

# Low Density Lipoprotein Receptor and Cation-independent Mannose 6-Phosphate Receptor Are Transported from the Cell Surface to the Golgi Apparatus at Equal Rates in PC12 Cells

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**Abstract.** Efficient transport of cell surface glycoproteins to the Golgi apparatus has been previously demonstrated for a limited number of proteins, and has been proposed to require selective sorting in the endocytic pathway after internalization. We have studied the endocytic fate of several glycoproteins that accumulate in different organelles in a variant clone of PC12, a regulated secretory cell line. The cation-independent mannose 6-phosphate receptor and the low density lipoprotein receptor, both rapidly internalized from the cell surface, and the synaptic vesicle membrane protein synaptophysin, were transported to the Golgi apparatus with equivalent, nonlinear kinetics. Transport to the Golgi apparatus ( $t_{1/2} = 2.5\text{--}3.0$  h) was several times faster than turnover of these proteins ( $t_{1/2} \geq 20$  h), indicating that transport of these proteins to the Golgi apparatus occurred on average

several times for each protein. In contrast, Thy-1, a protein anchored in the membrane by a glycosylphosphoinositide group, was internalized and transported to the Golgi apparatus more slowly than the three transmembrane proteins. Since each of the transmembrane proteins studied showed the same  $t_{1/2}$  for transport to the Golgi apparatus, we conclude that transport of these proteins from the cell surface to the Golgi apparatus does not require sorting information specific to any one of these proteins. These results suggest that one of the functions of late endosomes is constitutive recycling of cell surface receptors through the Golgi apparatus if they fail to recycle to the cell surface directly from early endosomes, and that the late endosome recycling pathway is followed frequently by many rapidly internalized proteins.

**E**FFICIENT transport of specific plasma-membrane proteins or their bound ligands from the cell surface to the Golgi apparatus has recently been documented in a variety of cell types (12, 14, 24, 52, 53, 56). This transport pathway has been presumed to be selective for a subset of cell surface proteins since the majority of plasma membrane glycoproteins rarely return to the Golgi apparatus (12, 53). Since endocytic transport of fluid phase or bulk plasma membrane markers to the Golgi apparatus cannot be detected morphologically in many cell types (6, 23, 49, 50, 57, 61), it has been concluded that endocytic traffic to the Golgi apparatus represents a very small fraction of the vesicular traffic in the endocytic pathway. These results have given rise to the idea that efficient transport of membrane proteins such as the mannose 6-phosphate receptors from the cell surface to the Golgi apparatus requires the presence of specialized sorting determinants not present on proteins such as the low density lipoprotein receptor (27).

Cell types specialized for protein secretion exhibit significantly more endocytic traffic directed to the Golgi apparatus than do the non-secretory cell types that have been used to study receptor traffic to the Golgi apparatus. There

is indirect biochemical evidence that many secretory granule membrane proteins in the exocrine pancreas are recycled to the Golgi apparatus after exocytosis (40). Also, the Golgi apparatus is readily labeled with bulk endocytic tracers in cells that are actively secreting protein (13, 22, 47, 48, 54), suggesting that total vesicular traffic from the cell surface to the Golgi apparatus is increased during and after active protein secretion. These observations suggested to us that secretory granule membrane proteins may have sorting determinants that permit them to be selectively transported from the cell surface to the Golgi apparatus.

To test this hypothesis, we established a system based on the method of Duncan and Kornfeld (12) to quantitate the transport of cell surface glycoproteins to the Golgi apparatus in the regulated secretory cell line PC12. We showed that many cell surface glycoproteins of PC12 cells recycle through the Golgi apparatus efficiently (19). To establish a basis for comparison of secretory granule membrane proteins and other membrane proteins, we have now examined endocytic transport to the Golgi apparatus of several specific membrane proteins exhibiting distinct steady state distributions in these cells. These markers are: Thy-1, a glycosyl-

phosphoinositide (GPI)<sup>1</sup>-anchored membrane protein which is found predominantly on the cell surface (32); low density lipoprotein receptor (LDL-R), a plasma membrane receptor that recycles rapidly through early endosomes (17); synaptophysin, a synaptic vesicle marker in neuroendocrine cells (10, 44) which is found in dense core granules at low or undetectable levels (11, 51); and the cation-independent mannose 6-phosphate receptor (CI-MPR), which is concentrated in late endosomes (27) and has been shown to undergo transport from the cell surface to the Golgi apparatus in other cell types (12, 24). All three transmembrane proteins recycle to varying extents through the cell surface and early endosomes (17, 27, 33, 51). We have so far been unable to measure transport of SGM110 (21), a dense core granule membrane marker.

We have found that in PC12 cells Thy-1 was transported to the Golgi apparatus slowly, similar to the delivery of total plasma membrane glycoproteins to the Golgi apparatus. In contrast, and to our surprise, three transmembrane proteins (LDL-R, synaptophysin, and CI-MPR) were transported from the cell surface to the Golgi apparatus with rapid, non-linear kinetics at virtually identical rates ( $t_{1/2} \sim 2.5-3$  h), suggesting that this endocytic pathway is not selective. We postulate that membrane proteins that normally recycle from early endosomes to the cell surface, and are transported to late endosomes when they fail to recycle, can exit late endosomes with high efficiency and return to the cell surface via the Golgi apparatus. Recycling from early and late endosomes may operate with similar efficiency and possibly by a similar mechanism to remove functional receptors from the lysosomal pathway.

## Materials and Methods

### Radioisotopes

UDP-[<sup>3</sup>H]galactose and [<sup>125</sup>I]sodium iodide were purchased from Amersham Corp. (Arlington Heights, IL). UDP-[<sup>3</sup>H]galactose was also purchased from American Radiochemical Corporation (St. Louis, MO), as was [<sup>3</sup>H]glucosamine. Translabel <sup>35</sup>S-amino acid mixture was from ICN Bio-medicals, Inc. (Irvine, CA).

### Enzymes

*Flavobacterium meningosepticum* peptide: N-glycosidase F (PNGase F) and *Diplococcus pneumoniae*  $\beta$ -galactosidase were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Bovine milk galactosyltransferase was from Sigma Chemical Co. (St. Louis, MO). *Bacillus thuringiensis* phosphoinositide-specific phospholipase C (37) was a gift of Martin Low (Columbia University, New York).

### Cells

Ricin-resistant PC12 clone A1 cells were grown as originally described (19). Since variant PC12 cells are less adherent than wild-type cells, they were generally plated for experiments on dishes precoated with poly-D-lysine. C7 hybridoma cells (2) producing mAbs that recognize the human LDL-R were obtained from Geoffrey Davis (University of California, San Francisco, CA) and grown in  $\alpha$ -modified MEM (Cell Culture Facility, University of California, San Francisco, CA) containing 10% heat-inactivated FBS.

1. *Abbreviations used in this paper:* CI-MPR, cation-independent mannose 6-phosphate receptor; LDL-R, low density lipoprotein receptor; NHS-SS-biotin, sulfo-succinimidyl 2-(biotinamido) ethyl-1,3'-dithiopropionate; PNGase F, peptide:N-glycosidase F; TGN, trans-Golgi network.

## Antibodies

Ascites fluid containing mAb OX-7 recognizing rat Thy-1 (39) was purchased from Serotec Ltd. (Oxford, U.K.). Polyclonal rabbit antisera against bovine or rat CI-MPR (7) were the gift of William Brown (Cornell University, Ithaca, NY). Both antisera gave identical results and were used interchangeably. Rabbit antiserum recognizing synaptophysin was raised against antigen that was gel purified from rat brain synaptic vesicles (10). mAb SY38 recognizing synaptophysin was purchased from Boehringer Mannheim Corp. Ascites fluid containing mAb C7 recognizing human LDL-R (2) was produced in pristane-primed BALB/c mice and was used unfractionated. Affinity-purified rabbit anti-mouse IgG and affinity-purified goat anti-rabbit IgG were from Organon Teknika-Cappel (Malvern, PA). Streptavidin for detection of biotinylated proteins was from Gibco-BRL (Gaithersburg, MD). Rhodamine-conjugated sheep anti-rabbit IgG was from Cappel Laboratories (Malvern, PA), biotin-conjugated horse anti-mouse IgG was from Vector Labs., Inc., (Burlingame, CA), and fluorescein-conjugated streptavidin was from Molecular Probes, Inc. (Eugene, OR).

## Expression of Human LDL-R in PC12 A1 Cells by Transfection

The plasmid pLDLR2 containing the cDNA encoding human LDL-R under a cytomegalovirus promoter (63) was kindly provided by Dr. C. Geoffrey Davis (University of California, San Francisco, CA). Ricin-resistant PC12 A1 cells on two poly-D-lysine-coated 10-cm dishes were cotransfected with 36  $\mu$ g of pLDLR2 plasmid and 6  $\mu$ g of pRSVneo, encoding aminoglycoside phosphotransferase (18), per dish using the lipofection protocol of Muller et al. (42). Cells were passaged into medium containing 0.4 mg/ml G418 (Gibco Laboratories, Grand Island, NY) two days after transfection. Drug-resistant clones were picked using cloning rings, expanded, and screened for surface expression of human LDL-R as follows. Clones were plated in poly-D-lysine-coated tissue culture wells and grown to 70-80% confluence. The medium was replaced with ice cold medium containing 1  $\mu$ Ci <sup>125</sup>I-C7 mAb (provided by Dr. Yasufumi Kaneda, Kelly Lab, University of California, San Francisco, CA) and the cells were incubated for 2 h on ice. The cells were washed three times with PBS containing 1% BSA, once with PBS, and then collected on cotton swabs. Swabs were counted in a Gamma 4000 counter (Beckman Instruments, Inc., Fullerton, CA). Five of 46 screened clones showed binding of labeled C7 antibody to the cell surface, and were rescreened by metabolic labeling with <sup>35</sup>S-amino acids followed by immunoprecipitation as described below. One clone, PC12 A1-L14 expressed 2.5-4-fold higher levels of human LDL-R than the others in both screens, and was used for all subsequent experiments. Expression of human LDL-R in these cells decreased with time in culture, so cells from the initial expansion of the clone were passaged 15-20 times and then discarded.

## Immunofluorescence Labeling and Microscopy

PC12 clone A1 or clone A1-L14 cells were grown on poly-D-lysine-coated coverslips. Some coverslips were incubated for 30 min in serum-free medium containing 0.2 mg/ml BSA and 500 mU/ml phosphoinositide-phospholipase C before fixation. The cells were washed twice in PBS and fixed for 15 min at room temperature in 3% formaldehyde in PBS. The cells were rinsed and washed in PBS; 50 mM glycine for 5 min and then permeabilized in PBS; 0.01% saponin; 5 mg/ml ovalbumin (immunofluorescence buffer) for 30 min. Primary and secondary antibodies, and fluorescein-streptavidin were diluted in immunofluorescence buffer. Incubations were for 1.5 h in primary antibodies diluted 1:150 followed by 1 h in secondary antibodies diluted 1:150 (biotin-horse anti-mouse IgG) or 1:600 (rhodamine-sheep anti-rabbit IgG), and 1 h in fluorescein-streptavidin diluted 1:125, with 3  $\times$  15 min washes in IF buffer between each incubation. After the final wash, cells were washed for 5 min in PBS, rinsed in distilled water, and mounted in 90% glycerol, 7.5% PBS, 2.5% 1,4-diazabicyclo (2.2.2) octane (Sigma Chemical Co.) (25).

## Cell Surface Biotinylation and Internalization Assay

The internalization of cell surface membrane proteins was measured using the reducible biotinylation reagent sulfo-succinimidyl 2-(biotinamido) ethyl-1,3'-dithiopropionate (NHS-SS-biotin) (Pierce Chemical Co., Rockford, IL) as previously described (31). Cells were washed three times with ice-cold PBS and then biotinylated with 1 mg/ml NHS-SS-biotin in PBS (from a 200 mg/ml stock in DMSO) twice for 15 min. Cells were then washed twice with ice-cold MEM lacking bicarbonate and containing 10 mM Hepes, pH 7.2, and 0.2% BSA (MEM/BSA). Some cells were then warmed

to 37°C in MEM/BSA for varying times, then returned to 0°C. Cells were then washed with ice-cold PBS containing 10% horse serum, and the biotin remaining on the cell surface was removed by two 20-min incubations on ice in 75 mM NaCl, 15 mg/ml reduced glutathione, 10% horse serum, 0.3% NaOH. After reduction, cells were washed in MEM/BSA, and the remaining glutathione was quenched with a 15-min incubation in PBS containing 1% horse serum and 5 mg/ml iodoacetamide. Cells were then harvested in PBS and lysed in 1% NP-40, 150 mM NaCl, 10 mM Tris, pH 7.8, 1 mg/ml iodoacetamide, and 1% BSA.

### Exogalactosylation

Exogalactosylation of cells was carried out as described (5, 12, 59). Cells on polylysine-coated 15-cm tissue culture plates were labeled at 75–90% confluence, usually 1–2 d after plating. Cells were cooled to 0°C and washed twice with ice-cold exogalactosylation buffer (5) (MEM without glucose or bicarbonate containing 1% FBS, 2 mM glutamine, and 10 mM Hepes, pH 7.3) or buffer B (26) (MEM without glucose, bicarbonate, or phosphate containing 0.2% BSA and 10 mM Hepes, pH 7.3). The labeling medium contained Exogalactosylation buffer or buffer B with the following additions: 4 mM MnCl<sub>2</sub>, 1.0–1.5 U/ml galactosyltransferase, and 180–250  $\mu$ Ci/ml UDP-[<sup>3</sup>H]galactose. Cells were labeled with 3 ml per 15-cm dish for 50 min on ice. At the end of the labeling all cells were washed twice with ice-cold PBS containing 1 mg/ml glucose. Cells were recultured in complete culture medium at 37°C. All cells were washed twice with ice-cold PBS and once with PBS lacking divalent cations before harvesting in ice-cold PBS containing 5 mM EDTA by trituration. Cells were lysed in 600  $\mu$ l 1.3% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.8, 5 mM EDTA containing freshly added proteinase inhibitor cocktail (added from a 1,000 $\times$  stock containing 10 mg/ml each of pepstatin, chymostatin, leupeptin, and aprotinin in DMSO, and a 200 $\times$  stock containing 200 mM PMSF, 2 mM benzamidine, and 200  $\mu$ g/ml *o*-phenanthroline in ethanol).

### Metabolic Labeling

10-cm dishes of cells at  $\sim$ 70% confluence were metabolically labeled overnight with [<sup>3</sup>H]glucosamine by addition of 0.2 mCi/ml of the labeled compound to growth medium containing 0.5 mg/ml glucose with or without 6 mM sodium butyrate. Addition of sodium butyrate was necessary to increase expression of human LDL-R driven from a cytomegalovirus promoter to a level that could be detected by fluorography of [<sup>3</sup>H]glucosamine-labeled precipitates within a reasonable time. Since sodium butyrate alters the pattern of gene expression in PC12 cells (8, 43), it was not used in any other experiments presented in this paper. Metabolic labeling of 10-cm dishes of cells at  $\sim$ 50% confluence with <sup>35</sup>S-amino acids was carried out for 1 h in MEM lacking methionine and cysteine, and containing 10% dialyzed FBS and 0.25 mCi/ml Translabel <sup>35</sup>S-amino acid mixture. Chase incubations were initiated by washing the labeled cells once in growth medium and reculturing in growth medium supplemented with 3 mM methionine and 3 mM cysteine. All metabolically labeled cells were washed twice with ice-cold PBS and harvested by trituration in PBS containing 1 mM EDTA before lysis. [<sup>3</sup>H]glucosamine-labeled cells were lysed in 300  $\mu$ l 1% NP-40, 150 mM NaCl, 10 mM Tris, pH 7.8. <sup>35</sup>S-amino acid-labeled cells were lysed in 400  $\mu$ l 1% Triton X-114, 150 mM NaCl, 10 mM Tris, pH 7.8.

### Immunoprecipitation

Lysates were centrifuged at 30,000 *g* for 15 min. Cleared <sup>35</sup>S-amino acid-labeled Triton X-114 lysates were phase partitioned at 37°C (4) and the detergent phase diluted to the original lysate volume (with 150 mM NaCl, 10 mM Tris, pH 7.8) was carried forward. All other lysates were adjusted to 2% (wt/vol) BSA. Since low stringency conditions were used to maximize recovery of labeled antigens, lysates for all immunoprecipitation experiments were pre-cleared three times with fixed *Staphylococcus aureus* cells (Zymed Laboratories, Inc., South San Francisco, CA) suspended in PBS containing 1% NP-40 and 0.2% SDS. Primary antibodies were added to the pre-cleared lysates and incubated for 2–18 h at 4°C. Appropriate secondary antibodies were added and incubated for 1–2 h. Fixed *S. aureus* cells were then added and incubated for 1 h with constant mixing. After recovery of the precipitates, lysates were pre-cleared with additional *S. aureus* cells for 30 min before the addition of the subsequent primary antibody to preclude possible contamination of later precipitations with earlier antibodies. Sequential precipitation of the four antigens studied was performed in different orders, in several experiments, with identical results. Precipitates were washed twice with PBS, 1% NP-40, 0.2% SDS, once with 50 mM

NaCl, 5 mM Tris, pH 7.4, and eluted by heating in a boiling water bath for 3 min either into gel sample buffer containing either 40 mM DTT (<sup>35</sup>S-amino acid label) or 1 mg/ml iodoacetamide (biotinylation experiments), or into PNGase F buffer (1% octylglucopyranoside, 0.2% SDS, 40 mM Tris, pH 8.0, 5 mM EDTA, 1%  $\beta$ -mercaptoethanol) (exogalactosylation and [<sup>3</sup>H]glucosamine-labeling experiments). [<sup>3</sup>H]glucosamine-labeled proteins in PNGase F buffer were mixed with an equal volume of 2 $\times$  concentrated gel sample buffer containing DTT and 0.5 vol of 10% SDS, and heated in boiling water again before SDS-PAGE.

### Analysis of N-linked Oligosaccharides After Exogalactosylation

N-linked oligosaccharides were analyzed using a minor modification (19) of the method of Duncan and Kornfeld (12). 2  $\mu$ l of the centrifuged cell lysate from exogalactosylated cells was removed before pre-clearing, mixed with 40  $\mu$ l PNGase F buffer, and heated in boiling water for 3 min for analysis of total oligosaccharides. This sample, as well as immunoprecipitated proteins eluted into PNGase F buffer, were cooled to room temperature and incubated 14–18 h at 37°C with 5 mU PNGase F. The samples were then cooled to 0°C, precipitated with 25  $\mu$ l 20% TCA, 1% phosphotungstic acid, 0.5 M HCl for 5 min, and centrifuged at 16,000 *g* for 8 min at 4°C. To minimize loss of  $\alpha$ -linked sugar residues by acid hydrolysis, the supernatants were immediately adjusted to pH 7.5–9.0 with 4  $\mu$ l 10 N NaOH and 1.6–1.8  $\mu$ l saturated aqueous Tris base, and desalted into distilled water by gel filtration chromatography on a 0.6  $\times$  22-cm column of Sephadex G25 (Pharmacia Fine Chemicals, Piscataway, NJ). Radioactive fractions were pooled, dried under vacuum, and dissolved in 40  $\mu$ l 50 mM MES, pH 6.0. Redissolved oligosaccharides were incubated for 15–18 h at 37°C with 10 mU  $\beta$ -galactosidase. An additional 5 mU  $\beta$ -galactosidase was then added, and the incubation continued for 3–5 h. Samples were boiled for 3 min to inactivate the enzyme, sucrose was added to a final concentration of 5–10% (wt/vol). The samples were then analyzed by gel filtration chromatography on Sephadex G25. 350- $\mu$ l fractions were counted in 4 ml Ecolume (ICN Biomedicals, Inc., Costa Mesa, CA) scintillation fluid.

### Electrophoresis, Fluorography, and Quantitation of Radioactivity in Gel Bands

SDS-polyacrylamide gels were run as described (28). For fluorography, gels were fixed in 10% methanol/5% acetic acid, rinsed with water, and impregnated with 0.5 M sodium salicylate according to the method of Chamberlain (9). The dried gels were exposed to preflashed (30) XAR5 film (Eastman Kodak Company, Rochester, NY) at  $-70^\circ\text{C}$ . For quantitation of radioactivity in <sup>35</sup>S-labeled gel bands, gels were fixed and dried without salicylate treatment, and analyzed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

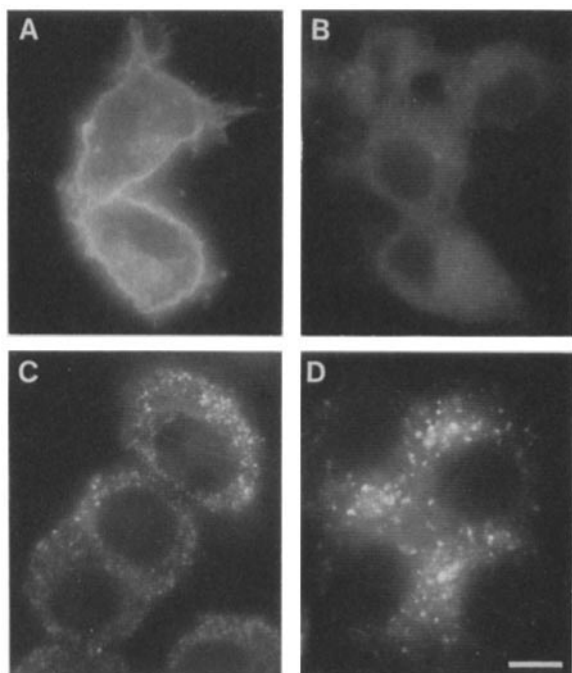
### Detection of Biotinylated Cell Surface Proteins

Immunoprecipitates from the cell surface biotinylation experiments were resolved on SDS-polyacrylamide gels polymerized from 12% (wt/vol) (synaptophysin and Thy-1) or 8% (wt/vol) (LDL-R and CI-MPR) acrylamide, the proteins were transferred to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, NH), and probed with <sup>125</sup>I-streptavidin. Iodination of the streptavidin with chloramine T and blocking and probing the transfer were carried out as described by Lisanti et al. (34). The dried filters were exposed to film with an intensifying screen for photography, and were analyzed on the phosphorimager for quantitation of radioactivity.

## Results

### Expression of Human LDL-R in Ricin-resistant PC12 Cells

We set out to identify membrane proteins that were selectively targeted to the Golgi apparatus after endocytosis in PC12 cells. We therefore sought markers for the plasma membrane and for other endocytic compartments as a basis for comparison. LDL-R is one of the best characterized receptors that undergoes constitutive endocytosis. Because the available antibodies recognize human LDL-R and not the

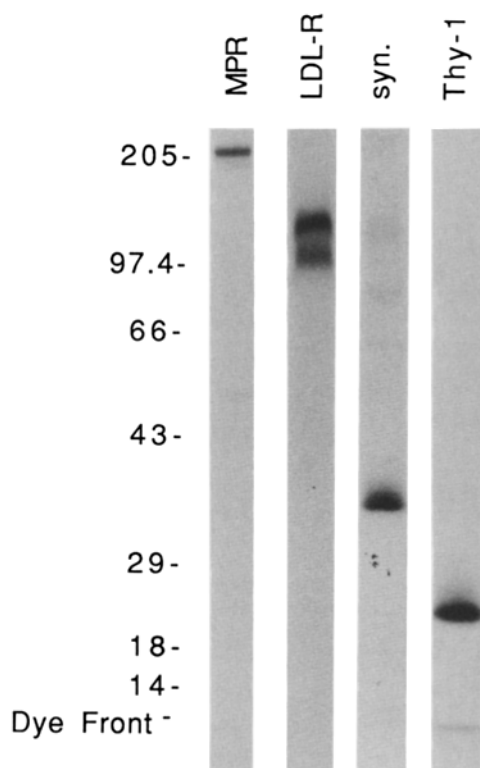


**Figure 1.** Immunofluorescence localization of CI-MPR, synaptophysin and Thy-1 in PC12 A1-L14 cells. PC12 A1-L14 cells were processed for indirect immunofluorescence microscopy either after treatment for 30 min at 37°C with phosphoinositide-specific phospholipase C (*B*) or without pretreatment (*A*, *C*, and *D*). The cells were labeled with mouse mAbs recognizing Thy-1 or synaptophysin, or with rabbit polyclonal antibodies recognizing CI-MPR. mAbs were localized with biotinylated second antibody and fluorescein-conjugated streptavidin, and polyclonal antibodies with rhodamine-conjugated second antibody. Thy-1 labeling (*A*) was localized primarily to the cell surface, and was largely eliminated by prior incubation of the cells with phosphoinositide-specific phospholipase C (*B*). Synaptophysin labeling was found in numerous small intracellular structures distributed throughout the cytoplasm (*C*). CI-MPR was found in structures of heterogeneous size that were concentrated in the juxtannuclear region (*D*). Thus, each of these proteins displayed a distinct steady-state distribution in these cells. Bar, 2  $\mu$ m.

endogenous rat receptor, we generated a clone of the ricin-resistant PC12 A1 clone expressing human LDL-R. PC12 A1 cells are deficient in the incorporation of galactose residues into glycoproteins, making them suitable for measurement of transport of glycoproteins from the cell surface to the Golgi apparatus (see below). The clone, designated PC12 A1-L14, expressed LDL-R that was readily detected by immunoprecipitation of receptor from lysates of metabolically labeled cells with C7 antibody. These cells were used for all experiments presented in this study.

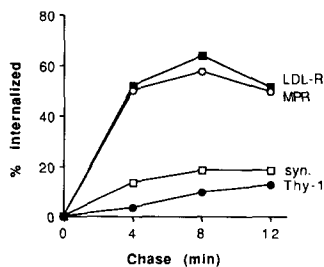
#### **Immunofluorescence Localization of Antigens in PC12 A1-L14 Cells**

We wished to determine whether endocytic transport to the Golgi apparatus was selective for any of several organelle markers. Indirect immunofluorescence labeling of PC12 A1-L14 cells was performed to determine whether the steady state distributions of the marker proteins chosen for study were distinct in PC12 cells. As in other cell types, Thy-1 immunofluorescence was found predominantly at the cell sur-



**Figure 2.** Immunoprecipitation of [ $^3$ H]glucosamine-labeled proteins from PC12 A1-L14 cells. PC12 A1 cells expressing human LDL-R (clone L14) were labeled with [ $^3$ H]glucosamine in the presence (*LDL-R*) or absence (*MPR*, *syn.*, *Thy-1*) of 6 mM sodium butyrate for 20 h before detergent lysis. MPR, LDL-R, synaptophysin (*syn.*) and Thy-1 were sequentially immunoprecipitated from the lysates and eluted from the adsorbent as described in Materials and Methods. The eluted proteins were resolved by electrophoresis on a 7–15% SDS-polyacrylamide gel, and visualized by fluorography. Exposure times varied from 1 d (*MPR*, *LDL-R*, and *Thy-1*) to 4 wk (synaptophysin). Numbers indicate the position of molecular mass markers in kilodaltons. Virtually all of the radioactivity immunoprecipitated by each antibody migrated as a single band of the appropriate mobility, except LDL-R which migrated as two bands.

face (Fig. 1 *A*). Thy-1 cell surface labeling was virtually abolished by pretreating the cells with phosphoinositid-specific phospholipase C (Fig. 1 *B*), demonstrating that Thy-1 expressed in PC12 cells is associated with the plasma membrane via a GPI moiety. Synaptophysin antibodies labeled small punctate structures distributed throughout the cytoplasm (Fig. 1 *C*), consistent with the previously described localization of this protein in small synaptic vesicles in neuroendocrine cells (10, 44). CI-MPR antibodies labeled less numerous structures that were heterogeneous in size and showed a juxtannuclear accumulation (Fig. 1 *D*). This distribution is similar to that seen in a wide variety of cell types (27), and is consistent with localization of CI-MPR in late endosomes. Human LDL-R could not be localized by immunofluorescence labeling in these cells because of high background signals obtained in nontransfected PC12 A1 cells. Since immunoprecipitation of LDL-R with C7 antibody was specific for transfected cells, the receptor was readily labeled at the cell surface, and it was rapidly internalized (see below), we assume that human LDL-R in these cells, as in many others (17), was primarily localized to the



**Figure 3.** Internalization of membrane proteins from the cell surface. Cell surface proteins of PC12 A1-L14 cells were derivatized with NHS-SS-biotin at 0°C. The cells were recultured at 37°C for the times indicated, and the biotin groups remaining at the surface were removed by incubation in glutathione solution at 0°C. The cells were lysed, and MPR, LDL-R, synaptophysin and Thy-1 were sequentially immunoprecipitated. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with <sup>125</sup>I-streptavidin. Radioactivity in the appropriate bands was quantitated by phosphorimager analysis. The data are expressed as a percentage of the radioactivity recovered from cells that were neither warmed to 37°C following labeling nor incubated in glutathione solution. Radioactivity recovered from cells that were not warmed to 37°C before glutathione treatment (0 chase) was subtracted from all values for each protein.

cell surface and early endosomes. These experiments show that each of the proteins examined has a distinct steady-state distribution.

### Immunoprecipitation of Membrane Proteins

The specificity of each of the antibodies was assessed by immunoprecipitation of metabolically labeled proteins. PC12 A1-L14 cells were labeled metabolically with [<sup>3</sup>H]glucosamine so that any contamination of the immunoprecipitates with *N*-glycosylated proteins would be detected. The four glycoproteins followed in this study were sequentially immunoprecipitated from the cell lysates, and the immunoprecipitates analyzed by SDS-PAGE and fluorography (Fig. 2). The bulk of the radioactivity in each immunoprecipitate migrated as a single band of the appropriate mobility, except LDL-R which migrated as a doublet. (All glycoproteins examined to date in the ricin-resistant PC12 A1 cells and their derivatives show higher mobility on polyacrylamide gels than the proteins expressed in the parent PC12 cell line due to the reduced incorporation of terminal sugar residues in clone A1 cells.) Because of the specificity of the sequential immunoprecipitations, the extent of modification of labeled oligosaccharides from these proteins can be determined by analyzing the entire immunoprecipitate directly, without isolation from gels (see below).

### Internalization of Cell Surface Glycoproteins in A1-L14 Cells

The relative efficiencies and rates of internalization of the four proteins were studied by reversible biotinylation of the cell surface. Surface proteins were derivatized with NHS-SS-biotin, a reagent containing a biotin moiety separated from the cross-linking group by a disulfide bond (31). After incubation at 37°C for varying times to allow internalization, biotin groups remaining at the cell surface were removed by incubation in an ice-cold glutathione solution. The proteins of interest were immunoprecipitated sequentially from detergent lysates of the cells, and the biotinylated fraction of each protein was identified by SDS-PAGE, transfer to nitrocellulose, and probing the transfer with <sup>125</sup>I-streptavi-

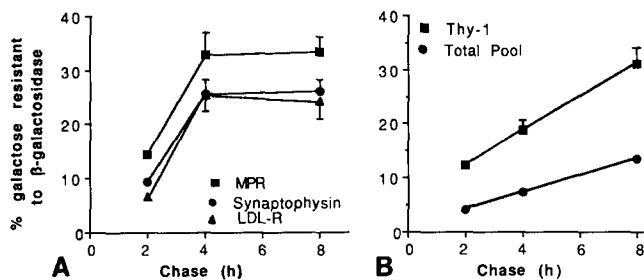
din. All of the glycoproteins examined were internalized, although the rates and extents of internalization were not the same (Fig. 3). LDL-R and CI-MPR were rapidly internalized, reaching a maximum in the level of internalized molecules within 8 min at 37°C. Synaptophysin was also internalized to a maximum extent at 8 min of chase, although a smaller fraction of synaptophysin was internalized relative to the receptors. A possible explanation of this result is that a fraction of the synaptophysin at the cell surface is rapidly internalized, while another fraction is slowly internalized. Although synaptophysin is rapidly internalized when expressed in fibroblasts (33), the transport of synaptophysin in neuroendocrine cells may be different, and is currently the subject of detailed investigation (51). Thy-1 was internalized much more slowly than the receptor proteins, and the percentage of molecules internalized continued to increase for at least 12 min. Thy-1 is not internalized to a significant extent in lymphoid cells (32), and it was surprising to find readily detectable internalization in PC12 cells.

### Transport of Glycoproteins from the Cell Surface to the Golgi Apparatus

To determine whether transport of any of these membrane proteins from the cell surface to the Golgi apparatus is selective, the rates of this transport process were compared for the four membrane glycoproteins, and for the total cell surface *N*-linked oligosaccharide pool. The assay is a minor modification (19) of the method of Duncan and Kornfeld (12). PC12 A1-L14 cells, which are deficient in the incorporation of galactose into glycoproteins, were exogalactosylated with UDP-[<sup>3</sup>H]galactose at 0°C and recultured at 37°C. Cells were lysed, the glycoproteins immunoprecipitated, and eluted under the same conditions as in Fig. 2. Immunoprecipitated proteins and a sample of the detergent lysate were digested exhaustively with PNGase F to release *N*-linked oligosaccharide chains. Proteins were removed by acid precipitation, and the oligosaccharides in the supernatant were desalted by gel filtration chromatography. The isolated oligosaccharide chains were digested exhaustively with  $\beta$ -galactosidase, the free galactose liberated by  $\beta$ -galactosidase separated from intact oligosaccharides by gel filtration chromatography, and the radioactivity in the two peaks quantitated. Only terminal galactose residues are sensitive to  $\beta$ -galactosidase, so subsequent terminal glycosylation of these residues renders the labeled galactose resistant to removal from the oligosaccharides.

Oligosaccharides isolated from all of the glycoproteins studied acquired resistance to  $\beta$ -galactosidase with time, reflecting terminal glycosylation of the galactose residues that had been added at the cell surface (Fig. 4). LDL-R, CI-MPR, and synaptophysin oligosaccharides underwent terminal glycosylation with similar nonlinear kinetics, reaching a plateau by 4 h (Fig. 4 A). To test whether terminal glycosylation of the galactose residues on all three of these proteins has been completed by 4 h of reculture, the data collected in four independent experiments at 4-h chase and in three independent experiments at 8-h chase were compared for each protein using a pooled variable *t* test. No significant difference was found between the 4- and 8-h measurements for any of the three transmembrane proteins (*P* > 0.2).

To allow a comparison of the relative efficiencies of transport to lysosomes and transport from the cell surface to the



**Figure 4.** Transport of cell surface glycoproteins to the Golgi apparatus. Cell surface glycoproteins on PC12 A1-L14 cells were labeled with galactosyltransferase and UDP-[ $^3$ H]galactose at 0°C and re-cultured for the indicated intervals at 37°C. MPR, LDL-R, synaptophysin, Thy-1, and total labeled oligosaccharides were immunoprecipitated from the cell lysates and analyzed according to the assay described in the text to determine the fraction of galactose that had acquired terminal sugars, as measured by resistance to removal with  $\beta$ -galactosidase, during reculture. The values represent the mean  $\pm$  SEM for four (4 h) or three (8 h) independent experiments (error bars shown in one direction for each point). The percentage of galactose resistant to  $\beta$ -galactosidase on oligosaccharides from Thy-1 increased significantly between 4 and 8 h ( $P < 0.05$ ), while the percentage of resistant galactose was unchanged after 4 h for MPR, LDL-R, and synaptophysin ( $P > 0.2$ ).

Golgi apparatus, the half-lives of these proteins were measured by metabolic labeling and immunoprecipitation. The half-lives of CI-MPR, LDL-R, and synaptophysin were at least several times longer than the rate of transport of these proteins from the cell surface to the Golgi apparatus (Table I), suggesting that transport to the Golgi apparatus occurs on average several times for each protein molecule.

The percentage of galactose residues resistant to  $\beta$ -galactosidase varied between the proteins, and was in no case greater than 42% of the total. These results are similar, both in rate and extent of terminal glycosylation, to the results obtained by Duncan and Kornfeld (12) for transport of CI-MPR from the cell surface to the Golgi apparatus. We (and others) assume that the low plateau levels of terminal glycosylation detected by resistance of galactose residues to  $\beta$ -galactosidase reflects the inability of the appropriate glycosyltransferases to recognize all of the galactose residues added at the cell surface. The finding that there is no significant change in the level of terminal glycosylation of the receptors after 4 h of chase suggests that all molecules that will pass through the Golgi apparatus have done so by that time. We therefore assume that the kinetics we have measured reflect the kinetics for the transport of the entire cell surface population.

In contrast to the three transmembrane proteins, terminal glycosylation of Thy-1 oligosaccharides increased for at least 8 h, at twice the apparent rate observed for the total oligosaccharide pool (Fig. 4B). The difference between 4- and 8-h chase was significant for Thy-1 ( $P < .05$ ). Since we did not observe a plateau in the terminal glycosylation of Thy-1 or total oligosaccharides and can therefore only place a minimum limit on the half time of this process ( $\geq 4$  h), we can not determine whether the rates of transport to the Golgi apparatus are different, or whether only the efficiency of terminal glycosylation differs. The half-life of Thy-1 (13 h, Table I) was at most two to three times longer than the half time of its transport from the cell surface to the Golgi apparatus, suggesting that Thy-1 may be less efficiently transported to

**Table I. Half-lives of MPR, LDL-R, Synaptophysin, and Thy-1 in PC12 A1-L14 Cells**

Protein	Half-life <i>h</i>
MPR	42
LDL-R	20
Synaptophysin	37
Thy-1	13

PC12 A1-L14 cells were metabolically labeled with  $^{35}$ S-amino acids and recultured for 1.5 h to allow for complete processing of N-linked oligosaccharides. Cells were harvested at intervals of up to 20 h, lysed in Triton X-114 buffer, and phase partitioned. MPR, LDL-R, synaptophysin, and Thy-1 were sequentially immunoprecipitated from the reconstituted detergent phase of the lysates and resolved by SDS-PAGE. Radioactivity in the gel bands corresponding to the terminally glycosylated proteins was quantitated by phosphorimager analysis.

the Golgi apparatus than the transmembrane proteins, once it has been internalized.

## Discussion

We have shown that CI-MPR was rapidly internalized from the surface of PC12 cells and returned to the Golgi apparatus, as in other cell types (12, 24). Unexpectedly, however, two other transmembrane proteins, LDL-R and synaptophysin, were also transported through the Golgi apparatus with identical kinetics in PC12 cells. Thy-1, a GPI-anchored protein, was also internalized and transported to the Golgi apparatus, although rates were significantly slower than the rates observed for the transmembrane proteins, and were too slow to be determined from our data. Endocytic transport of transmembrane proteins to the Golgi apparatus occurred at a rate that was several times more rapid than their rates of turnover. Since CI-MPR and LDL-R are rapidly internalized and show the same respective steady-state distributions in a wide variety of cell types, the results we have obtained for the endocytic transport of these two proteins to the Golgi apparatus in PC12 cells most likely reflect their trafficking in other cell types. The results suggest that this endocytic pathway to the Golgi apparatus is not a specialized route followed by a subset of receptors, but rather a general endocytic recycling pathway followed repeatedly by many internalized proteins. It remains possible that dense core granule membrane proteins may undergo endocytic transport to the Golgi apparatus more rapidly than the markers we have studied.

Snider and co-workers (24, 56) have previously analyzed transport of transferrin receptor and CI-MPR from the cell surface to the Golgi apparatus in K562 erythroleukemia cells. In these studies, cell surface receptors were desialylated with neuraminidase and radiolabeled, and the isoelectric shift of the receptors because of resialylation upon reculture was measured (24, 56). Normalizing the isoelectric distribution to that obtained from cell surface receptors that were not desialylated, these authors concluded that CI-MPR is resialylated more rapidly than transferrin receptor in the same cells, and therefore these receptors are transported differently within the endocytic pathway (24). However, consistent with our findings, the maximum extent of sialylation was different for the two receptors. When half times of this transport process are estimated based on the maximum isoelectric shift obtained for desialylated receptors, the rates

appear to be very similar for both receptors ( $t_{1/2} \sim 1.5$  h). Differences between cell types in the actual half times for transport to the Golgi apparatus and the rates of protein turnover may reflect differences in total endocytic activity. The striking similarity within a single cell type in the rates of transport and turnover for CI-MPR and receptors found predominantly on the cell surface suggests strongly that CI-MPR is not sorted from transferrin receptor or LDL-R during transport from the cell surface to the Golgi apparatus.

Available evidence suggests that endocytic traffic to the Golgi apparatus occurs via the late endosome or prelysosome. Vesicles that fuse specifically with the TGN in an in vitro reconstitution system are enriched in CI-MPR relative to transferrin receptor (16). Since CI-MPR is enriched in late endosomes, and transferrin receptor is enriched in early endosomes, it is inferred that the donor vesicles originate primarily from late endosomes. In addition, Stoorvogel et al. (58) showed that labeled transferrin taken up by endocytosis reaches the biosynthetic pathway (presumably via the Golgi apparatus) primarily from late, not early endocytic compartments. Accordingly, the kinetics of transport to the Golgi apparatus that we have observed are much slower than endocytosis, and are comparable to the slow kinetics with which cross-linked cell surface membrane markers are delivered to lysosomes (20, 41, 60). Thus, glycosylation assays such as the one used in this study measure delivery of molecules that have been transported through early endosomes and late endosomes before arrival in the Golgi apparatus.

We have shown that galactose incorporated into cell surface oligosaccharides acquires resistance to  $\beta$ -galactosidase in PC12 A1 cells because of the addition of sialic acid, fucose and *N*-acetylglucosamine (19). Since addition of *N*-acetylglucosamine to generate polyactosamine structures must occur in a compartment containing galactosyltransferase, generally the *trans* cisterna(e) (55), it is possible that a small fraction of the internalized receptor proteins are transported to Golgi cisternae in PC12 cells. However, until analysis of the terminal glycosylation of the oligosaccharides on specific proteins in PC12 A1 cells is completed, we assume that the major endocytic pathway to the Golgi apparatus in PC12 cells, as in other cell types (12, 45), is to the TGN.

### *A Model for Traffic through Late Endosomes*

The first specific assays for transport of cell surface proteins through the TGN (12, 14, 52, 56), demonstrated that this pathway represents a larger fraction of endocytic traffic than had previously been appreciated. However, the rate of transport from the cell surface to the Golgi apparatus is significantly slower than the endocytic rate (12, 14, 56), and is a rare event for most plasma membrane glycoproteins (12, 53). The observed transport has therefore been interpreted either as missorting, or as evidence for the presence of a specific signal that mediates the targeting of specialized proteins to the TGN, exemplified by CI-MPR. Because the endocytic rate is so much faster than the turnover rate of receptor proteins, recycling of receptors from early endosomes to the cell surface has been assumed to be extremely efficient, and receptors that are transported to late endosomes from early endosomes have been assumed to be destined for lysosomal delivery if they lack specific sorting determinants.

The experiments discussed above provide evidence that a

variety of internalized receptors are transported through the TGN on average several times before they are degraded in lysosomes. If this is true, then the major receptor pathway from late endosomes is to the TGN, not to lysosomes. These results suggest that receptors are sorted in late endosomes and early endosomes in very similar ways, that is, by retention of receptors bound for lysosomes, and removal of any receptors that are not actively retained (15, 41, 60, 62). Sorting in the early endosome allows a large fraction of the volume to be directed to late endosomes while the majority of the membrane receptors return to the cell surface. From late endosomes most of the volume carrying, for example, fluid phase tracers and LDL particles, is transferred to lysosomes while recycling receptors in late endosomes may again be diverted from the lysosomal pathway by recycling through the TGN. Two membrane recycling pathways in tandem, each of which separates membrane proteins from the bulk fluid pathway, would allow for minimal loss of internalized receptors to lysosomal degradation. Presumably, membrane proteins returning to the TGN from late endosomes would be sorted and exit the TGN in the same manner as newly synthesized molecules.

If CI-MPR and LDL-R are not sorted from each other in endosomes, the observed accumulation of CI-MPR in late endosomes may be due solely to selective targeting of CI-MPR from the TGN directly to endosomes, a sorting event that is well documented and clearly signal mediated (27, 35). The rapidly internalized fraction of the receptor found on the cell surface, up to 10% of the total at steady state, may be the pool that is "missorted" upon exit from the TGN into the constitutive secretory pathway, or delivered to early endosomes (38). CI-MPR will accumulate in late endosomes at steady state if transport from late endosomes to the TGN is less efficient than the combined delivery of receptors to late endosomes from the TGN and from the cell surface via early endosomes.

Postulating a constitutive vesicular shuttle from late endosomes to the TGN suggests a simple mechanism for the trafficking of major histocompatibility complex class II proteins from late endosomes to the cell surface. It has been shown that invariant chain contains sorting information which targets newly synthesized class II proteins from the TGN to a late endocytic compartment (1, 36). Invariant chain is degraded before the appearance of  $\alpha$  and  $\beta$  chains on the cell surface (3, 46). After degradation of invariant chain in late endosomes, molecules may return to the Golgi apparatus without invariant chain but with antigenic peptide. These molecules would lack the TGN-to-late endosome signal, and would therefore be carried to the plasma membrane via the constitutive secretory pathway. Such a transport mechanism is consistent with the observed kinetics of MHC class II biosynthesis, acquisition of antigenic peptide, and appearance at the cell surface (29).

The model presented here for traffic through late endosomes postulates that recycling membrane proteins are sorted from membrane proteins bound for lysosomes by similar mechanisms and possibly with similar efficiencies in early and late endosomes. This model is conservative in that it minimizes the number of pathways and sorting determinants needed to account for the available data. In particular, it obviates the need to propose additional sorting signals or new organelles to account for observations that are other-

wise difficult to explain if one assumes that endocytic transport of membrane proteins to the Golgi apparatus is a highly specialized or quantitatively trivial pathway. The model predicts that transport of any recycling receptors from late endosomes to the TGN would be efficient and fast relative to lysosomal delivery, much as receptor recycling from early endosomes to the cell surface is fast and efficient relative to endocytic transport to the TGN.

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