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 than 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-[1-3H]N-acetylgalactosamine (10.7 Ci/mmol) and UDP-[1-3H]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/
pared by mild acid treatment of the starting mucin, and (c) 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 \( \alpha \)-N-acetylgalactosaminidase, an enzyme known to cleave the \( \alpha \)-linkage between GalNAc and the peptide (30). The sugar alcohol released by the base

### TABLE I

| Acceptor | GalNAc Transferase | Gal Transferase |
|----------|--------------------|-----------------|
|          | 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 pg protein, was used as enzyme. When investigating the possible presence of UDP-Gal-N-acetylgalactosamine 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 radioactive 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**

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

Assays and treatment of the reaction products were carried out as described in Materials and Methods. 20 pg total microsomal protein was added as enzyme.
Lactalbumin inhibited the transfer of ferases present in membrane preparations. Consistent with this, the fact that lactalbumin inhibited the transfer of 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, 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. 1D). 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. 1C). 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 BWS147 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. 3A, 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,
UDP-Gal:polypeptide N-acetylgalactosaminyltransferase (A and C) and UDP-Gal:N-acetylgalactosaminyl 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 into 8 2.4-ml fractions while 16 1.2-ml fractions were collected from the upper half. The distribution profiles for the enzyme activities have 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:GalN-acetyl-galactosamine galactosyltransferase; (□) glucosidases I and II; (■) UDP-Gal:GalN-acetylglucosamine galactosyltransferase; (▲) % sucrose.

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. 3B 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 adenylyl 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 di-galactosylated 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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REFERENCES

1. Kornfeld, R., and S. Kornfeld. 1980. Structure of glycoproteins and their oligosaccharide units. In The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lennarz, editor. Plenum Press, New York. 6-12.

2. Gajewski, A., F. C. Westfall, J. S. Whitehead, and E. H. Eylar. 1971. Glycophorin of the A1 protein from myelin by a polypeptide N-acetylgalactosaminyltransferase. Identification of the receptor sequence. J. Biol. Chem. 246:2519-2523.

3. Niemann, H., D. Boschek, M. Evans, T. Rosing, and H.-D. Klenk. 1982. Post-translational glycosylation of coronavirus glycoprotein E1. EMBO (Eur. Mol. Biol. Organ.) J. 1:1495-1504.

4. Funakoshi, O., and I. Yamashina. 1982. Structure of O-glycosidically linked sugar units from plasma membranes of an ascites hepatoma, AH66. J. Biol. Chem. 257:3782-3787.

5. Johnson, D. C., and P. G. Spear. 1983. O-linked oligosaccharides are acquired by herpes Simplex virus glycoproteins in the Golgi apparatus. Cell. 32:987-997.

6. Sprio, R. G., and V. D. Bhoyroo. 1974. Structure of the O-glycosidically linked carbohydrate units of fucan. J. Biol. Chem. 249:5704-5717.

7. C. K. Schneeberger, H. van Halbeek, P. J. Kemerling, J. J. Smits, and J. F. G. Vliegenthart. 1981. Structural carbohydrate chains of human platelet glycoconjugates. Proceedings of the 7th International Symposium on Glycoconjugates. 186-187.

8. Cummings, R. D., S. Kornfeld, W. J. Schneider, K. K. Hobgood, H. Tolleshaug, M. S. Brown, and J. L. Goldstein. 1983. Biosynthesis of N- and O-linked oligosaccharides of the low density lipoprotein receptor. J. Biol. Chem. 258:15261-15273.

9. Rosenman, S. 1970. The synthesis of complex carbohydrates by multicytoplasmic transfer systems and their potential function in intercellular adhesion. Chem. Phys. Lipids. 5:270-280.

10. Lucass, J. J., C. J. Waechter, and W. J. Lennarz. 1973. The participation of lipid-linked oligosaccharide in synthesis of membrane glycoproteins. J. Biol. Chem. 250:1992-2002.

11. Struck, D. K., and W. J. Lennarz. 1980. The function of saccharide-lipids in the synthesis of glycoproteins. In The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lennarz, editor. Plenum Press, New York. 35-83.

12. Kornfeld, S., E. L. and I. Tabas. 1978. The synthesis of complex-type oligosaccharides. II. Characterization of the processing intermediates in the synthesis of the complex oligosaccharides of the gastrointestinal tract. J. Biol. Chem. 253:7771-7778.

13. Strous, G. J. A. M. 1979. Initial glycosylation of proteins with acetylgalactosaminyltransferase II. Structure and linkage. Proc. Natl. Acad. Sci. USA 76:2691-2698.

14. Kim, Y. S., J. Perdomo, and J. Nordberg. 1971. Glycoprotein biosynthesis in small enzymes and glycosyltransferases involved in lysosomal enzyme biosynthesis. J. Cell Biol. 52:993-1007.

15. Roth, J. 1984. Cytochemical localization of terminal N-acetylgalactosamine residues in cellular compartments of intestinal goblet cells: implications for the topology of O-glycosylation. J. Cell Biol. 98:390-399.

16. Bitz, R. H., and G. E. Palade. 1980. Distribution of terminal glycosyltransferases in hepatic Golgi fractions. J. Cell Biol. 84:87-101.

17. Hanover, J. A., W. J. Lennarz, and J. D. Young. 1980. Synthesis of N- and O-linked glycopeptides in oviduct membrane preparations. J. Biol. Chem. 255:6713-6716.

18. Shida, H., and S. Matsumoto. 1983. Analysis of the hemagglutinin glycoprotein from mutants of vaccinia virus that accumulates on the nuclear envelope. Cell. 33:423-434.

19. Hanover, S. A., S. Eilting, G. R. Mintz, and W. J. Lennarz. 1982. Temporal aspects of the N- and O-glycosylation of human chorionic gonadotropin. J. Biol. Chem. 257:10172-10177.

20. Roth, J. 1984. Cytochemical localization of terminal N-acetylgalactosamine residues in cellular compartments of intestinal goblet cells: implications for the topology of O-glycosylation. J. Cell Biol. 98:390-399.

21. Bitz, R. H., and G. E. Palade. 1980. Distribution of terminal glycosyltransferases in hepatic Golgi fractions. J. Cell Biol. 84:87-101.

22. Deutsch, S. L., K. E. Creek, M. Merion, and C. B. Hirschberg. 1983. Subfractionation of rat liver Golgi apparatus: distribution of enzymes involved in the biosynthesis of the phosphomannosyl recognition marker in lysosomal enzymes. Proc. Natl. Acad. Sci. USA 80:3938-3942.

23. Deutscher, S. L., K. E. Creek, M. Merion, and C. B. Hirschberg. 1983. Subfractionation of rat liver Golgi apparatus: distribution of enzymes involved in the biosynthesis of the phosphomannosyl recognition marker in lysosomal enzymes. Proc. Natl. Acad. Sci. USA 80:3938-3942.

24. Deutscher, S. L., K. E. Creek, M. Merion, and C. B. Hirschberg. 1983. Subfractionation of rat liver Golgi apparatus: distribution of enzymes involved in the biosynthesis of the phosphomannosyl recognition marker in lysosomal enzymes. Proc. Natl. Acad. Sci. USA 80:3938-3942.

25. Deutscher, S. L., K. E. Creek, M. Merion, and C. B. Hirschberg. 1983. Subfractionation of rat liver Golgi apparatus: distribution of enzymes involved in the biosynthesis of the phosphomannosyl recognition marker in lysosomal enzymes. Proc. Natl. Acad. Sci. USA 80:3938-3942.