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Evidence for Extensive Subcellular Organization of Asparagine-linked Oligosaccharide Processing and Lysosomal Enzyme Phosphorylation*

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Membranes prepared from mouse lymphoma BW5147.3 cells and P388D$_1$ macrophages were fractionated on a continuous sucrose gradient and assayed for enzymes involved in the processing of asparagine-linked oligosaccharides. The order in which these enzymes distributed from dense to light membranes correlated with the established sequence of events in glycoprotein biosynthesis. A number of enzymes which have been previously localized to the Golgi separated into four regions on the gradient. UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase, the enzyme which catalyzes the selective phosphorylation of the high mannose oligosaccharides of lysosomal enzymes, was present in the densest membranes. *N*-Acetylglucosamine 1-phosphodiester α-*N*-acetylglucosaminidase was in the next region. Several enzymes involved in the late stages of asparagine-linked oligosaccharide processing were localized to the third region. UDP-galactose:*N*-acetylglucosamine galactosyltransferase was present in the lightest membranes (region IV).

Pulse-chase experiments utilizing [2-^{3}H]mannose demonstrated that the distribution of *in vivo* labeled asparagine-linked oligosaccharide intermediates correlates with the distribution of these processing enzymes. Analysis of the phosphorylated oligosaccharides of lysosomal enzymes which were bound to the phosphomannosyl receptor indicated that these enzymes had already passed through the region of the Golgi which contains galactosyltransferase and sialyltransferase.

These findings are consistent with there being a high degree of organization within the Golgi complex. The physical separation of processing enzymes could serve as one mechanism for the control of asparagine-linked oligosaccharide biosynthesis.

The participation of the Golgi complex in glycoprotein biosynthesis was first indicated by the [^{3}H]hexose autoradiographic studies of Neutra and Leblond (1-3). Since then numerous morphological and biochemical studies have added to our understanding of the role of the Golgi in oligosaccharide processing (reviewed in Ref. 4). Galactosyltransferase was the first enzyme to be localized to a Golgi subcellular fraction (5), and since then sialyltransferase (6), fucosyltransferase (7), *N*-acetylglucosaminyltransferase I (6), α-mannosidase I (8), *N*-acetylglucosamine 1-phosphodiester α-*N*-acetylglucosaminidase[1] (9, 10), and *N*-acetylglucosamine 1-phosphotransferase (10) have all been shown to fractionate with Golgi membranes.

Evidence for functional specialization of different regions of the Golgi has come from 5 separate approaches: 1) morphological studies which showed differences between the *cis* and *trans* faces of the Golgi in appearance and in proximity to transitional endoplasmic reticulum and condensing vacuoles (4); 2) cytochemical studies of thin tissue slices and Golgi subfractions which revealed differential staining of the *cis* or *trans* face of Golgi stacks for thiamine pyrophosphatase (11), acid phosphatase (11), and NADP phosphatase (12); 3) immunological studies of Ito and Palade (13) showing that Golgi membranes containing NADPH-cytochrome P-450 reductase could be immunoprecipitated without co-precipitation of galactosyltransferase activity; 4) immunocytochemical localization of galactosyltransferase to a few cisternae on the *trans* side of the Golgi by Roth and Berger (14); 5) biochemical characterization of Golgi subfractions by Dunphy *et al.* (15) which demonstrated that galactosyltransferase and α-mannosidase I are located on different membranes.

Thus, a variety of studies have led to a picture of the directional movement of glycoproteins through the cell starting with translation and glycosylation in the rough endoplasmic reticulum, followed by movement through an ill-defined transitional region to the Golgi. Next, transit through the Golgi takes place, during which time oligosaccharide processing and other post-translational modifications occur. The processing of asparagine-linked oligosaccharides requires a great number of enzymes. Fig. 1 shows the steps involved in the conversion of high mannose-type units to complex-type units and to the phosphorylated species present on lysosomal enzymes. Finally, at or near the *trans* face of the Golgi, sorting occurs and proteins are packaged for secretion, transport to lysosomes, or targeting to various membranes.

Lysosomal enzymes are a special class of glycoproteins that possess phosphorylated high mannose units in addition to the usual neutral high mannose and complex-type units. Their oligosaccharides are phosphorylated by the two step reaction shown in Fig. 1 as reactions 3 and 4. First *N*-acetylglucosamine 1-phosphate is transferred to an acceptor mannose by UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (*N*-acetylglucosaminylphosphotransferase), resulting in a phosphate group in diester linkage between the outer "blocking" *N*-acetylglucosamine and the

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[1] Formerly called α-*N*-acetylglucosaminylphosphodiesterase. New name: *N*-acetylglucosamine 1-phosphodiester α-*N*-acetylglucosaminidase, or phosphodiester glycosidase for short. (A. Varki, W. Sherman, and S. Kornfeld (1983) *Arch. Biochem. Biophys.*, in press.)

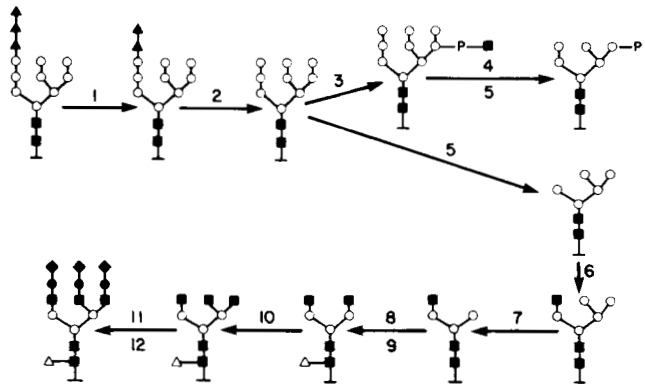

FIG. 1. **Steps in the processing of asparagine-linked oligosaccharides.** *1*, glucosidase I; *2*, glucosidase II; *3*, *N*-acetylglucosaminylphosphotransferase; *4*, phosphodiester glycosidase; *5*, mannosidase I; *6*, *N*-acetylglucosaminyltransferase I; *7*, mannosidase II; *8*, *N*-acetylglucosaminyltransferase II; *9*, core fucosyltransferase; *10*, *N*-acetylglucosaminyltransferase IV; *11*, galactosyltransferase; *12*, sialyltransferase. Symbols: ▲, glucose; ■, GlcNAc; ○, mannose; ●, galactose; △, fucose; ♦, sialic acid; *P*, phosphate.

inner mannose (16, 17). Then *N*-acetylglucosamine 1-phosphodiester α-*N*-acetylglucosaminidase (phosphodiester glycosidase) removes the *N*-acetylglucosamine, generating a Man-6-P[2] recognition marker (18–20). Enzymes containing this recognition marker bind to a specific intracellular receptor which mediates translocation to the lysosomes (21, 22). The primary lysosomes are thought to bud from the GERL (23) or *trans*-Golgi (24, 25) regions, perhaps as coated vesicles.

In this study we have attempted to gain further insight into the functional organization of the Golgi with respect to the biosynthesis of asparagine-linked oligosaccharides. Using a modification of the sucrose density gradient procedure of Rothman and co-workers (15, 26), we have fractionated cell membranes and assayed them for many of the oligosaccharide processing activities shown in Fig. 1. The data obtained have been correlated with kinetic studies of the synthesis of asparagine-linked oligosaccharides, especially those present on lysosomal enzymes. The results suggest that there is an extensive organization within the Golgi.

EXPERIMENTAL PROCEDURES

Materials

[2-^{3}H]Mannose (15.8 Ci/mmol), UDP-[1-^{3}H]galactose (11.6 Ci/mmol), UDP-[6-^{3}H]*N*-acetylglucosamine (24 Ci/mmol), and GDP-[U-^{14}C]fucose (0.22 Ci/mmol) were from New England Nuclear. [β-^{32}P]UDP-*N*-acetylglucosamine (20 mCi/mmol) was generously provided by Dr. Marc Reitman, Washington University. Its synthesis and use have been described (16). Quaternary aminoethyl (QAE)-Sephadex (Q-25-120), Sephadex G-25-80, α-methylmannoside, α-methylglucoside, α-methylgalactoside, glucose 6-phosphate, mannose 6-phosphate, *N*-acetylglucosamine, and *N*-acetylmannosamine were from Sigma. Pronase was from Calbiochem. Sucrose (ultra pure crystalline sucrose density gradient grade) was from Schwarz/Mann, Inc. ConA-Sepharose was from Pharmacia. Lentil lectin-Sepharose, prepared as described (27), contained 5.5 mg of lectin/ml of Sepharose 4B. Endo H was from Miles. The 3a70 scintillation mixture was from Research Products International Corp., Mt. Prospect, IL. α-MEM was from Flow Laboratories, Rockville, MD. GBFM was prepared as previously described (22). Fetal calf serum (heat-inactivated) was from K. C. Biological, Inc., Kansas City, MO. All other reagents were from standard sources.

[2] The abbreviations used are: Man-6-P, mannose 6-phosphate; ConA, concanavalin A; GlcNAc, *N*-acetylglucosamine; endo H, endo-β-*N*-acetylglucosaminidase H; α-MEM, α-minimal essential medium; GBFM, glucose/bicarbonate-free medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TBS, Tris-buffered saline; BSA, bovine serum albumin; ER, endoplasmic reticulum.

Fractionation of P388D$_1$ Macrophage Membranes

The fractionation scheme was modified from the procedure of Fries and Rothman (26). P388D$_1$ macrophages (kindly provided by Dr. John Atkinson, Washington University) were grown in suspension culture to a density of 1×10^6 cells/ml in α-MEM plus 10% fetal calf serum. 1.5×10^8 cells were harvested by centrifugation, washed in ice-cold phosphate-buffered saline, and suspended in 10 ml of 15 mM KCl, 1.5 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 10 mM HEPES, adjusted to pH 7.5 with KOH. After centrifugation, 5 ml of supernatant fluid was removed and discarded; the cells were resuspended in the remaining 5 ml. The macrophages were disrupted by nitrogen cavitation in a Parr Nitrogen bomb (Parr Instrument Co., Moline, IL) using a pressure of 150 p.s.i. applied for 15 min. One-tenth volume of 400 mM HEPES, 700 mM KCl, 40 mM $Mg(OAc)_2$, 1 mM dithiothreitol, pH 7.5 was added. Nuclei were removed by centrifugation at $600 \times g$ for 5 min, and the supernatant fluid was diluted to 15 ml with 10 mM Tris HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$. After centrifugation in a SW 50.1 rotor at 40,000 rpm for 60 min, the membrane pellet was resuspended in 5 ml of 1 mM Tris/1 mM EDTA, pH 8.0 (Buffer A), with 8 strokes in a Dounce homogenizer. The suspension was centrifuged again for 60 min at 40,000 rpm and the pellet was resuspended in 1.5 ml 45% (w/w) sucrose in Buffer A with the Dounce. 3 ml of 60% sucrose in Buffer A was added and mixed well. The mixture was placed over a 1-ml cushion of saturated sucrose and was overlaid with a 33-ml sucrose gradient from 45% to 15% in Buffer A. The gradient was centrifuged in a SW 27 rotor at 25,000 rpm for 16 h. Twenty-four 1.6-ml fractions were collected from the bottom of the tube. Each was diluted to 5 ml with Buffer A and membranes were sedimented by centrifugation at 50,000 rpm in a Ti-50 rotor for 60 min. Pellets were resuspended in 0.3 ml of 10 mM Tris, pH 7.0, 5 mM $MgCl_2$, 0.5% Triton X-100, and assayed for enzyme activities. All centrifugations were performed at 1 °C.

Fractionation of BW5147.3 Lymphoma Cell Membranes

The fractionation was similar to that described above for macrophages with the following modifications: Murine BW5147.3 lymphoma cells were grown in roller bottles to a density of 2×10^6 cells/ml in α-MEM supplemented with 10% fetal calf serum and 10 mM HEPES, pH 7.0. 1×10^9 cells were harvested and worked up as for the macrophages. Resuspension was in 50 ml of KCl/$Mg(OAc)_2$/dithiothreitol/HEPES buffer instead of 10 ml. All but 10 ml of supernatant buffer was removed after centrifugation, and the 10 ml of cells were disrupted at 750 p.s.i. in the nitrogen bomb. The postnuclear supernatant fluid was diluted to 20 ml instead of 15 ml. Twelve 3.2-ml fractions were collected from the sucrose gradient, were diluted with 5 ml of Buffer A, and the membranes were sedimented. The membranes were finally resuspended in 0.3 ml of 5 mg/ml BSA, 4 mM Tris, 1% Triton X-100, pH 7.0, and assayed for enzyme activities.

Enzyme Assays

All assays were performed with two concentrations of protein. Product formation was proportional to protein concentration in each assay on each gradient fraction except for GlcNAc transferase IV, where lower concentration points gave values too low to assess linearity.

Glucosidases I and II—This assay, measuring cleavage of glucose from $Glc_3Man_9GlcNAc_1$ or $Glc_1Man_9GlcNAc_1$ substrates, respectively, has been described (28). Values of controls lacking enzyme have been subtracted. The recovery of activity on the gradient was 31% for glucosidase I and 16% for glucosidase II relative to the starting membranes.

UDP-N-acetylglucosamine:Lysosomal Enzyme N-Acetylglucosamine-1-phosphotransferase (N-Acetylglucosaminylphosphotransferase)—This assay, measuring transfer of GlcNAc 1-phosphate to α-methylmannoside, has been described (29). Incubations were carried out at 37 °C for 30 min. Controls with α-methylgalactoside as acceptor showed little activity in all regions of the gradient. Values of controls lacking enzyme have been subtracted. The recovery of activity ranged from 75 to 85% relative to the starting membranes.

N-Acetylglucosamine 1-Phosphodiester α-N-Acetylglucosaminidase (Phosphodiester Glycosidase)—This assay, measuring cleavage of *N*-acetylglucosamine from oligosaccharides with one phosphodiester moiety, is described in Ref. 18. The final assay volume was 0.035 ml and the incubation was carried out for 2 h at 37 °C. Values of controls lacking enzyme have been subtracted. The recovery ranged from 85 to 95%.

α-1,2-Mannosidase (α-Mannosidase I)—The assay conditions were identical to those used by Tabas and Kornfeld (8).

UDP-N-acetylglucosamine:α-Mannoside β-1,2-N-Acetylglucosaminyltransferase (N-Acetylglucosaminyltransferase I)—This assay measures transfer of [^{3}H]GlcNAc from UDP-GlcNAc to fetuin which has been treated with exoglycosidases to remove outer sialic acid, galactose, and GlcNAc, exposing the mannose core. The procedure was identical with that of Gottlieb and Kornfeld (30) except that 5 mM ATP was added to the reaction mixture and the final incubation volume was 65 μl. Acid-precipitated pellets were solubilized by incubation in 2 mg/ml of pronase, 0.1 M Tris, pH 8, 20 mM $CaCl_2$ for 1 h at 56 °C before measuring the radioactivity. Recovery ranged from 60 to 95%. Endogenous transfer in the absence of fetuin acceptor was negligible across the gradient. Values of controls lacking enzyme have been subtracted. It is possible that *N*-acetylglucosaminyltransferase II activity is also being detected in this assay.

α-1 → 3, α-1 → 6 Mannosidase (α-Mannosidase II)—This assay, which measures cleavage of mannose from GlcNAc([^{3}H]Man)$_5$GlcNAc$_2$ peptide, was performed as described previously (31). Recovery ranged from 90 to 95%. Values of controls lacking enzyme have been subtracted.

GDP-Fucose:β-N-Acetylglucosamine 1 → N-Asparagine Fucosyltransferase (Core Fucosyltransferase)—This assay, measuring transfer of fucose from GDP-fucose to the asparagine-linked GlcNAc of fibrinogen glycopeptides, was developed by Dr. Richard Cummings of our laboratory.[3] The 20-μl reaction mixture contained 2.1 mM fibrinogen glycopeptides (32), 23 μM [^{14}C]GDP-fucose, (2×10^5 cpm), 40 mM $MgCl_2$, 5 mM ATP, 1% Triton X-100, 5 mg/ml BSA, 4 mM Tris-HCl, pH 7.0. The samples were incubated at 37 °C for 30 min, heated in a boiling water bath for 5 min, and resuspended in 0.2 ml of 10 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, pH 8.0 (TBS). The samples were then passed over 0.5-ml columns of lentil lectin-Sepharose in pasteur pipettes. Lentil lectin binds fibrinogen glycopeptides only after they are substituted with core fucose residues (33). The columns were washed with 12 ml of TBS and were eluted with 2 ml of 0.5 M α-methylmannoside in TBS. The radioactivity in the eluate was measured. Controls without fibrinogen acceptor or without added membranes yielded no detectable product. The recovery of enzyme activity was 36%.

UDP-GlcNAc:α-Mannoside β-1,6-N-Acetylglucosaminyltransferase (N-Acetylglucosaminyltransferase IV)—The details of this assay, measuring transfer of GlcNAc β1,6 to α-linked mannose on complex-type glycopeptides, are described elsewhere (34). Briefly, fractions were assayed for their ability to convert a ^{125}I-asialo, agalactoglycopeptide which binds to ConA-Sepharose and binds to lentil lectin-Sepharose, to a form which does not bind to ConA-Sepharose but still binds to lentil lectin-Sepharose. Only glycopeptides which have been converted from biantennary to triantennary form by substitution of the hydroxyl group at C-6 of an α-linked mannose with an *N*-acetylglucosamine will behave this way. Controls without UDP-GlcNAc have been subtracted. The recovery of activity was 49%.

UDP-Galactose:N-Acetylglucosamine Galactosyltransferase (Galactosyltransferase)—This assay was modified from the procedure of Bretz and Staubli (35) by including 2 mM ATP, reducing the incubation volume to 50 μl (36), and using [^{3}H]UDP-galactose instead of the ^{14}C form, at a higher specific activity (65,000 cpm per assay, at 0.5 mM). The assay measures transfer of galactose from UDP-galactose to *N*-acetylglucosamine. Values of controls without acceptor or without enzyme have been subtracted. The recovery ranged from 55 to 70%. During the assay less than 15% of the UDP-Gal was broken down in any gradient fraction, as measured by charcoal absorption.

Man-6-P Receptor Assay—To perform this assay, each sucrose gradient fraction was divided into two parts. One part (25%) was processed in the usual fashion to use for enzyme assays. The other part (75%) was diluted with buffer and the membranes pelleted for use in the receptor assay. The membrane pellets were resuspended with a pasteur pipette in 1 ml of 25 mM Tris, pH 7, 100 mM NaCl, 5 mM PO_4, 0.5% saponin, 10 mM Man-6-P, and 0.2 unit/ml of Trasylol. After incubation for 30 min at room temperature, membranes were pelleted in a Ti-50 rotor at 230,000 × *g* for 60 min and then resuspended in 2 ml of the same buffer without Man-6-P. Membranes were pelleted again and resuspended with a Dounce homogenizer in 1.2 ml of buffer without Man-6-P. Two 0.5-ml aliquots were removed, Man-6-P was added to 10 mM in one set of tubes, and 300,000 cpm of *D. discoideum* ^{125}I-β-hexosaminidase at 10^4 cpm/ng (generously provided by Dr. Christopher Gabel of this laboratory) was added to both sets of tubes. After 30 min at 4 °C, the reaction mixtures were diluted with 2 ml of 25 mM NaCl, 25 mM Tris, pH 7, 0.1% saponin, and 0.2 unit/ml of Trasylol. The membranes were pelleted as before, washed once with 2 ml of dilution buffer, solubilized in 1 ml of 1 N NaOH and assayed for γ radioactivity. The radioactivity associated with the membranes in the presence of Man-6-P was subtracted from the membrane-bound radioactivity in the absence of Man-6-P. This value was deemed specific binding. The recovery of receptor activity was assessed in one preparation and was 93%.

[3] R. Cummings, unpublished data.

Cell Labeling

10^9 BW5147.3 lymphoma cells were suspended in 40 ml of GBFM, distributed between four 50-ml plastic conical tubes and incubated at 37 °C for 10 min. The cells in each tube were then pelleted, resuspended in 15 ml of GBFM containing 0.5 mCi of [2-^{3}H]mannose, and rotated gently for 20 min at 37 °C. Chase was initiated by addition of glucose and mannose to a concentration of 10 mM each. The cells were incubated for an additional 25 min in some experiments and for 60 min in others. Membranes were then prepared and subjected to sucrose gradient fractionation as described above for unlabeled lymphoma cells. The sucrose gradient fractions were divided into two portions with 25% used for enzyme assays and 75% used for analysis of endogenously-labeled oligosaccharides. Each aliquot was diluted and the membranes pelleted as before. The portion for oligosaccharide analysis was digested with pronase and the resultant glycopeptides were fractionated on ConA-Sepharose. The high mannose-type oligosaccharides were treated with endo H and then analyzed on QAE-Sephadex as previously described (37). Enzyme assays utilizing ^{14}C, ^{32}P, or ^{125}I substrates were performed as detailed above. The presence of the endogenous radiolabel in the membranes interfered with assays involving [^{3}H]substrates. This problem was overcome in the case of the galactosyltransferase assay by stopping the reaction with ice-cold ethanol (1 ml), sedimenting the precipitate, evaporating the ethanol supernatant to dryness, and continuing with the normal assay.

Lectin-Sepharose Fractionation of Glycopeptides

Fractionation of glycopeptides on ConA-Sepharose (37) and lentil lectin-Sepharose (33) has been described.

RESULTS

Fractionation of Enzymes Involved in the Biosynthesis of Phosphorylated Oligosaccharides in P388D$_1$ Cells—Total cell membranes were prepared from P388D$_1$ cells and subjected to equilibrium centrifugation on a continuous sucrose gradient. The various fractions were then assayed for a number of enzymes involved in the processing of lysosomal enzyme oligosaccharide units (see Fig. 1). As shown in Fig. 2, the membranes containing glucosidase I, the first enzyme in asparagine-linked oligosaccharide processing, distribute near the bottom (dense) part of the gradient. This enzyme has been localized to the rough endoplasmic reticulum in hen oviduct (38) and to the rough and smooth endoplasmic reticulum in rat liver (39). The activity profile of glucosidase II, the second processing enzyme, was indistinguishable from that of glucosidase I (data not shown). In hen oviduct this enzyme has been shown to fractionate with smooth membranes (38). *N*-Acetylglucosaminylphosphotransferase activity was intermediate in distribution between the glucosidases and galactosyltransferase, a late processing enzyme and a marker of the *trans* Golgi membranes (14). In this cell type the phosphodiester glycosidase was situated between the *N*-acetylglucosaminylphosphotransferase and galactosyltransferase, and could not be adequately resolved from either enzyme.

The gradient fractions exhibited very low mannosidase I activity (less than 1% of the applied value) and all attempts to improve the recovery of this enzyme activity were unsuccessful.

Fractionation of Asparagine-linked Oligosaccharide Processing Enzymes in BW5147.3 Cells—When BW5147.3 cell membranes were fractionated on the continuous sucrose gradient, the distribution of enzyme activities shown in Fig. 3 was observed. In this cell line, *N*-acetylglucosaminylphospho-

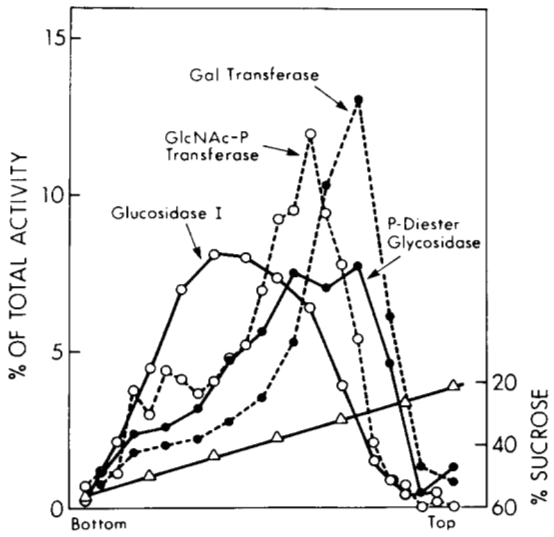

FIG. 2. **Fractionation of asparagine-linked oligosaccharide processing enzymes in P388D$_1$ cells.** Membranes were prepared and fractionated on a continuous sucrose gradient as described under "Experimental Procedures." Enzymes were assayed as described under "Experimental Procedures." The same qualitative separation was obtained in three different experiments. The figure shows the best separation. ○——○, glucosidase I; ○---○, *N*-acetylglucosaminylphosphotransferase; ●——●, phosphodiester glycosidase; ●---●, galactosyltransferase; △——△, % sucrose.

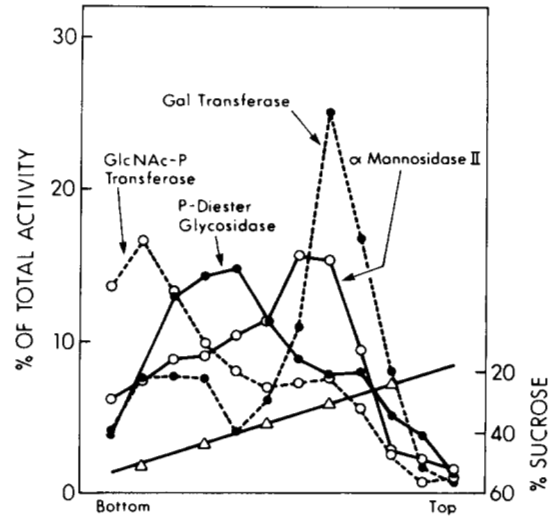

FIG. 3. **Fractionation of asparagine-linked oligosaccharide processing enzymes in BW5147.3 cells.** Membranes were prepared and fractionated on a continuous sucrose gradient as described under "Experimental Procedures." The same qualitative separation was obtained in six different experiments. The figure shows one of the best separations. ○---○, *N*-acetylglucosaminylphosphotransferase; ●——●, phosphodiester glycosidase; ○——○, α-mannosidase II; ●---●, galactosyltransferase; △——△, % sucrose.

transferase activity peaked in the dense membrane fraction and could not be separated from glucosidase I and II (not shown). The phosphodiester glycosidase peaked in a lighter region of the gradient while galactosyltransferase peaked at the top of the gradient, clearly distinct from the other two activities. Mannosidase II, an intermediary enzyme in the conversion of asparagine-linked oligosaccharides to complex-type units, fractionated between phosphodiester glycosidase and galactosyltransferase. As with the P388D$_1$ cells, the poor recovery of mannosidase I activity prevented analysis of its distribution.

Fig. 4 shows the distribution of a number of late stage processing enzymes. While all of these activities are concentrated in the lighter membranes, it is apparent that galactosyltransferase is partially resolved from α-mannosidase II, *N*-acetylglucosaminyltransferases I and IV, and the core fucosyltransferase. The data in Figs. 3 and 4 provide evidence that the membranes containing asparagine-linked oligosaccharide processing enzymes can be separated into at least four regions.

Distribution of Newly Synthesized Asparagine-linked Oligosaccharides in BW5147.3 Membranes—The distribution of the various enzymes in the sucrose gradients is consistent with the stepwise processing of asparagine-linked oligosaccharides as newly synthesized glycoproteins move from dense membranes to lighter membranes. To verify that this actually occurs, BW5147.3 cells were labeled with [2-^{3}H]mannose for 20 min and chased for either 25 or 60 min. The cell membranes were then fractionated on the sucrose gradients and analyzed for total radioactivity and for the distribution of high mannose and complex-type oligosaccharides. To do this, aliquots of the membranes from each fraction were digested with pronase and the resultant glycopeptides were subjected to serial lectin affinity chromatography on ConA-Sepharose and lentil lectin-Sepharose (33). Under the conditions used, triantennary fucosylated complex-type oligosaccharides which have an α-linked mannose substituted at positions C-2 and C-6 by *N*-acetylglucosamine residues pass through ConA-Sepharose and bind to lentil lectin-Sepharose. Biantennary fucosylated complex-type species bind to both ConA-Sepharose and lentil lectin-Sepharose and can be eluted with 10 mM α-methylglucoside. High mannose-type oligosaccharides bind to ConA-Sepharose and require 0.1 M α-methylmannoside for elution. The results of this experiment are summarized in Fig. 5. At the end of the 25-min chase period the great majority of the [^{3}H]mannose-labeled oligosaccharides were associated with

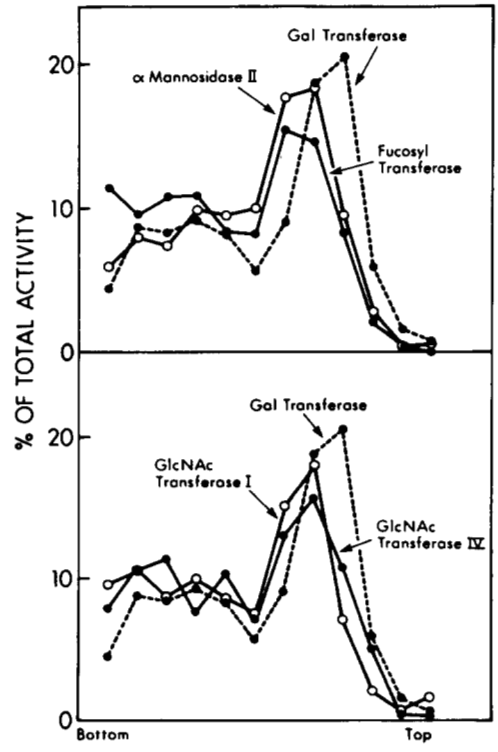

FIG. 4. **Fractionation of late-stage processing enzymes in BW5147.3 cells.** The *top* and *bottom* depict different activities measured on the same gradient. It should be noted that the assay for *N*-acetylglucosaminyltransferase I may also detect *N*-acetylglucosaminyltransferase II activity.

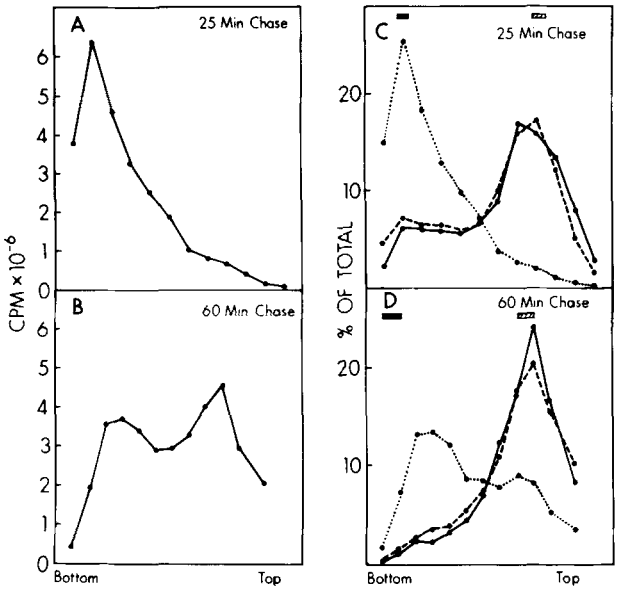

FIG. 5. **Distribution of newly synthesized glycopeptides labeled with [^{3}H]mannose.** Labeled glycopeptides obtained from the two pulse-chase experiments were analyzed by serial lectin affinity chromatography as described under "Experimental Procedures." *A* and *B*, total incorporation of [^{3}H]mannose into each membrane fraction. *C* and *D*, ●···●, high mannose-type glycopeptides; ●---●, triantennary fucosylated glycopeptides; ●——●, biantennary fucosylated glycopeptides. *Bars* at the top of *C* and *D* indicate the peak fraction or fractions of *N*-acetylglucosaminylphosphotransferase activity (bottom of gradient) and galactosyltransferase (top of gradient).

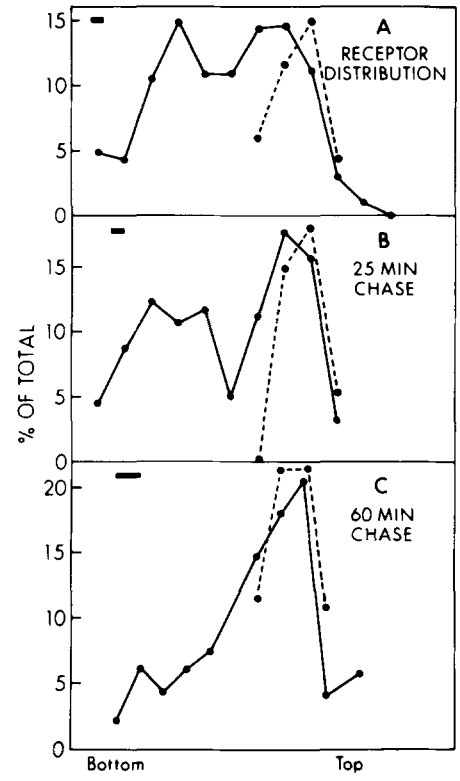

FIG. 6. **Fractionation of Man-6-P receptor and receptor-bound endogenous phosphorylated oligosaccharides.** *A*, distribution of the mannose 6-phosphate receptor assessed by quantitating the binding of ^{125}I-β-hexosaminidase as described under "Experimental Procedures." *B*, distribution of oligosaccharides with two phosphomonoesters on membranes from cells labeled with a 20-min pulse of [^{3}H]mannose followed by a 25-min chase, as described under "Experimental Procedures." *C*, similar to *B*, but from cells chased for 60 min. The data in *B* and *C* are derived from the same experiments shown in Fig. 5. The total radioactivity in oligosaccharides with two phosphomonoesters on the gradient was: 25-min chase, 8.1×10^3 cpm; 60-min chase; 2.8×10^4 cpm. *Bars* at the top of each panel indicate the peak fraction or fractions for *N*-acetylglucosaminylphosphotransferase activity. The *dotted lines* show the distribution of peak fractions of galactosyltransferase.

glycoproteins in the dense membranes (Fig. 5*A*). However, the bi- and triantennary fucosylated complex-type oligosaccharides were localized to the light membranes in the same region as the late stage processing enzymes (Fig. 5*C*). After 60 min of chase there was a substantial movement of the [^{3}H] mannose-labeled oligosaccharides to the light region of the gradient, consistent with the migration of newly synthesized glycoproteins from dense membranes to lighter membranes (Fig. 5*B*). The distribution of the complex-type species (Fig. 5*D*) was unchanged from the earlier time point, as one would expect if these species are formed only in the light membranes. High mannose-type oligosaccharides were found predominantly in the dense membranes at both time points, although a portion shifted to the light membranes in the 60-min chase (Fig. 5, *C* and *D*). This is consistent with the fact that some mature glycoproteins contain high mannose oligosaccharides.

Localization of the Man-6-P Receptor and Receptor-bound Phosphorylated Oligosaccharides—The distribution of the Man-6-P receptor in the BW5147.3 membranes is shown in Fig. 6*A*. The receptor is concentrated in the middle and top of the gradient. Very little receptor is present in the densest membranes which contain glucosidase I and II, indicating that in this cell type the receptor is not located in the endoplasmic reticulum membranes.[4]

Whereas the data in Fig. 6*A* provide information about the distribution of the steady state Man-6-P receptor, the data in *B* and *C* show the distribution of receptor containing newly synthesized lysosomal enzymes. As a measure of receptor-bound lysosomal enzyme, we determined the quantity of high mannose-type oligosaccharides containing two phosphomonoesters on the membrane fractions from the pulse-chase experiment of Fig. 5. This species was used as an indicator of receptor-bound lysosomal enzyme since our previous study had shown that all of the membrane-bound oligosaccharides with two phosphomonoesters are specifically eluted with Man-6-P (22). At the end of the 25-min chase, membrane-bound phosphorylated oligosaccharides distributed in two peaks, corresponding to the distribution of the receptor (Fig. 6*B*). After the 60-min chase, these oligosaccharides accumulated in the lighter peak (Fig. 6*C*). The distribution of this peak was close to the distribution of galactosyltransferase activity, but the correspondence was not exact (see Fig. 6*B*). In another experiment, there was a clearer separation of the lighter peak of membrane-bound phosphorylated oligosaccharides and galactosyltransferase (Fig. 7).

We have recently discovered the existence of sialylated, phosphorylated, hybrid-type oligosaccharides in BW5147.3 cells.[5] These oligosaccharides have the usual trimannosyl core with a phosphorylated, high mannose-type branch attached to the mannose that is linked α-1,6 to the β-linked mannose and a sialic acid → galactose → *N*-acetylglucosamine sequence attached to the mannose that is linked α-1,3 to the β-linked mannose. The details of the structural analysis of these hybrid

[4] With rat liver, using the fractionation procedure of Adelman *et al.* (40), we have also succeeded in separating almost all of the Man-6-P receptor activity away from the glucose 6-phosphatase-containing endoplasmic reticulum fractions.

[5] Varki, A., and Kornfeld, S. (1983) *J. Biol. Chem.* **258**, 2808–2818.

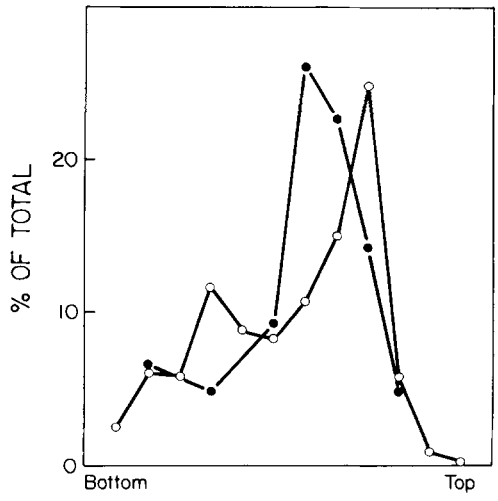

FIG. 7. **Fractionation of receptor-bound endogenous phosphorylated oligosaccharides and galactosyltransferase activity.** This experiment is identical with the one shown in Fig. 6*C*, except that 1×10^8 cells were labeled rather than 1×10^9 cells. ●——●, distribution of oligosaccharides containing two phosphomonoesters; ○——○, distribution of galactosyltransferase activity.

species will be described elsewhere.[5] Since these molecules contain residues suggesting passage through the late Golgi (galactose, sialic acid), we analyzed the receptor-bound phosphorylated oligosaccharides from the 25-min chase for the presence of such species. It was found that ligand in both the dense and light Man-6-P receptor peaks of Fig. 6*B* contained sialylated, phosphorylated hybrids by the following criteria: 1) susceptibility to endo-β-*N*-acetylglucosaminidase H; 2) elution position on QAE-Sephadex columns as expected for molecules with one phosphomonoester and one additional negative charge; 3) change in elution position on QAE-Sephadex, after mild acid hydrolysis under conditions which release sialic acid, to that of oligosaccharides with one phosphomonoester; 4) subsequent conversion to a neutral oligosaccharide by alkaline phosphatase digestion; 5) partial resistance to α-mannosidase digestion after removal of the phosphate. Thus, α-mannosidase released only 55% of the radioactivity as free mannose and the residual oligosaccharide behaved as a large fragment on paper chromatography. In contrast, similar treatment of the usual high mannose-type oligosaccharide releases all of the radioactivity as free mannose and Man-GlcNAc. Both dense and light peaks of the sucrose gradient contained about 50% as much phosphorylated, sialylated hybrid as oligosaccharide with two phosphomonoesters. These phosphorylated hybrids are assumed to be bound to the Man-6-P receptor since they are membrane-associated and elutable with Man-6-P (22).

DISCUSSION

The data presented here indicate that the events of asparagine-linked oligosaccharide processing occur in multiple regions within the cell. We believe that most of these regions represent areas within the Golgi complex for the following reasons. First, with the exception of glucosidases I and II, most of the enzyme activities measured in these experiments have been shown to fractionate with the Golgi marker, galactosyltransferase, in other systems (5–10). By analogy it seems likely that these enzymes are also associated with the Golgi complex in the cell lines used in this study. Second, there is a considerable body of autoradiographic evidence which indicates that the Golgi complex is involved in glycoprotein synthesis, at least in terms of the addition of the terminal sugars galactose, fucose, and sialic acid (3). We have been able to separate these putative Golgi enzymes from glucosidases I and II which have previously been shown to fractionate with the rough and smooth endoplasmic reticulum (38, 39). The present data confirm and extend the findings of Dunphy *et al.* (15). Using Chinese hamster ovary cells, these workers demonstrated that α-mannosidase I was localized to a heavier Golgi membrane fraction than galactosyltransferase.

The striking observation reported here is that the order of fractionation of the various Golgi enzymes on the sucrose gradients is identical with the sequence in which these enzymes appear to act *in vivo* (see Fig. 1). *N*-Acetylglucosaminylphosphotransferase, the first Golgi enzyme in the pathway, is found in the densest membranes. Galactosyltransferase, one of the terminal enzymes in the pathway, is in the lightest membranes. Intermediary enzymes of the processing pathway are found in membranes of intermediate density. These data are consistent with there being a functional order to the different regions of the Golgi.

Studies of the lipid composition of rat liver subcellular fractions have indicated that there is a gradient of cholesterol content from the endoplasmic reticulum (low) to the Golgi to the plasma membrane (high) (41, 42). In addition, electron microscopic studies using filipin as a cholesterol probe (43) and density shift experiments using digitonin (44) have suggested that there is a gradient of cholesterol across the Golgi apparatus itself, being lowest at the *cis* face and highest at the *trans* face. Galactosyltransferase, which has been localized to the *trans* face by immunologic techniques (14), is found in the most cholesterol-rich region of the Golgi (44). It seems reasonable to suggest that a similar gradient of cholesterol content may be the basis of the membrane separation observed on our sucrose gradients.

An indication that these separations are relevant to the *in vivo* situation comes from the pulse-chase experiments in which we analyzed endogenously labeled oligosaccharide intermediates fractionated on the same sucrose gradients. The dramatic shift of [^{3}H]mannose-labeled glycoproteins from the dense membranes to the lighter membranes during the chase period is consistent with the proposed movement of newly synthesized glycoproteins through the Golgi complex. The stage of processing and terminal glycosylation of the asparagine-linked oligosaccharides in the fractions correlated quite well with the localization of the various processing enzymes. Thus, fucosylated complex-type oligosaccharides were primarily localized to the membranes which contained the fucosyltransferase. Likewise, triantennary complex-type species which have an α-linked mannose substituted at positions C-2 and C-6 by *N*-acetylglucosamine residues were found in these same membranes which also contain *N*-acetylglucosaminyltransferase IV, the enzyme which converts the biantennary species to this particular triantennary species.

The finding that *N*-acetylglucosaminylphosphotransferase and phosphodiester glycosidase are distributed in membranes of different densities indicates that the Man-6-P recognition marker of lysosomal enzymes is generated in two regions, both of which are distinct from the membrane region that contains galactosyltransferase. However, the fact that the receptor-bound lysosomal enzymes contain sialylated, phosphorylated hybrid oligosaccharides indicates that newly synthesized lysosomal enzymes pass all the way through the Golgi complex prior to binding to the Man-6-P receptor. The recent report of Vladutiu (45) that newly synthesized β-hexosaminidase contains highly sialylated oligosaccharides is consistent with this proposal. Subsequent to sialylation, the lysosomal enzymes presumably bind to the Man-6-P receptor and are targeted to lysosomes although we cannot exclude the alter-

nate possibility that these molecules bind to the receptor and then pass through the late Golgi region. The membranes containing the receptor distributed into two peaks on the sucrose gradients. The pulse-chase experiment demonstrated that receptor-bound ligand appears early in both regions, but with subsequent chase the receptor-bound molecules accumulate in the light peak. This light compartment appears to be distinct from the *trans* Golgi compartment which contains the galactosyltransferase since the two regions can be partially resolved on the sucrose gradients. This finding, along with the observation that the receptor-bound ligand contains sialylated oligosaccharides, leads us to conclude that the majority of the receptor is present in post-Golgi organelles. While the subcellular origin of these dense and light receptor-containing membranes is unknown, one possibility is that they represent transport vesicles and the post-Golgi staging area described by Novikoff (46) as GERL.

The separation of various enzymatic activities on the sucrose density gradients can be explained in two ways. The first is that these enzymes are located in the same Golgi cisternae but are associated with different 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 interpretation is consistent with the cytochemical studies which 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 (47, 48). An alternate explanation, and the one we feel is most likely, is that the enzyme separations reflect heterogeneity in the location of the various enzymes across the Golgi stack. If this is in fact the case, it would indicate that asparagine-linked oligosaccharide processing occurs in a series of regions as the newly synthesized glycoproteins move from one region to the next. Based on the known localization of some of the processing enzymes, it is possible to suggest the following morphologic correlates. The first region is the endoplasmic reticulum where glycosylation occurs and glucosidases I and II are located. Newly synthesized glycoproteins then enter an early area of the Golgi complex (at the *cis* face) where phosphorylation of the oligosaccharide units of lysosomal enzymes occurs. In the next region, phosphodiester glycosidase "uncovers" the mannose 6-P recognition marker. The glycoproteins then move to an area which contains a series of late stage processing enzymes (including *N*-acetylglucosaminyltransferases I, II, and IV, α-mannosidase II, fucosyltransferase). Finally, they enter a late compartment in the *trans*-most region of the Golgi which contains galactosyltransferase and sialyltransferase. Lysosomal enzymes with oligosaccharide units containing phosphomonoester groups bind to the Man-6-P receptor here or in a still later compartment. This is followed by sorting of the various glycoproteins and packaging for secretion, transport to lysosomes, or targeting to the plasma membrane. It must be stressed that in the absence of immunolocalization of the various processing enzymes we can only speculate about the possible morphologic correlates to these studies.

The observed organization of the Golgi enzymes indicates that there must be a targeting mechanism to allow intrinsic Golgi enzymes to reach their specific position within the Golgi complex. The basis for this targeting is unknown.

One potential benefit of compartmentalization is that it provides a mechanism for the control of oligosaccharide processing. For example, the physical separation of *N*-acetylglucosaminylphosphotransferase from later processing enzymes would ensure that the former enzyme can act before its substrate high mannose oligosaccharides are converted to complex-type species. The localization of *N*-acetylglucosaminyltransferase IV in a compartment proximal to that which contains galactosyltransferase could have functional implications. This enzyme only transfers *N*-acetylglucosamine residues to biantennary oligosaccharides which have not yet been substituted with galactose (34). If galactosyltransferase were in the same compartment as *N*-acetylglucosaminyltransferase IV, the action of the latter transferase might be impaired. Therefore, compartmentalization may regulate competing enzyme reactions, providing a control mechanism complementary to the established mechanisms of enzyme specificity.

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