Compartimentation of Asparagine-linked Oligosaccharide Processing in the Golgi Apparatus

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ABSTRACT

Golgi-associated processing of complex-type oligosaccharides linked to asparagine involves the sequential action of at least six enzymes. By equilibrium sucrose density gradient centrifugation of membranes from Chinese hamster ovary cells, we have partially resolved the set of four initial enzymes in the pathway (Mannosidase I, N-acetylglucosamine (GlcNAc) Transferase I, Mannosidase II, and GlcNAc Transferase II) from two later-acting activities (galactosyltransferase and sialyltransferase). In view of the recent demonstration that galactosyltransferase is restricted to the trans face of the Golgi complex in HeLa cells (Roth, J., and E. G. Berger, 1982, J. Cell Biol., 93:223-229), our results suggest that removal of mannose and attachment of peripheral N-acetylglucosamine may occur in some or all of the remaining cisternae on the cis side of the Golgi stack.

After the removal of glucose in the endoplasmic reticulum (ER),1 asparagine-linked oligosaccharides destined to become complex chains are processed by a battery of Golgi complex-associated enzymes (21). The processing steps that yield a two-branched, complex-type sugar chain are summarized in Fig. 1. We recently reported a partial separation, by density gradient centrifugation, of Chinese hamster ovary (CHO) cell membranes containing the earliest enzyme in the pathway (Mannosidase I) from those possessing the two late-acting glycosyltransferases galactosyltransferase and sialyltransferase (6). On the basis of this and related findings, we proposed a spatial separation or compartmentation of these enzymes within the Golgi complex. Here we extend these studies to include all six enzymatic steps described in Fig. 1.

MATERIALS AND METHODS

MATERIALS: UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-galactose, CMP-sialic acid, a-methylmannoside, Concanavalin A-Sepharose, and ovalbumin were obtained from Sigma Chemical Co. (St. Louis, MO). [2-3H]-Mannose (15 Ci/mmol), [1-3H]galactose (11.6 Ci/mmol), UDP-[6-3H]-N-acetylglucosamine (24 Ci/mmol), UDP-[1-3H]galactose (11.6 Ci/mmol), and CMP-[9-3H]sialic acid (18.9 Ci/mmol) were obtained from New England Nuclear (Boston, MA). AGI-X8 (formate from 200--400 mesh) was obtained from Bio-Rad Laboratories (Richmond, CA). Endo-β-N-acetylglucosaminidase H (Endo H) was purchased from Health Research, Inc. (Albany, NY). Purified rabbit liver GlcNAc Transferase I (sp act, 2.5 U/mg protein; see reference 30) was generously supplied by Dr. R. Hill (Duke University).

CELLS AND VIRUSES: Wild-type, clone 15B, and clone 1021 CHO cells and clone 6 of L cells (kindly provided by Dr. S. Kornfeld, Washington University) were cultivated as before (6). Vesicular stomatitis virus (VSV) infections were carried out as described (6).

MEMBRANE FRACTIONATION: In all fractionation experiments, membranes from postnuclear supernatants of CHO clone 1021 cells (4) were used. The membranes were washed as described before (6) and fractionated on a six-step sucrose gradient consisting of 4.5 ml of 55%; 1.5 ml of 40%; 2.5 ml of 35%; 2.5 ml of 30%; and 1.0 ml of 20% (wt/wt) sucrose containing 1 mM Tris-HCl/l mM EDTA, pH 8.0. After centrifugation (20 h at 2°C in the Beckman SW 27.1 rotor), 14 fractions (~1 ml) were collected. The bottom two fractions (which routinely contained no protein or enzyme activity) were discarded. Membranes from the remaining fractions were harvested (6), resuspended in 0.1 ml of H2O, and assayed for enzyme activity.

PREPARATION OF TRITIATED OLIGOSACCHARIDES: We isolated ([3H]-Man, GlcNAc, a nearly equimolar mixture of ([3H]Man)GIcNAc and ([3H]Man)GIcNAc, and ([3H]Man)GIcNAc from Endo H-treated glycopeptides prepared from VSV-infected clone 15B CHO cells labeled with [3H]mannose (39, 40). ([3H]Man)GIcNAc was also isolated from VSV-infected clone 6 of mouse L cells as before (6, 40). GlcNAc([3H]Man)GIcNAc, the product of GlcNAc Transferase I action, was prepared as described by Tabas and Kornfeld (39) except that 0.02 U of purified rabbit liver GlcNAc Transferase I was used and incubation time was lengthened to 12 h. ([3H]GIc)ManGIcNAc was prepared from the lipid-linked oligosaccharide fraction (4) isolated from VSV-infected clone 15B cells labeled with [3H]galactose (25). The oligosaccharide was released from the lipid carrier by mild acid treatment (37), digested with Endo H, and partially purified by passage through a 0.5 x 2.5-cm AGI-X8 column equilibrated in 0.15 M NH4HCO3 (pH 8.0).

UNLABELED GLYCOSYLTRANSFERASE ACCEPTORS: Man(GlcNAc)2-asparagine (ovalbumin glycopeptide V) was prepared from ovalbumin as described (20). Aloxosotransferrin (transferrin with an exposed three-mannose core generated by exhaustive treatment with neuraminidase, β-galactosidase, and N-acetylglucosaminidase; see reference 32) was kindly provided by Dr. R. Hill. To prepare the substrate for GlcNAc Transferase II assays, we treated aloxosotransferrin exhaustively with purified rabbit liver GlcNAc Transferase I (30). The incubation contained 0.1 M Mes (pH 6.3), 10 mM MnCl2, 0.2% Triton X-100, 24 mM UDP-GlcNAc, 5 mg of aloxosotransferrin, and 0.007

1 Abbreviations used in this paper: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; Endo H, Endo-β-N-acetylglucosaminidase H; GlcNAc, N-acetylglucosamine; VSV, vesicular stomatitis virus; Mes, 2[N-Morpholino]ethanesulfonic acid.

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270
U GlcNAc Transferase I in a 0.75-ml vol. After a 48-h incubation at 37°C, the enzyme was inactivated by heating (90 min at 56°C). Two lines of evidence indicated that the reaction had gone to completion. A parallel incubation containing UDP-[3H]GlcNAc demonstrated that 1.07 mol of GlcNAc had been incorporated into transferrin oligosaccharide. Also, further treatment of the reaction product with GlcNAc Transferase I resulted in no additional GlcNAc transfer. Agalactofetuin was prepared from desialated fetuin (38) by digestion with jack bean β-galactosidase (Sigma Chemical Co.) as described in reference 26.

Enzyme Assays

MANNOSIDASE I: We measured this activity as described by Tabas and Kornfeld (40), with some modifications. Assays contained in a 0.03-ml vol: 0.1 M sodium acetate (pH 6.0), 0.3% Triton X-100, 1 mM MgCl₂, 1 mM CaCl₂, and 5.000 cpm of [³H]Man₆GlcNAc. Assay with [³H]Man₆GlcNAc as substrate gave the same results. After incubation (60 min at 37°C), released [³H]mannose was quantitated as described (40).

GlcNAc TRANSFERASE I: For assay of GlcNAc Transferase I, we measured transfer of [³H]GlcNAc to Man₆GlcNAc-asparagine (ova-lubumin glycopeptide V). Incubation conditions were based on those described by Oppenheimer and Hill (30). The labeled glycopeptide was separated by binding to Concanavalin A-Sepharose. Assays contained 0.1 M Mes (pH 6.5), 10 mM MnCl₂, 0.2% Triton X-100, 2 mM ATP, 1 μCi UDP-[³H]GlcNAc, 1 mM nonradioactive UDP-GlcNAc, and 10 nmol of ovalbumin glycopeptide V in a final volume of 0.05 ml. After incubation (30 min at 37°C), samples were boiled for 5 min, mixed with 1 ml of phosphate-buffered saline (PBS) (see reference 6 for composition), and centrifuged for 2 min at 12,800 g in an Eppendorf microfuge. The supernastant was applied to a 0.5-ml (0.5 x 2.5-cm) Concanavalin A-Sepharose column (equilibrated with PBS). The column was first washed with 7 ml of PBS, bound glycopeptides were then eluted over a 5-min period with 2.5 ml of 0.1 M α-methylmannoside containing 10 mM Tris-HCl (pH 8.0). Greater than 90% of the added glycopeptide was routinely recovered in this eluate. Negligible [³H]GlcNAc incorporation in the absence of glycopeptide acceptor was detected.

MANNOSIDASE II: We assayed this enzyme by measuring the release of [³H]mannose from GlcNAc-[³H]Man₆GlcNAc as described by Tabas and Kornfeld (39) except that the incubation buffer was that used by Tsulisani et al. (43). Assays contained 0.1 M sodium acetate (pH 6.0), 0.3% Triton X-100, and GlcNAc-[³H]Man₆GlcNAc oligosaccharide (5,000 cpm) in a 0.03-ml vol. Incubations were carried out for 30 min at 37°C.

GlcNAc TRANSFERASE II: For this assay, we used GlcNAc Transferase I-treated aspergmin-linked oligosaccharide (21). Enzymes are: (1) Mannosidase I; (2) GlcNAc Transferase I; (3) Mannosidase II; (4) GlcNAc Transferase II; (5) Galactosyltransferase; and (6) Sialyltransferase. M, mannosae; Gal, galactose; SA, sialic acid; R, GlcNAc-asparagine (polypeptide chain).

RESULTS

Membranes from CHO clone 1021 cells were washed, centrifuged in a sucrose density gradient, and the distributions of the enzymes catalyzing the six enzymatic steps depicted in Fig. 1 were determined. As before (6), we observed that Mannosidase I resided in somewhat denser membranes than galacosyltransferase (Fig. 2A). Qualitatively, the same separations were observed in wild-type and clone 15B CHO cells, but clone 1021 cells routinely gave the best results. Prolonged centrifugation (20 h at 85,000 g) to apparent equilibrium was required to effect this separation. After a 4-h centrifugation, both activities were diffusely distributed throughout the gradient. EDTA (1 mM) could be removed from the procedure with little effect. We previously demonstrated that the glycoprotein products newly processed in vivo by Mannosidase I and galactosyltransferase could also be separated in this manner (6). [³H]Palmitate-labeled (36) VSV G protein with five mannose-containing oligosaccharides followed the distribution of Mannosidase I, whereas G protein pulse-labeled in VSV with [³H]glucosamine distributed with galactosyltransferase.

The enzymes catalyzing steps 2–4 (Fig. 1) (GlcNAc Transferase I, Mannosidase II, and GlcNAc Transferase II) all codistributed with Mannosidase I in the gradient. Sialyltransferase, the last enzyme in the pathway, closely paralleled galactosyltransferase activity (Fig. 2E). These results suggest that removal of mannose and attachment of at least two terminal GlcNAc residues takes place in a Golgi complex region(s) distinct from the site of galactose and sialic acid transfer. Our data do not, however, exclude the possibility that further compartmentation might exist.
Figure 2 Distributions of oligosaccharide-processing enzymes in membranes from CHO clone 1021 cells. For each gradient, membranes from 4 ml of postnuclear supernatant were fractionated as described in Materials and Methods. Results from three different gradients are depicted in panels A–C, D, and E–F, respectively. (A–C) Distribution of Glucosidase I, Mannosidase I, GlcNAc Transferase I, Mannosidase II, and galactosyltransferase measured in the same gradient. For reference, the distribution for galactosyltransferase is presented in each panel. Recoveries of enzyme activity (relative to levels in the washed membranes applied to the gradient) and the levels of enzyme activity in peak fractions (activity per total fraction) were respectively: Glucosidase I (40%; 4.8 x 10^-2 U); Mannosidase I (43%; 0.39 U); GlcNAc Transferase I (65%; 2.8 nmol of GlcNAc transferred/h); Mannosidase II (54%; 1.03 U); and galactosyltransferase (98%; 1.29 nmol of galactose transferred/h). The definitions for units of Glucosidase I and Mannosidases I and II are in references 16 and 40, respectively. (D) Codistribution of GlcNAc Transferase I and II activities. Galactosyltransferase (peak in activity indicated by arrow) was measured in this gradient as an internal control. Recovery of GlcNAc Transferase II was 81%; total enzyme activity in the peak fraction was 2.4 nmol of GlcNAc transferred/h. (E) Codistribution of galactosyltransferase and sialyltransferase. Mannosidase I and GlcNAc Transferase I (peak in activity indicated by arrow) were measured in the same gradient as internal controls. Recovery of sialyltransferase was 26%; total enzyme activity in the peak fraction was 0.19 nmol of sialic acid transferred/h. (F) Protein content (mg/fraction) (measured by the Lowry et al. (27) method) and sucrose densities (g/ml) of the fractions from the same gradient shown in E.
The Mannosidase I and II activities we have measured had the expected properties (40, 43). Mannosidase I was strongly inhibited (Table I) by 5 mM EDTA or 50 mM Tris-maleate (pH 6.0); Mannosidase II was unaffected by these treatments (Table II). In both assays, hydrolysis of Man₉GlcNAc, a substrate for neither enzyme, was only slightly above background levels (Tables I and II). To be certain that the two N-acetylglucosaminyltransferase assays indeed measure distinct enzymes, we compared levels of the two activities in wild-type CHO cells and the mutant clone 15B line that selectively lacks GlcNAc Transferase I (13). Membranes from wild-type but not clone 15B CHO cells possessed GlcNAc Transferase I (Table III), as expected (13). GlcNAc Transferase II levels in both cells lines were comparable (13, 28).

All the Golgi complex activities floated to densities much lighter than Glucosidase I (Fig. 2A), the enzyme that removes the outermost glucose (15) from the glycosylated precursor oligosaccharide transferred from dolichol. Kinetic (22, 24) and cell fractionation (15) studies have indicated that this enzyme resides in the ER. The distribution of Glucosidase I is quite similar to that reported for glucose-containing VSV G protein pulse-labeled in vivo with [³⁵S]methionine (6).

Pohlmann et al. (31), using a variation of our procedure (6), recently reported an apparent separation of Mannosidase I and galactosyltransferase in rat liver Golgi complex fractions. However, using this procedure (Dunphy, W., and J. Rothman, unpublished data) we have not observed a separation of Mannosidases I and II from galactosyltransferase in rat liver Golgi complex subfractions prepared by three separate methods (described in references 3, 19, and 40). In measurement of galactosyltransferase in these experiments, we had to take care to inhibit a pyrophosphatase (by including 2 mM ATP in the assay; see reference 3) present in rat liver in order to avoid the erroneous underestimation of galactosyltransferase in certain pyrophosphatase-rich gradient fractions. This difficulty was not encountered with CHO membrane fractions. ATP (2 mM) had no effect on galactosyltransferase levels in the gradient fractions of CHO membranes. Also, mixing experiments ruled out the presence of an inhibitor (or activator) of either galactosyltransferase or Mannosidase I in the CHO membrane fractions. Upon mixing peak fractions, we observed that levels of both galactosyltransferase and Mannosidase I were linearly additive (not shown). In a separate experiment, we ruled out a potential inhibition of GlcNAc Transferase I in fractions containing high galactosyltransferase levels by mixing the fractions with a known amount of purified rabbit liver GlcNAc Transferase I (Table IV).

| Condition | GlcNAc[^3H] | Man₉GlcNAc[^3H] | Man₉GlcNAc[^3H] | Difference |
|-----------|-------------|-----------------|-----------------|------------|
| Control   | 2.03 (1.00) | 0.22 (0.11)     |                 |            |
| +5 mM EDTA| 2.15 (0.07) | 0.10 (0.05)     |                 |            |
| 0.05 M Tris-maleate (pH 6.0) | 0.15 (0.07) | 0.13 (0.06)     |                 |            |

Membranes from clone 1021 CHO cells were washed as for gradient fractionation (see Materials and Methods) and assayed for hydrolysis of [³⁵S]Man₉GlcNAc or [³⁵S]Man₉GlcNAc under Mannosidase I assay conditions (see Materials and Methods). Both substrates (5,000 cpm per assay) were obtained from the same glycopeptide preparation (see Materials and Methods). Control incubations contained 0.1 M sodium acetate (pH 6.0), and all incubations contained 1 mM MgCl₂, 1 mM CaCl₂, and 0.3% Triton X-100. Values are the average of determinations at two separate protein concentrations (45 and 90 µg per assay) within the linear assay range. Analysis of the pooled peak fractions from gradients such as those depicted in Fig. 2 gave identical results.

* A unit of activity was defined as 1 unit (see Materials and Methods). GlcNAc[^3H]Man₉GlcNAc was prepared from the batch of [³⁵S]Man₉GlcNAc used in this experiment, and is thus of identical specific radioactivity. Control incubations contained 0.1 M sodium acetate (pH 6.0), and all incubations contained 0.3% Triton X-100 and 5,000 cpm of the appropriate tritiated oligosaccharide.

† Values in parentheses indicate activity as a fraction of that observed for hydrolysis of GlcNAc[^3H]Man₉GlcNAc under control conditions.

Table I: Properties of Mannosidase I in CHO Membranes

Table II: Properties of Mannosidase II in CHO Membranes

Table III: Distinction between GlcNAc Transferase I and II Activities

Table IV: GlcNAc Transferase I is not Inhibited in the Presence of CHO Membrane Fractions
DISCUSSION

A large body of electron microscopic evidence has established that the stacked cisternae of the Golgi complex are heterogeneous in composition (see references 8, 34, and 42 for recent reviews). But a few examples are selective osmium deposition in cis Golgi cisternae (19); restriction of thiamine pyrophosphatase activity to trans saccules (5); and a graded increase in cholesterol concentration from cis to trans Golgi membranes (29).

We have described the resolution of membranes containing the late-acting sugar transferases galactosyltransferase and sialyltransferase from those housing four earlier-acting oligosaccharide-processing enzymes (Mannosidase I, GlcNAc Transferase I, Mannosidase II, and GlcNAc Transferase II). All of these enzymes have been shown to be enriched greatly in total Golgi fractions (9, 17, 28, 35, 40, 44). Using a variation of our procedure (6), Goldberg and Kornfeld (12) recently observed a separation of GlcNAc Transferase I and Mannosidase II (as well as fucosyltransferase and GlcNAc Transferase IV) from galactosyltransferase in murine macrophage and lymphoma cell lines.

To what morphologically defined elements of the Golgi complex might the sequentially-acting membrane fractions that we have identified correspond? Two recent reports have furnished compelling evidence supporting the conclusion (6, 34) that galactose transfer occurs in trans Golgi cisternae. By immunocytochemistry, Roth and Berger (33) have directly localized galactosyltransferase to the cisternae at the trans side of the Golgi stack in HeLa cells. Also, Griffiths et al. (14) have shown that the galactose-specific lectin ricin binds to trans saccules that would be expected to contain the products of galactosyltransferase action. The distribution defined by galactosyltransferase (and sialyltransferase) in our fractionation experiments therefore most likely corresponds to remnants of these trans cisternae. We have shown here that four earlier-acting enzymes (the two mannosidases and N-acetylglucosaminyltransferases) reside, at least in part, in a different region of the Golgi complex. An ample and tenable hypothesis, therefore, is that removal of mannos and attachment of peripheral GlcNAc takes place in some or all of the remaining cis saccules, while galactose and sialic acid transfer occurs in the trans Golgi. A direct test of this model awaits immunoelectron microscopic localization of early-acting enzymes in the processing pathway.

Numerous investigators have reported methods for subfractionation of rat liver Golgi apparatus. Among the techniques described have been immunoaffinity adsorption (23), counter-current separation in aqueous polymer two-phase systems (19), and sucrose density gradient separation based upon peripheral oligosaccharide-processing enzymes that act at distinct temporal stages in the maturation of glycoproteins is needed to delineate the intragolgi complex transport pathway with precision. The initial step in this task, localization of galactosyltransferase to the trans Golgi complex cisternae (33), has corroborated the notion that glycoproteins exit at the trans face. Since precursors destined for the plasma membrane, lysosomes, and secretion granules all can contain terminal galactose (11, 18, 21), these classes of protein must all have passed through the trans cisternae. The implication, as we pointed out earlier (6, 34), is that the sorting of these three types of protein most likely occurs within or during departure from the trans Golgi cisternae.

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