Two Enzymes Involved in the Synthesis of O-linked Oligosaccharides Are Localized on Membranes of Different Densities in Mouse Lymphoma BW5147 Cells

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ABSTRACT Microsomal membranes from mouse lymphoma BW5147 cells were fractionated on a continuous sucrose gradient and assayed for two enzymes involved in the synthesis of O-linked oligosaccharides. Both enzymes were recovered in membranes that were less dense than the membranes containing the endoplasmic reticulum marker enzymes, glucosidase I and II. UDP-Gal:*N*-acetylgalactosamine-β1,3-galactosyltransferase had a distribution that coincided with that of the galactosyltransferase that acts on asparagine-linked oligosaccharides. This latter enzyme has been immunolocalized to the *trans* Golgi elements. The UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase was recovered in a membrane fraction of intermediate density, between the endoplasmic reticulum and trans Golgi markers. These findings are consistent with the assembly of O-linked oligosaccharides occurring in at least two different Golgi compartments.

Many membranes and secretory proteins contain oligosaccharide units linked O-glycosidically to serine and/or threonine residues (1–8). In most instances, the linkage sugar is *N*-acetylgalactosamine, which is then substituted by galactose and/or *N*-acetylglucosamine. A number of different structures can then be formed from this basic core by the stepwise addition of monosaccharides that are donated directly from nucleotide sugars (9). This is in contrast to the synthesis of N-linked oligosaccharides that are formed by the en bloc transfer of an oligosaccharide from a lipid carrier to the nascent protein (10, 11). This oligosaccharide is then processed as the protein passes through the endoplasmic reticulum and the Golgi apparatus (12).

The subcellular location of the initial reactions in the synthesis of O-linked oligosaccharides is not yet firmly established. Strous (13) concluded that the attachment of the *N*-acetylgalactosamine residues to Ser/Thr takes place while the nascent peptide is still associated with ribosomes, indicating that this reaction is a co-translational event. In contrast to this conclusion, there is an increasing body of evidence that the initial step in O-linked glycosylation occurs posttranslationally in the smooth endoplasmic reticulum or the Golgi apparatus (3, 5, 14–20). Studies of the biosynthesis of the low density lipoprotein receptor in A431 cells have revealed that GalNAc is added to the protein before processing of the receptor's asparagine-linked high mannose oligosaccharide (8). Subsequently the high mannose oligosaccharide is converted to a complex-type unit and the assembly of the O-linked units is completed by the addition of galactose and sialic acid residues. This indicates that the synthesis of O-linked oligosaccharides occurs in more then one subcellular compartment.

One approach for analyzing the subcellular localization of oligosaccharide processing enzymes is to fractionate total microsomal membranes on linear sucrose gradients and then to determine the distribution of the various enzyme activities in the gradients (21–27). Using this approach, the Golgi enzymes involved in the late stage processing of N-linked oligosaccharides have been separated into two regions on the gradient, a finding that is consistent with these enzymes being localized in distinct compartments of the Golgi apparatus (26).

In this study we fractionated total microsomal membranes on continuous sucrose gradients and assayed them for two of the enzymes involved in the synthesis of O-linked oligosaccharides. The distribution of these enzyme activities was compared to that of several of the enzymes involved in N-linked oligosaccharide processing. The results indicate that the *N*-acetylgalactosaminyltransferase and the galactosyltransferases occupy separate compartments within the Golgi apparatus.

MATERIALS AND METHODS

Materials: UDP-[1-^{3}H]*N*-acetylgalactosamine (10.7 Ci/mmol) and UDP-[1-^{3}H]-galactose (12.0 Ci/mmol) were from New England Nuclear (Boston, MA). ATP, UDP-galactose, bovine submaxillary mucin, *Escherichia coli* β-galactosidase and lactalbumin were from Sigma Chemical Co. (St. Louis). Sucrose (ultrapure crystalline sucrose density gradient grade) was from Schwarz/

Mann Inc. (Spring Valley, NY). Jack bean β-galactosidase, *Aspergillus niger* α-*N*-acetylgalactosaminidase and *A. niger* β-galactosidase were prepared as previously described (28–30). α-Minimal essential medium was from Flow Laboratories (Rockville, MD). Fetal calf serum (heat inactivated) was from K. C. Biological, Inc. (Kansas City, MO). All other reagents were from standard sources.

Preparation of Acceptors: The asialomucin (desialized mucin) was prepared by heating intact mucin in 10 mM HCl (5 mg protein/ml) at 80°C for 2 h. Apomucin (deglycosylated mucin) was prepared from bovine submaxillary mucin by the method of Hagopian and Eylar (31) with minor modifications. The carbohydrate content of the various acceptors was determined by the method of Reinhold (32). Sialic acid content was measured by the thiobarbituric acid procedure (33).

Enzyme Assays: UDP-Galactose:*N*-acetylglucosamine galactosyltransferase and glucosidases I and II were assayed essentially as described (24).

The enzyme UDP-*N*-Acetylgalactosamine:apomucin *N*-acetylgalactosaminyltransferase was assayed by the method of Sugiura et al. (34) with the following modifications: UDP[1-^{3}H]GalNAc (170,000 cpm/assay) was used instead of UDP[1-^{14}C]GalNAc and ATP (2 mM) was included to inhibit breakdown of the UDP-GalNAc. The incubation time was extended to 20 min, and the final trichloroacetic acid–precipitated reaction product was solubilized by sonication and boiling for 2 h in 1 ml of 5% SDS. The solubilized product was subsequently supplemented with 10 ml scintillation fluid and counted for radioactivity.

The assay of UDP-Galactose:asialomucin galactosyltransferase used an adaptation of the method of Cheng and Bona (35). The incubation mixture contained the following components in a final volume of 50 μl: 50 mM *N*-morpholino ethanesulphonic acid, pH 7.5, 30 mM $MnCl_2$, 2 mM ATP, 0.1 mM UDP-[1-^{3}H]galactose (130,000 cpm), 1 mg Triton X-100, 50 μg bovine serum albumin, 300 μg asialomucin (~0.25 μmol available GalNAc sites), and varying amounts of enzyme protein (see individual experiments). Control assays without acceptor were done to correct for endogenous acceptor activity. The reaction mixture was incubated for 20 min at 37°C and the reaction product was precipitated, solubilized, and counted for radioactivity by the same procedure as used in the GalNAc transferase assay.

Enzyme Digestions: All enzyme digestions were performed in a 50 mM citrate-phosphate buffer, pH 4.6, except *E. coli* β-galactosidase where 50 mM phosphate buffer, pH 7.3, containing 4 mM $MgCl_2$ was used. The samples were dialyzed against the appropriate buffer for 6 h and 10 mU of enzyme was added (8 U in the case of *E. coli* β-galactosidase). The final volume was adjusted to 200 μl and the samples were incubated at 37°C for 48 h. The digested material was precipitated with trichloroacetic acid as described above and the radioactivity in the precipitate determined.

Fractionation of Mouse Lymphoma BW5147 Membranes: The procedures for growing and harvesting the cells, isolating the microsomal fraction, and separating the membranes on a linear sucrose gradient have been described (24). The membrane pellet from each sucrose gradient fraction was suspended in 300 μl of 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5% Triton to give a final protein concentration of 1.5–2 mg/ml.

The recovery of activity of the enzymes assayed in the various fractions ranged from 150 to 180% of that applied.

Analysis of the Reaction Products: The O-linked oligosaccharides were released from the protein with alkaline sodium borohydride as described by Carlson (36). Sodium was removed by passing the incubation mixture over a column of Dowex 50-H$^+$. The borate was removed from the eluate of this column by repeated evaporation with 0.4 M glacial acetic acid in methanol. The released sugar alcohols were subjected to paper chromatography using the following solvent systems: (*a*) ethyl acetate/pyridine/acetic acid/water (5:5:1:3); (*b*) ethyl acetate/pyridine/water (8:2:1).

Protein Determination: Protein was measured as described by Lowry et al. (37).

RESULTS

Characterization of the Enzyme Assay Systems

Since several *N*-acetylgalactosaminyl- and galactosyltransferases are known to exist, it was necessary to establish that the assay systems were specific for the glycosyltransferases involved in O-linked oligosaccharide assembly. This was particularly important since membrane extracts were used as the enzyme source rather than purified enzymes. Three different acceptors were tested: (*a*) intact bovine submaxillary mucin, which contains sialic acid α2→6GalNAc as the predominant O-linked oligosaccharide, (*b*) asialomucin, which was prepared by mild acid treatment of the starting mucin, and (*c*) apomucin, which was obtained by performing a Smith periodate degradation on the mucin (31). The asialomucin had 13% of the original sialic acid content and the apomucin contained <5% of the original GalNAc.

As shown in Table I, the apomucin is the only efficient acceptor in the *N*-acetylgalactosaminyltransferase assay. After subtracting the incorporation into endogenous acceptors, it can be seen that the intact mucin and the asialomucin are only 6% and 8% as effective, respectively, as apomucin in this assay. These data indicate that the transfer of GalNAc residues to Ser/Thr is the predominant reaction that is occurring in this assay system. This was confirmed by analyzing the product formed when apomucin served as acceptor. As shown in Table II, >90% of the radioactivity transferred to apomucin could be released with either alkaline borohydride treatment or incubation with α-*N*-acetylgalactosaminidase, an enzyme known to cleave the α-linkage between GalNAc and the peptide (30). The sugar alcohol released by the base

TABLE I

Substrate Specificity of UDP-GalNAc:Polypeptide N-Acetylgalactosaminyltransferase and UDP-Gal:N-Acetylgalactosamine Galactosyltransferase

	GalNAc Transferase		Gal Transferase	
Acceptor	cpm Transferred to protein	Acceptor* efficiency	cpm Transferred to protein	Acceptor* efficiency
		%		%
None	1,310		140	
Intact mucin	2,480	6	1,410	16
Asialomucin	2,950	8	7,850	100
Asialomucin + lactalbumin	—	—	6,515	81
Apomucin	24,200	100	1,130	12

Preparation of acceptors and enzyme assays were performed as described in Materials and Methods. Total microsomal fraction, 10 μg protein, was used as enzyme. When investigating the possible presence of UDP-Gal:*N*-acetylglucosamine galactosyltransferase, lactalbumin at a final concentration of 2 mg/ml was included in the assay.

* Acceptor efficiency in the GalNAc transferase and Gal transferase assays is defined as the percent of radioactivity transferred relative to the transfer to apomucin and asialomucin respectively. Transfer to endogenous acceptors has been subtracted in both assays.

TABLE II

Characterization of the Reaction Products from N-Acetylgalactosaminyltransferase and Galactosyltransferase

	GalNAc Transferase		Gal Transferase	
Treatment	cpm Remaining in Reaction Product	%	cpm Remaining in Reaction Product	%
None	45,750	100	13,600	100
Alkaline-sodium borohydride	230	0.5	1,100	8
α-*N*-Acetylgalactosaminidase (*A. niger*)	3,800	8.3	—	—
β-Galactosidase (*A. niger*)	—	—	12,200	90
β-Galactosidase (Jack bean)	—	—	10,150	78
β-Galactosidase (*E. coli*)	—	—	810	6

Assays and treatment of the reaction products were carried out as described in Materials and Methods. 20 μg total microsomal protein was added as enzyme.

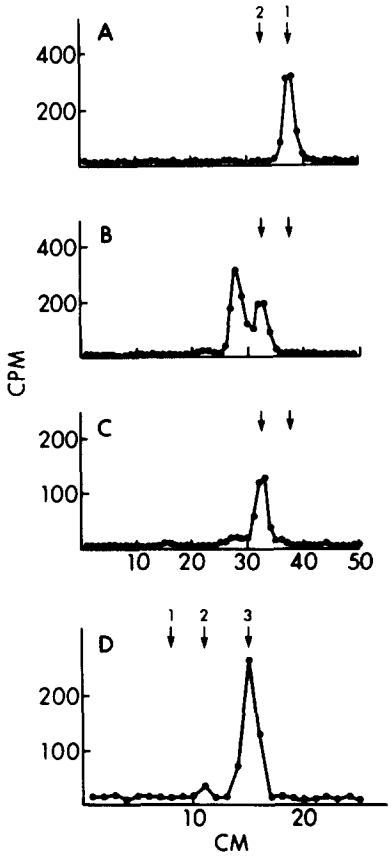

FIGURE 1 Paper chromatography of oligosaccharides released by alkaline sodium borohydride treatment of transfer reaction products. The reaction products from the UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase and the UDP-Gal:*N*-acetylgalactosamine galactosyltransferase assays were treated with alkaline sodium borohydride to release O-linked oligosaccharides. The procedure for preparing samples and running the paper chromatography has been described (8). *A*, *B*, and *C* were run in solvent *a*; *D* was run in solvent *b*. (*A*) Material released from the GalNAc transferase reaction product. (*B*) Oligosaccharides released from the Gal transferase reaction product. (*C*) Oligosaccharides released from the Gal transferase reaction product using Jack bean β-hexosaminidase-treated acceptor. (After the enzyme digestion, the β-hexosaminidase-treated acceptor was dialyzed against 3 changes of distilled water, lyophilized and resolubilized in 20 μl distilled water before addition to the assay.) (*D*) B after treatment with *E. coli* β-galactosidase (10 U β-galactosidase in 50 mM phosphate buffer, pH 7.3, 4 mM $MgCl_2$ at 37°C for 48 h; final volume 30 μl). The migration distances for standards are indicated with arrows: (*1*) *N*-acetylgalactosaminitol; (*2*) galactoseβ1-3*N*-acetylgalactosaminitol; (*3*) galactose.

treatment migrated with authentic *N*-acetylgalactosaminitol when subjected to paper chromatography (Fig. 1). These results would be expected if the assay is measuring the transfer of *N*-acetylgalactosamine to Ser/Thr residues of the apomucin.

The galactosyltransferase assay proved to be somewhat less specific. As shown in Table I, asialomucin was the best acceptor. However, the apomucin retained 12% of the acceptor activity that could not be explained by the presence of residual GalNAc (<5% of original value). This suggested that the asialomucin might contain some N-linked oligosaccharides that could serve as acceptors for other galactosyltransferases present in the membrane preparations. Consistent with this is the fact that lactalbumin inhibited the transfer of [^{3}H]galactose to asialomucin by 19%. This protein is a potent and selective inhibitor of the galactosyltransferase which acts on N-linked oligosaccharides (38).

When the product of the reaction with asialomucin was treated with alkaline borohydride, 92% of the radioactivity transferred to the acceptor was released (Table II). The remaining 8% was assumed to represent N-linked structures and was not characterized further. *A. niger* β-galactosidase and jack bean β-galactosidase released 10% and 22% of the radioactivity, respectively, whereas *E. coli* β-galactosidase released 94% of the label. This would be expected if the major product is galactose linked β1→3 to *N*-acetylgalactosamine since the *A. niger* and jack bean enzymes work very poorly on this linkage, whereas the *E. coli* enzyme is known to be active toward β1→3 linked galactose residues (28, 39, 40).

The alkaline borohydride-released oligosaccharides were also analyzed by paper chromatography. As shown in Fig. 1*B*, two peaks of radioactivity were obtained. The smaller one had the same mobility as the Galβ1→3GalNAcitol standard while the larger peak migrated as if it contained one additional sugar. The labeled galactose was released from both products by digestion with *E. coli* β-galactosidase (Fig. 1*D*). It has been reported that the bovine submaxillary mucin contains O-linked structures with the composition sialic acid α2→6 GalNAc β1→4GalNAc and sialic acid α2→6 GlcNAc β1→6GalNAc in addition to the sialic acid α2→6 GalNAc disaccharides (41). Upon desialization these structures would produce disaccharides that could serve as acceptors in the galactosyltransferase assay. To investigate this possibility, an aliquot of the asialomucin was treated with jack bean β-hexosaminidase to remove the terminal amino sugars and then tested in the standard galactosyltransferase assay system. When the oligosaccharide product of this reaction was released from the protein acceptor by alkaline-sodium borohydride treatment and analyzed by paper chromatography, a single peak of radioactivity was obtained that migrated in the position of the Galβ1→3GalNAcitol standard (Fig. 1*C*). Based on these data we conclude that 80–90% of the product formed in the reactions using asialomucin as acceptor represent the transfer of galactose to O-linked oligosaccharides.

The assays for both the *N*-acetylgalactosaminyltransferase and the galactosyltransferases were proportional to the amount of membrane protein added and the time of incubation (Fig. 2). There was essentially no transfer to endogenous acceptors in the galactosyltransferase assays, whereas there was a small amount of GalNAc transferred to endogenous acceptors. The transfer to endogenous acceptors was subtracted in all subsequent assays.

Fractionation of Membranes Containing the Enzymes Involved in O-Linked Oligosaccharide Biosynthesis

The total microsomal fraction from mouse lymphoma BW5147 cells was subfractionated by flotation through a linear sucrose gradient ranging from 15–60%. The various fractions were then assayed for the two enzymes involved in O-linked oligosaccharide biosynthesis as well as for several enzymes involved in the processing of N-linked oligosaccharides. As shown in Fig. 3*A*, the *N*-acetylgalactosaminyltransferase was detected in membranes with a higher density than the membranes that contain the galactosyltransferase. While there was considerable overlap of these two activities,

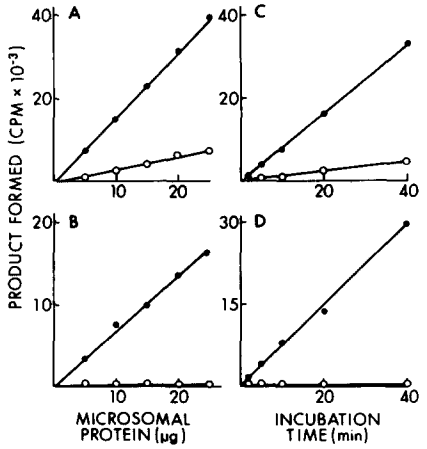

FIGURE 2 Effect of enzyme concentration and incubation time on the activities of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (*A* and *C*) and UDP-Gal:*N*-acetylgalactosamine Galactosyltransferase (*B* and *D*). The assays were performed as described in Materials and Methods. Total microsomal fraction was used as the source of enzyme. The amount of enzyme added in the incubation time experiments was 10 µg. (●) Radioactivity transferred to added exogenous acceptor. (○) Radioactivity transferred to endogenous acceptor.

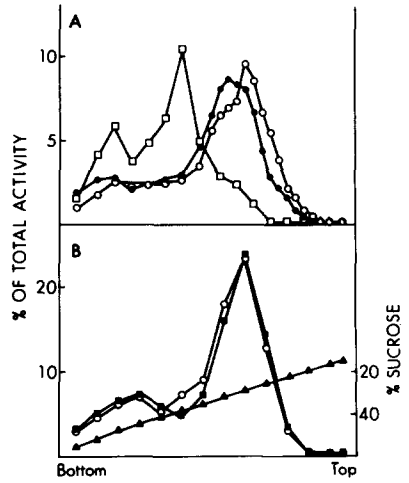

FIGURE 3 Distribution of enzyme activities in subcellular membrane fractions. The total microsomal fraction from BW5147 cells was isolated and subfractionated in a linear sucrose gradient as described in Materials and Methods. In experiment A, 24 fractions were collected: the lower, denser, half of the gradient was divided in 8 2.4-ml fractions while 16 1.2 ml fractions were collected from the upper half. The distribution profiles for the enzyme activities has been corrected for the differences in fraction volume. In experiment B, the gradient was divided into 14 equal fractions (~2.7 ml each). One of three separate experiments is shown. (●) UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferase; (○) UDP-Gal:N-acetyl-galactosamine galactosyltransferase; (□) glucosidases I and II; (■) UDP-Gal:*N*-acetylglucosamine galactosyltransferase; (▲) % sucrose.

this partial separation was observed in three separate experiments.

The activity of the glucosidases I and II distributed in a denser region of the gradient, consistent with the localization of these enzymes to the endoplasmic reticulum (42, 43). Fig. 3*B* shows that the galactosyltransferase involved in N-linked oligosaccharide biosynthesis has the identical distribution as the galactosyltransferase involved in O-linked oligosaccharide assembly. The former enzyme has been localized to the trans Golgi complex by immunocytochemical techniques (44).

DISCUSSION

The major finding in this study is that two glycosyltransferases involved in the sequential addition of sugars to O-linked oligosaccharides are present in membranes of different densities. We believe that these membranes probably arise from the Golgi complex since they separated from the membranes containing the enzyme markers for the endoplasmic reticulum (glucosidase I and II). There are two ways to interpret the separation of the GalNAc transferase and the Gal transferase activities. The first is that the two enzymes are located in the same Golgi cisternae but are associated with separate regions that have membranes of different densities. Upon disruption of the cell, vesicles from these regions may form and be separated on the sucrose gradients. This has, in fact, been demonstrated by Ehrenreich et al. (45) in fractionation experiments of rat liver Golgi complex. Furthermore, cytochemical studies have shown that both 5′ nucleotidase and adenylate cyclase activities are concentrated at the rims of isolated rat liver Golgi elements and are undetectable on the flattened centers of the cisternae (46, 47). The alternate explanation is that the enzymes are located in different cisternae of the Golgi stack. There is considerable evidence indicating that the cisternae of the Golgi stack differ in composition and enzyme content (reviewed in references 48–50). Of note is the electron microscopic study using filipin as a cholesterol probe (51) and the density shift experiments using digitonin (52), which have indicated that there is a gradient of cholesterol across the Golgi stack, being lowest at the *cis* face and highest at the *trans* face. The cholesterol gradient could account for the membrane separation observed on the sucrose gradients. In addition, both galactosyltransferase and thiamine pyrophosphatase have been localized to the *trans* face of the Golgi (44, 53).

While our data do not distinguish between these two possibilities, we favor the second explanation since it is most consistent with the published data on the assembly of O-linked oligosaccharides. Thus Roth (20) has performed an electron microscopy study that has localized GalNAc-containing glycoproteins to the cis Golgi cisternae of intestinal goblet cells. In these experiments that used *Helix pomatia* lectin bound to colloidal gold to detect the GalNAc-containing glycoproteins, no staining was observed in the endoplasmic reticulum, whereas dense staining occurred in the *cis* Golgi. This indicates that O-linked glycosylation is a posttranslational event that begins in the *cis* Golgi complex. A number of other studies examining the kinetics of N- and O-linked glycosylation in intact cells have also indicated that O-linked glycosylation occurs posttranslationally (14–20). Furthermore, Cummings et al. (8) have shown that the precursor form of the low density lipoprotein receptor of A431 cells has high mannose-type N-linked units and O-linked units that contain only GalNAc. By contrast, the mature form of the receptor contains complex -type N-linked units plus O-linked oligosaccharides that are mono- and disialylated species with Gal→GalNAc-Ser/Thr cores. These findings demonstrate that the transfer of GalNAc residues must occur before the entry of the glycoprotein acceptor into the region of the Golgi complex that contains the processing enzyme α-mannosidase I. This indicates that the assembly of the O-linked oligosaccharides occurs in more than one region of the Golgi complex.

In view of these findings, we suggest that the membranes containing the two glycosyltransferases involved in O-linked oligosaccharide biosynthesis are derived from different regions of the Golgi stack. According to this interpretation, the membranes that contain the *N*-acetylgalactosaminyltransferase would be derived from *cis* Golgi elements, whereas the membranes with the galactosyltransferase would be derived from the *trans* Golgi elements. Similar separation of the processing enzymes and glycosyltransferases involved in the assembly of N-linked oligosaccharides have been reported (22, 24, 27). These data are consistent with both N- and O-linked glycosylation occurring in a series of compartments as the newly synthesized glycoproteins pass through the Golgi stack.

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