Subfractionation of rat liver Golgi apparatus: Separation of enzyme activities involved in the biosynthesis of the phosphomannosyl recognition marker in lysosomal enzymes

(UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminyl-1-phosphotransferase/ α -*N*-acetylglucosaminylphosphodiesterase/Percoll/oligosaccharide processing)

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ABSTRACT A highly purified Golgi apparatus preparation from rat liver was subfractionated on a Percoll gradient into two major protein peaks of similar size that migrated at densities of 1.028 and 1.051 g/ml. The lighter protein peak contained 70–80% of the total activities of the oligosaccharide-processing enzymes α -1,2-mannosidase and mannosidase II and of UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminyl-1-phosphotransferase (α -*N*-acetylglucosaminylphosphotransferase), an enzyme involved in the biosynthesis of the mannose 6-phosphate recognition marker of lysosomal enzymes. These enzyme activities were enriched 2-fold in specific activity over that of the heavy protein peak. In contrast, 80% of the α -*N*-acetylglucosaminylphosphodiesterase, an enzyme that exposes 6-phosphomonoesters of mannose on the oligosaccharide chains of lysosomal enzymes, migrated in a region of slightly higher density than did the protein peak of density 1.051 g/ml. Sialyltransferase (SiaTase) and galactosyltransferase (GalTase) activities distributed almost equally among the two protein peaks. Controls rule out that the two protein peaks were the result of aggregation/deaggregation and that enzyme activities were altered by Percoll *per se*. Lysosomal enzyme activities migrated in a region essentially devoid of Golgi apparatus-associated enzyme activities. These results suggest a physical separation within the Golgi apparatus of some of the enzymes involved in the biosynthesis and processing of the oligosaccharides on glycoproteins, including those responsible for the formation of the mannose 6-phosphate recognition marker on lysosomal enzymes.

The Golgi apparatus (GA) plays a central role in the assembly and intracellular sorting of many secretory and membrane proteins (1). Reactions that have been shown to occur in this organelle include terminal glycosylation and sulfation of proteins and lipids (2–4), processing of the sugar moieties of glycoproteins (5, 6), and generation of the phosphomannosyl recognition marker that is responsible for the targeting of lysosomal enzymes to the lysosome (7, 8). The complexity and diversity of these reactions and functions coupled to the striking morphological polarity of this organelle have led to recent efforts to determine the mechanism(s) by which these events are sorted from one another within the organelle.

A current hypothesis is that those enzymes involved in glycoprotein biosynthesis, which occurs in the GA, have a sequential spatial arrangement within the organelle: thus, enzymes catalyzing the addition of sugars and the oligosaccharide-processing reactions that occur at early times after protein arrival from the rough endoplasmic reticulum (rough ER) would be localized in regions of the GA proximal to the rough ER,

whereas those enzymes of the GA catalyzing subsequent reactions, such as terminal glycosyltransferases, would be localized in regions distal from the rough ER (9). The side of the GA complex adjacent to the rough ER is referred to as *cis*, and the side closest to the plasma membrane is referred to as *trans* (1).

Attempts to substantiate this hypothesis have involved the isolation of biochemically distinct GA subfractions with the hope of relating them to the morphologically distinct *cis/trans* regions. Ehrenreich *et al.* (10), using rat liver, obtained three GA subfractions on a sucrose gradient that morphologically resembled *cis*, *trans*, and intermediate regions. However, biochemical analyses showed that the total enzymatic activities of the terminal glycosyltransferases in each subfraction were nearly identical, even though there was a 2-fold enrichment in the specific activity in the low density or *trans*-like fraction (11). With the same tissue, a similar result was obtained by Meritt and Morré for galactosyltransferase (GalTase) (12), although these authors did not directly correlate their lighter density fraction with *trans* GA elements, their assumption being that the lighter density fraction contained very low density lipoprotein vesicles derived from both *cis* and *trans* GA regions *in vivo* (13, 14). Bergeron *et al.* (15) generated GA subfractions by the method of Ehrenreich *et al.* (10) and reported a higher galactose transfer to endogenous acceptors in the *cis* region. Immunoelectron microscopy (16), cytochemistry (17), electron microscopy/autoradiography (18), and lectins (19–21) have been used to localize GalTase and oligosaccharide moieties to *trans* GA regions in a number of cell types.

More recently, the proposal has been made that α -1,2-mannosidase and the two enzymes involved in the biosynthesis of the phosphomannosyl recognition marker of lysosomal enzymes—namely, UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminyl-1-phosphotransferase (α -*N*-acetylglucosaminylphosphotransferase) and α -*N*-acetylglucosaminylphosphodiesterase—are markers of the *cis* GA region, whereas terminal glycosyltransferases, such as those catalyzing the addition of galactose and sialic acid, are markers of the *trans* GA region (22, 23). Upon sucrose density fractionation of crude liver membranes, it was found that α -1,2-mannosidase comigrated with the phosphomannosyl recognition marker biosynthetic enzymes in a peak of heavier density than did GalTase (23). Furthermore, fractionation of homogenates of Chinese hamster ovary cells on a sucrose gradient resulted in α -1,2-mannosidase ac-

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Abbreviations: GA, Golgi apparatus; ER, endoplasmic reticulum; SiaTase, sialyltransferase; GalTase, galactosyltransferase; α -*N*-acetylglucosaminylphosphotransferase, UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminyl-1-phosphotransferase; Endo H, endo- β -*N*-acetylglucosaminidase H.

tivity migrating at slightly higher density than did GalTase and sialyltransferase (SiaTase) activity (22).

In order to further substantiate the above hypothesis, we fractionated on an isosmotic, self-forming gradient of Percoll a highly purified GA fraction from rat liver prepared by the procedure of Morr (24). We found that the two enzymes involved in the biosynthesis of the phosphomannosyl recognition marker on lysosomal enzymes—i.e., α -N-acetylglucosaminylphosphodiesterase and -phosphotransferase—were separable with the latter, comigrating with other GA-associated oligosaccharide-processing activities, such as α -1,2-mannosidase and mannosidase II.

MATERIALS AND METHODS

Reagents. Percoll and Sephacryl-S-1000 were purchased from Pharmacia. AG 1-X8 (formate form, 200–400 mesh) and AG 1-X2 (chloride form, 100–200 mesh) were from Bio-Rad. Yeast hexokinase (EC 2.7.1.7) was from Boehringer Mannheim, and *p*-nitrophenyl- α -N-acetylglucosaminide was from Calbiochem. QAE-Sephadex (stock number Q-25-120) was from Sigma. Endo- β -N-acetylglucosaminidase H from *Streptomyces griseus* was purchased from Miles (endoglycosidase H; Endo H). NaB[3 H]₄ was purchased from Research Products International (Elk Grove, IL). [2- 3 H]Mannose-labeled Man₉GlcNAc oligosaccharide, prepared from Endo H digests of Pronase glycopeptides isolated from NIL-8 cells, was a gift of S. C. Hubbard (Massachusetts Institute of Technology, Cambridge, MA).

D-[1- 14 C]Glucose 6-phosphate (57.4 mCi/mol; 1 Ci = 3.7×10^{10} Bq), GDP-[14 C]mannose (150 mCi/mmol), CMP-[4- 14 C]sialic acid (1.6 mCi/mmol), UDP-[1- 3 H]galactose (10 Ci/mmol), and UDP-N-acetyl-D-[6- 3 H]glucosamine (24 Ci/mmol) were from New England Nuclear. All other chemicals were of reagent grade and obtained from commercial sources.

Preparation of GA Fraction and Subfractions. GA, rough ER, and smooth ER were isolated from the liver of 200- to 250-g male Sprague–Dawley rats (Sasco, Omaha, NE) as described by Morr (24). Electron microscopic examination of the GA fraction showed numerous stacks of cisternae and secretory vesicles and was similar to that published by Morr (24). Biochemical analyses of this fraction showed a 65-fold enrichment of GalTase over homogenate content and a 66-fold enrichment of SiaTase. The former GA marker activity was recovered in a 10% yield, and the latter, in a 9% yield. Markers for ER and plasma membrane were enriched less than 2-fold in the GA fraction. GA subfractions were prepared as follows. The isolated GA was resuspended by using a Dounce homogenizer with a loose-fitting pestle in 0.25 M sucrose in a final volume of 0.5 ml ($\approx$ 2 mg of protein/ml) and loaded onto a 7% solution of Percoll in 0.25 M sucrose (9 ml). A linear gradient was formed by centrifugation at $42,000 \times g$ for 20 min in a Ti 50 rotor at 5°C, following which 0.4-ml fractions were collected by upward flow displacement. The 7% Percoll gradient was made from a stock Percoll solution diluted with 0.25 M sucrose. The stock Percoll solution was prepared by adding solid sucrose to 45 ml of Percoll such that the final concentration of sucrose was 0.25 M in a final volume of 50 ml.

Preparation of [3 H]Oligosaccharide Substrate and Assay for α -N-Acetylglucosaminylphosphodiesterase. Oligosaccharides were released from human spleen β -glucuronidase by Endo H treatment and labeled by NaB[3 H]₄ reduction as described (25). Labeled high-mannose-type oligosaccharides containing a single phosphate in diester linkage were separated from the labeled oligosaccharide mixture by chromatography on QAE-Sephadex (26). α -N-Acetylglucosaminylphosphodiesterase was assayed by using 1,000 cpm of labeled oligosaccharide per assay containing a single phosphodiester as described by Varki and

Kornfeld (27). The assay mixture included N-acetylmannosamine (10 mM) and EDTA (5 mM) to inhibit any lysosomal α -N-acetylglucosaminidase and the GA specific α -1,2-mannosidase. One unit of activity is defined as cleavage of 1% of substrate per hour.

Other Enzyme Assays and Protein Estimation. Protein was estimated by the method of Peterson (28). When Percoll was present, it was removed by precipitation with 4 M NaOH prior to protein determinations. SiaTase with asialofetuin as acceptor was assayed by the procedure of Briles *et al.* (29). GalTase was measured with asialomucin (30) and ovalbumin (31) as acceptors. 5'-Nucleotidase (32), glucose 6-phosphatase (33), β -hexosaminidase (34), thiamine pyrophosphatase (35), and acid phosphatase with β -glycerophosphate as substrate (35) were assayed as described. Mannosyltransferase with dolichol phosphate as acceptor was measured by the procedure of Ravoet *et al.* (36). α -D-Mannosidase was measured by using both 4-methylumbelliferyl and *p*-nitrophenyl substrates (37, 38). Mannosidase II was assayed by using *p*-nitrophenyl- α -D-mannopyranoside in the presence of α -methylmannoside at pH 5.5 as described by Touster and co-workers (37, 39). α -1,2-Mannosidase was measured as the amount of [3 H]mannose released from ([3 H]Man)₉GlcNAc oligosaccharide as described by Tabas and Kornfeld (5). One unit of activity was defined as the release of 100 cpm of [3 H]mannose in 3 hr. Assay of α -N-acetylglucosaminidase by using *p*-nitrophenyl- α -N-acetylglucosaminide as substrate was done as described (40). α -N-Acetylglucosaminylphosphotransferase was assayed as described by Reitman and Kornfeld (41) with α -methylmannoside as acceptor and UDP-N-acetyl[6- 3 H]glucosamine as donor. After incubation at 37°C for 45 min, the reactions were terminated with the addition of 1 ml of 5 mM EDTA (pH 7.8). One unit of enzyme activity is defined as the transfer of 1 pmol of N-acetyl[6- 3 H]glucosamine per hr to α -methylmannoside.

RESULTS

In these studies a highly purified, morphologically preserved, homogeneous GA preparation was desired. Such a preparation was obtained by the procedure of Morr. This procedure has been shown previously to yield intact GA, derived primarily from hepatocytes, and not a subpopulation of vesicles and cisternae (24). Electron microscopic examination of a representative GA preparation showed, as expected, numerous stacks of cisternae and secretory vesicles (data not shown; ref. 24).

We next determined whether the GA-associated enzyme activities could be separated by further subfractionation of the GA fraction on a Percoll density gradient. As shown in Fig. 1A, two major protein peaks of nearly equal size were obtained at densities 1.028 and 1.051 g/ml. Several oligosaccharide-processing enzyme activities that act on glycoproteins at early times after their arrival from the ER were assayed across the gradient. We will present enzymatic activity profiles obtained from individual experiments (see figure legends) that are representative of several fractionations. Figs. 1C and 2A and B show that 70–80% of the activities of mannosidase II, α -1,2-mannosidase, and α -N-acetylglucosaminylphosphotransferase comigrated in a peak with a density of 1.028 g/ml, while the remainder of each activity migrated at a density of 1.051 g/ml. The specific activity of the enzymes was enriched in the former peak by at least 2-fold.

In marked contrast, 80% of the activity of α -N-acetylglucosaminylphosphodiesterase migrated in the region of the denser protein peak (Fig. 2C). The peak enzymatic activity was consistently two to three fractions heavier than the peak protein fraction. The specific activity of the phosphodiesterase was en-

FIG. 1. Distribution of enzyme activities and protein in GA sub-fractions. Isolated GA was subfractionated on a linear Percoll density gradient as described. Fractions (0.4 ml) were collected from the top and analyzed for protein and density (A), SiaTase activity (B), and mannosidase II activity (C). Results shown are from a representative experiment out of a total of 30 for protein profile, 30 for SiaTase activity profile, and 10 for mannosidase II activity profile.

riched 3-fold in this peak compared to that of the peak with density of 1.021 g/ml.

In order to rule out that lysosomal mannosidase or α - and β -N-acetylglucosaminidases were contributing to the enzymatic activity measured in the above assays, these activities were determined across the gradient. α -N-Acetylglucosaminidase activity was negligible throughout, and β -N-acetylglucosaminidase activity was found to comigrate with the mannosidase activity at the bottom of the gradient in a region essentially devoid of GA-associated enzyme activities (not shown). A similar distribution was found also for acid phosphatase activity, often used as a lysosomal marker (not shown).

SiaTase activity, a terminal glycosyltransferase, was found to be distributed nearly equally among the two protein peaks (Fig. 1B). GalTase activity, another terminal glycosyltransferase activity, also was distributed rather equally among the two protein peaks (not shown). The specific activities of both terminal glycosyltransferases were similar in both peaks.

Further characterization of the gradient was achieved by measuring various classical phosphatases throughout. Thiamine pyrophosphatase, an enzyme shown by cytochemistry to be localized in the *trans* GA region (35), was distributed 70% in the low density peak fraction (not shown). The specific activity was enriched 2-fold in this peak. Glucose 6-phosphatase, an ER marker enzyme (with some activity native to the GA) was of low

FIG. 2. Distribution of GA-associated oligosaccharide-processing enzymes. Isolated GA was subfractionated on a linear Percoll density gradient as described. Fractions (0.4 ml) were collected from the top and analyzed for α -1,2-mannosidase activity (A), α -N-acetylglucosaminylphosphotransferase activity (B), and α -N-acetylglucosaminylphosphodiesterase activity (C). Results shown are from a representative experiment out of a total of at least 30 separate experiments for the phosphodiesterase activity and four separate experiments for the other activities.

activity throughout and coincided with the protein distribution across the Percoll gradient. 5'-Nucleotidase activity, a plasma membrane marker activity, was negligible throughout.

The possibility that the two protein peaks (Fig. 1A) were the result of aggregation/deaggregation was next examined. Material from each peak was collected separately, homogenized in a Dounce apparatus, and reapplied to individual Percoll gradients. It was found that the migration of each GA peak did not change, making it unlikely that aggregation-deaggregation was contributing to the separations (Fig. 3).

It was necessary to rule out that the asymmetric distribution of the various enzyme activities across the Percoll gradient was a result of differential activation or inhibition of the activities by Percoll *per se* or that a component in one peak and not the other was preferentially altering the enzyme being assayed. Increasing concentrations of Percoll in enzyme assays did not result in changes of enzyme activities being measured. Also, 90–110% of each activity loaded onto the Percoll gradient could be recovered from the collected fractions, suggesting that Percoll did not preferentially change an activity in one peak over the other. The mixing of an aliquot of the light fraction with that of the heavy fraction did not result in inhibition or stimulation of enzyme activities (not shown).

It was of interest to determine whether the two different

FIG. 3. Isolation of individual peaks of SiaTase activity, followed by reapplication to separate Percoll gradients. Total SiaTase activity of GA subfractionated on a Percoll density gradient (A). Fractions 8–10 composing the low density peak were pooled, treated with a Dounce homogenizer, and rerun on Percoll, after which SiaTase activity was analyzed across the gradient (B). Fractions 19–21 composing the high density peak from A were pooled, homogenized, and rerun on Percoll, after which SiaTase was determined (C).

density GA peaks were morphologically distinct. Electron microscopy showed the light peak to consist mainly of small vesicles, mature very low density lipoprotein-containing secretory vesicles, and a few cisternal elements (not shown). In contrast, the heavy density peak consisted of membranous elements resembling cisternae and large vesicles (not shown). The very low density lipoprotein vesicles of this fraction, in addition to being larger than those of the lighter fraction, were less compact—a characteristic of secretory vesicles often associated with the dilated rims of the GA. The substantial heterogeneity within each fraction does not allow one to correlate them with specific regions of the GA *in vivo*.

DISCUSSION

The separation into two GA subfractions of α -N-acetylglucosaminylphosphodiesterase and -phosphotransferase, two enzymes involved in the biosynthesis of the phosphomannosyl recognition marker of lysosomal enzymes, suggests that they are physically separated within this organelle *in vivo*. Whether or not this means different membrane regions within the same cisternae, different cisternae, different vesicles, or a combination of these possibilities as well as different localization in different types of liver cells is not clear. It should be emphasized that the lack of separation of enzymes being assayed is not necessarily an indication that they occur in the same GA region *in vivo*.

Several controls were performed in these studies to rule out potential artifacts: (i) the distribution of both total and specific activities were measured across the gradient and were always found to follow one another, (ii) the most meaningful substrates were used for assaying α -N-acetylglucosaminylphosphodies-

terase, (iii) changes of enzyme activity in the gradient due to Percoll were ruled out, (iv) contamination by lysosomal enzymes or adsorption of soluble enzymes onto membranes appeared to be unlikely, (v) the amount of content proteins in vesicles appeared to be similar, and (vi) the GA preparation that was applied to the Percoll gradient had been shown previously, both morphologically and biochemically, to be representative of the whole organelle *in vivo* and was obtained in a relatively high yield, based on the recovery of classical GA marker activities such as GalTase and SiaTase.

The recovery yield of α -N-acetylglucosaminylphosphodiesterase activity was similar to that of the above named GA enzymes (8%). The specific activity was 16-fold higher than that in the homogenate. This enzyme activity was recovered in <1% yield in both rough and smooth ER-derived fractions; the enrichment in specific activity (over homogenate) was less than 1-fold in the former and 3-fold in the latter fraction. The α -N-acetylglucosaminylphosphotransferase activity was recovered in a 17% yield with a 43-fold enrichment in specific activity (over homogenate) in the GA fraction. The combined smooth and rough ER-derived fractions contained <2% of this activity; the enrichment in specific activity (over homogenate) was <2-fold in each fraction. We have not determined whether changes of activities of these two enzymes occurred during the obtainment of the unfractionated GA preparation.

Our results do not unequivocally rule out that specific regions of the GA were purified and that the fraction applied to the gradient was not truly representative of the whole organelle *in vivo*. Recent immunoelectron microscopic evidence strongly suggests that GA marker enzymes are *not* evenly distributed across the membranes of the organelle *in vivo* (16); this observation and the morphological heterogeneity of the organelle (42) suggest that one might expect to find in purified GA preparations different yields of different GA enzymes. Our results are consistent with this hypothesis, although until immunoelectron microscopic studies with antibodies against such putative GA enzymes are performed, one will not be able to rule out the possibility that some enzymes attributed to the GA are really part of a different morphological region that copurifies with the GA preparation.

Pohlmann *et al.* (23) observed that α -1,2-mannosidase, α -N-acetylglucosaminylphosphotransferase, and α -N-acetylglucosaminylphosphodiesterase activities comigrated upon sucrose density centrifugation of rat liver microsomal membranes. The authors speculated that these three enzymes occur within the *cis* GA cisternae. Our results suggest that the enzymes responsible for the biosynthesis of the phosphomannosyl recognition marker may reside in separate compartments of the GA *in vivo*. Although our results do not address the question as to where these two compartments lie morphologically, they are probably not contiguous in the same membrane, unless one assumes that vesicles with such restricted enzyme activity domains could be formed after tissue homogenization. We are not aware of such a precedent.

From our results we speculate that α -N-acetylglucosaminylphosphodiesterase is at the site of exit of lysosomal enzymes from the GA. We assume that once the mannose 6-phosphate group is exposed, lysosomal enzymes leave the GA. However, as previously mentioned, this approach does not allow one to define "compartment" and "site of exit" in a morphological sense. We also speculate that this GA exit region may be distinct from that where other GA reactions occur, including the "early reactions" such as those catalyzed by α -1,2-mannosidase and "late reactions" such as those catalyzed by SiaTase and GalTase. In keeping with this, we have consistently observed that the peak activity fraction of α -N-acetylglucosaminylphosphodiesterase is

somewhat heavier than the heavy profile peak fraction. Our observations do not rule out that additional biosynthetic or processing reactions occur on the carbohydrate moieties of lysosomal enzymes between the site and time of addition and removal of the *N*-acetylglucosamine moiety.

Current efforts are directed towards determining whether or not a kinetic relationship between the GA subfractions can be demonstrated by the movement of newly synthesized lysosomal enzymes from one subcompartment to another. While this manuscript was submitted to referees, Goldberg and Kornfeld (43) reported the separation of α -*N*-acetylglucosaminylphosphodiesterase from -phosphotransferase upon sucrose density centrifugation of membranes from lymphoma cells.

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