Lysosomal Enzyme Oligosaccharide Phosphorylation in Mouse Lymphoma Cells: Specificity and Kinetics of Binding to the Mannose 6-Phosphate Receptor In Vivo

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ABSTRACT   Phosphomannosyl residues on lysosomal enzymes serve as an essential component of the recognition marker necessary for binding to the mannose 6-phosphate (Man 6-P) receptor and translocation to lysosomes. The high mannose-type oligosaccharide units of lysosomal enzymes are phosphorylated by the following mechanism: N-acetylglucosamine 1-phosphate is transferred to the 6 position of a mannose residue to form a phosphodiester; then the N-acetylglucosamine is removed to expose a phosphomonoester. We examined the kinetics of this phosphorylation pathway in the murine lymphoma BW5147.3 cell line to determine the state of oligosaccharide phosphorylation at the time the newly synthesized lysosomal enzymes bind to the receptor. Cells were labeled with [2-^{3}H]mannose for 20 min and then chased for various times up to 4 h. The binding of newly synthesized glycoproteins to the Man 6-P receptor was followed by eluting the bound ligand with Man 6-P. Receptor-bound material was first detected at 30 min of chase and reached a maximum at 60 min of chase, at which time ~10% of the total phosphorylated oligosaccharides were associated with the receptor. During longer chase times, the total quantity of cellular phosphorylated oligosaccharides decreased with a half-time of 1.4 h, suggesting that the lysosomal enzymes had reached their destination and had been dephosphorylated. The structures of the phosphorylated oligosaccharides of the eluted ligand were then determined and compared with the phosphorylated oligosaccharides of molecules which were not bound to the receptor. The major phosphorylated oligosaccharide species present in the nonreceptor-bound material contained a single phosphodiester at all times examined. In contrast, receptor-bound oligosaccharides were greatly enriched in species possessing one and two phosphomonoesters. These results indicate that binding of newly synthesized lysosomal enzymes to the Man 6-P receptor occurs only after removal of the covering N-acetylglucosamine residues.

The targeting of lysosomal enzymes from their site of synthesis in the rough endoplasmic reticulum to their final destination in lysosomes involves a series of specific recognition events. The nascent polypeptides are glycosylated by transfer of a preformed high mannose-type oligosaccharide from a lipid carrier to specific asparagine residues (1, 2). Selected mannose residues of the oligosaccharide are then phosphorylated by the combined action of two enzymes, UDP-GlcNAc:lysosomal enzyme N-acetylglucosaminylphosphotransferase and N-acetylglucosamine 1-phosphodiester-α-N-acetylglucosaminidase (3–8). The former enzyme transfers N-acetylglucosamine 1-phosphate to mannose residues and the latter enzyme removes the "covering" N-acetylglucosamine residue to generate a phosphomonoester. The final product is an oligosaccharide with one or two phosphates that may be located on any of five different mannose residues (9), as shown in Fig. 1. The N-acetylglucosaminylphosphotransferase phosphorylates lysosomal enzymes at least 100-fold more efficiently than nonlysosomal glycoproteins and thereby can account for the selective phosphorylation of this class of glycoproteins (10).

In most cell types, the next step in the targeting process is believed to be the specific binding of the phosphorylated lysosomal enzymes to the mannose 6-phosphate (Man 6-P) receptor (11). Studies using intact fibroblasts and Chinese
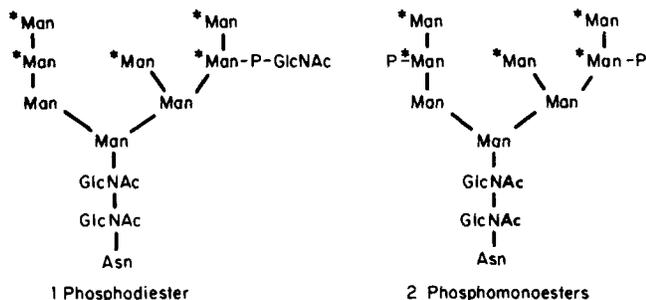
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  *Man            *Man              *Man            *Man
    |               |                 |               |
  *Man    *Man    *Man-P-GlcNAc     P*Man   *Man    *Man -P
    |       \      /                   \      \      /
   Man       Man                       Man     Man
      \      /                            \    /
       Man                                 Man
        |                                   |
      GlcNAc                              GlcNAc
        |                                   |
      GlcNAc                              GlcNAc
        |                                   |
       Asn                                 Asn

   1 Phosphodiester                  2 Phosphomonoesters
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FIGURE 1   Representative structures of high mannose oligosaccharides containing phosphodiesters and phosphomonoesters. The species shown contain eight mannose residues but the actual number may vary from six to nine; the asterisks indicate the mannose residues which may be phosphorylated (9). Oligosaccharides may contain one or two phosphate residues.

hamster ovary cells demonstrated that these cells contain surface receptors that mediate adsorptive pinocytosis of phosphorylated lysosomal enzymes (12–14). On this basis it was initially proposed that lysosomal enzymes are secreted and then recaptured by cell surface receptors which deliver the enzymes to lysosomes (15). However, the discovery that the majority of the Man 6-P receptors are located intracellularly (13), and the observation that addition of Man 6-P to fibroblast cultures does not inhibit lysosomal enzyme targeting (16, 17), has led to the suggestion that the bulk of the newly synthesized lysosomal enzymes bind to intracellular receptors. The receptor-ligand complex would then translocate to a lysosome where the low pH would cause dissociation and trapping of the enzyme in the lysosome (11). Support for this model has been obtained through the use of lysosomotropic amines such as ammonium chloride and chloroquine, which raise intralysosomal pH (18) and inhibit lysosomal enzyme targeting (19).

Previous studies of receptor-mediated endocytosis of lysosomal enzymes by fibroblasts have indicated that the uptake is prevented by predigestion with alkaline phosphatase (20–23). This suggests that phosphomonoesters are important for recognition by cell surface receptors. However, nothing is known about the structure of newly synthesized lysosomal enzyme oligosaccharides at the time the enzymes first bind to intracellular Man 6-P receptors. It seemed possible that lysosomal enzymes with phosphodiesters actually bind to the receptor and that conversion to phosphomonoesters occurs subsequently. In addition, if the Man 6-P receptor is operating intracellularly to transport lysosomal enzymes to lysosomes, then the binding of newly synthesized lysosomal enzymes should precede entry into lysosomes. Therefore, to further characterize the intracellular Man 6-P receptor, we examined the nature of the phosphorylated oligosaccharides on newly synthesized lysosomal enzymes both before and after binding to the receptor in Thy$^+$ mouse lymphoma cells. Our data indicate that lysosomal enzymes bind to the Man 6-P receptor shortly after their synthesis and that conversion of phosphodiesters to phosphomonoesters precedes binding.

MATERIALS AND METHODS

Cells and Labeling Conditions

Murine Thy$^+$ lymphoma cells (BW5147.3) and P388D$_1$ macrophages (kindly provided by Dr. John Atkinson, Washington University) were maintained in α-minimal essential medium containing 10% fetal calf serum in either roller bottles (Thy$^+$) or spinner flasks (P388D$_1$). For labeling, an aliquot of the appropriate stock culture (containing 1×10^8 cells) was removed, centrifuged at 600 g for 10

min, and the supernatant discarded. The cells were resuspended in 10 ml of modified minimal essential medium containing 10% dialyzed horse serum (GFMEM); the medium was prepared as described (Gibco manual, Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), except that glucose and bicarbonate were omitted and N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, pH 7.2, was added to a final concentration of 20 mM. After 10 min at 37°C, the suspension was centrifuged and the cells resuspended in 10 ml of GFMEM containing 1 mCi of [2-³H]mannose (New England Nuclear; 14 Ci/mmol in 95% EtOH; the solvent was evaporated before use by N$_2$ aeration). The labeling mixture was gently rotated for the indicated time at 37°C; 0.1 ml of a solution containing 1 M each of mannose and glucose was added to begin the chase. All manipulations up to this point were done using sterile conditions. After the appropriate chase time (0–240 min), the cells were placed on ice and washed twice with cold PBS.

Cell Fractionation

Labeled cells were resuspended in 3 ml of 50 mM Tris, pH 7.5, 5 mM PO$_4$, 1 mg/ml bovine serum albumin (BSA), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (lysis buffer), and ruptured by three 10-s bursts on a Bronwill sonicator (setting of 60). The sonicates were centrifuged at 800 g for 10 min and the supernatants recovered. The pellets were resuspended in 1 ml of lysis buffer, sonicated for two 10-s bursts, and recentrifuged. The 800 g supernatants were combined and centrifuged at 80,000 g for 30 min in a Ti50 rotor. The supernatants were removed (save, 80,000-g supernatant) and the pellets resuspended in 2.5 ml of 25 mM Tris, pH 7, 0.1 M NaCl, 5 mM PO$_4$, 0.2 mM PMSF, containing 0.5% saponin (wash buffer) by gentle homogenization in a Dounce homogenizer. The mixtures were incubated at 20°C for 30 min and then centrifuged at 80,000 g for 30 min. The supernatants were recovered (saponin wash) and the pelleted membranes washed twice (3 ml/wash) with wash buffer. The resulting membranes were resuspended by homogenization in 2.5 ml of wash buffer containing 10 mM Man 6-P, incubated at 20°C for 30 min, then centrifuged at 80,000 g for 30 min. The supernatants were recovered and used as the Man 6-P eluates; the membrane pellets were combined with the appropriate initial 80,000-g supernatant and saponin wash and used as the nonreceptor-bound fractions.

Oligosaccharide Fractionation

BSA was added to the Man 6-P eluate and to the nonreceptor-bound fraction, resulting in a final concentration of 3 mg/ml, and cold trichloroacetic acid was added to a final concentration of 10%. After 10 min on ice, the precipitated proteins were collected by centrifugation (10,000 g for 10 min) and the precipitates washed twice with cold ether (3 ml/wash). The pellets were resuspended in 0.9 ml of 0.1 M Tris, pH 8, 0.1 M glucose 6-P, 20 mM CaCl$_2$; 0.1 ml of 100 mg/ml pronase was added to each and the fractions placed at 56°C. After 14 h, an additional 5 mg of pronase were added and the digestions continued for 2 h. The pronase digests were boiled for 10 min, diluted with 4 ml of 10 mM Tris, pH 7.5, 0.15 M NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$ (TBS), and clarified by centrifugation. The supernatants were applied to 1.5-ml columns of concanavalin A (Con A)-Sepharose in TBS and the columns eluted in succession with 6 ml of TBS, 4.5 ml of 10 mM α-methylglucoside in TBS, and 9 ml of 0.1 M α-methylmannoside in TBS at 56°C. The glycopeptides eluted by 0.1 M α-methylmannoside were concentrated by evaporation under reduced pressure, desalted on Sephadex G-25, and digested with endo-β-N-acetylglucosaminidase H (endo H) as previously described (9). The endo H digests were diluted with 2 ml of 2 mM pyridinium acetate, pH 5.3, and applied to 5-ml columns of QAE-Sephadex in 2 mM pyridinium acetate. Each column was eluted with a 200 ml gradient of pyridinium acetate, pH 5.3, from 2 to 500 mM as described elsewhere (24).

The purity of the phosphorylated oligosaccharides recovered from quaternary aminoethyl (QAE)-Sephadex was examined using QAE-Sephadex minicolumns as previously described (9). Fractions suspected of containing phosphodiesters were treated with 0.01 N HCl at 100°C for 30 min then lyophilized, diluted with 2 mM Tris base, and applied to QAE-Sephadex. Radioactivity that required a higher concentration of NaCl for elution from QAE-Sephadex after the mild acid treatment was considered to be derived from oligosaccharides possessing phosphodiesters. Fractions suspected of containing phosphomonoesters were digested with Escherichia coli alkaline phosphatase for 2 h at 37°C and then applied to QAE-Sephadex minicolumns. The radioactivity which did not bind to QAE-Sephadex after phosphatase digestion was considered to be derived from oligosaccharides possessing phosphomonoesters. The phosphodiester-containing fractions were generally 50–70% pure and the phosphomonoester fractions, 60–85% pure. The contaminants which bound to QAE-Sephadex are presumed to result either from the incomplete digestion of the glycopeptides by endo H or by the presence of oligosaccharides that possess sialic acid residues.

Oligosaccharides containing phosphodiesters that were to be analyzed by paper chromatography were treated with mild acid, fractionated on QAE-Sephadex, desalted, and digested with alkaline phosphatase; phosphomonoesters were

treated only with alkaline phosphatase. The phosphatase digests were reapplied to QAE-Sephadex and the neutralized radioactivity recovered, desalted by passage over Amberlite MB-3, and spotted on Whatman #1 paper. The descending chromatograms were developed for 4 d in the following solvent: ethyl acetate/ pyridine/acetic acid/water (5/5/1/3). After drying, each chromatogram was cut into 1-cm strips, the strips soaked in 0.4 ml of water, and 4 ml of scintillation fluid added.

RESULTS

Characterization of Endogenous Receptor-bound Phosphorylated Oligosaccharides

To characterize the oligosaccharide units of newly synthesized lysosomal enzymes bound to the Man 6-P receptor, Thy^+ mouse lymphoma cells were labeled for 1 h with [2-^{3}H]mannose and total cell membranes isolated. The membranes were washed with saponin to remove material that was trapped inside vesicles and then treated with Man 6-P to elute the receptor-bound lysosomal enzymes. Previous investigations (13, 25) have shown that Man 6-P accelerates the release of receptor-bound lysosomal enzymes. The two fractions (the Man 6-P eluate and the pooled nonreceptor-bound material), were digested with pronase and applied to columns of Con A-Sepharose. The high mannose-type glycopeptides were eluted with α-methylmannoside and treated with endo H to release the oligosaccharides from the underlying peptides. The digests were then fractionated on QAE-Sephadex using an elution system which separates neutral oligosaccharides and the various phosphorylated oligosaccharide species (24).

Fig. 2 shows typical QAE-Sephadex profiles for the components eluted from the membranes with Man 6-P (Fig. 2 B) and for the nonreceptor-bound material (Fig. 2 A). The major phosphorylated species recovered from the cells was an oligosaccharide containing a single phosphodiester. In addition, smaller amounts of oligosaccharides containing one phosphomonoester, two phosphodiesters, and two phosphomonoesters were present. The material eluted from the receptor with Man 6-P contained the same four phosphorylated species (Fig. 2 B); however, relative to the nonreceptor-bound material there was a great enrichment in species containing phosphomonoesters. Since the phosphorylated species in the different peaks from the QAE-Sephadex columns account for only 50–85% of the radioactivity, the various fractions were pooled and analyzed further to correct for the purity of each species (see Materials and Methods).[1] The results are summarized in Table I. The Man 6-P eluate contained 8% of the total cellular phosphorylated oligosaccharides. In terms of the individual species, the eluate contained 35% of the oligosaccharides with two phosphomonoesters, 12% of the oligosaccharides with one phosphomonoester, and only 3% of the species with one and two phosphodiesters. The neutral oligosaccharides eluted by Man 6-P presumably are not bound directly to the Man 6-P receptor; their presence in the eluate most likely results either from: (a) the elution of lysosomal enzymes containing multiple oligosaccharides of which only a few are phosphorylated (24); or (b)

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[1] The results presented in this paper focus only on the four major phosphorylated oligosaccharides synthesized by mouse lymphoma cells (i.e., high mannose-type oligosaccharides containing one phosphodiester, one phosphomonoester, two phosphodiesters, and two phosphomonoesters). We recently identified another class of phosphorylated oligosaccharides that elute from QAE-Sephadex in the same region of the gradient as molecules containing two phosphodiesters. These hybrid-type oligosaccharides contain residues of sialic acid and galactose in addition to phosphomannose.
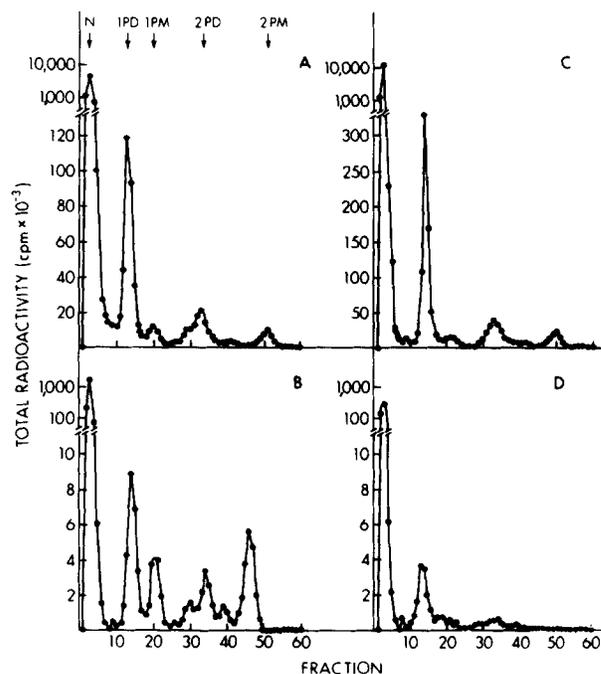
FIGURE 2   QAE-Sephadex fractionation of phosphorylated oligosaccharides. The endo H digests of the nonreceptor-bound ^{3}H-labeled glycopeptides (A and C) and those obtained from the Man 6-P eluates (B and D) were applied to QAE-Sephadex columns (5 ml) equilibrated in 2 mM pyridinium acetate, pH 5.3 The columns were eluted with linear pyridinium acetate gradients (200 ml) from 2 to 500 mM, and 2-ml fractions were collected; no radioactive species eluted beyond fraction 60, so that abbreviated profiles are shown. The elution positions of neutral oligosaccharides (N) as well as oligosaccharides with one phosphodiester (1PD), one phosphomonoester (1PM), two phosphodiesters (2PD), and two phosphomonoesters (2PM) are shown in A. The fractions were obtained from Thy$^+$ cells (A and B) and P388D$_1$ cells (C and D) after a 60-min labeling with [2-^{3}H]mannose. The Man 6-P eluate contained 6.3% of the total ^{3}H-mannose-labeled radioactivity in Thy$^+$ cells and 5.3% of the radioactivity in P388D$_1$ cells. The break in the ordinate represents a shift between two linear scales.

TABLE I

Distribution of Phosphorylated Oligosaccharides in Mouse Lymphoma Cells

Species	Total cpm		% of Total	
	Nonreceptor	Receptor	Nonreceptor	Receptor
1 Phosphodiester	3.7×10^5	9.7×10^3	97	3
1 Phosphomonoester	5.4×10^4	7.6×10^3	88	12
2 Phosphodiesters	1.1×10^5	3.6×10^3	97	3
2 Phosphomonoesters	5.8×10^4	3.1×10^4	65	35

nonspecific release from the membranes, as saponin washing alone causes release of radioactivity (data not shown). However, saponin in the absence of added Man 6-P does not release any phosphorylated oligosaccharides.

To further establish that the phosphorylated oligosaccharides eluted from Thy$^+$ membranes with Man 6-P represent material bound to the Man 6-P receptor, a similar experiment was performed using the murine macrophage cell line P388D$_1$. This cell line phosphorylates lysosomal enzymes to a similar extent as Thy$^+$ cells, but has no detectable Man 6-P receptor (Gabel, C., D. Goldberg, and S. Kornfeld, manuscript in preparation).

Therefore, if Man 6-P is specifically eluting receptor-bound phosphorylated oligosaccharides from Thy$^+$ cells, a similar eluate from P388D$_1$ cells should not contain these structures. Fig. 2 C and D show the QAE-Sephadex profiles obtained after labeling macrophages for 1 h with [2-^{3}H]mannose and then fractionating as described for the lymphoma cells. The macrophages actively form the recognition signal as evidenced by the recovery of all the various species of phosphorylated oligosaccharides in the nonreceptor-bound fraction (Fig. 2 C). While Man 6-P elution did release a small amount of glycoprotein from macrophage membrane (as evidenced by a release of radioactivity), the eluate lacked detectable amounts of oligosaccharides with phosphomonoesters and had only a trace of material with phosphodiesters (Fig. 2 D). Separate analysis of the macrophage and Thy$^+$ membranes after Man 6-P elution indicated that there were no phosphorylated oligosaccharides still membrane-associated, ruling out the possibility that the hapten elution was ineffective. These results indicate that Man 6-P elution of Thy$^+$ cell membranes specifically releases phosphorylated oligosaccharides which are bound to the Man 6-P receptor.

Kinetics of Receptor Binding

The kinetics of binding of endogenously labeled glycoproteins to the Man 6-P receptor were determined by labeling Thy$^+$ cells for 20 min with [2-^{3}H]mannose and chasing for various periods of time in the presence of unlabeled mannose. At each time point, cells were harvested and analyzed as described above. Fig. 3 shows the QAE-Sephadex profiles of the oligosaccharides released by Man 6-P from the Thy$^+$ membranes after the 20-min pulse and chase times of 30, 60, 120, 180, and 240 min. These results, along with the data obtained from the corresponding nonreceptor-bound fractions (not shown), are summarized in Fig. 4. At the end of the 20-min labeling period, the amount of total cellular phosphoryl-
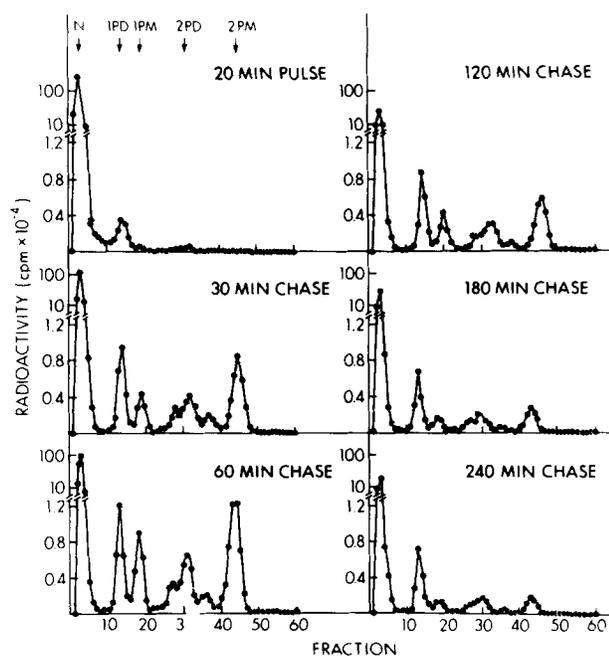
ated oligosaccharides was quite small, and there were few phosphorylated species in the Man 6-P eluate (Fig. 4 A). This is consistent with our finding in mouse P388D$_1$ cells that phosphorylation is just beginning after a 20-min pulse (24). During the first 60 min of the chase period, the quantity of phosphorylated oligosaccharides progressively increased as did the amount of material eluted from the membranes with Man 6-P (Fig. 3). It is evident that only a small amount (maximum of 10%) of the total phosphorylated oligosaccharides are present on the receptor at any given time. However, at each chase time a high percentage of the total cellular content of species with phosphomonoesters is associated with the receptor. Thus, the Man 6-P eluate contains 40–48% of the oligosaccharides with two phosphomonoesters and 15–27% of the oligosaccharides with one phosphomonoester (Fig. 4 B). In contrast, only a few percent of the species with phosphodiesters are eluted from the membranes with Man 6-P. These data indicate that throughout the pulse-chase analysis oligosaccharides with phosphomonoesters bind to the Man 6-P receptor much more efficiently than oligosaccharides with phosphodiesters.

After 60 min of chase, there is a progressive decrease in the total amount of cellular phosphorylated oligosaccharides (Fig. 4 A) and a corresponding decrease in receptor-bound phosphorylated oligosaccharides (Fig. 3). When these data are plotted in a semilog fashion (not shown), it can be calculated that the quantity of cellular phosphorylated species decreases with a $t_{1/2}$ of 1.4 h. The phosphorylated species are lost from the receptor at a slightly faster rate, $t_{1/2}$ of 1 h.

Size of the Underlying Oligosaccharides

Although only a small percentage of the total oligosaccharides possessing a single phosphodiester are eluted from the Man 6-P receptor at any specific time (Fig. 4 B), analysis of the size of the underlying high mannose units suggests that these oligosaccharides are bound to the receptor and not contaminants. For example, following 60 min of chase the majority of the nonreceptor-bound oligosaccharides with one phosphodiester contain nine and eight mannose residues (Fig. 5 A). In contrast, the receptor-bound species with one phosphodiester have oligosaccharides that are more processed as evidenced by the predominance of the Man$_7$GlcNAc species (Fig. 5 D). With increasing chase time the species with one phosphodiester both on and off the receptor show evidence of mannose processing, but even after 240 min of chase the receptor-bound oligosaccharides contain a higher percentage of the smaller oligosac-

FIGURE 3 QAE-Sephadex fractionation of Man 6-P-eluted phosphorylated oligosaccharides obtained from a pulse-chase labeling of Thy$^+$ cells. The QAE-Sephadex columns were developed as described in the legend to Fig. 1. Cells were labeled for 20 min with ^{3}H-mannose and then chased for the indicated times.
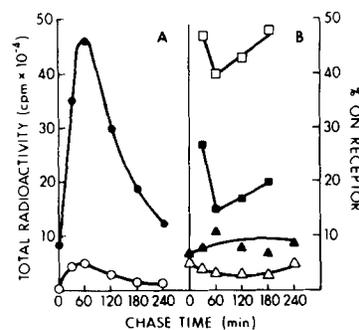

FIGURE 4 Kinetics of oligosaccharide phosphorylation and receptor binding. A shows the total radioactivity recovered as phosphorylated oligosaccharides as a function of chase time both in the nonreceptor-bound fraction (●) and the Man 6-P eluate of Thy$^+$ cells (O). In B, the percent of each phosphorylated species recovered in the Man 6-P eluate is shown as a function of chase time. The species are: △, 1 phosphodiester; ▲, 2 phosphodiesters; ■, 1 phosphomonoester; □, 2 phosphomonoesters. Due to the low quantity of phosphomonoesters present on the receptor at 0 and 240 min of chase, it was not possible to calculate accurate percentages.
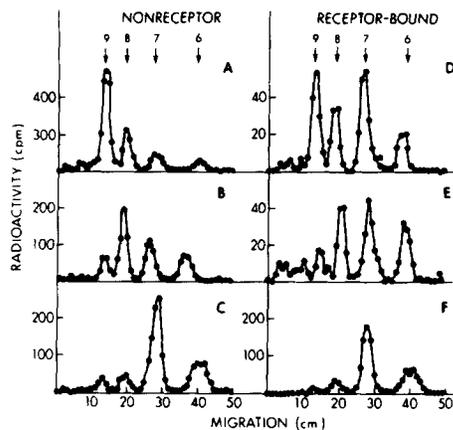
FIGURE 5 Size of the underlying high mannose-type oligosaccha-ride units derived from nonreceptor-bound (A, B, and C) and receptor-bound (D, E, and F) phosphorylated oligosaccharides. Spe-cies containing one phosphodiester, obtained after 60 (A and D) and 240 (B and E) min of chase, and one phosphomonoester, obtained after 120 min of chase (C and F) were neutralized and fractionated by paper chromatography. The migration position of standard high mannose-type oligosaccharides containing Man₉GlcNAc (9), Man₈GlcNAc (8), Man₇GlcNAc (7), and Man₆GlcNAc (6) are indicated in A and D.

charides compared to the nonreceptor-bound fractions (com-pare Fig. 5 E to B). The oligosaccharides with phosphomon-oesters show evidence of mannose processing at all times of the chase, regardless of receptor occupancy (Fig. 5 C and F).

Control for Nonspecific Rebinding to the Man 6-P Receptor

We considered the possibility that the material eluted from the Man 6-P receptor was not bound in the intact cell but, rather, only became receptor-associated during lysis of the cells and preparation of the membranes. To examine this, unlabeled Thy⁺ cells were lysed in the presence of an exogenous radio-labeled ligand and the membranes were prepared in the usual fashion. The membranes were treated with Man 6-P, and the amount of labeled phosphorylated oligosaccharides released was quantified. The exogenous ligand was derived from Thy⁺ cells that had been incubated with [2-³H]mannose for 20 min and chased for 60 min. After the chase the labeled cells were fractionated and their membranes eluted with Man 6-P. Two fractions were selected as a source of labeled ligand: (a) the Man 6-P eluate and (b) the initial 80,000-g supernatant ob-tained after lysing the cells and sedimenting the membranes. The latter fraction contains the majority of nonreceptor-bound phosphorylated oligosaccharides (data not shown). The results of these experiments are summarized in Table II. Using either ligand, <5% of the oligosaccharides containing phosphomon-oesters became receptor-associated. These experiments are complicated by the fact that the exogenously-added ligand must compete with unlabeled material released by the cells during lysis. In addition, it is impossible for the exogenously added ligand to achieve the same physical location as endog-enous molecules at the precise instant of cell lysis. Nevertheless, the results suggest that Thy⁺ Man 6-P receptors are occupied intracellularly and that rebinding during preparation of the membranes did not occur to a great extent.

DISCUSSION

The involvement of the Man 6-P receptor in the targeting of

TABLE II

Binding of Exogenously Added ³H-Ligand to Mouse Lymphoma Cell Membranes

	% of each species recovered in Man 6-P eluate			
Source of labeled ligand	One phos-pho-diester	One phos-pho-mon-oester	Two phos-pho-diesters	Two phos-pho-mon-oesters
80,000-g supernatant	0.6	2.2	1.4	3.5
Man 6-P eluate	2.7	5.0	3.4	5.0

Thy⁺ cells (1 × 10⁸) were labeled for 60 min with [2-³H]mannose and chased for 60 min, then fractionated as described in Materials and Methods. Two fractions were recovered from the preparation for use as labeled ligand in the rebinding experiments: the initial 80,000-g supernatant and the Man 6-P eluate. For analysis of rebinding, 1 × 10⁸ unlabeled Thy⁺ cells were washed twice with PBS and resuspended in either the 80,000-g supernatant or the Man 6-P eluate. The Man 6-P eluate was dialyzed extensively against lysis buffer to remove Man 6-P before its application to the unlabeled cells. The cells were disrupted by sonication and membranes prepared and eluted with Man 6-P as described in Materials and Methods except that the lysates were adjusted to 0.5% saponin immediately after sonication to allow the exogenous ligand access to all possible receptor sites.

newly synthesized lysosomal enzymes to lysosomes has previ-ously been inferred from in vitro uptake studies (12–14), inhib-itor studies (19), and more recently by the isolation of mutants that possess a reduced number of cell surface receptors (26). Our results extend these previous observations by establishing that the intracellular receptor binds newly synthesized glyco-proteins carrying the Man 6-P recognition signal and demon-strating that the kinetics of binding are consistent with the receptor functioning in lysosomal enzyme targeting.

In previous studies (9, 24, 27) we established that oligosac-charide phosphorylation is a post translational event and that the mechanism of phosphorylation involves the formation of a phosphodiester which is subsequently converted to a phos-phomonoester. The major goal of the present study was to determine the state of the oligosaccharide units of newly syn-thesized lysosomal enzymes at the time they bind to the Man 6-P receptor. To do this, we took advantage of the fact that the receptor is a membrane protein and that ligand bound to the receptor can be selectively eluted with Man 6-P (13, 25, 28). We assumed that the glycoproteins released from the receptor by Man 6-P represent newly synthesized lysosomal enzymes since these molecules appear to be the only glycoproteins which contain the Man 6-P recognition signal (10). This approach, combined with the use of [2-³H]mannose to label the oligosac-charide units of newly synthesized glycoproteins, allowed us to follow the various events in the oligosaccharide phosphoryla-tion pathway. Our data indicate that conversion of the phos-phodiesters to phosphomonoesters by removal of the covering N-acetylglucosamine residues is required for efficient ligand recognition by the Man 6-P receptor. Quantification of the various species eluted from the receptor demonstrates that, relative to the nonreceptor-bound material, there is a great enrichment for phosphorylated oligosaccharides containing one and two phosphomonoesters. This finding is consistent with previous studies of receptor-mediated uptake by fibro-blasts which have suggested that phosphomannosyl residues in the form of phosphomonoesters are required for high-affinity binding to the cell surface receptor (20–23).

The glycoproteins eluted from the receptor also contain small but significant amounts of oligosaccharides with one and

two phosphodiesters which cannot be accounted for on the basis of contamination with nonreceptor-bound material. The most likely explanation for this finding is that these oligosaccharides are derived from receptor-bound lysosomal enzymes that contain multiple phosphorylated oligosaccharides. For instance, β-glucuronidase appears to have 12 glycosylation sites, each of which may be phosphorylated (24). When a sufficient number of these oligosaccharides are converted to the phosphomonoester form, the enzyme may bind to the receptor even though it still contains some oligosaccharides with phosphodiesters and others that have not been phosphorylated at all. The data do demonstrate, however, that the majority of the phosphodiesters are converted to phosphomonoesters before the newly synthesized lysosomal enzymes bind to the Man 6-P receptor. The observation that lysosomal enzymes eluted from the Man 6-P receptor contain hybrid-type oligosaccharides possessing galactose, sialic acid, and Man 6-P[1] indicates that these molecules had migrated through the *trans* face of the Golgi apparatus, where galactosyl transferase is localized (32), before binding to Man 6-P receptor.

After the 20-min pulse with [2-³H]mannose, the total amount of phosphorylated oligosaccharides in the cell reached a maximum at 60 min of chase and then declined with a $t_{1/2}$ of 1.4 h. This loss cannot be accounted for by the secretion of lysosomal enzymes since Thy⁺ cells secrete <5% of their newly synthesized enzyme (data not shown). Rather, it most likely results from the removal of phosphate from the lysosomal enzymes upon their entry into lysosomes or another compartment where they encounter an acid phosphatase. This finding is consistent with the observation that high uptake β-glucuronidase loses its recognition signal after it has been endocytosed by fibroblasts (29). Skudlarek and Swank (30) recently measured the time necessary for newly synthesized lysosomal enzymes to be converted from the proenzyme to mature forms in mouse peritoneal macrophages. This proteolytic processing is thought to occur as lysosomal enzymes reach lysosomes (31). The proteolytic conversion was shown to begin at about 1 h after synthesis of the protein and to proceed with a $t_{1/2}$ of 1 h. Our findings are in good agreement with these data and together indicate that in these two cell types ~1 h is required for newly synthesized lysosomal enzymes to be incorporated into lysosomes.

Maximal binding of lysosomal enzymes to the Man 6-P receptor occurs after 60 min of chase, at which time ~10% of the total cellular phosphorylated oligosaccharides are present on glycoproteins that can be eluted from the receptor. Since the lysosomal enzymes which bind to the receptor probably contain more phosphorylated oligosaccharides than the molecules which are still in the process of being phosphorylated and "uncovered", it would appear that somewhat <10% of the newly synthesized lysosomal enzymes may be present on the receptor at any one time. Yet the receptor would appear to be near maximal occupancy as judged by the inability of exogenously-added ligand to bind to the Thy⁺ membranes upon cell lysis (Table II). This could also explain why only a portion of the total cellular phosphomonoester-containing oligosaccharides are receptor-associated at any given time. If the Man 6-P receptor is limiting, then receptor recycling most likely occurs in order to account for the flow of newly synthesized lysosomal enzymes through this pathway. Evidence for recycling of the cell surface Man 6-P receptor has been presented by Gonzalez-Noriega et al. (19).

Although the Man 6-P receptor-mediated pathway is oper-

ative in Thy⁺ lymphoma cells as well as several other cell lines, evidence is accumulating that alternate mechanisms exist for segregating newly synthesized lysosomal enzymes to lysosomes. This is illustrated by our findings with the murine macrophage P388D₁ cell line. These cells phosphorylate lysosomal enzymes but have no demonstrable Man 6-P receptor or Man 6-P-elutable ligand. Despite this lack of a Man 6-P receptor, P388D₁ cells form dense bodies possessing hydrolase activities that sediment as lysosomes on colloidal silica gradients (Gabel, C., D. Goldberg, S. Kornfeld, manuscript in preparation). In addition, it has been found that several cell types and tissues isolated from patients with I-cell disease and pseudo-Hurler polydystrophy contain normal hydrolase activity despite the lack of N-acetylglucosaminylphosphotransferase activity (33–36). These data indicate that in some cell types alternative mechanisms must exist for the targeting of lysosomal enzymes to lysosomes.

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