Sorting of Lysosomal Membrane Glycoproteins lamp-1 and lamp-2 into Vesicles Distinct from Mannose 6-Phosphate Receptor/γ-Adaptin Vesicles at the trans-Golgi Network*

Katrin Karlsson and Sven R. Carlsson‡

From the Department of Medical Biochemistry and Biophysics, University of Umeå, S-901 87 Umeå, Sweden

The trans-Golgi network (TGN) is the main sorting station in the secretory pathway of mammalian cells. At this site, newly synthesized proteins are targeted to their appropriate destinations by incorporation into transport vesicles, mainly through the recognition of specific signals in the proteins. The general view is that the lack of a sorting signal in a protein leads to vesicular transport by default to the cell surface, referred to as the constitutive pathway. All other pathways originating at the TGN, such as those leading to endosomes/lysosomes, storage granules, and apical and basolateral plasma membranes in polarized cells, are believed to require specific structures in the proteins to be sorted (for recent reviews, see Refs. 1 and 2). A common theme for selective vesicular transport is that cargo molecules to be sorted are concentrated in budding structures in the membrane through interaction with receptor molecules. On the cytoplasmic side of the membrane, coat proteins are assembled which are believed to drive the formation of the vesicle. At least in some cases, a direct interaction between cargo and coat proteins seems to be responsible for the specificity of the sorting reaction (3, 4).

The sequence of events leading to the generation of a transport vesicle has been a subject of intense studies during recent years. A key factor for initiation of the assembly of several types of coats is the small cytosolic G-protein ARF (ADP-riboseylating factor) (5–7). When activated by exchange of GTP for GDP by a brefeldin A-sensitive GTP exchange factor, ARF binds to the membrane and triggers the assembly of the coat by a mechanism that is not yet fully elucidated. Several studies have indicated the active involvement of lipid molecules in the formation of transport vesicles. One class of molecules which has a putative role in different vesicle systems is the 3-phosphorylated phosphatidylinositol(3,4,5)P3. Several types of phosphatidylinositol-3-kinases have been identified, some of which are inhibited by the drug wortmannin (11, 12). When added to cells, this drug affects, among other processes, the sorting of lysosomal hydrolases at the TGN, resulting in the secretion of the enzymes to the medium (13, 14). The mechanism by which wortmannin affects endosomal sorting at the TGN is not known.

Of several types of vesicles known to form at the TGN, only the clathrin-coated vesicles (CCVs) have been reasonably well characterized (reviewed in Refs. 15–17). The assembly of clathrin is triggered by the binding of heterotetrameric complexes of adaptor proteins (AP) to the membrane. The adaptor complex specific to the TGN is designated AP-1 and consists of β1, γ, μ1, and σ1 chains (called adaptins). The μ1-adaptin of AP-1 is thought to directly bind to signal sequences in the cytoplasmic domains of cargo molecules, thereby trapping them in the growing bud. A similar system, involving clathrin and an analogous adaptor complex (AP-2), is responsible for the uptake of molecules from the plasma membrane by endocytosis. The best described cargo molecules in AP-1-containing vesicles are the trans-membrane mannose 6-phosphate receptors (MPRs) (18, 19). These receptors recycle between the TGN and endosomes, and act as a shuttle system for newly synthesized, mannose 6-phosphate-modified, lysosomal hydrolases. Two different MPRs, MPR46 and MPR300, both have in their cytoplasmic tails signal sequences that are responsible for the accumulation of the molecules in CCVs. Recent studies have shown that the cytoplasmic tails of MPRs are important determinants for the generation of clathrin vesicles at the TGN per se, indicating that the cargo molecules may drive the formation of its own

* This work was supported by Swedish Medical Research Council Grant 03X-07886 and by the Medical Faculty at Umeå University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom all correspondence should be addressed: Dept. of Medical Biochemistry and Biophysics, University of Umeå, S-901 87 Umeå, Sweden. Tel.: 46-90-7866743; Fax: 46-90-136310; E-mail: sven.carlsson@med kem.umu.se.

The abbreviations used are: TGN, trans-Golgi network; AP, adaptor protein; ARF, ADP-riboseylation factor; CCV, clathrin-coated vesicle; GTPγS, guanosine 5′-3′-O-(thio)triphosphate; FITC, fluorescein isothiocyanate; lamp, lysosome-associated membrane protein; MPR, mannose 6-phosphate receptor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PNS, post-nuclear supernatant.
transport vesicle (20, 21).

Apart from the water-soluble hydrolases, several newly synthesized lysosomal membrane proteins are also sorted at the TGN (22–26). Lysosome-associated membrane proteins 1 and 2 (lamp-1 and lamp-2) are related major molecules in the lysosomal membrane, and consist of two heavily glycosylated amino-terminal luminal domains, a single trans-membrane domain, and a short (11 amino acids) cytoplasmic tail (18, 27–29). The signal for sorting is contained within the carboxy-terminal tail and has the following features critical for recognition by the sorting machinery: a tyrosine located as the fourth residue from the end, a large hydrophobic amino acid as the last residue, and a glycine residue amino-terminal to the tyrosine residue (25, 26, 30). In addition, the distance from the tyrosine to the membrane seems to be of importance for the signal to be functional at the TGN (31). In certain cells, the sorting of lambs at the TGN is inefficient and a fraction of the molecules follows the default pathway to the cell surface. There, the lambs are taken up by endocytosis through a less strict recognition of the same tyrosine-containing signal, and are finally delivered to lysosomes (24, 25, 32, 33).

The mechanism for recognition of the tyrosine signal of lambs at the TGN is not known. A key question is whether lambs utilize the same vesicles as MPRs, or if other pathways exist for intracellular transport of molecules from the TGN to the endosomal system. Although MPRs do have tyrosine-containing signal sequences in their cytoplasmic tails, they seem not to be important for TGN sorting (34–36). Instead, the major sorting motif in MPRs is a di-hydrophobic sequence, or a recently described acidic sequence, located close to the carboxyl terminus (37, 38). In order to investigate the relationship between MPR and lamp sorting in living cells, we took advantage of the finding that the intracellular MPR-dependent sorting of lysosomal hydrolases is sensitive to wortmannin (13, 14). We could show that lambs are incorporated in vesicles that are g-adaptin-containing vesicles. Therefore, at least in the hematopoietic cells investigated, vesicular carrier different from that used by lysosomal hydrolases and MPRs transport lambs from the TGN to the endosomal system.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit antibodies against lamp-1, lamp-2, and leuksialin were as described (39, 40). Mouse anti-galactosyltransferase monoclonal antibody was a generous gift from Dr. E. G. Berger (University of Zürich, Zürich, Switzerland). Goat anti-MPR46 polyclonal antibody was a generous gift from Dr. K. von Figura (Georg-August-University, Göttingen, Germany). Monoclonal antibodies against g-adaptin was purchased from Sigma. Anti-(cathepsin D) was from Calbiochem. Secondary peroxidase-conjugated rabbit anti-(mouse immunoglobulins) and rabbit anti-(goat immunoglobulins) antibodies were both from DAKO, and donkey anti-(rabbit immunoglobulin) was from Amersham Pharmacia Biotech.

Determination of Subcellular Markers—Endosomes were labeled by incubation of HL-60 cells with the fluid phase marker FITC-dextran (Mf 70,000, Sigma) (41). FITC-dextran was dialyzed overnight against PBS and used at a final concentration of 10 mg/ml in RPMI 1640 medium. The cells were incubated at 37 °C for 5 min (to label early endosomes) or 20 min (to label late endosomes). At the end of the labeling period the cells were immediately cooled and washed with ice-cold PBS. FITC-dextran in gradient fractions was measured by fluorescence spectroscopy. Endocytic reticulum was determined by immunoprecipitation of newly synthesized leuksialin from cells pulse-labeled for 10 min with 35Smethionine, as described (40). Plasma membrane was determined by immunoblotting with antibodies against leuksialin, which predominantly is displayed at the cell surface at steady state (40). Golgi membranes were determined by immunoblotting with antibodies against galactosyltransferase. trans-Golgi network was measured by sialyltransferase activity, using asialofetuin as substrate, as described (42), and lysosomes were measured by β-N-acetylgalactosaminidase activity, using 4-methylumbelliferyl-N-acetyl-b-glucosaminide as substrate (20).

Preparation and Labeling of Membranes—HL-60 cells (0.5–1 ml of packed cells), cultured in RPMI 1640 medium containing 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), were washed twice in PBS and once in HES buffer (15 mM Hepes, pH 7.0, 1 mM EDTA, 0.25 mM sucrose). The cells were resuspended in HES buffer and homogenized in a ball-bearing disintegrator (43). The homogenate was centrifuged at 800 x g for 10 min, and the post-nuclear supernatant (PNS) was collected and centrifuged at 20,000 x g for 10 min. The pellet was washed twice with SHM buffer (0.25 mM sucrose, 25 mM Hepes, pH 7.0, 2.5 mM MgAc2) by centrifugation at 20,000 x g for 10 min. The membranes were resuspended in 80 µl of SHM buffer and incubated for 40 min at 30 °C with 600 pmol of dried CMP-[3H]sialic acid (21.3 Ci/mmol, NEN Life Science Products). The radiolabeled membranes were washed twice with SHM buffer before further use.

The radiolabeled membranes were either used directly for generation of vesicles (see below), or further purified. The membranes, resuspended in SHM buffer (150–200 µl), were placed on top of a 4 ml 5–20% (w/v) continuous sucrose gradient in 10 mM Hepes, pH 7.0, 1.8 mM CaCl2, 5.4 mM KCl, 0.8 mM MgCl2, pH 7.8, and centrifuged at 50,000 x g, for 16 h in a SW 41 rotor. The gradient was fractionated into 17 fractions (250 µl each) and analyzed for organelle markers. Fractions containing protein-bound radioactivity (fractions 4–6 from the bottom) were combined, diluted 4-fold with water, and centrifuged at 20,000 x g for 10 min. The pellet was resuspended in 100 µl of SHM buffer and placed on top of an 11-ml 10–50% (w/v) continuous gradient of Nycodenz (Sigma) in 10 mM Hepes, pH 7.0, 0.25 mM sucrose. After equilibrium centrifugation for 18 h at 150,000 x g, in a SW 41 rotor, the gradient was fractionated into 15 fractions (800 µl each) and analyzed for organelle markers and radioactivity. Fractions containing the highest protein-bound radioactivity (fractions 7–8 from the bottom) were pooled and diluted 4-fold with water, centrifuged at 20,000 x g for 10 min, and resuspended in SHM buffer for further use. The pool of TGN membranes from the Nycodenz gradient (banding at 1.17–1.19 g/cm3) contained 28% of the protein-bound label, 13% of late endosomes, and 4% of early endosomes, compared with PNS. Plasma membranes, endoplasmic reticulum, and lysosomes were removed in the sucrose gradient and were not detected in Nycodenz fractions (>1%).

Preparation of Cytosol—HL-60 cells were washed twice in PBS and once in HES buffer, and the cell pellet was resuspended in one volume of HES buffer. The cells were frozen in an ethanol bath at −50 °C and thawed in a 37 °C water bath before being pulled through a 21-gauge needle 30 times. The suspension was centrifuged at 100,000 x g for 1 h in a SW 60 rotor, and the supernatant was collected. The cytosol was stored in aliquots in liquid nitrogen.

Generation and Analysis of Vesicles—Incubations (200 µl) were carried out in Eppendorf tubes for 40 min at 37 °C in the presence of labeled membranes (added as a 50-µl suspension, corresponding to material from 100–200 µl of packed cells, see above), 25 mM KCl, 2.5 mM MgAc2, 10 mg/ml cytosol (or 10 mg/ml bovine serum albumin), together with ATP and an ATP regeneration system (final concentrations 1 mM ATP, 8 mM creatine phosphate, 50 µg/ml creatine kinase, reagents from Boehringer Mannheim). In experiments with no ATP, 30 units/ml apyrase (Sigma) was added to remove ATP in the cytosol. When used, GTP-SγS was at 100 µM and brefeldin A was at 25 µM. After incubation, 1 ml of HES buffer was added and the samples were centrifuged for 10 min at 20,000 x g. Either the vesicle supernatant was centrifuged for 2 h at 100,000 × g in a SW 60 rotor and the pelleted vesicles solubilized in 200 µl of lysis buffer (0.5% Nonidet P-40 and 1 mM phenylmethanesulfonyl fluoride in PBS), or it was subjected to density gradient centrifugation. The vesicle supernatant was placed on top of an 11-ml continuous 5–25% (w/v) Nycodenz gradient in 10 mM Hepes, pH 7.0, 0.25 mM sucrose. After equilibrium centrifugation for 18 h at 150,000 × g, in a SW 41 rotor, the gradient was fractionated into approximately 16 fractions (750 µl each) from the bottom of the tube. The retrieved index was reduced by 25% for each fraction before 4-fold dilution with water and centrifugation for 2 h at 100,000 × g in a SW 60 rotor. The pellets were solubilized as described above.

[35S]Sulfate Labeling of Cells—Cells were washed twice in PBS and once in 116 mM NaCl, 26 mM NaHCO3, 6 mM glucose, 1 mM NaH2PO4, 1.8 mM CaCl2, 5.4 mM KCl, 0.8 mM MgCl2, pH 7.8, and resuspended in 1.5 ml of the same buffer. The cells were pre-incubated for 30 min at
30 °C, after which 0.5 mCi of [35S]sulfate (1600 Ci/mmol, NEN Life Science Products) was added and the cells were pulse-labeled for 3.5 min at 37 °C. The labeling was stopped by the addition of sodium sulfate to 5 mM, and the label was chased by further incubation at 37 °C. At various time points, cells and medium were separated by centrifugation at 250 × g for 5 min. Protein-bound radioactivity was determined by scintillation counting after precipitation with cetylpyridinium chloride (44).

Preparation of [35S]Sulfate-labeled membranes for use in vesicle generation was made by pulse labeling of cells as above, after which the cells were homogenized and membranes were isolated as for [3H]sialic acid-labeled membranes.

Chondroitinase digestion was performed by incubation of a cell lysate, prepared as above, at 37 °C for 2 h in the presence of 1 unit/ml of chondroitinase ABC (Boehringer Mannheim). The reaction was stopped by boiling in sample buffer for electrophoresis.

Pulse-Chase Experiments—K562 cells were chased twice in methionine-cysteine-free RPMI 1640 medium supplemented with 10% fetal calf serum, and pre-incubated for 10 min at 37 °C in 1 ml of the same medium. Fifty μl (0.5 mCi) of PRO-MIX (Amersham Pharmacia Biotech) was added, and the cells were pulse-labeled for 10 min. The labeling was stopped by the addition of 10 ml of complete medium, and the incubation was continued for various periods of time. In cathepsin D experiments, the chase medium was supplemented with 5 mM mannose-6-phosphate to prevent binding to cell surface-exposed MRPs. When present, wortmannin (Sigma) was at 2 μM in both pulse and chase medium. At harvest, cells and medium were separated by centrifugation.

For determination of cell-associated cathepsin D, cells were washed twice in PBS and lysed in 1% Nonidet P-40 in PBS with protease inhibitors (10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin, 10 μM benzamidine, 1 μM o-phenanthroline, and 1 mM phenylmethylsulfonfyl fluoride). After incubation on ice for 30 min, the lysate was cleared by centrifugation at 20,000 × g for 10 min and the supernatant was used for immunoprecipitation. The medium was adjusted to 1% Nonidet P-40, and used for immunoprecipitation after centrifugation as for the cells.

For determination of the total amount of lamp-1 in cells and in lysosomes, cells were homogenized and PNS was prepared as described above. To part of the PNS (one-fourth) was added Nonidet P-40 to 0.5% together with protease inhibitors, and the samples were treated as for cells described above. Lysosomes were isolated from the rest of the PNS (three-fourths) by density centrifugation in 25% (v/v) Percoll (Amersham Pharmacia Biotech) and methanesulfonic acid. After incubation on ice for 30 min, the lysate was cleared by centrifugation at 20,000 × g for 10 min and the supernatant was used for immunoprecipitation. The medium was adjusted to 1% Nonidet P-40, and used for immunoprecipitation after centrifugation as for the cells.

Radioactive proteins in gels were detected either by fluorography, by the Molecular Imager system, or by cutting of the gel in small pieces followed by extraction in Solvable (NEN Life Science Products) and scintillation counting as described (24).

RESULTS

The effect of wortmannin on the sorting of cathepsin D and lamp-1 was studied by pulse-chase experiments on K562 cells. Cathepsin D is normally synthesized as a proenzyme of 53 kDa, which, after sorting at the TGN by recognition of its mannose 6-phosphate residues, is processed to a 47-kDa intermediate form, and subsequently to a two-chain mature form of 31 kDa/14 kDa in the lysosomes (46). Confirming previously published results (13, 14), we found that wortmannin caused the secretion of cathepsin D (Fig. 1). In the presence of the drug, the form of cathepsin D started to accumulate in the medium after 2 h of chase and reached a maximum after 4 h of chase. Without wortmannin, the majority of cathepsin D was present intracellularly at all time points (Fig. 1), and processing to the mature form indicated its arrival to the lysosomes after 4 h of chase (data not shown). Since a previous study showed that the synthesis of mannose 6-phosphate and its recognition by MPR is unaffected by the drug (14), the result indicates an effect of wortmannin on the mechanism of MPR sorting at the TGN. If lysosomal membrane proteins were using the same mechanism for transport, wortmannin would affect the kinetics for arrival to the lysosomes of newly synthesized lamps. A consequence of mis-sorting of lamp-1 at the TGN would be that the molecules are forced to use a slower route to the lysosomes through the cell surface (24). However, in contrast to the result on cathepsin D, no difference was found in the transport kinetics of lamp-1 to the lysosomes without or with the presence of wortmannin (Fig. 1). This may indicate that lamp-1 and MPRs are using different mechanisms for vesicle incorporation at the TGN.

The results on the effect of wortmannin in living cells argue for the presence of two different intracellular routes for transport of molecules from the TGN to the endosomal compartments. If lamps and MPRs are incorporated into different transport vesicles at the TGN, it may be possible to separate vesicles with different properties in vitro. We therefore set up a system for generation of vesicles from the TGN, taking advantage of the fact that the last modification on the glycan chains on glycoproteins, the addition of sialic acid, takes place in this compartment (47, 48). When intracellular membranes are in-
fluorography, and in C shown to the material corresponding to the total amount obtained from a typical extracted and measured by scintillation counting. The four panels show membranes and vesicles by SDS-PAGE. Isolated membranes were collected in the supernatant after centrifugation for 10 min at 18,000 g. Vesicles were formed, which were incubated at 37 °C in the presence of cytosol and ATP. The lamp-1 (C) and lamp-2 (D) proteins were immunoprecipitated from vesicles after solubilization in detergent. Radioactive material in A and B was detected by fluorography, and in C and D the gels were cut in small pieces that were extracted and measured by scintillation counting. The four panels show material corresponding to the total amount obtained from a typical preparation. Migration and molecular weights of standard proteins are shown to the left, and apply to all four panels.

When these membranes were incubated at 37 °C in the presence of cytosol and ATP, vesicles were formed, which were collected in the supernatant after centrifugation for 10 min at 20,000 × g. At optimal conditions, as described below, approximately 25% of the protein-bound radioactivity was released into vesicles. The pattern of glycoproteins in vesicles is shown in Fig. 2B. Although most of the label in vesicles also was in high-molecular weight glycoproteins, the pattern differed somewhat from that of total membranes, showing that incorporation in vesicles was a selective process. The intactness of formed vesicles was shown by the resistance to neuraminidase, and the label could only be removed from the vesicle glycoproteins in the presence of detergent (results not shown).

Both lamp-1 (Fig. 2C) and lamp-2 (Fig. 2D) were immunoprecipitated from the vesicles after solubilization in detergent, and showed the expected molecular weights of 120–150 kDa (27). Due to the low specific radioactivity of glycoproteins obtained with this method, lamps could not be visualized by fluorography but were instead measured by scintillation counting. The results from Fig. 2 (C and D) show that the immunoprecipitates were pure enough to be counted directly in subsequent experiments. Proteins lamp-1 and lamp-2 amounted to approximately 1% each of the total protein-bound radioactivity in vesicles.

The requirements for generation of lamp-containing vesicles were investigated (Fig. 3). For each condition tested, part of the formed vesicles were analyzed for total labeled protein by precipitation with trichloroacetic acid (most likely representing several types of vesicles, see below), and the rest of the vesicles was sequentially immunoprecipitated with anti-lamp-1 and anti-lamp-2 antibodies. The formation of both total vesicles and lamp vesicles was dependent on temperature, ATP, and cytosol. In initial experiments, the dose-response for vesicle production was determined by varying the amount of cytosol. For lamp vesicles, no plateau was obtained by applying up to 10 mg/ml cytosol (which was the upper limit for technical reasons). This indicates that cytosolic factors are limiting in the assay. In subsequent experiments, 10 mg/ml cytosol was used, and in control experiments without cytosol, the total protein concentration was kept constant by the addition of albumin. The time required to obtain maximal response was determined to be 30–40 min, after which there was no further increase in vesicle production (not shown). Therefore, in all experiments, 40-min incubations were used. GTPγS caused a slight reduction in the amount of vesicles produced (Fig. 3). This is similar to the results obtained by Huttner and co-workers (50) for [35S]sulfate-containing vesicles formed from TGN, and may be a reflection of the involvement of both stimulatory and inhibitory trimeric G-proteins. Brefeldin A caused a complete inhibition of vesicle production (Fig. 3). This effect has been demonstrated.

![Fig. 2. Analysis of [3H]sialic acid-labeled glycoproteins in TGN membranes and vesicles by SDS-PAGE. Isolated membranes were labeled with CMP-[3H]sialic acid as described under "Experimental Procedures." A, total labeled glycoproteins from membranes. B, total labeled glycoproteins from vesicles generated by incubation of membranes at 37 °C in the presence of cytosol and ATP. The lamp-1 (C) and lamp-2 (D) proteins were immunoprecipitated from vesicles after solubilization in detergent. Radioactive material in A and B was detected by fluorography, and in C and D the gels were cut in small pieces that were extracted and measured by scintillation counting. The four panels show material corresponding to the total amount obtained from a typical preparation. Migration and molecular weights of standard proteins are shown to the left, and apply to all four panels.](image1)

![Fig. 3. Generation of vesicles from [3H]sialic acid-labeled TGN membranes. Isolated membranes were labeled with CMP-[3H]sialic acid and incubated for 40 min under the conditions indicated. Membranes and vesicles were separated by centrifugation, and the vesicle fraction was analyzed for total labeled glycoprotein by trichloroacetic acid precipitation (total sialoglycoprotein), and for labeled lamp-1 and lamp-2 by immunoprecipitation. Radioactivity was measured by scintillation counting. The results were expressed as percent of the radioactivity in vesicles formed under optimal conditions (complete mixture containing ATP and cytosol incubated at 37 °C; left bar in each panel), and are the means from three independent experiments. The error bars are standard deviations.](image2)
Leukosialin (CD43) is a mucine-like cell surface glycoprotein used for the detection of constitutive vesicles. One such glycoprotein, lamp-1 and lamp-2, were analyzed together, yielding a stronger signal in the gradient fractions. The rationale for this is that it is likely that the two glycoproteins are using the same pathways and vesicles for transport since their trans-membrane domains and cytoplasmic tails are very similar (28). In addition, the kinetics of transport for lamp-1 and lamp-2 is almost identical in HL-60 cells (24). Both total vesicles and lamp-1 and lamp-2 were analyzed together, yielding a stronger signal in the gradient fractions. The rationale for this is that it is likely that the two glycoproteins are using the same pathways and vesicles for transport since their trans-membrane domains and cytoplasmic tails are very similar (28). In addition, the kinetics of transport for lamp-1 and lamp-2 is almost identical in HL-60 cells (24). Both total vesicles and lamp-1 and lamp-2 were analyzed together, yielding a stronger signal in the gradient fractions. The rationale for this is that it is likely that the two glycoproteins are using the same pathways and vesicles for transport since their trans-membrane domains and cytoplasmic tails are very similar (28).

The background in our assay (i.e. the material produced at 4 °C, without ATP, or without cytosol) was high, especially for lamp-1 and lamp-2 (see Fig. 3). In order to examine this background further, material produced at 37 °C with cytosol, with or without ATP, was analyzed by density centrifugation on sucrose gradients (Fig. 4). In this and subsequent experiments, lamp-1 and lamp-2 were analyzed together, yielding a stronger signal in the gradient fractions. The rationale for this is that it is likely that the two glycoproteins are using the same pathways and vesicles for transport since their trans-membrane domains and cytoplasmic tails are very similar (28). In addition, the kinetics of transport for lamp-1 and lamp-2 is almost identical in HL-60 cells (24). Both total vesicles and lamp-1 and lamp-2 were analyzed in the presence of ATP appeared as broad peaks in the middle of the gradients (peak fraction at approximately 1.1 m sucrose), whereas the background material (obtained by omitting ATP and including apyrase to hydrolyze ATP in the cytosol) was found in the lighter fractions. This material probably represents fragments caused by the manipulation of the TGN membranes. The specific peak of total glycoproteins appeared at a slightly higher density than the specific peak of lamps (fraction 5 versus fraction 6 in Fig. 4), but no separation of different vesicles in the total material could be obtained on sucrose gradients.

The population of vesicles formed in the present assay is anticipated to include both vesicles with proteins destined for the plasma membrane and CCVs. To specifically detect different vesicles by immunoprecipitation, only highly sialylated marker glycoproteins could be used. One such glycoprotein, leukosialin, was used for the detection of constitutive vesicles. Leukosialin (CD43) is a mucine-like cell surface glycoprotein and contains more than 100 sialic acid residues (40). In HL-60 cells, newly synthesized leukosialin is transported with fast kinetics to the plasma membrane, most likely by a direct pathway from the TGN (the time for transport from the site of O-glycan addition to the cell surface is approximately 10 min) (40). By immunoprecipitation it was determined that approximately 4% of the protein-bound label was associated with leukosialin in the vesicles generated in the present assay. For CCVs, no good marker with a high content of sialic acid was found. To specifically detect these vesicles, the assay was modified so that the reaction was performed on purified, radioactive TGN membranes, allowing for detection of TGN-derived MPRs and γ-adaptin by immunoblotting.

The generation of glycoprotein-vesicles from purified membranes was as efficient as from crude membranes described above. Addition of GTPγS to the reaction was found to increase the resolution in the analysis of vesicles. This effect was most profound on the migration of MPR/γ-adaptin vesicles, which may indicate that clathrin uncoating of CCVs was prevented by GTPγS. We found that a 5–25% Nycozend gradient in 0.25 m sucrose gave an optimal separation of the detected vesicles when centrifuged to equilibrium (Fig. 5A). Total labeled sialoglycoproteins distributed over almost the whole gradient, with peaks at densities 1.12, 1.13, and 1.20 g/cm³. When analyzed for specific proteins, the least dense peak (ρ = 1.12 g/cm³) was found to contain the majority of leukosialin, and therefore most likely represents plasma membrane-destined vesicles. Certain amounts of lamp-1 and lamp-2 was also detected in light vesicles, but a greater fraction of these glycoproteins migrated at a slightly higher density (ρ = 1.13 g/cm³). The background material described above can also be seen in fractions 11–13. Analysis of the same gradient fractions for MP/46 and γ-adaptin by immunoblotting, revealed that CCVs had a much higher density, banding at approximately 1.19–1.20 g/cm³. This position corresponds to the densest peak in the total vesicle analysis for radioactivity, showing that only a minor part of the sialoglycoproteins is incorporated into CCVs.

The results from density-based separations indicate the presence of at least three different types of vesicles: plasma membrane-destined vesicles, CCVs, and lamp-containing vesicles. Although MPR46 and γ-adaptin were totally absent from the lighter vesicles, lamp and leukosialin were clearly distributed in positions of the gradient corresponding to both types of light vesicles, albeit with different preference. In three separate experiments, the distribution in percentage between the 1.12 g/cm³ and 1.13 g/cm³ peaks was 38/62, 41/59, 46/54 for lamp-1 plus lamp-2, and 59/41, 61/39, 63/37 for leukosialin. It is possible that the sorting of these molecules is not absolute, and that leakage into several types of vesicles may occur. That this may be the case for lamp-1 in living cells is corroborated by the finding of two different transport pathways from the TGN, referred to above.

In order to confirm the position of constitutive vesicles in the gradient, another TGN-specific labeling method was used that admitted detection of secretable proteoglycans (51). In this procedure, living cells were given a short pulse with [35S]sulfate, allowing the label to be incorporated into glycosaminoglycans and proteins by sulfotransferases in the TGN. Control experiment showed that the majority of the labeled material (approximately 65%) could be chased to the medium, with a half-time of 10 min. Almost all (>90%) of the label was sensitive to chondroitinase ABC, showing that it was mainly incorporated into chondroitin sulfate proteoglycans (results not shown). When vesicles formed from [35S]sulfate-treated membranes were analyzed by Nycozend gradient centrifugation (Fig. 5B), the majority of the label migrated to a position with
a density of 1.12 g/cm$^3$. In addition, an even lighter ($\rho \sim 1.10$ g/cm$^3$) putative vesicle population was detected with this system. Leukosialin, which was previously shown to contain sulfated carbohydrate chains (52), distributed similarly to chondroitin sulfate proteoglycans and to $[^3H]$sialic acid-labeled leukosialin (Fig. 5, compare A and B). It is therefore likely that vesicles represented by leukosialin correspond to intermediates in the direct pathway from the TGN to the plasma membrane. Neither lamp-1 nor lamp-2 was detected by $[^35S]$sulfate labeling.

If the effect of wortmannin treatment of cells, shown in Fig. 1, is due to inhibition of CCV formation at the TGN, this effect should be possible to reproduce in the in vitro system described here. As shown in Fig. 6, the generation of vesicles containing MPR46 and γ-adaptin was inhibited by wortmannin, showing a half-maximal response at $<300$ nM. In contrast, neither lamp- nor leukosialin-containing vesicles were affected by the drug up to $3 \mu M$. This result confirms that constitutive vesicles are not sensitive to wortmannin, and that lamps are incorporated into vesicles at the TGN that are separate from those carrying MPRs.

**DISCUSSION**

The present study shows that lysosomal membrane proteins lamp-1 and lamp-2 can be packaged into vesicles at the TGN that are different from AP-1-decorated vesicles carrying MPRs and lysosomal hydrolases. This finding is in discordance with the results of several recent reports, indicating that lamps and MPRs are co-transported in the same type of vesicle from the TGN. The view that lysosomal membrane proteins may be assembled into CCVs is based on the following observations. First, peptides corresponding to the cytoplasmic tail of lamp-1 specifically bound to AP-1 (and AP-2) in vitro, both in column experiments and as detected by surface plasmon resonance (53). Second, in a yeast two-hybrid system, the $\mu$-chain of AP-1 (and the $\mu$-chain of AP-2) was shown to interact when the lamp-1 sorting signal was used as the binding partner (54, 55). Third, immune electron microscopy visualized vesicles containing both lamp-1 and γ-adaptin in the trans region of the Golgi complex (53).

Even though the above observations are strong arguments for CCVs as the responsible vesicles for lamp transport, we are challenging this view by showing that lamps can be found in separate vesicles when generated from the TGN in vitro. In addition, we show that this separation of pathways also operates in vivo, inasmuch as the pathways are differently sensitive to wortmannin. How, then, can these apparently contradictory results be explained? There is no doubt that tyrosine-signals have the ability to specifically interact with AP-1 and AP-2 in vitro. At the plasma membrane, it is indeed very likely that...
trans-membrane proteins are recognized through a direct interaction between their tyrosine signals and the μ-2-chain of AP-2 (10). These membrane proteins often have their signals far out from the trans-membrane domains, in a spatially favorable position for this interaction. If the signal in the lamp cytoplasmic tails is recognized by an AP complex, this would probably require interactions of the complex also with the phospholipid membrane (the maximal distance from the tyrosine to the membrane is only approximately 15 Å). Such an interaction would in turn require distinctive properties of the complex for lamp recognition that is not needed for binding of tyrosine signals located farther out or for the signals in MPRs. It would appear more plausible that proteins in the membrane serve as receptors for lamp signals; such sorting receptors could in addition interact with the well conserved trans-membrane domains of lamps. Indeed, a recent study showed that the nature of the trans-membrane domain influences the trafficking of lamp-1 in cells (56). Nevertheless, since μ-adaptins obviously have a recognition site for tyrosine signals, peptides corresponding to the lamp cytoplasmic sequence are expected to bind in vitro. However, it is possible that this interaction never occurs in vivo, or that the affinity is much lower than that measured in vitro due to the proximity of the signal to the membrane.

An alternative explanation for the difference between our results and the observations described in the literature is that sorting events may be cell-specific. It is well known that specialized cells behave differently in terms of organelle biogenesis (2). The hematopoietic cell lines used in the present study, HL-60 and K562, are characterized as promyelocytes and pro-erythroblasts, respectively, which both have the capacity to differentiate into granulocytes and macrophages (20). The secretions of lysosomal hydrolases caused by wortmannin treatment of living cells would then be a consequence of a lack of receptors at the TGN. However, in an in vitro system, we obtained a clear effect by wortmannin not only on MPRs, but also on γ-adaptin in generated vesicles. This would also be the expected result in vivo if MPRs indeed are important for the recruitment of γ-adaptin to the membrane (20, 21). In the study of Gaffet et al. (64), a reduction of γ-adaptin vesicles was found by prolonged incubation (2 h) of living cells with wortmannin. In any case, since the production of γ-adaptin-containing vesicles was inhibited by wortmannin in the in vitro system presented here whereas lamp-containing vesicles were not, this observation gives additional evidence for two separate pathways regardless of the mechanism of inhibition.

So far we have no clue as to the nature of lamp-containing vesicles formed at the TGN, and work is under way to define its coat components. Due to the increasing number of transport pathways identified in cells, one can predict that the few types of structures hitherto known will be followed by the identification of several new types of coats. In yeast cells, three parallel pathways mediate transport from the Golgi body to the cell surface (2), and two pathways are identified that carry molecules to the endocytic system (66). The packaging of molecules into various vesicular structures at the TGN must involve distinct mechanisms and many specific protein interactions for the sorting to be effective. It will be important in the future to elucidate these processes at the molecular level.

Acknowledgments—We thank Dr. H. Semb for critically reading the manuscript, and Drs. K. von Figura and E. G. Berger for antibodies.

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FIG. 6. The effect of wortmannin on the generation of TGN vesicles in vitro. Membranes were labeled with CMP-[3H]sialic acid and incubated at 37 °C with ATP and cytosol in the absence or presence of indicated concentrations of wortmannin, or with apyrase and cytosol as a negative control. Formed vesicles were collected and proteins were quantified as described in the legend to Fig. 5. The amount of the different molecules in vesicles generated in the presence of wortmannin is expressed as the specific release relative to the uninhibited reaction and to the reaction inhibited by apyrase, which was set to 100% and 0%, respectively, for each data set. The data are the means from three independent experiments, and error bars are standard deviations.
