Intracellular Movement of Two Mannose 6-Phosphate Receptors: Return to the Golgi Apparatus

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Abstract. We have used Chinese hamster ovary (CHO) cells and a murine lymphoma cell line to study the recycling of the 215-kD and the 46-kD mannose 6-phosphate receptors to various regions of the Golgi to determine the site where the receptors first encounter newly synthesized lysosomal enzymes. For assessing return to the *trans*-most Golgi compartments containing sialyltransferase (*trans*-cisternae and *trans*-Golgi network), the oligosaccharides of receptor molecules on the cell surface were labeled with [³H]galactose at 4°C. Upon warming to 37°C, the [³H]galactose residues on both receptors were substituted with sialic acid with a $t_{1/2}$ ~3 hrs. Other glycoproteins acquired sialic acid at least 8–10 times slower. Return of the receptors to the *trans*-Golgi cisternae containing galactosyltransferase could not be detected. Return to the *cis*/middle Golgi cisternae containing α-mannosidase I was measured by adding deoxymannojirimycin, a mannosidase I inhibitor, during the initial posttranslational passage of [³H]mannose-labeled glycoproteins through

the Golgi, thereby preserving oligosaccharides which would be substrates for α-mannosidase I. After removal of the inhibitor, return to the early Golgi with subsequent passage through the Golgi complex was measured by determining the conversion of the oligosaccharides from high mannose to complex-type units. This conversion was very slow for the receptors and other glycoproteins ($t_{1/2}$ ~20 h). Exposure of the receptors and other glycoproteins to the dMM-sensitive α-mannosidase without movement through the Golgi apparatus was determined by measuring the loss of mannose residues from these proteins. This loss was also slow.

These results indicate that both Man-6-P receptors routinely return to the Golgi compartment which contains sialyltransferase and recycle through other regions of the Golgi region less frequently. We infer that the *trans*-Golgi network is the major site for lysosomal enzyme sorting in CHO and murine lymphoma cells.

T is now well established that newly synthesized lysosomal enzymes are sorted from secretory proteins and routed to lysosomes by means of the mannose 6-phosphate (Man-6-P)[1] recognition system (39, 54, 62). The lysosomal enzymes are synthesized in the rough endoplasmic reticulum and translocated to the Golgi apparatus where they acquire Man-6-P residues which act as recognition markers for binding to the Man-6-P receptors. The receptor-ligand complex then exits the Golgi region and moves to an acidified compartment where the low pH causes the complex to dissociate. The receptor can recycle back to the Golgi region or to the cell surface while the lysosomal enzymes are packaged into lysosomes. Since mature lysosomes lack detectable Man-6-P receptor (24, 51, 64), it is assumed that an acidified pre-lysosomal compartment exists. A small percent of newly synthesized lysosomal enzymes are secreted by most cells and a portion of these molecules may bind to the Man-6-P receptors present on the cell surface. These receptor-ligand complexes are concentrated in coated pits where they are in-

ternalized into endosomes. Endosome acidification dissociates the complexes, freeing the receptor to recycle while the lysosomal enzymes continue to the lysosome.

Two distinct Man-6-P receptors have been identified and their primary structures determined through cDNA cloning (17, 35, 42, 50, 57). One is a 215-kD *trans*-membrane glycoprotein which binds lysosomal enzymes in a cation-independent manner, while the other is a 46-kD *trans*-membrane glycoprotein which requires divalent cations for optimal lysosomal enzyme binding. The receptors have similar, but not identical, binding specificities for various phosphorylated oligosaccharides (36). Both receptors bind ligands best at slightly acidic pHs and release the ligands at pH 5.5 or below (23, 34, 36, 52), a property characteristic of receptors which deliver ligands to acidified compartments.

While these aspects of the pathway are generally accepted, conflicting results have been presented as to where in the Golgi region the newly synthesized lysosomal enzymes first encounter the Man-6-P receptors. Brown and Farquhar, based on their immunolocalization of the 215-kD Man-6-P receptor in pancreatic, hepatic, and epididymis epithelial

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1. *Abbreviations used in this paper:* Man-6-P, mannose 6-phosphate; TGN, *trans*-Golgi network.

cells, concluded that this receptor is concentrated in the *cis*-Golgi complex (12, 14). On this basis they proposed that lysosomal enzymes bind to the receptor in the *cis*-Golgi complex, and exit at this level. However, other groups studying different cell types with immunolocalization techniques found the receptor to be concentrated mainly in the *trans*-Golgi network (TGN) (also termed *trans*-Golgi reticulum, *trans*-tubular network, and GERL) with only very low levels being detected in the Golgi stack (25-28, 33, 64). These data have led to the alternative hypothesis that the lysosomal enzymes pass through the entire Golgi apparatus before being segregated to lysosomes. Additional evidence in support of this second pathway comes from the finding that several lysosomal enzymes have been shown to contain terminally processed complex-type N-linked oligosaccharides in addition to their phosphorylated high mannose-type oligosaccharides (5, 22, 29, 41, 46, 61). Since the glycosyltransferases responsible for terminal processing are located in the *trans*-Golgi cisternae and the TGN (6, 47, 48), this finding indicates that at least a portion of the lysosomal enzymes pass through the entire Golgi stack enroute to the lysosome. It is still not clear, however, exactly where lysosomal enzymes first come in contact with the Man-6-P receptors.

In an attempt to resolve this controversy, we set out to develop methods for analyzing the kinetics of receptor recycling to the different regions of the Golgi complex. The approach takes advantage of the fact that the various enzymes involved in oligosaccharide processing are distributed among specific regions of the Golgi stack and the TGN (2-4, 6, 19-21, 30, 44, 47-49). Consequently, these enzymes have the potential to mark receptors which move through specific Golgi compartments, provided that the oligosaccharides on the mature receptors are suitable substrates for the Golgi enzymes. Two experimental approaches have been used to accomplish this. First, the oligosaccharide units of receptor molecules present on the cell surface were modified so that they would become substrates for sialyltransferase, a marker of the *trans*-Golgi cisternae and the TGN (6, 48, 49). If these receptors then returned to the compartment containing sialyltransferase, the oligosaccharide should gain a sialic acid residue. The other method uses deoxymannojirimycin, a reversible inhibitor of the early Golgi enzyme, α-mannosidase I (8), to prevent the processing of oligosaccharides on newly synthesized glycoproteins during their initial pass through the Golgi apparatus. Subsequent removal of the inhibitor allows Golgi α-mannosidase I to act upon those molecules which return to the early Golgi complex. If the molecules continue through the Golgi apparatus, they would then become substrates for the later stage processing enzymes. Our results indicate that in the cell types studied the two Man-6-P receptors routinely return to the TGN but rarely recycle through the entire Golgi apparatus.

Materials and Methods

Materials

All reagents were of analytical grade. [2-^{3}H]Mannose (15 Ci/mmol) and [6-^{3}H]galactose (20 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO). UDP-[6-^{3}H]galactose (17 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). Sephadex G-25-80, bovine milk UDP-galactose/D-glucose β1-4 galactosyltransferase, UDP-galactose, aprotinin, antipain, leupeptin, chymostatin, pepstatin, α-methylglucoside, α-methyl-

mannoside, glucose 6-phosphate, and mannose 6-phosphate were from Sigma Chemical Co. (St. Louis, MO). *Vibrio cholera* neuraminidase and pronase were from Calbiochem-Behring Corp. (San Diego, CA). Deoxymannojirimycin was from Miles Laboratories (Naperville, IL). Con A-Sepharose was from Pharmacia Fine Chemicals (Piscataway, NJ). RCA-I agarose was from E-Y Labs (San Mateo, CA). Phosphomannan-Sepharose was prepared as previously described (35). Jack bean β-galactosidase was provided by Walter Gregory of Washington University (St. Louis, MO). New Castle disease virus containing an α 2-3 specific neuraminidase was a kind gift of Dr. Jacques Baenziger, Washington University (St. Louis, MO).

Cells

BW 5147 mouse lymphoma cells and the galactose-deficient cell line BW 5147 PHA^{R}1.8 derived from it (60) were grown as a suspension culture in α-MEM containing 10% FCS and antibiotics (complete medium). Two Chinese hamster ovary (CHO) galactose-deficient lines were used. The WGA-resistant CHO clone 13 line (11) was grown in suspension culture while the CHO ldl-D line (37) obtained from M. Kreiger (MIT, Cambridge, MA) was grown as a monolayer. Cells were harvested as they neared maximal density.

While the three galactose-deficient cell lines are similar in phenotype, they have two different underlying defects. CHO clone 13 cells synthesize normal amounts of UDP-Gal, but are unable to transport this nucleotide sugar from the cytosol into the lumen of the Golgi cisternae (11, 18). In the absence of its sugar donor, galactosyltransferase cannot function. The BW 5147 PHA^{R}1.8 cells appear to have a similar defect (60). In contrast, CHO ldl-D cells are unable to form UDP-Gal from UDP-Glc due to a deficiency of the UDP-Glc/UDP-Gal epimerase enzyme (37). The effects of this mutation can be circumvented by adding galactose to the culture medium since a pathway exists for forming UDP-Gal directly from galactose.

Surface Labeling of CHO Clone 13 and BW 5147 PHA^{R}1.8

Cells were cooled on ice, collected by centrifugation and resuspended to a density of 1 × 10^8 cells/ml in ice-cold glucose and bicarbonate-free MEM containing 20 mM Hepes, pH 7.4 (GBFM). To begin the surface labeling, MnCl$_2$, galactosyltransferase and UDP-[^{3}H]Gal were added to final concentrations of 10 mM, 0.5-1.0 U/ml and 0.05-0.1 mCi/ml, respectively. After a 30-min incubation at 4°C with end-over-end mixing, unlabeled UDP-galactose was added to a final concentration of 2.5 mM and the incubation continued for 10 min. The cells were then collected by centrifugation, washed once with complete medium, and when desired, warmed to 37°C by diluting 200-fold into pre-warmed complete medium containing 20 mM Hepes, pH 7.4. After the indicated times at 37°C, the cells were collected by centrifugation at 4°C, washed once with cold PBS, and the cell pellets frozen.

Surface Labeling of CHO ldl-D

Cells were grown to confluence in 150-mm dishes. Where indicated, cyclo-heximide was added to 50 μg/ml 1 h before cooling the cells to 4°C. The cultures were washed twice with cold GBFM and then 9.75 ml of GBFM was added to each dish followed by the addition of MnCl$_2$, galactosyltransferase and UDP-[^{3}H]Gal to final concentrations of 10 mM, 0.025 U/ml and 5 μCi/ml. After a 1-h incubation at 4°C, the medium was removed and the cultures washed once with GBFM. Each dish then received 10 ml GBFM containing 10 mM MnCl$_2$, 0.012 U galactosyltransferase, and 0.25 mM UDP-Gal. After a 10-min incubation at 4°C, the cultures were washed twice with cold complete medium. The cultures were then warmed to either 20°C or 37°C by adding 25 ml of pre-warmed complete medium containing 20 mM Hepes, pH 7.4. After the indicated time, the cells were washed twice with cold PBS, scraped, collected by centrifugation, and the cell pellets frozen. This surface labeling procedure is similar to that used by other groups to label a variety of cell types (58, 59).

Neuraminidase Treatment of CHO ldl-D Cells

Cells were plated into 60-mm dishes and grown in complete medium. 3 h before labeling the medium was replaced with 2 ml of α-MEM lacking serum (a source of galactose) to decrease the intracellular pool of galactose. The labeling was initiated by replacing the serum-free medium with 2 ml of MEM containing 1% dialyzed calf serum, 20 mM Hepes pH 7.4 and 0.1 mCi/ml [^{3}H]galactose. After a 20-h incubation, the medium was removed and the cells washed three times. The chase was then begun by the addition

of 5 ml of medium containing 1% calf serum, 10 μM galactose, 100 μM GalNAc, and 20 mM Hepes pH 7.4 (chase medium). After a 2-h chase, the medium was removed and replaced with 2 ml of chase medium containing 0.1 U/ml *V. cholerae* neuraminidase. After 3–4 h at 37°C, the cells were washed four times to remove the neuraminidase, and then incubated in chase medium lacking neuraminidase. At the indicated times throughout the experiments, the cells were cooled to 4°C, washed three times with cold PBS, scraped, pelleted by centrifugation, and cell pellets frozen.

Analysis of Endogenous Galactose Addition to the Man-6-P Receptors in BW 5147 PHA^{R}1.8

Cells were collected by centrifugation and suspended to a density of 1 × 10^7 cells/ml in GBFM containing 0.1 mCi/ml [^{3}H]mannose. After a 40-min incubation, the cells were centrifuged, and resuspended at a density of 1 × 10^6 cells/ml in complete medium to initiate the chase. At the indicated times, cells were collected by centrifugation at 4°C, washed once with cold PBS and the cell pellets frozen.

The Man-6-P receptors were purified (see below) and the fraction containing galactose assessed by RCA-I agarose chromatography. The receptors in column buffer (50 mM Imidazole pH 6.5, 150 mM NaCl, 0.05% Triton, 10 μg/ml BSA and either 5 mM EDTA or 10 mM MnCl$_2$) were applied to a 1.0 ml RCA-I agarose column equilibrated in the same buffer. The column was washed and eluted with column buffer containing 0.2 M galactose. Fractions of the run through, wash and eluate were monitored for radioactivity. To release terminal sialic acid, the 46-kD Man-6-P receptor (in column buffer) and the 215-kD Man-6-P receptor (in column buffer containing 10 mM CaCl$_2$) were incubated with 0.1 U/ml *V. cholerae* neuraminidase for 6 h at 37°C before chromatography on RCA-I agarose.

Deoxymannojirimycin Inhibition of Oligosaccharide Processing in BW 5147 Cells

Cells were collected by centrifugation and resuspended to a density of 1 × 10^7 cells/ml in GBFM containing 0.25 mM deoxymannojirimycin. After a 60-min incubation at 37°C, [^{3}H]mannose was added to a final concentration of 0.1 mCi/ml, and the incubation continued for 40 min. The cell suspension was then diluted 3-fold with complete medium containing 20 mM Hepes pH 7.4, 10 mM mannose and 0.25 mM dMM to initiate the chase. After 6 h at 37°C, the cells were sedimented, washed three times with complete medium lacking dMM, and resuspended to a density of 1 × 10^6 cells/ml in complete medium containing Hepes and mannose but lacking dMM. After the indicated times at 37°C, the cells were collected by centrifugation in the cold, washed once with cold PBS and the cell pellet frozen.

Purification of the Mannose 6-Phosphate Receptors

The two Man-6-P receptors were purified by a modification of our previously described method (35). The frozen cells were resuspended in 6 ml of 50 mM Hepes pH 7.4, 5 mM NaPO$_4$, 5 mM EDTA, 0.34 TIU/ml aprotinin, 1:200 dilution of a cocktail of protease inhibitors (2.5 mg/ml of antipain, pepstatin, chymostatin, leupeptin, and 10 TIU/ml aprotinin), 2 mM Man-6-P and sonicated by 3 10-s bursts (power level 2) from a Branson Sonifer model 350 (Danbury, CT). Saponin was added to a final concentration of 0.5% and the mixture held on ice for 30 min. Membranes were sedimented at 80,000 g for 30 min, the supernatant fluid removed, and the pellet resuspended in 6 ml of 25 mM Hepes pH 7.4, 0.1 M NaCl, 5 mM NaPO$_4$, 0.17 TIU/ml Trasylol, 0.5% saponin. The membranes were incubated on ice for 15 min, resedimented and the wash repeated. Membrane proteins were solubilized by resuspending the membrane pellet in 3 ml of 50 mM Imidazole pH 6.5, 150 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 0.1% SDS, 0.02% NaN$_3$, 1:200 dilution of protease inhibitors and incubating overnight on ice. The solution was sedimented at 80,000 g for 30 min to remove insoluble material and the supernatant applied to a phosphomannan-Sepharose column (0.6 ml bed volume). The column was then washed with 15 ml of solubilization buffer. Under these conditions, the 215-kD Man-6-P receptor binds to the column while the 46-kD Man-6-P receptor runs through. The first 15 ml of the column run through, containing the 46-kD Man-6-P receptor, was brought to 20 mM MnCl$_2$ and applied to a second phosphomannan column-Sepharose equilibrated in solubilization buffer containing 20 mM MnCl$_2$. Under these conditions, the 46-kD Man-6-P receptor binds to this affinity matrix. Each column was then washed with 15 ml of solubilization buffer containing 0.1 mg/ml BSA and either EDTA or MnCl$_2$, followed by 100 ml of 50 mM Imidazole pH 6.5, 150 mM NaCl, 0.05% Triton X-100, 10 μg/ml BSA and either 5 mM EDTA or 10 mM

MnCl$_2$ (column buffer). After a final wash with column buffer containing 2 mM glucose 6-phosphate, the receptors were eluted from the affinity matrix using column buffer containing 5 mM Man-6-P. All steps were done at 4°C. Analysis of each receptor fraction by SDS–PAGE revealed a single band of appropriate size (data not shown).

TCA Precipitation and Pronase Digestion

The purified receptors or the run through fraction obtained from the second phosphomannan column (total membrane glycoproteins) were precipitated with cold TCA (20% final concentration) and centrifuged at 11,000 g for 5 min. The pellets were washed with acetone and then with Et$_2$O/EtOH (3:1). The dried pellets were suspended in 0.2 ml of 100 mM Tris pH 8.0, 20 mM CaCl$_2$, 10 mg/ml pronase and incubated overnight at 56°C to degrade the glycoproteins to glycopeptides. The samples were then boiled for 10 min, clarified by centrifugation and the supernatant fluid recovered.

Structural Analysis of Glycopeptides

Details of Con A-Sepharose chromatography, endo H digestion and HPLC sizing of the high mannose oligosaccharides are described elsewhere (30, 40, 43). To assess the extent of sialylation, [^{3}H]galactose-labeled glycopeptides were desalted by Sephadex G-25 chromatography, evaporated to dryness and resuspended in 0.15 ml of 40 mM NaOAc pH 4.6 containing 0.05 U β-galactosidase. The reaction mixtures were incubated at 37°C for 72 h under a toluene atmosphere with additional β-galactosidase (0.025 U) added at 24 and 48 h. The [^{3}H]galactose released by this treatment was then quantified by Sephadex G-25 chromatography. To release sialic acid, samples were treated with a 1:100 dilution of New Castle disease virus (which contains an α 2-3 specific neuraminidase) in 50 mM NaCacodylate pH 6.0 for 24 h at 37°C under a toluene atmosphere or with 2N HOAc for 1 h at 100°C.

Results

Receptors Return to the Golgi Compartment Containing Sialyltransferase

Sialyltransferase is a late acting Golgi enzyme which has been immunolocalized to the *trans*-cisternae and the TGN in rat liver hepatocytes (48). Therefore, this enzyme was selected as a marker of these compartments. To determine whether Man-6-P receptors present on the cell surface return to the Golgi compartments containing sialyltransferase, two approaches were used. The first is schematized in Fig. 1. Intact cells were cooled to 4°C to arrest endocytosis and incubated with UDP-[^{3}H]Gal and galactosyltransferase to transfer [^{3}H]galactose residues to the Man-6-P receptor molecules present on the cell surface. To increase the extent of the labeling, three mutant cell lines, BW 5147 PHA^{R}1.8 cells, CHO clone 13 cells and CHO ldl-D cells were used. Each of these cell lines is impaired in the incorporation of galactose into N-linked oligosaccharides and therefore the glycoproteins are enriched in terminal GlcNAc residues and serve as much better substrates for the galactosyltransferase. Preliminary experiments demonstrated that both Man-6-P receptors could be labeled at the cell surface of each cell type and were internalized upon warming to 37°C with a half time of ~2 min, as judged by resistance to external proteases (data not shown). To assess whether they acquired sialic acid after internalization, the receptors were purified, digested with pronase to generate glycopeptides and the glycopeptides treated with β-galactosidase. β-Galactosidase is an exoglycosidase that only releases terminal galactose residues and therefore any addition of sialic acid will block cleavage by this enzyme. After digestion with β-galactosidase, the released galactose was separated from the resistant sialylated
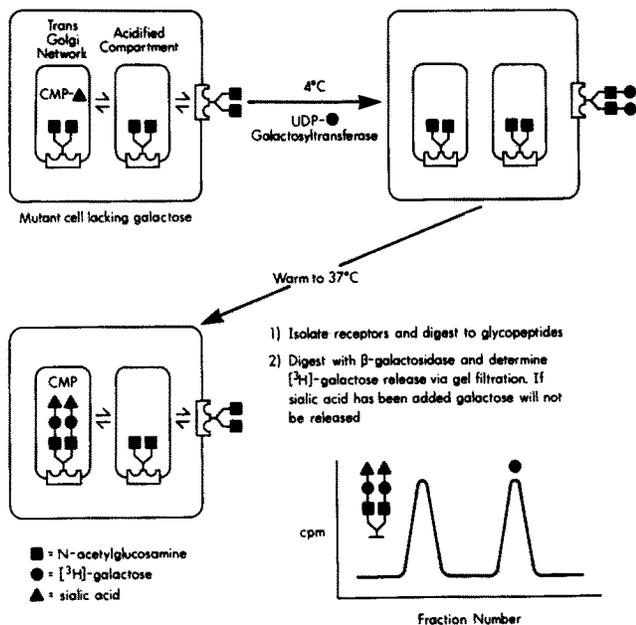
Figure 1. Experimental protocol used for cell surface labeling and subsequent analysis of sialic acid addition.
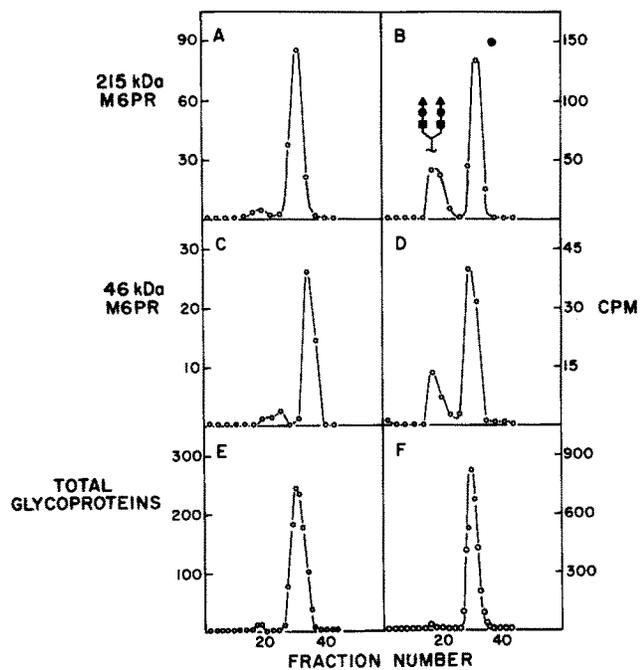

Figure 2. The Man-6-P receptors acquire sialic acid following surface labeling. Surface glycoproteins of BW 5147 PHA^{R}1.8 cells were labeled with [^{3}H]galactose using galactosyltransferase as described in Materials and Methods. The cells were then kept at 4°C (A, C, and E) or warmed to 37°C for 3.3 h (B, D, and F). The 215-kD Man-6-P receptor (A and B), 46-kD Man-6-P receptor (C and D) and total glycoproteins (E and F) were prepared, digested with pronase, treated with β-galactosidase and analyzed by gel filtration chromatography. The gel filtration profiles are shown and the relative positions of released [^{3}H]galactose (●) and glycopeptides containing sialylated [^{3}H]galactose are indicated by the symbols.
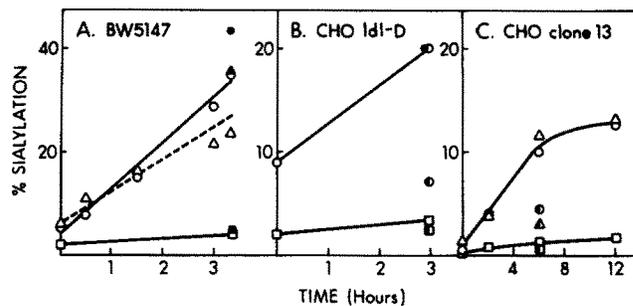

Figure 3. Man-6-P receptors acquire sialic acid in three different cell lines. Surface glycoproteins were labeled with [^{3}H]galactose using galactosyltransferase as described in Materials and Methods. The cells were kept at 4°C or warmed to 37° or 20°C for the times indicated. The 215-kD Man-6-P receptor (O, ●), 46-kD Man-6-P receptor (△, ▲), and total membrane glycoproteins (□, ■) from BW 5147 PHA^{R}1.8 (A), CHO ldl-D (B), or CHO clone 13 (C) were then prepared and analyzed as described in Materials and Methods. The extent of sialylation was calculated as the fraction of galactose residues possessing a terminal sialic acid, and was determined from the amount of radioactivity eluting as the glycopeptide species after exhaustive digestion with β-galactosidase. In A, the filled symbols indicate the fraction that was sialylated when the cells were labeled at 4°C and then warmed to 37°C for 3.3 h in medium containing 0.2 mM cycloheximide. This concentration of cycloheximide inhibited protein synthesis by greater than 95% (data not shown). In B, the filled symbols indicate the fraction that was sialylated when cells were treated with 0.2 mM cycloheximide 1 h before labeling and then warmed to 37°C for 3 h in medium again containing cycloheximide. In B and C, the half-filled symbols (◐, ◮, ◪) indicate the fraction that was sialylated when cells were labeled at 4°C and then warmed to 20°C. Panel A represents the combined results of two experiments (Time = 0, 0.5, 1.5, and 3 h; Time = 0, 3.3 h).

glycopeptide species by gel filtration, and the extent of sialylation calculated from the fraction of radioactivity eluting as glycopeptide.

Fig. 2 shows the gel filtration profiles of [^{3}H]galactose-labeled glycopeptides obtained from purified 215-kD Man-6-P receptor (panels A and B), 46-kD Man-6-P receptor (panels C and D), and total membrane glycoproteins (panels E and F) isolated from the BW 5147 PHA^{R}1.8 cell line. β-galactosidase digestion of the samples obtained from cells incubated at 4°C resulted in nearly all of the [^{3}H]galactose migrating as free galactose (panels A, C, and E), indicating that virtually no terminal sialic acid residues were added to the [^{3}H]galactose-labeled carbohydrate chains under these conditions. When the cells were incubated at 37°C for 200 min before lysis and analysis, a significant fraction of the oligosaccharides from both Man-6-P receptors was sialylated. This is shown by panels B and D, in which 30 and 23% of the [^{3}H]galactose residues on the 215-kD and the 46-kD Man-6-P receptors, respectively, are resistant to β-galactosidase treatment. Since sialylation is thought to occur in the trans-Golgi cisternae and the TGN (6, 48), these data indicate that at least a portion of both Man-6-P receptors found at the cell surface can recycle to these compartments. This phenomenon is not shared by most cell surface glycoproteins, however, since there was no detectable sialylation of total membrane glycoproteins (Fig. 2, panel F).

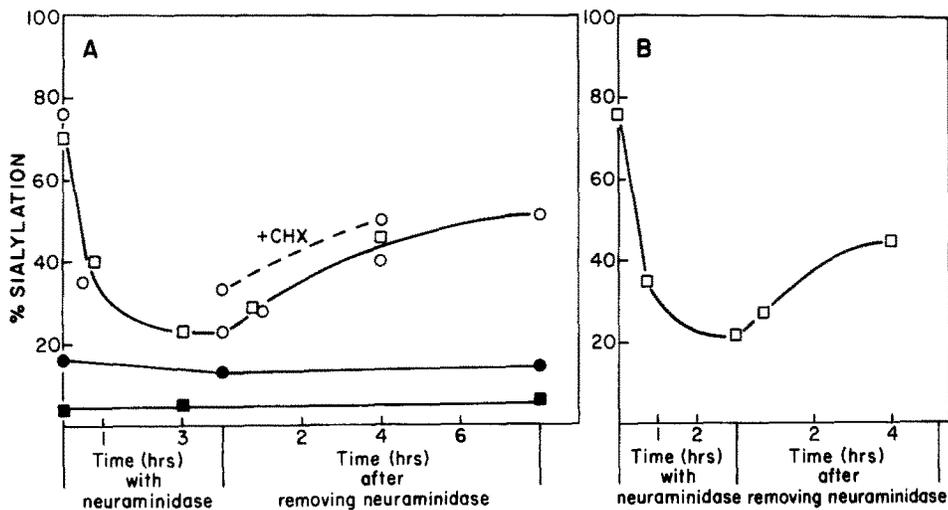Internalization and sialylation of the Man-6-P receptors

Figure 4. The Man-6-P receptors lose sialic acid during incubation with neuraminidase but regain it once the neuraminidase is removed. CHO ldl-D cells were metabolically labeled with [^{3}H]galactose for 20 h, then chased 2 h before adding *V. cholerae* neuraminidase to the culture medium. After 3 or 4 h at 37°C, the neuraminidase was removed and the 37°C incubation continued. At various times, receptors were prepared and the extent of sialylation of the receptor glycopeptides determined as before. Panel *A*, 215-kD Man-6-P receptor prepared from two separate experiments where neuraminidase was present for 3 (□, ■), or 4 h (○, ●) before it was removed. Filled symbols (■, ●) represent glycopeptides treated with mild acid (■) or an α 2-3 specific neuraminidase (●) to remove sialic acid; these glycopeptides were then treated with β-galactosidase and the fraction sialylated determined. Where indicated (+CHX), 0.2 mM cycloheximide was added to the cells at the end of the chase and was present during the remainder of the 37°C incubations. (*B*) The 46-kD Man-6-P receptor prepared from the same experiment where neuraminidase was present for 3 h.

was also observed in CHO clone 13 cells and CHO ldl-D cells, although the extent of sialylation was greater in the BW 5147 PHA^{R}1.8 cells than in the CHO cells (Fig. 3). In all three cell lines, the rates of sialylation of both receptors were 8–10 times greater than the rates observed for the total cell surface glycoproteins.

When the cells were warmed to 20°C instead of 37°C after the surface labeling, little or no sialylation was observed (Fig. 3, panels *B* and *C*), consistent with the findings of Snider and Rogers that this temperature blocks some aspect of the plasma membrane to Golgi pathway (55). Receptor recycling does not appear to require the synthesis of lysosomal enzymes since cycloheximide treatment had no effect on receptor sialylation (Fig. 3, panels *A* and *B*), regardless of whether the inhibitor was added 1 h before surface labeling or following the labeling.

When ldl-D cells are grown in the presence of [^{3}H]galactose, the sugar is incorporated exclusively into glycoproteins and glycolipids as galactose residues. This property allowed us to use a second approach to study the sialylation of the receptors. ldl-D cells were grown in medium containing [^{3}H]galactose for 20-h to generate Man-6-P receptors with complex-type N-linked oligosaccharides terminating in the sequence sialic acid-[^{3}H] galactose-N-acetylglucosamine. After a 2-h chase, *V. cholerae* neuraminidase was added to the culture medium for 3–4 h to remove sialic acid residues from the receptors which appeared on the cell surface. The neuraminidase was then removed, and the chase continued to allow those receptors which has lost sialic acid to regain it upon return to the Golgi region. The loss or gain of sialic acid was assessed as in the previous experiments. Fig. 4 shows the results of two experiments done in this manner. Before the addition of the neuraminidase to the cultures, ~75% of the galactose residues present on the receptors were sialylated. The addition of the neuraminidase caused a rapid decrease in sialic acid content which leveled off at 20–23%

sialylation.[2] After removal of the neuraminidase, both receptors began to regain sialic acid. The resialylation of the 215-kD Man-6-P receptor continued for the next 6–8 h before reaching a plateau where 51% of the galactose residues contained sialic acid (Fig. 4, panel *A*). Similar results were obtained with the 46-kD Man-6-P receptor (Fig. 4, panel *B*). The half times for resialylation in these experiments were ~3 h (Table I). The reason why the resialylation does not return to the initial level of 75% is not clear. One possibility that we cannot exclude is that a trace amount of added neuraminidase remains bound to the cell surface in spite of the extensive washing to remove it. Interestingly, Rogers and Snider using a similar experimental protocol to analyze the resialylation of the transferrin receptor also observed that the extent of resialylation was less than the extent of sialylation of the native receptor (55). When cycloheximide was present during the neuraminidase treatment and the subsequent chase, desialylation was slightly decreased and resialylation was unaffected (Fig. 4, panel *A*). This again suggests that the synthesis of lysosomal enzymes is not required for receptor recycling.

Two control experiments were performed to demonstrate that the resistance of the receptor glycopeptides to β-galactosidase was actually due to the presence of blocking sialic acid residues. The glycopeptides were treated with either mild acid or an α2-3 specific neuraminidase to remove sialic acid prior to the β-galactosidase digestion. Glycopeptides treated in this way were almost completely susceptible to the

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2. The most likely explanation for this plateau is that it represents the steady state between two competing processes: The loss of sialic acid due to the added neuraminidase and resialylation due to receptor recycling to the TGN. If true, the plateau value of 23% should approximate the $t_{1/2}$ for exposure to neuraminidase divided by the $t_{1/2}$ for resialylation. The $t_{1/2}$ for exposure to neuraminidase calculated from the initial rate of sialic acid loss is 30 min. Solving for the $t_{1/2}$ of sialylation yields a value of 2.5 h. This calculated sialylation half-time is similar to the value of 3 h actually observed in the experiment.

Table I. Estimated Half-Times

	215-kD Receptor	46-kD Receptor	Total membrane glycoproteins
Sialic acid addition			
BW 5147 PHA^{R}1.8	3	3	≫3
CHO ldl-D	3	2.5	≫4
CHO clone 13	3.5	4	≫12
Galactose addition (without added galactosyltransferase or UDP-Gal)			
BW 5147 PHA^{R}1.8	≫12	≫12	
Mannosidase processing in BW 5147			
With conversion to complex-type	20	20	21
Without conversion to complex-type	*	14	12

The data shown in Figs. 3,4,6,9, and 10 were used to estimate the relative rates of receptor movement through the compartments containing sialyltransferase, galactosyltransferase or a deoxymannojirimycin-sensitive mannosidase. In each experiment, the extent of processing at each time point was calculated according to the general formula:

$$\frac{\text{Extent of processing at}}{\text{Time } (T)} = (T_x) = \frac{(\% \text{ at } T_x - \% \text{ at } T_0)}{(\% \text{ at } T_\infty - \% \text{ at } T_0)}$$

The values used for percentage of oligosaccharides processed at $T = \infty$ were obtained as follows: The maximal extent of sialic acid addition was estimated to be 80% in BW 5147 PHA^{R}1.8 cells (31), 51% in CHO ldl-D cells (Fig. 4) and 14% in CHO clone 13 cells (Fig. 3). The maximal extent of galactose addition was estimated to be 95% based on receptor binding to RCA-1 Agarose (Fig. 7). The maximal extent of conversion of high mannose units to complex-type units was estimated from the ratio of these two types of structures on the receptors of control cells (Fig. 9, panels A and B). The actual cpm in each type of oligosaccharide was divided by its content of mannose and fucose residues (average of 4 for complex-type and 8 for high mannose units) to calculate molecules of oligosaccharide. The maximal extent of mannose processing without conversion to complex-type units was calculated by adding the amount of Man$_{5-6}$GlcNAc$_2$ species present on the proteins in control cells (Fig. 9, panels A-C and Fig. 10, panels A-C) plus the amount of Man$_5$GlcNAc$_2$ expected due to the percentage of complex-type species formed in control cells (the complex-type species being derived from the Man$_5$GlcNAc$_2$ intermediate).

The extent of processing at each time point was used to calculate a half-time assuming the processing obeyed first order kinetics, and the individual half times were averaged within an experiment. In the cases of galactose addition to the receptors and sialic acid addition to total glycoproteins, little or no oligosaccharide processing was observed over the course of the experiment. In these instances, the half-time of processing was estimated to be much longer than the longest available time point.

* A $t_{1/2}$ could not be accurately calculated since the data do not fit a first order decay reaction.

β-galactosidase (Fig. 4, panel A).[3] These results demonstrate that the β-galactosidase-resistant glycopeptides contain sialic acid.

Receptors do not Return to the Golgi Compartment Containing Galactosyltransferase

Since the above results demonstrated that the receptors recycle to the *trans*-Golgi/TGN compartment containing sialyltransferase, we next asked whether they also recycle to the Golgi compartment containing galactosyltransferase and acquire galactose. Galactosyltransferase is a late acting enzyme which has been immunolocalized to the trans-Golgi cisternae in HeLa cells (47) and to this same region as well as the TGN

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3. The slightly greater effect observed with the mild acid treatment most likely reflects the fact that acid treatment is more efficient than neuraminidase in releasing all of the sialic acid. Alternatively, the differences could be due to the presence of some α2-6 linked sialic acid that would not be cleaved by the α2-3 specific neuraminidase.
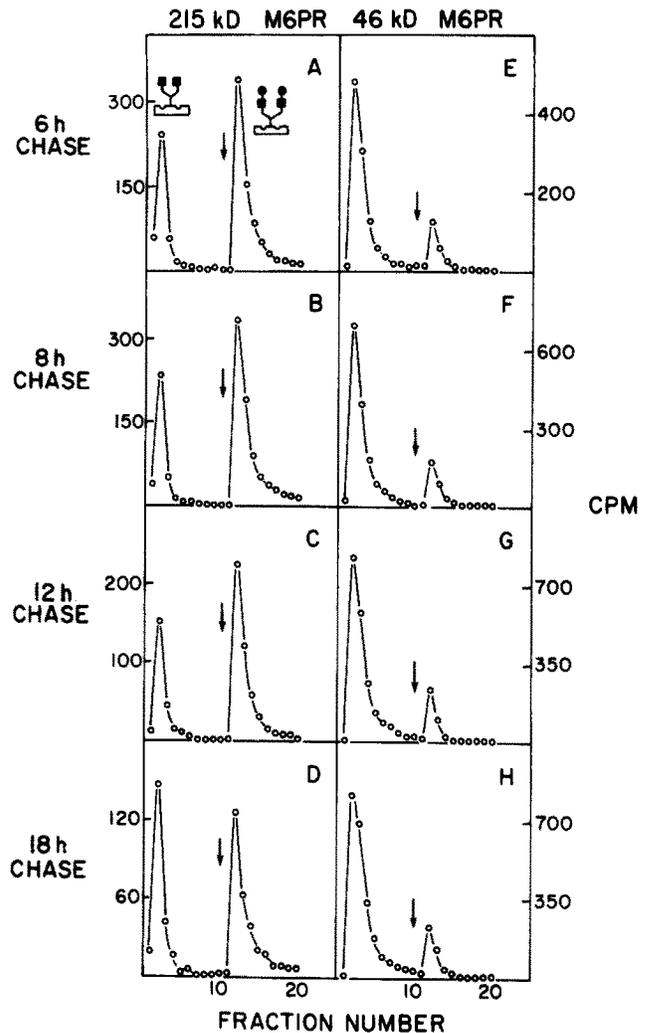
Figure 5. The mature Man-6-P receptors do not acquire galactose. BW 5147 PHA^{R}1.8 cells were labeled with [^{3}H]mannose and chased for the times indicated. Both Man-6-P receptors were then isolated and the fraction containing galactose assessed by chromatography on RCA-I Agarose. The arrows indicate where the 0.2 M galactose was included in the column buffer to elute those receptors containing galactose. (A-D) 215-kD Man-6-P receptor isolated from cells chased 6, 8, 12 and 18 h, respectively. (E-H) 46-kD Man-6-P receptor isolated from cells chased 6, 8, 12 and 18 h, respectively.

in HepG$_2$ cells (28). In the biosynthesis of oligosaccharides, it acts just before sialyltransferase. BW 5147 PHA^{R}1.8 cells were used for this experiment since preliminary experiments had indicated that the defect in galactose addition in these cells was not complete, i.e., the glycoproteins synthesized by this cell line acquired a small amount of galactose during their initial passage through the Golgi complex. Therefore, in these cells, if the receptors returned to the region of the Golgi containing galactosyltransferase, they should acquire additional galactose residues with each pass. The cumulative effect of multiple trips could then be measured as increased binding of the receptors to the galactose-specific lectin, RCA-1 (1).

To test this possibility, cells were grown in the presence of [^{3}H]mannose for 40 min to metabolically label the receptors and then chased for 6-18 h before harvesting. The mini-
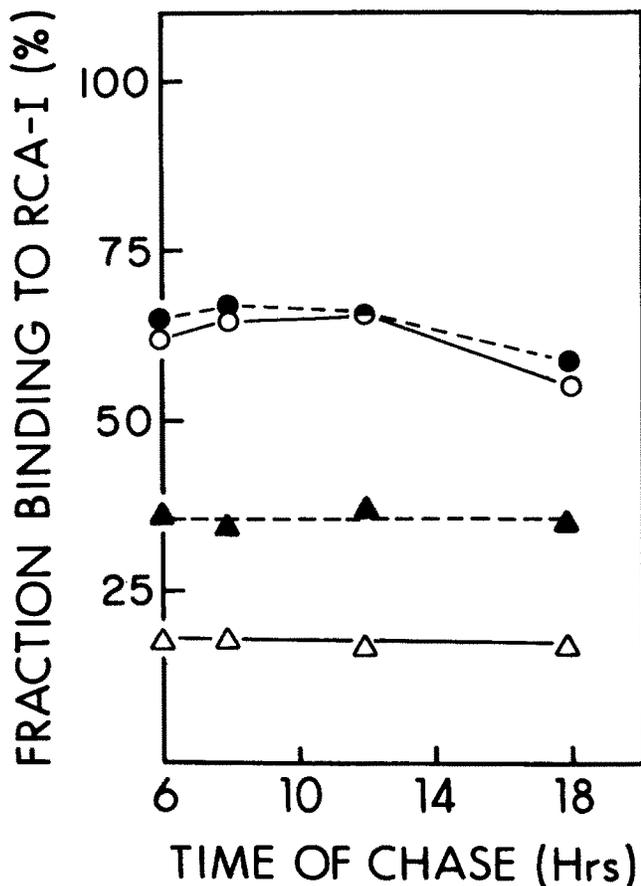
Figure 6. Effect of neuraminidase treatment on Man-6-P receptor binding to RCA-1 Agarose. Aliquots of the 215-kD and 46-kD Man-6-P receptors isolated in the experiment described in Fig. 5 were treated with *V. cholerae* neuraminidase and analyzed for binding to RCA-I Agarose. (○, ●) 215-kD receptor; (△, ▲) 46-kD receptor. (*Open symbols*) no neuraminidase treatment; (*closed symbols*) neuraminidase treatment.

Figure 7. The Man-6-P receptors can acquire galactose when galactosyltransferase and UDP-galactose are added to the culture medium. BW 5147 PHA^{R}1.8 cells were labeled with [^{3}H]mannose and chased 6 h. Galactosyltransferase, UDP-Gal and MnCl$_2$ were then added (final concentrations 0.05 U/ml, 0.54 mM and 10 mM, respectively) to the medium and the 37°C incubation continued. Additional UDP-Gal (0.5 μmol per ml) was added every 30 min. The fraction of the 215-kD (*A*) or 46-kD (*B*) Man-6-P receptors capable of binding to RCA-1 Agarose with (●, ▲) or without (○, △) prior *V. cholerae* neuraminidase treatment was determined as described in Materials and Methods. Where indicated (4°C), cells were labeled with [^{3}H]mannose and then chased 6 h at 37°C. The cells were cooled to 4°C, then UDP-Gal, galactosyltransferase and MnCl$_2$ were added and the 4°C incubation continued for 2 h. Thereafter, the fraction of each receptor containing galactose was determined as above.

mum chase of 6 h insured that the radiolabeled receptors had completed their first pass through the Golgi region (31). The two receptors were purified and the fraction capable of binding to RCA-1 Agarose determined. As shown in Fig. 5, a portion of both receptors obtained from cells at the end of the 6-h chase bound to the lectin, demonstrating partial galactosylation (panels *A* and *E*). This binding did not increase during the next 12 h of chase, indicating that neither receptor acquired additional galactose residues (Fig. 5, panels *B–D* and *F–H*). Since substitution of galactose residues by sialic acid can reduce the affinity of the oligosaccharide for the lectin (1), the binding of RCA-1 Agarose was redetermined after *V. cholerae* neuraminidase treatment. As shown in Fig. 6, this treatment had no effect on binding of the 215-kD Man-6-P receptor to the lectin and only a small effect on binding of the 46-kD Man-6-P receptor. Therefore, the failure to observe a progressive increase in lectin binding was not due to the acquisition of "blocking" sialic acid residues.

To prove that the mature receptors could serve as substrates for galactosyltransferase and that galactose acquisition would cause an observable increase in RCA-1 Agarose binding, the experiment was repeated with UDP-Gal and galactosyltransferase added to the culture medium during the chase. Under these conditions, both receptors acquired suf-
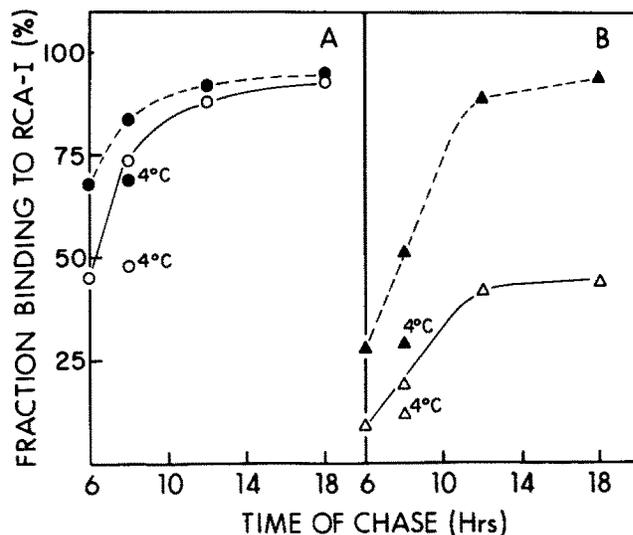
ficient galactose so that greater than 90% of each bound to the lectin (Fig. 7). The half-time of this process was 2 h for the 215-kD Man-6-P receptor and 2.5 h for the 46-kD Man-6-P receptor. This galactosylation was temperature-dependent since cells incubated with UDP-Gal and galactosyltransferase at 4°C for 2 h manifested only a slight increase in lectin binding (Fig. 7), presumably due to galactose addition to the small fraction of receptors present on the cell surface (23). Since almost all of the receptor molecules acquired galactose residues, the total pool of receptors would appear to be in equilibrium even though only a small percent is present on the cell surface at any point in time.

In similar experiments performed with CHO clone 13 cells, the same result was obtained in that the receptors only acquired galactose when the cells were exposed to exogenous UDP-Gal and galactosyltransferase. However, in this cell line the rate of galactose addition to the receptors was significantly faster with the half-time being 30 min for the 215-kD receptor and <1 h for the 46-kD receptor (data not shown). We conclude from these experiments that neither receptor acquires additional galactose residues after its initial pass through the Golgi region even though the receptors are good substrates for galactosyltransferase. These data indicate that the receptors do not recycle to the Golgi compartment containing galactosyltransferase.

As shown in Fig. 7, panel *B*, the 46-kD Man-6-P receptor isolated from cells incubated with galactosyltransferase and

1) Treat cells with
deoxymannojirimycin (dMM)

2) Label with [³H] mannose for
1 hr in presence of dMM

3) Chase 6 hrs in presence of
dMM

Golgi   Acidified
         Compartment
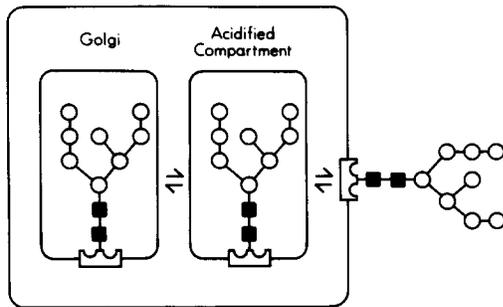
4) Remove dMM
5) Continue chase
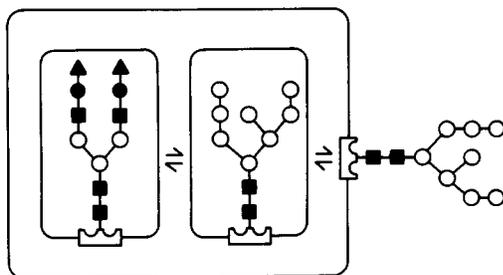
6) Isolate receptor and prepare glycopeptides
7) Analyze glycopeptides on Con A Sepharose
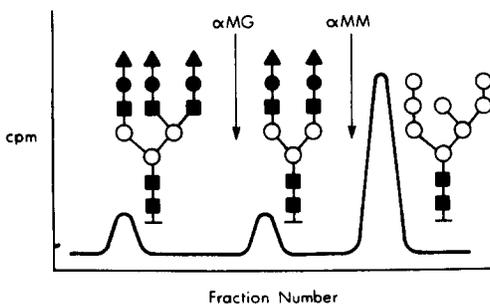
αMG   αMM

cpm

Fraction Number

Figure 8. Experimental protocol used to determine whether receptor molecules return to the early Golgi compartment containing α-mannosidase I. The symbols represent: (■) N-acetylglucosamine; (○) mannose; (●) galactose; (▲) sialic acid.

215 kDa M6PR   46 kDa M6PR   Total

FRACTION NUMBER

Figure 9. Con A-Sepharose chromatography of glycopeptides isolated from BW 5147 cells incubated with or without dMM. Parent BW 5147 cells were labeled with [³H]mannose for 40 min and chased 6 h in the presence (*D-O*) or absence (*A-C*) of 0.25 mM dMM. The inhibitor was removed and the cells recultured for 3 (*G-I*), 6 (*J-L*) or 12 (*M-O*) h. Both Man-6-P receptors as well as total glycoproteins were isolated and digested to glycopeptides. Conversion of high mannose-type to complex-type oligosaccharide units was assessed by Con A-Sepharose chromatography. The columns were washed with Tris-buffered saline and eluted with 10 mM α-methylglucoside (*G*) and 100 mM α-methylmannoside (*M*) at the indicated points. (*A, D, G, J,* and *M*) 215-kD Man-6-P receptor; (*B, E, H, K,* and *N*) 46-kD Man-6-P receptor; (*C, F, I, L,* and *O*) total membrane glycoproteins.

UDP-Gal bound completely to RCA-1 Agarose only after it was treated with neuraminidase. This indicates that the receptor had acquired sialic acid after the addition of galactose, as would be predicted from the previous experiments on resialylation of the receptor. The effect of neuraminidase on binding of the 215-kD receptor to the RCA-1 Agarose was much smaller (Fig. 7, panel *A*). This most likely reflects the fact that the 215-kD receptor has more glycosylation sites which might allow binding to the lectin even when the galactose residues are sialylated (17, 42).

Receptors Rarely Recycle Through the Golgi Region

The approach used to determine whether the Man-6-P receptors or other glycoproteins return to the early Golgi complex
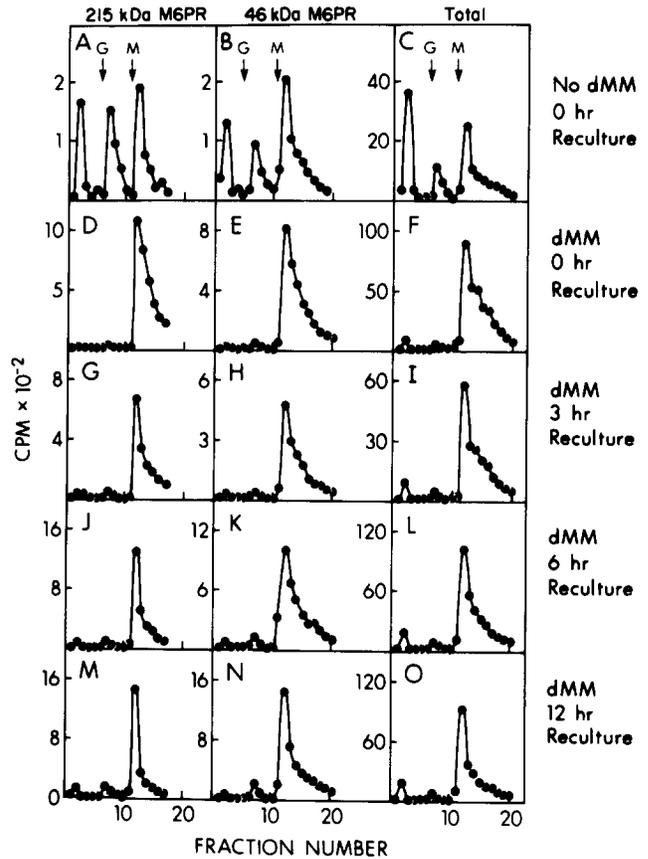
and encounter Golgi α-mannosidase I is outlined in Fig. 8. This approach takes advantage of a reversible inhibitor of Golgi α-mannosidase I, deoxymannojirimycin (dMM) (8). Cells incubated in the presence of the inhibitor produce glycoproteins which retain their high mannose structures as they pass through the Golgi complex whereas under normal circumstances many of these oligosaccharides would be processed to complex-type units. After allowing sufficient time for the glycoproteins to complete their initial pass through the Golgi region, the inhibitor is removed. During the subsequent incubation, any glycoprotein that recycles back through the Golgi region should encounter an active Golgi α-mannosidase I and have its high mannose oligosaccharides trimmed. Subsequent processing enzymes would then convert the trimmed high mannose units to complex-type units (38). The conversion from high mannose to complex-type
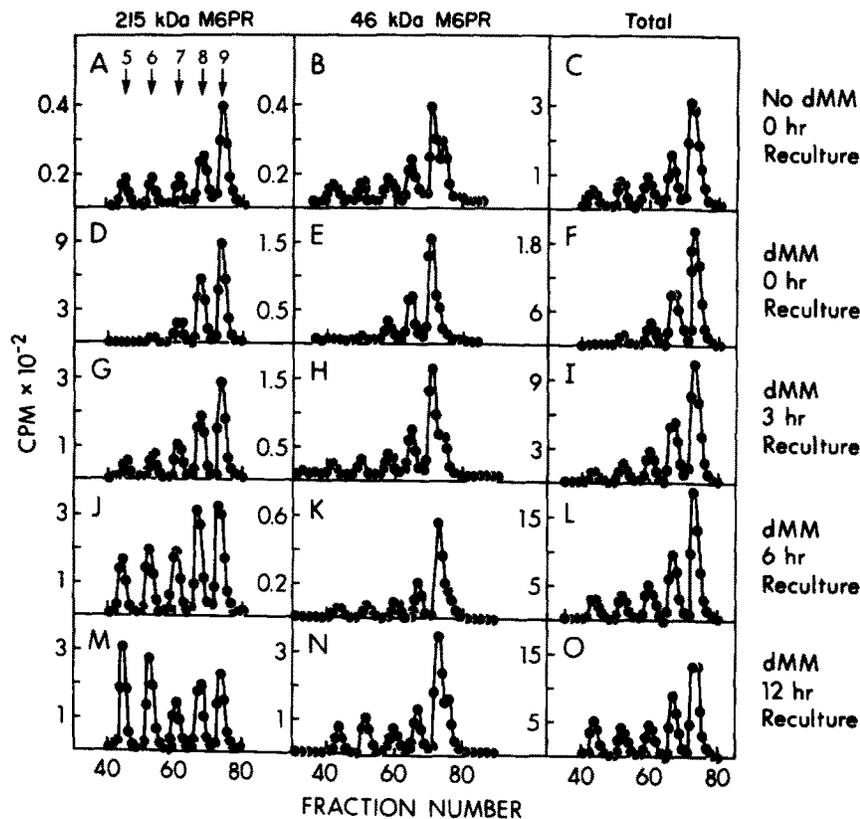
Figure 10. Size of the high mannose oligosaccharides isolated from Man-6-P receptors and total glycoproteins. The 0.1-M α-methylmannoside eluates from the Con A-Sepharose columns (Fig. 9) were desalted, concentrated by evaporation under reduced pressure, and digested with 2.5 mU of endo H. The digests were applied to Amberlite MB3 minicolumns in water and an aliquot of the released, neutral oligosaccharides was taken to dryness, dissolved in 35% aqueous acetonitrile, and applied to an AX5 micropak high performance liquid chromatography column (Varian). The oligosaccharides were eluted using a 60-ml linear gradient from 35 to 65% aqueous acetonitrile; 0.6-ml fractions were collected and diluted with 4 ml of scintillation fluid for radioactivity determination. The arrows in *A* indicate the elution position of the following high mannose oligosaccharide units: $Man_5GlcNAc$ (5), $Man_6GlcNAc$ (6), $Man_7GlcNAc$ (7), $Man_8GlcNAc$ (8), and $Man_9GlcNAc$ (9). The labeling of the panels is identical to that in Fig. 9.

units can be conveniently followed by analyzing the Asn-linked oligosaccharides on Con A-Sepharose columns. Under the elution conditions employed, complex-type units either pass through the column (peak I) or are eluted with 10 mM α-methylglucoside (peak II) while high mannose-type oligosaccharides bind tightly and require 100 mM α-methylmannoside for elution (peak III) (31, 40, 43). Thus, processing of high mannose to complex-type units would be reflected by a shift of material from peak III to peaks I and II. Preliminary experiments showed that 250–500 µM dMM inhibited oligosaccharide processing of total cellular glycoproteins by greater than 80% and that the effect was fully reversed within 30 min after the dMM was removed from the medium (data not shown). These results are in agreement with those reported by Snider and Rogers (56).

Using the protocol shown in Fig. 8, parent BW 5147 cells were incubated with [³H]mannose for 40 min in the presence of 0.25 mM dMM and then chased for 6 h in the presence of dMM. The 6-h chase allowed the newly synthesized receptors to exit the rough endoplasmic reticulum and complete their initial pass through the Golgi region (31). The dMM was then removed and the chase continued for 12 more h. At various intervals, cells were harvested and the Man-6-P receptors isolated. Glycopeptides were prepared from the receptors and analyzed on Con A-Sepharose columns. The results are shown in Fig. 9. In the absence of dMM, both receptors contain high mannose and complex-type oligosaccharide units (Fig. 9, panels *A* and *B*). The addition of dMM to the medium during [³H]mannose labeling and chase inhibited complex-type oligoscaccharide formation (Fig. 9, panels *D* and *E*). After removal of the dMM, the high mannose oligosaccharides in peak III were converted to complex-

type species (peaks I and II) extremely slowly (Fig. 9, panels *G*, *H*, *J*, *K*, *M*, and *N*). The half-times of conversion from high mannose to complex-type oligosaccharides was ∼20 h for both receptors. Similar results were obtained with the total membrane glycoproteins where the half-time of processing was estimated to be 21 h (Fig. 9, panels *C*, *F*, *I*, *L*, and *O*). These results, which are summarized in Table I, indicate that the Man-6-P receptors as well as other membrane glycoproteins rarely recycle through the *cis*/middle Golgi region where the enzymes required to convert high mannose to complex-type structures are concentrated.

We next considered the possibility that the receptors might only be exposed to Golgi α-mannosidase I, i.e., they might enter and exit the compartment containing α-mannosidase I without proceeding to the compartments containing the subsequent oligosaccharide-processing enzymes. If this occurred the high mannose oligosaccharides would be processed from the $Man_{8-9}GlcNAc_2$ species to $Man_5GlcNAc_2$ without conversion to complex-type molecules (38). To address this possibility, the peak III glycopeptides were treated with endo H to release the oligosaccharides and the distribution of sizes (Man_5 to Man_9) determined by HPLC analysis. As shown in Fig. 10, there was a gradual shift in oligosaccharide size from $Man_{8-9}GlcNAc$ to smaller forms for both Man-6-P receptors and total glycoproteins. In the case of the 46-kD receptor, the shift was quite slow and very similar to that observed for the total membrane glycoproteins. By taking the conversion of the $Man_{8-9}GlcNAc_2$ species to $Man_{5-6}GlcNAc_2$ as a measure of Golgi α-mannosidase I action, the processing could be shown to occur by a first order reaction, with a calculated $t_{1/2}$ of 14 h for the processing of the 46-kD receptor oligosaccharides and 12 h for the pro-

cessing of the total membrane glycoprotein oligosaccharides. While the rate of processing of the high mannose units of the 215-kD receptor was somewhat faster than that observed with the 46-kD receptor, the kinetics of the processing were complex and did not obey a first order decay curve. For this reason, we could not calculate a valid $t_{1/2}$ for this processing. However, by comparing these data with the values obtained for the 46-kD receptor and the total membrane glycoproteins, we estimate the $t_{1/2}$ of the mannose processing to be between 6–13 h. Thus, the shortest $t_{1/2}$ is twice as long as the $t_{1/2}$ for sialic acid addition in this cell type. These data indicate that neither Man-6-P receptor routinely enters and exits the early Golgi compartment that contains the α-mannosidase I.

In a separate experiment, swainsonine was added to determine if the mannose loss could be attributed to the swainsonine-sensitive lysosomal mannosidase (8). For oligosaccharides prepared from the 215-kD receptor, swainsonine did not affect the shift from $Man_{8-9}GlcNAc_2$ to $Man_{5-6}GlcNAc_2$ during a 6-h reculture (data not shown). This indicates that a dMM-sensitive, swainsonine-resistant α-mannosidase mediates this processing.

Discussion

We have used the compartmentalization of oligosaccharide processing enzymes in the Golgi apparatus as a means for studying the cycling of the Man-6-P receptors to various regions of that organelle. The methods used in this study measure the flux of receptor through the various Golgi compartments in contrast to immunolocalization and subcellular fractionation techniques which measure steady state concentrations. Hence this approach gives a better indication of the actual movement of receptors to the various regions of the Golgi. Using these methods, several significant findings have emerged. First, both the 215-kD and the 46-kD Man-6-P receptors cycle between the cell surface and the Golgi region and do so at a similar rate. The finding that the 46-kD Man-6-P receptor cycles to the Golgi region suggests that this protein actually functions as a receptor rather than serving as a static-binding protein. The surface galactosylation experiment (Fig. 7) resulted in virtually all of the receptor molecules acquiring galactose residues. One explanation for this is that the total pool of receptor molecules is in equilibrium even though only a small percentage of the molecules are on the cell surface at any point in time. Alternatively, since the experiments were carried out at 37°C, it is possible that some of the galactosyltransferase and UDP-galactose entered endosomal compartments and acted on a subset of receptors that cycle between these compartments and the Golgi region without passing through the cell surface. Since the sialylation experiments document that the receptors cycle between the cell surface and the Golgi complex, presumably by passing through intermediate endosomal compartments, we favor the concept that there is only one pool of receptor molecules which cycle between all the compartments. Previous investigators using different techniques have also concluded that all of the 215-kD Man-6-P receptor is in equilibrium (16, 53, 63). However, these studies share the same potential pitfall as the galactosyltransferase experiments. The cycling of the 46-kD Man-6-P receptor was not analyzed in these studies.

To study whether receptor cycling is affected by ligand

binding, cycloheximide was added in several experiments to inhibit the synthesis of lysosomal enzymes, thereby removing the source of ligand. Under these conditions, receptor flux through the various compartments was not significantly altered, indicating that both receptors cycle constitutively. Braulke et al. and Pfeffer came to the same conclusion using different methods to analyze the cycling of the 215-kD Man-6-P receptor (10, 45). However, these experiments do not rule out the possibility that ligand binding might facilitate certain steps in receptor recycling, thereby changing the steady state concentration of receptor in specific compartments (14, 15).

The major conclusion from these studies is that both Man-6-P receptors return to the Golgi compartment containing sialyltransferase much more frequently than to the Golgi regions containing galactosyltransferase or α-mannosidase I. Since sialyltransferase has been immunolocalized to the trans-Golgi cisternae and the TGN (48), whereas galactosyltransferase has been immunolocalized to the trans-Golgi cisternae (47) and α-mannosidase I is considered to be a cis/middle Golgi marker (2–4), we conclude that the receptors are returning to the TGN. On this basis we propose that in the cell types examined the TGN is the major site where lysosomal enzymes encounter the Man-6-P receptors. This proposal is consistent with a wide range of morphologic and biochemical data. Geuze et al. (28) and Griffiths et al. (33) have shown that the 215-kD Man-6-P receptor is present in the TGN with lesser amounts being detected in the Golgi stack. In agreement with our observation that the receptors do not return to the compartment containing galactosyltransferase, Geuze et al. found little overlap in the distribution of galactosyltransferase and the 215-kD Man-6-P receptor when both were immunolocalized in HepG2 cells (28). Furthermore, Berger et al. using double immunofluorescence labeling in HeLa cells found galactosyltransferase and sialyltransferase to be localized in different subcellular compartments (7). The finding that several lysosomal enzymes contain terminally processed complex-type N-linked oligosaccharides indicates that these enzymes traversed the Golgi complex as would be expected if sorting occurred in the TGN (5, 22, 29, 41, 46, 61).

Brown and Farquhar have recently used the immunoperoxidase technique to localize the 215-kD Man-6-P receptor in three cell types (13). They found the receptor to be concentrated in the cis Golgi region of Clone 9 cells, in the TGN of CHO cells and throughout the Golgi region of NRK cells. They concluded that the distribution of the 215-kD receptor within the Golgi complex varies from one cell type to another and may reflect variations in lysosomal enzyme trafficking among different cell types. It should be noted that their data on the localization of the 215-kD Man-6-P receptor in CHO cells is in agreement with our kinetic studies of this cell type.

As part of this study we also examined the return of total membrane glycoproteins (minus the Man-6-P receptors) to the Golgi compartments containing sialyltransferase and α-mannosidase I. The sialylation of the cell surface glycoproteins was detectable but was extremely slow when compared with the Man-6-P receptors. This indicates that either the total cell surface glycoproteins cycle through the TGN at a very slow rate or that a small subset of these molecules cycles at a rate comparable to that of the Man-6-P receptors with the remainder of the molecules not returning to this

compartment. The latter possibility is favored by the observation that in K562 erythroleukemia cells the transferrin receptor is resialylated with a $t_{1/2}$ of 2–3 h (55). We also found that the return of total glycoproteins to the Golgi compartment containing α-mannosidase I was quite slow, whether measured by the conversion of high mannose units on the glycoprotein to complex-type units (e.g., return to early Golgi stacks followed by transit through the Golgi region) or as determined by the processing of $Man_{7-9}GlcNAc$ species to $Man_{5-6}GlcNAc$ species. The calculated $t_{1/2}$'s for these events were 20 and 12 h, respectively. The $t_{1/2}$ of 12 h for mannose processing may actually be an overestimate of the true rate of return to the early Golgi region since dMM-inhibitable α-mannosidases are known to exist in other compartments, such as the endoplasmic reticulum (9). Based on the paucity of sialic acid addition and the slow rate of conversion from high mannose to complex-type oligosaccharides, we conclude that glycoproteins as a whole do not routinely recycle through the Golgi region in murine BW 5147 lymphoma cells. This conclusion differs significantly from that drawn from similar experiments reported by Snider and Rogers on the return of the transferrin receptor and other glycoproteins to the early Golgi compartments in K562 erythroleukemia cells (56). These workers calculated that the transferrin receptor and total glycoproteins return to the Golgi compartment containing α-mannosidase I with $t_{1/2}$'s of >6 and 4 h, respectively. These differences may reflect the method used for calculating half times, some variations in experimental design and cell-to-cell variation.

Griffiths and Simons have recently proposed that the TGN is the site where newly synthesized proteins destined for the plasma membrane, secretory vesicles and lysosomes are sorted and packaged into the correct transport vesicles (32). Our finding that both Man-6-P receptors return to the Golgi compartment containing sialyltransferase and rarely recycle through other regions of the Golgi is totally consistent with this model.

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