern blot analysis using bovine clone 7A as the probe (Shaper et al., 1985). This analysis showed that CHO cells transfected with either pRRSGT or pRRLGT expressed the anticipated 2.1-kb transcript. In contrast, CHO cells transfected with the parent vector, or plasmids containing the coding region of SGT and LGT in the antisense orientation, did not express the 2.1-kb transcript (data not shown).

Two single cell clones, 4B and 6E, derived from the pRRSGT and pRRLGT transfectants, respectively, were analyzed in more detail. As shown in Fig. 2, a 2.1-kb transcript is detected in RNA isolated from both 4B and 6E clones (lanes 2 and 3), whereas CHO cells transfected with the pSV₂ parental vector do not express a transcript of this size (lane 1). The level of expression of the 2.1-kb transcript is similar for both
constructs and is comparable with the level of the endogenous 4.1-kb CHO β1,4-GT transcript (data not shown).

Localization of the Short and Long Forms of Bovine β1,4-Galactosyltransferase in Stably Transfected CHO Cells—In order to determine the subcellular localization of the expressed short and long form of bovine β1,4-GT, CHO cells derived from clones 4B (SGT) and 6E (LGT) were fixed, permeabilized, and stained with either mAb H162 or H5. As discussed under “Experimental Procedures,” these monoclonal antibodies are species specific and recognize two distinct polypeptide epitopes located in the luminal domain of bovine β1,4-GT. After incubation with the mAb, cells were counterstained with WGA, a lectin which has routinely been used as an histochemical marker for the Golgi region (Virtanen et al., 1980). As seen in Fig. 3, mAb H162 stains an asymmetric perinuclear region in both clone 4B (SGT) cells (panel 2) and clone 6E (LGT) cells (panel 5) that is coincident with WGA staining (Fig. 3, panels 3 and 6). This co-localization suggests that both forms of β1,4-GT are directed to and retained in the Golgi region. Control CHO cells, stably transfected with the parent vector, exhibit no specific staining pattern with mAb H162 in a region coincident with WGA staining. Similar staining patterns were also obtained when mAb H5 was used (data not shown).

In order to determine if either form of bovine β1,4-GT was also transported to the cell surface in detectable levels, fixed, non-permeabilized cells (from clone 4B or 6E) were decorated with our monospecific polyclonal antiserum or alternatively with mAbs H5 and H162. We were unable to detect either form of the expressed bovine β1,4-GT on the cell surface of CHO cells transfected with either pRRSGT or pRRLGT (data not shown).

Both SGT and LGT Are Restricted to the trans-Golgi Region of Transfected CHO Cells—Results from light level immunofluorescence were confirmed and extended by immunolocalization at the electron microscopic level. β1,4-GT immunoreactivity detected with an H5/H162 mAb mixture followed by protein A-gold was found only in the Golgi apparatus of both SGT (Fig. 4, panel 1) and LGT (Fig. 4, panel 2) cells. No immunoreactivity was detectable at the cell surface of either cell type with either the H5/H162 mAb mixture or with the polyclonal antiserum. Within the Golgi apparatus, the immunostaining was subcompartmentalized. The structure of the Golgi apparatus in CHO cells consists of a stack of flattened cisternae, with the cisternal elements increasing in length from the cis to the trans side (Fig. 4). The trans side of the stack is characterized by the presence of numerous secretory vesicles and tubules, forming part of the trans-Golgi network. Aside from morphological appearance as a criterion for establishing polarity of the Golgi apparatus, we also used lectin cytochemical methods. While the presence of sialic acid is normally a good indicator of the trans-Golgi apparatus, we found that the sialic acid-specific Limax flavus lectin stained the entire Golgi apparatus cis/trans stacks. These results agree with those published by Lee et al. (1989) obtained on sections transfected CHO cells embedded in Lowicryl K4M. However, we found that staining with R. communis lectin I followed by asialofetuin-gold is limited to trans cis stacks, portions of the trans-Golgi network (tGN), and secretory vesicles. Bar = 0.15 μm (panel 1), 0.11 μm (panel 2), 0.14 μm (panel 3).

Expression of β1,4-GT/PK Chimeric Proteins in Stably Transfected CHO Cells—In the second parallel set of experiments we asked if the NH2-terminal region of each form of β1,4-GT contained within its primary sequence, sufficient information to direct and retain a non-resident membrane-bound protein in the Golgi compartment.

To address this question we have fused the NH2-terminal region from each form of β1,4-GT to the reporter protein, chicken muscle PK and then stably expressed the chimeric proteins in CHO cells. PK is a soluble, cytoplasmic enzyme from the glycolytic pathway. Our selection of chicken PK as a reporter protein was based on the results of two previous studies. Kalderon et al. (1984) demonstrated that when the

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**Fig. 3. Immunolocalization of bovine β1,4-GT in stably transfected CHO cells.** Cells transfected with pRRSGT (clone 4B; panels 1-3) or pRRLGT (clone 6E; panels 4-6) were formalin-fixed, permeabilized, and stained as described under “Experimental Procedures.” Panels 2 and 5 show the staining patterns obtained with mAb H162 followed by FITC-conjugated sheep anti-mouse IgG. The same fields counterstained with Texas Red-conjugated WGA are shown in panels 3 and 6. Corresponding phase photographs are shown in panels 1 and 4. Bar, 12 μm.

**Fig. 4. Immuno-electron microscopic localization of bovine β1,4-galactosyltransferase and lectin cytochemical detection of galactose residues in transfected CHO cells.** β1,4-GT immunoreactivity is localized to two or three trans ciserna of the Golgi apparatus in both SGT (panel 1) and LGT (panel 2) cells. The Golgi apparatus in panel 1 is horseshoe-shaped, with cis regions at both the bottom and top of the micrograph and the trans region in the middle. Note the prominent secretory vesicles (arrowheads) associated with the trans side of the stack. Polarity of the Golgi apparatus is demonstrated by lectin cytochemical methods in panel 3. Detection of galactose residues with R. communis lectin I followed by asialofetuin-gold is limited to trans ciserna, portions of the trans-Golgi network (tGN), and secretory vesicles. Bar = 0.15 μm (panel 1), 0.11 μm (panel 2), 0.14 μm (panel 3).
Terminus of chicken muscle PK (Kalderon et al., 1984). The vector XR30PK8 which encodes a chimeric protein containing signal for either the endoplasmic reticulum or the Golgi. This observation is particularly relevant for our study in that it demonstrated that PK does not contain an intrinsic retention signal for either the endoplasmic reticulum or the Golgi.

To make the β1,4-GT/PK construct we used the expression vector XR30PK8 which encodes a chimeric protein containing a region of the SV40 large T antigen fused to the NH2 terminus of chicken muscle PK (Kalderon et al., 1984). The large T antigen sequence from this vector was removed and replaced with a sequence encoding the NH2-terminal cytoplasmic and transmembrane domain of either SGT or LGT. In addition, the 8 amino acids immediately following the transmembrane domain of bovine β1,4-GT were present in each construct (see Fig. 1B). This short sequence was included in order to maintain the cluster of charged residues that occur in juxtaposition to the transmembrane domain. It has been suggested that these positively charged residues in the regions adjacent to both sides of the transmembrane domain serve to establish topological orientation of a membrane-bound protein (von Heijne and Gavel, 1988; Hartmann et al., 1988; Parks and Lamb, 1991). The predicted amino acid sequence across the β1,4-GT/PK fusion site is Gln-Asn-Ser-Leu-., in which Gln is amino acid residue 52 of LGT (Russo et al., 1990), Asn and Ser are encoded by the filled-in EcoRI site, and Leu is amino acid residue 17 of chicken muscle PK (Lonberg and Gilbert, 1983). The resultant chimeric constructs, pRRSPK and pRRLPK, encode two closely related fusion proteins which are 552 and 565 amino acid residues in length.

CHO cells were transfected with pRRSPK, pRRLPK, or the parent vector XR30PK8. RNA was isolated from G418-resistant transfected cells and Northern blot analysis was performed as described above. When the blot was hybridized with a 32P-labeled probe derived from PK coding sequence, a band of 2.2 kb was detected in RNA from cells transfected with XR30PK8, pRRSGT, and pRRLGT (Fig. 5, lanes 1–3). When the same blot was stripped and rehybridized with a 32P-labeled probe derived from a sequence corresponding to the NH2-terminal and transmembrane domains of SGT, a 2.2-kb band was only detected in RNA isolated from the pRRSPK and pRRLPK transfected cells (Fig. 5, lanes 5 and 6). It is also apparent that the level of expression of the transcript synthesized by these constructs is significantly greater (at least 20-fold) than that seen with the pRRSGT and pRRLGT constructs (Fig. 2). This is probably due to the fact that the latter constructs use the SV40 late promoter for expression and since large T antigen is absent in CHO cells, high levels of expression are not achieved. In contrast the β1,4-GT/PK constructs use the SV40 early promoter for expression which is not dependent on large T antigen.

The Amino-terminal and Transmembrane Domains of the Short and Long Forms of β1,4-Galactosyltransferase Are Sufficient for Golgi Retention—Translation and immunolocalization of the β1,4-GT/PK chimeric proteins was assessed by indirect immunofluorescence using a rabbit polyclonal antibody directed against chicken muscle PK. Fixed and permeabilized cells were stained with the anti-PK antibody and counterstained with WGA in order to visualize the Golgi region. When cells transfected with either pRRSPK (Fig. 6, panels 2 and 3) or pRRLPK (Fig. 6, panels 5 and 6) were examined, strong perinuclear fluorescence in a region coincident with WGA staining was readily seen. Low levels of diffuse staining in the cytoplasm, suggestive of the ER, were also seen, which may be due to the over expression from these constructs. Control CHO cells transfected with the parent vector XR30PK8 (containing the SV40 large T antigen/PK chimera) exhibit a nuclear staining pattern that is virtually identical to the staining seen with XR30PK8-transfected Vero cells (Kalderon et al., 1984) (data not shown). These results demonstrate that the chimeric proteins SGT/PK and LGT/PK are expressed in stably transfected CHO cells, and more importantly, that the NH2-terminal sequence contributed by β1,4-GT is sufficient to redirect PK from its normal cytoplasmic location to the Golgi.

In order to determine if significant levels of either chimeric protein were also transported to the cell surface, non-permeabilized, stably transfected CHO cells were stained with the polyclonal anti-PK antibody. However, no staining above background was observed (data not shown). Thus, if any chimeric protein is present on the cell surface, the level is too low to be detected by indirect immunofluorescence.

**DISCUSSION**

Both SGT and LGT Are Retained in the Golgi Region—We have demonstrated previously that the gene for bovine and murine β1,4-GT predicts the biosynthesis of two related forms of this galactosyltransferase that differ only in the length of their respective NH2-terminal, cytoplasmic domains. The short and long forms of β1,4-GT have NH2-terminal, cyto-

**FIG. 5.** RNA blot analysis of transcripts encoding chimeric proteins. Ten µg of total RNA from CHO cells transfected with XR30PK8 (lanes 1 and 4), pRRSPK (lanes 2 and 5), or pRRLPK (lanes 3 and 6) was electrophoresed on a 1% agarose-formaldehyde gel. After transfer to Nytran the blot was hybridized with a 32P-labeled fragment corresponding to the coding region of PK (lanes 1–3). After probe removal the blot was rehybridized with a 32P-labeled fragment corresponding to the NH2-terminal and transmembrane domains of SGT (lanes 4–6).

**FIG. 6.** Immunolocalization of chimeric proteins in stably transfected CHO cells. Cells transfected with pRRSPK (panels 1–3) or pRRLPK (panels 4–6) were fixed, permeabilized, and stained as described under “Experimental Procedures.” Panels 2 and 5 show the staining patterns obtained with the rabbit polyclonal anti-PK antibody followed by FITC-conjugated goat anti-rabbit F(ab’)2. The same fields counterstained with Texas Red-conjugated WGA are shown in panels 3 and 6. Corresponding phase photographs are shown in panels 1 and 4. Bar = 9 µm (panels 1–3), 10 µm (panels 4–6).
plasmatic domains of 11 and 24 amino acids, respectively. Interestingly, of the different glycosyltransferase cDNA clones reported to date, only bovine, murine, and apparently human β1,4-GT (Strous, 1986) are synthesized with structurally different cytoplasmic domains. The demonstration of two forms of the enzyme coupled with the dual localization of β1,4-GT in the Golgi and on the cell surface of a variety of cells and tissues has invited the question of whether the long and short forms are differentially directed to these two subcellular compartments. To test this premise we have stably transfected CHO cells with an expression plasmid encoding either the short or long form of bovine β1,4-GT. Subcellular distribution of each form of the enzyme was determined at both the light and electron microscope level using a series of species specific mAbs that recognize distinct polypeptide epitopes of the recombinant bovine enzyme expressed in E. coli.

This analysis demonstrated that both forms of β1,4-GT are localized to, and retained in, the Golgi region of CHO cells. However, non-permeabilized cells or cryosections of intact cells were analyzed for the presence of cell surface β1,4-GT, we were unable to detect either form of the enzyme on the plasma membrane of the stably transfected CHO cells. Similar results were obtained with our monospecific polyclonal antiserum. These results are in direct contrast with our results using the bovine kidney epithelial cell line (MDBK) where we were able to detect a cell surface antigen with our polyclonal antiserum (Shaper, 1985). Although we cannot eliminate the possibility that cell surface transport of β1,4-GT is cell-type-specific and that β1,4-GT is found only in the Golgi in CHO cells, this would imply that the general cellular machinery to direct any cell surface resident type II membrane protein to the plasma membrane is not operative in CHO cells. In summary, the most direct interpretation of these experiments is that both the short and long form of β1,4-GT are trans-Golgi resident membrane-bound proteins and that neither form is differentially directed to the cell surface of CHO cells.

The Signal for Golgi Retention Is Contained within the Amino-Terminal Cytoplasmic and Transmembrane Domains of β1,4-Galactosyltransferase—Chimeric proteins, constructed from the NH2-terminal and transmembrane domains of SGT or LGT, fused to the cytoplasmic protein chicken muscle PK, were stably expressed in CHO cells and localized to determine if the information necessary for Golgi retention was located within these β1,4-GT-derived domains. Co-localization of the chimeric proteins with WGA lectin was consistent with the conclusion that the NH2-terminal and transmembrane domains of both SGT (39 amino acids) and LGT (52 amino acids) contain the information necessary to direct and retain the cytoplasmic protein PK in the Golgi. Of particular interest is the question of whether these β1,4-GT derived domains also contain the required information to direct these chimeric proteins to the trans-Golgi. We have attempted to address this question by immunoelectron microscopy. However, to date, we have not obtained an unambiguous answer, due primarily to the fact that the anti-PK polyclonal antiserum has consistently given us a high background.

The Status of Golgi Retention Signals Is a “Mixed Signal”—To date, only a limited number of studies have addressed the molecular basis of Golgi retention. Payne and Schekman (1989) have characterized a series of yeast mutants deficient in clathrin heavy chain. Analysis of this defect demonstrated that a Golgi resident membrane-bound endoprotease (Kex2p), which is responsible for initiating proteolytic maturation of the α-factor precursor, was unexpectedly present at the cell surface in mutant cells. This observation suggested that the cytoplasmic protein clathrin is required for retention of Kex2p in the Golgi, presumably by interaction with the cytoplasmic domain of the protease.

In a series of studies, Machamer et al. (1987, 1990) demonstrated that the avian coronavirus E1 glycoprotein, a polylaminar membrane protein with three transmembrane domains, is targeted to the cis-Golgi complex and that the first transmembrane domain is required for cis-Golgi retention; a mutant E1 polypeptide lacking this domain is found on the cell surface. Swift and Machamer (1991) have subsequently demonstrated that two different type I plasma membrane proteins can be redirected and retained within the Golgi by substituting their single transmembrane domain with the first transmembrane domain of the E1 glycoprotein. Significantly, single amino acid substitutions within the transmembrane domain, particularly at charged polar residues, result in release of the chimeric protein from the Golgi compartment.

These results should be contrasted with the recent study of Armstrong and Patel (1991) on a second coronavirus glycoprotein. This protein, which is about 30% similar to the E1 glycoprotein, exhibits an identical protein domain structure. However, in this case removal of the COOH-terminal 18 amino acids from the cytoplasmic domain also resulted in translocation from the Golgi to the cell surface.

Comparison with Other Glycosyltransferases—These results are interesting when compared with the recent studies of Colley et al. (1989) on rat α2,6-sialyltransferase. This sialyltransferase is also a type II membrane-bound enzyme that has been localized to both the trans-Golgi, trans-Golgi network, and the plasma membrane (Taatjes et al., 1988). In these studies the NH2-terminal 57 amino acids, comprising the cytoplasmic domain (9 amino acids), transmembrane domain (17 amino acids), and 31 amino acids of the luminal stem region, were replaced with the cleavable signal sequence from γ-interferon. In stably transfected CHO cells, it was observed that this soluble form of α2,6-sialyltransferase was efficiently secreted, indicating that a large segment of the COOH-terminal catalytic domain of this enzyme (345 amino acids) did not contain sufficient information for Golgi retention. By inference, one would predict that the retention signal for this glycosyltransferase was located in the missing NH2-terminal segment.

Based on this result one might anticipate that a comparison of the primary sequence of the NH2-terminal region from β1,4-GT and α2,6-ST (and other trans-Golgi type II membrane proteins) would reveal a common sequence motif that could function as a Golgi retention signal. For example, this strategy has been successful for deducing the KDEL retention signal for soluble ER proteins (reviewed by Pelham (1989)), a COOH-terminal cytoplasmic signal for type I endoplasmic reticulum membrane proteins (Jackson et al., 1990), and the context of a required tyrosine for endocytotic uptake of cell surface receptors (Kistakis et al., 1990) and apparent targeting of resident type I proteins (LAMPS) to the lysosome (Williams and Fukuda, 1990).

The primary structures of the NH2-terminal regions for β1,4-GT (Russo et al., 1990), α2,6-sialyltransferase (Weinstein et al., 1987) and α1,3-GT (Joziass et al., 1989) are shown below, where the underlined segment represents the transmembrane domain (TMD).

With the possible exception of the cluster of basic amino acids flanking the TM and the aromatic amino acids at the TMD-luminal interface, the most striking feature is the lack
of similarity in the primary structures of the corresponding regions of these three trans-Golgi enzymes. From the point of view of identifying a common Golgi retention signal, this lack of obvious sequence similarity raises several fundamental questions. 1) Is the Golgi retention signal highly degenerate as has been observed for the cleavable signal sequence? 2) Is the retention signal dependent on a threedimensional structure, a "signal patch" (Pfeffer and Rothman, 1987), rather than a linear amino acid sequence? 3) Does each glycosyltransferase have its own unique retention signal? 4) Are the retention signals for different glycosyltransferases located in the same domain?

The expression and subsequent localization in the Golgi of a chimeric protein constructed from a short NH2-terminal segment of 3,4-GT fused to the reported protein PK establishes a system to analyze in detail the molecular bases of targeting and retention of this resident trans-Golgi type II membrane-bound protein.

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