

Transport and Topology of Galactosyltransferase in Endomembranes of HeLa Cells

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ABSTRACT HeLa cell membranes were studied for the distribution and orientation of the Golgi marker enzyme uridine diphosphate-galactose: β -D-N-acetylglucosamine β , 1-4 transferase (GT). Short pulse labeling in the presence of [35 S]methionine resulted in two precursor species ($M_r = 44,000$ and $47,000$), present in a microsomal fraction with a density of 1.18 g/ml in sucrose, presumably derived from the rough endoplasmic reticulum. Processing of the N-linked oligosaccharide(s) occurred only after the precursor molecules migrated to lighter density fractions, presumably derived from the Golgi complex. The mature GT molecules ($M_r = 54,000$) contain O-linked oligosaccharides as shown by β -elimination of metabolically incorporated [3 H]galactose. The O-glycosylation occurred mainly in the light density fractions. The topology of GT was studied on membrane fractions after labeling with [35 S]methionine as well as immunocytochemically on ultrathin cryosections at the electron microscope level. Our results indicate that both the antigenic determinants of GT as well as polypeptide chain are present intramembraneously and at the luminal side of the membranes of the Golgi complex and rough endoplasmic reticulum.

Galactosyltransferase (GT¹) catalyzes the transfer of galactose from uridine diphosphate-galactose to glycoproteins with N-acetylglucosamine as the terminal residue (1). The enzyme has only recently been visualized in the Golgi complex by electron microscope immunocytochemistry (2), despite being used for many years as a biochemical marker enzyme for this organelle (3–6). In a previous report we demonstrated that in HeLa cells GT is synthesized similarly to plasma membrane and secretory glycoproteins (7, 8). GT is synthesized in the rough endoplasmic reticulum (ER), core-glycosylated, and transported to the Golgi complex in ~ 20 min. However, in contrast to the short intracellular half-life of 20–60 min for secretory proteins, GT is retarded at the level of the trans-Golgi cisternae for an average of 20 h (7). Latency of GT activity in Golgi fractions has been reported by Fleischer (9) and was taken as evidence for luminal orientation of the active site. In this orientation it catalyzes the transfer of galactose to glycoprotein-bound glcNAc and, in the presence of α -lactalbumin, to glucose as shown by Kuhn and White

(10) in the Golgi vesicles of rat mammary gland.

In this paper we provide direct evidence that GT as well as its precursors is oriented towards the lumen of the rough ER and cisternae of the Golgi complex. No protease-cleavable polypeptide could be detected at the outside of microsomal vesicles. In addition, we show that GT transport from the rough ER to the Golgi complex precedes complex N-glycosylation as well as O-glycosylation.

MATERIALS AND METHODS

Cell Culture and Labeling: HeLa cells were grown in Eagle's minimal essential medium containing 10% decomplexed fetal bovine serum. Near-confluent 60-mm dishes were labeled with [35 S]methionine (800–1,200 Ci/mmol), The Radiochemical Center, Amersham, England, 70 μ Ci/ml in medium without unlabeled methionine and serum as described (7). After the labeling, cells were lysed in 0.3 ml of 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride in PBS or scraped off the dish and homogenized in a tight-fitting Dounce homogenizer in low salt buffer (0.01 M 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.5, 15 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride). For radioactive labeling with monosaccharides, 10-cm dishes were used. Cells were labeled in Eagle's minimal essential medium containing 10% dialyzed fetal bovine serum, 0.1 mg/ml glucose, 0.5 mM pyruvate and 100 μ Ci/ml [3 H]-galactose or [3 H]mannose (8.2 Ci/mmol and 16 Ci/mmol, respectively, The Radiochemical Center) for 16 h. Then the medium was substituted with [35 S]-

¹ Abbreviations used in this paper: ER, endoplasmic reticulum; GT, uridine diphosphate-galactose: β -D-N-acetylglucosamine β , 1-4 transferase.

methionine labeling medium and the cells were grown for an additional 60 min (7). The cells were lysed and GT was isolated by immunoprecipitation and SDS-gel electrophoresis. After autoradiography of the ^{35}S -label the GT band was cut out and used for β -elimination.

Analysis of Monosaccharide-labeled Oligosaccharides: After labeling with [^3H]galactose or [^3H]mannose, followed by [^{35}S]methionine, the 54,000-mol wt-GT band was cut out of the gel and subjected to mild alkaline hydrolysis (0.05 M NaOH) in the presence of 1 M NaBH_4 for 16 h at 50°C (11). The gel pieces were neutralized, washed with water, and the combined washings were treated with Dowex-50 (H^+) to remove amino acids and peptides, and chromatographed on a Biogel P-2 column in 0.05 M ammonium acetate. Fractions were counted in a liquid scintillation counter. The β -eliminated oligosaccharides of porcine submaxillary mucin, with or without removal of *N*-acetylneuraminic acid, were used as oligosaccharide standards. The structure of the standards was determined after gas chromatographic sugar analyses as compared with the known structures of these oligosaccharides (12).

Cell Fixation and Immunocytochemistry: Cells were fixed in 0.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4. After 60 min the cells were washed with PBS, scraped from the dish, and stored in PBS containing 2% paraformaldehyde and 1 M sucrose for up to 2 wk. Ultrathin cryosections were prepared according to Tokuyasu (13) and indirectly labeled with 8-nm gold particles as described previously (14, 15). Affinity-purified rabbit anti-GT antibodies (16) were used at 40 $\mu\text{g}/\text{ml}$. After immunolabeling, the sections were stained in uranyl acetate and embedded in 1.5% methyl cellulose (13). Background labeling as judged from sections labeled with affinity-purified anti rat amylase was negligible.

Miscellaneous: Gel electrophoresis, fluorography, and immunoprecipitation of GT were carried out as described previously (7). Fluorograms were scanned with a Joyce-Loebl microdensitometer. Limited proteolytic digestion was performed according to the procedure of Cleveland et al. (17) using 1 $\mu\text{g}/\text{ml}$ protease V-8 (Miles Laboratories, Elkhart, IN). For this purpose [^{35}S]methionine-labeled bands were cut out from a 10% SDS gel and the digests were run on a 15% gel. Cell fractionation of HeLa cells and GT enzyme assays were done as described (7).

RESULTS

Biosynthetic Pathway of GT

To study the orientation of GT in the membranes of the rough ER and the Golgi complex, it is important to know how GT is transported from the rough ER to its main site of localization, the trans-Golgi cisternae. In an earlier study we established that it takes ~ 20 min for GT to become resistant to the glycosidase endo- β -*N*-acetylglucosaminidase H (Endo H). The enzyme Endo H cleaves between the two core GlcNAc residues, leaving a single GlcNAc residue attached to the asparagine residue (18). Substrates for Endo H include $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, the initial oligosaccharide, as well as the processed intermediates $\text{Man}_9\text{GlcNAc}_2$, $\text{Man}_8\text{GlcNAc}_2$, and $\text{Man}_5\text{GlcNAc}_2$. Later intermediates are resistant to Endo H (18, 19). The conversion of oligosaccharides to Endo H-resistant form occurs in the Golgi complex (20). Thus the rate at which newly made glycoproteins acquire resistance to Endo H is a measure of the movement through the Golgi complex. Additional information about this transfer process can be acquired from cell fractionation studies in combination with pulse-chase labeling.

In HeLa cells GT is synthesized as two species ($M_r = 45,000$ and 47,000). After labeling cells for 8 min in the presence of [^{35}S]methionine, this double band was present in the heavier fractions of an equilibrium density sucrose gradient (Fig. 1*a*). This density (1.18 g/ml) is consistent with that of rough ER. After an additional chase period of 8 min the double band was no longer present exclusively in the heavier fractions; part of the immunoprecipitable material co-sedimented with the lighter 1.13 g/ml fractions (Fig. 1*b*), which contain the GT enzymatic activity (Fig. 1*d*). So the precursor GT polypeptides migrated to the light fraction in an Endo H-sensitive

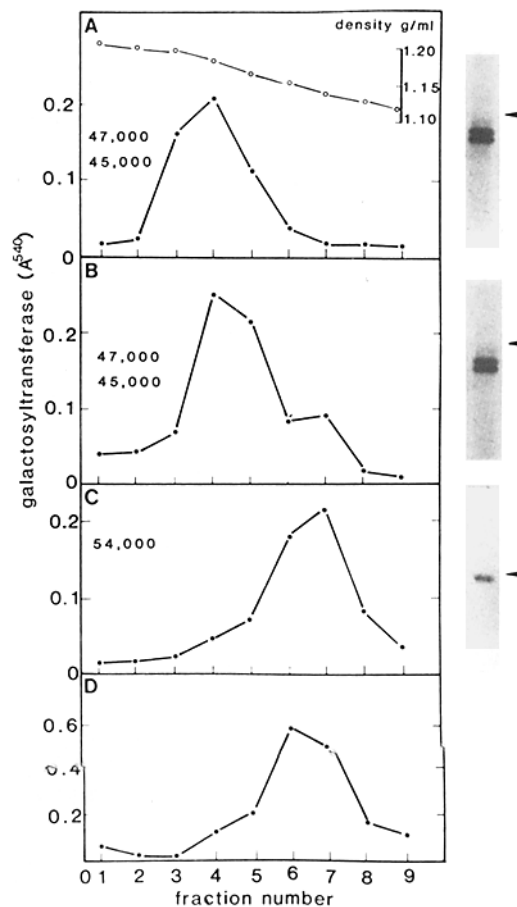


FIGURE 1 Distribution in a sucrose gradient of GT after pulse (-chase) labeling. 10-cm plates were labeled with [^{35}S]methionine (100 $\mu\text{C}/\text{ml}$ for 8 min) and chased for 0 (A), 8 (B), and 30 min (C). After centrifugation, equal fractions were collected from the bottom of the tubes and GT was immunoprecipitated and analyzed on a 10% SDS polyacrylamide gel. The gel was exposed and the GT bands were scanned so that the full scale deflection was 1.16 A units. In the margin of the figure the pattern of GT species is shown in A, fraction 4; in B, fraction 7; in C, fraction 7. In D the GT enzyme activity (nanomoles galactose transferred per hour) is depicted as measured with ovomucoid as exogenous acceptor in a parallel gradient. The arrowheads indicate the position of the 55,000 IgG heavy chain.

form. Following 30 min of chase, all of the pulse label GT had moved to the 1.13 g/ml density fractions (Fig. 1*c*). Moreover, the molecular weight of GT had increased to 54,000. This is consistent with our earlier finding that, 20 min after biosynthesis, there is a conversion of molecular weight from 45,000 and 47,000 to $\sim 54,000$. Furthermore, the 54,000-mol-wt species is resistant to hydrolysis with Endo H (7). Therefore, we conclude that the transport of GT from the rough ER to a compartment of lighter density takes place prior to the acquisition of Endo H resistance and also prior to an apparent increase in molecular weight of 7,000–9,000. The processing of *N*-linked oligosaccharide occurred in the light fraction, presumably derived from the Golgi complex. The close relationship between the precursor and “mature” GT species is illustrated in Fig. 2. The products of simultaneous partial degradation with protease V-8 were identical, except for one extra band derived from the 47,000-mol wt-precursor. *N*-linked oligosaccharides do not lead to the extra band; as in the presence of tunicamycin GT precursors show

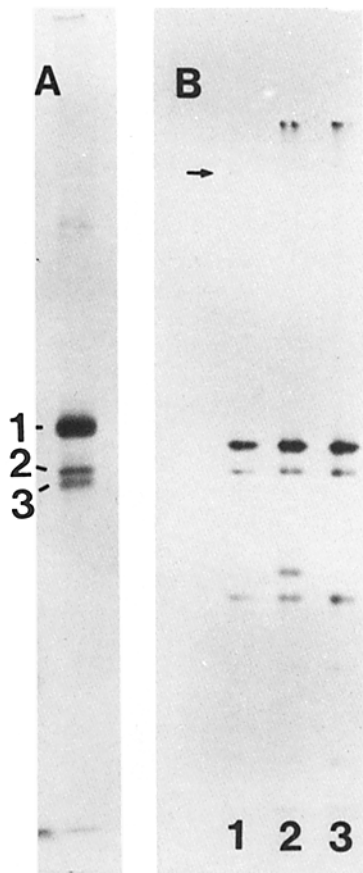


FIGURE 2 Partial digestion of GT species. After 30-min labeling with [³⁵S]-methionine, GT was immunoprecipitated and the different species were separated on a 10% SDS gel (A). After autoradiography the bands, designated 1, 2, and 3, were cut out, digested with V-8 protease, and analyzed on a 15% SDS gel according to the procedure of Cleveland et al. ([17]; B). The arrow indicates the position of the parent bands.

a similar 2,000-mol wt-difference (7). Although a difference in the presence of *O*-linked sugars cannot be excluded, we could not detect any radioactivity in the precursor bands after continuous labeling of the cells in the presence of [³H]galactose. Therefore, the most probable explanation for the extra band in lane 2 is a difference in amino acid sequence between the two precursor polypeptides, which disappears later during the maturation of the protein.

O-Glycosylation of GT

As pointed out in the previous section, GT loses its susceptibility to Endo H digestion in an intracellular compartment with a relative low density, which is distinct from the rough ER. Concomitantly, the GT precursor molecules are converted to a 54,000-mol wt-species, an apparent molecular weight increase of 7,000–9,000. Glycosylation most likely contributes to this increase in molecular weight. *N*-glycosylation, as previously shown by tunicamycin treatment, results in an apparent molecular weight increase of ~2,000 (7). Thus, the remaining difference raises the possibility that GT is provided with *O*-linked oligosaccharides. A direct approach to this question relies on metabolically labeling with [³H]-galactose and subsequent β -elimination as galactose occurs in virtually all *O*-linked oligosaccharides thus far examined (1). Therefore, HeLa cells were labeled for 16 h, after which GT was isolated by immunoprecipitation as described in Materials and Methods, and the 54,000-mol wt-band was cut out from a 10% SDS-gel. Treatment with mild alkali was used to cleave oligosaccharides *O*-glycosidically linked to serine or threonine residues. The presence of 1 M BH₄⁻ during this cleavage (β -elimination) converted the linking GalNAc to an acetylgal-

tosaminitol residue. After removal of amino acids and peptides with Dowex-50 (H⁺) resin the galactose-containing oligosaccharides were analyzed on a Biogel P-2 column (Fig. 3). Two major peaks eluted at fractions 38 and 44. Comparison with two β -eliminated oligosaccharides derived from porcine submaxillary mucin after mild acid treatment (to remove sialic acids) indicates that GT contains galacto-oligosaccharides in the order of four to eight sugar residues per oligosaccharide. Precursor GT bands cut out from the gel after continuous labeling in the presence of [³H]galactose did not contain ³H-radioactivity. As a control, GT was labeled with [³H]mannose in the same way. Analysis of mannose-containing β -eliminated oligosaccharides failed to demonstrate any labeling on the P-2 column except for a very small amount, present in the void volume of the column. This indicates that the mild hydrolysis indeed was selective for *O*-linked oligosaccharides.

Topology of GT

Until now, conclusions relating to the orientation of GT could be drawn only from the enzymatic activity of the molecule (9, 10). To study the membrane topology more precisely we have used two independent approaches: (a) immunocytochemistry with a high resolution probe, and (b) biochemical examination of the metabolically labeled molecular species in the membranes of rough ER and Golgi complex following immunoprecipitation.

Electron microscope immunocytochemistry of ultrathin cryosections treated with monospecific anti-GT antibodies and protein A/gold complexes (8 nm) showed the presence of GT molecules in only one or two cisternae located at the trans (maturing) side of the Golgi stack (Fig. 4). Small vesicles surrounding the Golgi stack were essentially devoid of label. 75% of all 8-nm gold particles were present over the lumen of the cisternae, 20% lie over the membranes, and only 5% were at the cytoplasmic face of the cisternal membrane. Taking into account that a protein A/gold particle (r is ~5 nm) linked to an IgG (r ~5 nm) can never span more than a distance of 15 nm, all gold particles must be located within

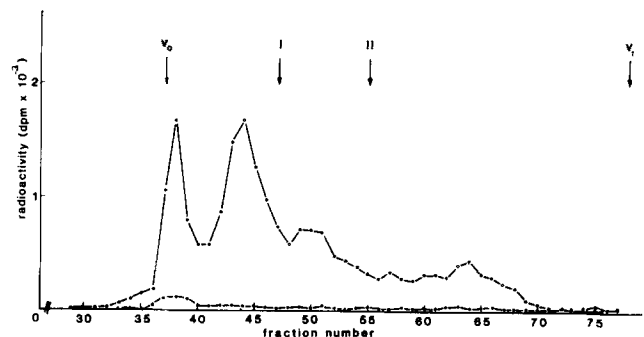


FIGURE 3 Elution profile of [³H]galactose-labeled β -eliminated oligosaccharides from GT. A 10-cm plate was labeled overnight, GT was isolated by immunoprecipitation and SDS gel electrophoresis, and β -eliminated as described in Materials and Methods. The labeled oligosaccharides were chromatographed on a Biogel P-2 column (90 × 1.5 cm) and the radioactivity was counted. The column was calibrated with two oligosaccharides derived from porcine submaxillary mucin: I, GalNAc-Gal-GalNAcol and II, Gal-GalNAcol. The tetrasaccharide stachyose eluted in fraction 55. O, [³H]galactose; ●, GT labeled according to the same procedure with [³H]mannose. V_o, void volume; V_t, total volume.

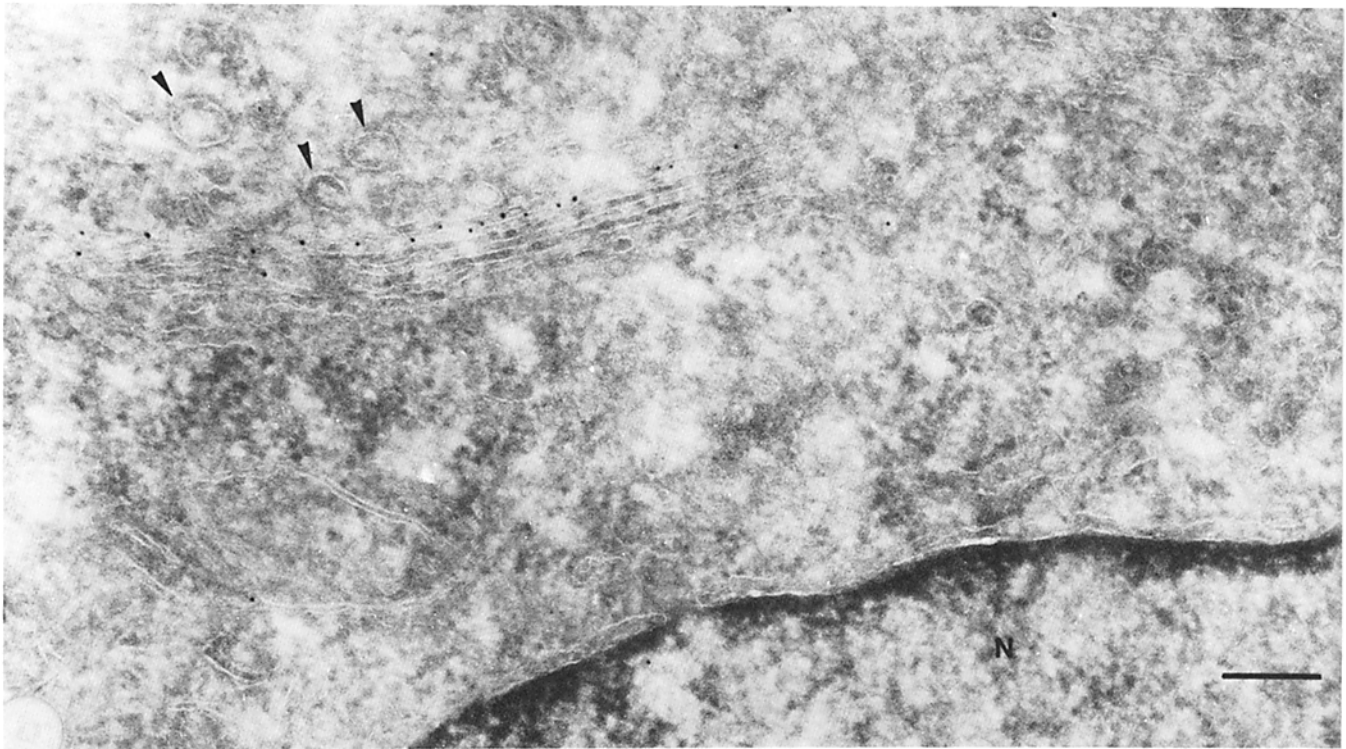


FIGURE 4 Immunoelectron micrograph of ultrathin cryosections of Golgi complex, indirectly labeled with 8-nm colloidal gold particles, coated with protein A for the demonstration of GT. Immunolabel can be seen over one or two cisternae. These are trans-cisternae, since many vesicles can be seen to be formed at this side of the stack (arrowheads). N, nucleus. Bar, 0.2 μm . \times 65,000.

15 nm from the antigen. As the thickness of the Golgi membranes was ~ 10 nm in our sections, this consideration has led us to the conclusion that the antigenic sites of GT are present at the inner side of the Golgi cisternal membrane. As all GT molecules detectable intracellularly are 54,000 mol wt (7) and as the 54,000 species are membrane-associated as determined by ultracentrifugation after sonication (data not shown), it is likely that all the protein A/gold label is bound to membrane-associated GT molecules. Experiments with monensin-treated cells, which showed highly dilated cisternae, gave an identical membrane localization of GT indicating that all GT molecules present in the Golgi complex are membrane-bound (pictures not shown).

Further evidence for the intraluminal localization of GT was obtained from studies of the effect of trypsin and pronase on the total microsomal fraction after [^{35}S]methionine labeling for 60 min (Fig. 5). In such an experiment one can expect labeled GT species to be present within the membranes of both the rough ER ($M_r = 45,000$ and $47,000$) and the Golgi complex ($M_r = 45,000$, $47,000$, and $54,000$). Treatment with trypsin (lane 2), pronase (lane 5), or proteinase K (not shown) did not alter the molecular weight of any of the GT species. As expected, similar incubations performed in the presence of detergent demonstrated marked sensitivity of the GT species to the proteases under these conditions (Fig. 5, lane 3, 6). These results, therefore, demonstrate that GT is protected by the membranes of the rough ER and the Golgi complex. Apparently, neither the type of membrane nor the stage of posttranslational processing influences the topology of GT as a membrane protein.

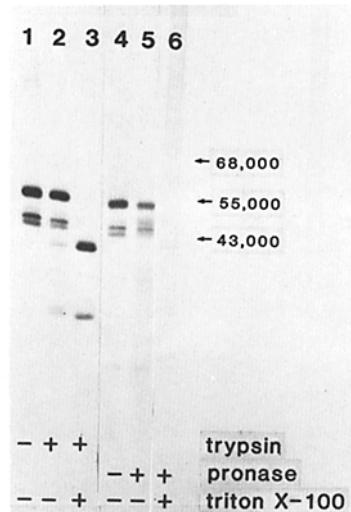


FIGURE 5 Latency of GT species in vesicles derived from Golgi complex and rough ER. Microsomes were isolated from HeLa cells, and labeled with [^{35}S]methionine for 60 min. The 100,000-g pellet was resuspended in PBS and digested with trypsin (15 $\mu\text{g}/\text{ml}$) or pronase (1.2 $\mu\text{g}/\text{ml}$) for 60 min on ice. In case of pronase digestion, 0.3 mM CaCl_2 was added. The reactions were terminated by adding 1 mM phenylmethylsulfonyl fluoride, soybean trypsin inhibitor (50 $\mu\text{g}/\text{ml}$), and 5 mM EGTA (for pronase inhibition). Controls without proteolytic enzymes and in the presence of 1% Triton X-100 were as indicated in the figure.

DISCUSSION

The transport of GT from the rough ER to the Golgi complex apparently takes place in the precursor (Endo H sensitive) form. This finding is consistent with the localization in the Golgi membranes of $\alpha(1-2)$ mannosidase 1A and 1B as described by Tabas and Kornfeld (21) and Tulsiani et al. (22). The molecule retains the "high mannose" configuration until

its arrival in the Golgi complex. Thus, GT shares this feature with other plasma membrane proteins such as erythrocyte band 3 (23), transferrin receptor (24), and with lysosomal enzymes (25). The transport for all these classes of proteins appears to be similar although the transport rates may differ substantially (26).

Studies performed with biosynthetic labeling *in vivo* have clearly demonstrated that at least the macromolecular weight acceptors for GT activity are predominantly localized in the Golgi complex (27, 28). The present subcellular fractionation studies performed in combination with metabolic labeling of the enzyme provide a more defined picture of the intracellular localization. Since GT moves from the rough ER to the Golgi complex in about 20 min and since its half-life in the HeLa cell is 20 h (7), it can be estimated that in a steady-state situation ~1–2% of GT is in an Endo H-sensitive form (present in the membranes of rough ER and cis-Golgi). This fact also explains the inability of GT in the rough ER and cis-Golgi to be detected immunocytochemically (2, this paper).

In the low density microsomal fraction (presumably derived from the Golgi complex) GT becomes Endo H resistant and, at the same time, and with identical kinetics, gains a molecular weight of 7,000–9,000. Calculation from preliminary experiments with [³H]galactose and [³H]mannose in the presence and absence of tunicamycin indicates that, when mannose incorporation is completely inhibited, galactose incorporation into GT is still 70% of that found in the absence of tunicamycin (Strous and Berger, unpublished results). This is consistent with a substantial number (5–10) of galactose-containing *O*-linked oligosaccharides. Furthermore, these results also imply that galactose incorporation into *O*-linked oligosaccharides takes place at the same time as galactose addition to *N*-linked oligosaccharides.

Both the immunocytochemical localization and the sequestration from added proteases strongly suggest that most if not all of the GT polypeptide including the antigenic sites and catalytic portions lie within the membranes of the Golgi cisternae. This is an important conclusion as it makes it unlikely that cytoplasmic matrix or cytoskeletal elements play a major role in the arrest of GT in the trans-cisternae of the Golgi complex. The residency of GT in the Golgi complex is most probably related to the interaction of the anchoring peptide of GT with the specific membrane environment in this specialized portion of the Golgi complex. This hypothesis is in agreement with our earlier finding that the "secreted" GT species is present as a soluble molecule with a lower molecular weight than the intracellular (membrane-associated) species (7). The nature of this membrane-protein interaction will be the objective of further study.

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