# Recognition and Receptor-mediated Uptake of Phosphorylated High Mannose-type Oligosaccharides by Cultured Human Fibroblasts

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ABSTRACT The intracellular transport of newly synthesized lysosomal hydrolases to lysosomes requires the presence of one or more phosphorylated high mannose-type oligosaccharides per enzyme. A receptor that mediates mannose-6-$PO_4$-specific uptake of lysosomal enzymes is expressed on the surface of fibroblasts and presumably accounts for the intracellular transport of newly synthesized enzymes to the lysosome. In this study, we examined the internalization of lysosomal enzyme-derived phosphorylated oligosaccharides by cultured human fibroblasts. Oligosaccharides of known specific activity bearing a single phosphate in monoester linkage were internalized with a $K_{uptake}$ of $3.2 \times 10^{-7}$ M, whereas oligosaccharides bearing two phosphates in monoester linkage were internalized with a $K_{uptake}$ of $3.9 \times 10^{-8}$ M. Thus, phosphorylated high mannose-type oligosaccharides appear to be the minimal structure required for recognition and uptake by the fibroblast receptor. The finding that the $K_{uptake}$ for monophosphorylated oligosaccharides is 100-fold less than the reported $K_i$ for mannose-6-phosphate indicates that the fibroblast phosphomannosyl receptor contains a binding site that recognizes features of the oligosaccharide in addition to mannose-6-phosphate.

Considerable progress has been made in developing an understanding of how lysosomal enzymes become localized in lysosomes. It has been established that a common structural feature present on a large number of lysosomal enzymes consists of one or more high mannose-type oligosaccharides bearing mannose-6-$PO_4$ (Man-6-P)[1] residues (1–10). These phosphorylated oligosaccharides are required for the transport of lysosomal enzymes to lysosomes (11, 12), and a deficiency of the N-acetylglucosamine-1-phosphotransferase results in an intracellular deficiency of multiple fibroblast lysosomal enzymes (13–16). Although the biosynthetic pathway (7, 9, 13–19) as well as the structures of these phosphorylated oligosaccharides (8, 10) have been extensively characterized, two important issues are unresolved. First, it is not yet apparent which phosphorylated oligosaccharide(s) interact with the lysosomal enzyme receptor. Second, it is currently not known whether all of the information necessary for targeting to lysosomes is present on the phosphorylated oligosaccharide(s) or whether the protein is also required. The first issue is of interest since recent structural analyses of the phosphorylated high mannose-type oligosaccharides (8, 10) reveal that a number of types of structural heterogeneity exist that could affect the ligand:receptor interaction. Thus, it has been shown that phosphorylated oligosaccharides may contain either one or two mannose-6-phosphate moieties per oligosaccharide, that the phosphate may be present in either phosphomonoester or phosphodiester linkage, and that the phosphates may be located in any of several positions (7–10). These findings raise the interesting questions of the relative affinities of mono- versus di-phosphorylated oligosaccharides, and of phosphomonoester- versus phosphodiester-containing ligands for the receptor, as well as whether the location of the phosphate moiety influences affinity for the receptor.

Recent work with two other mammalian lectins, the galactose/N-acetylgalactosamine receptor of hepatocytes and the mannose/N-acetylglucosamine receptor of hepatic reticuloendothelial cells, has established that all of the information necessary for recognition and endocytosis resides in individual oligosaccharides (20, 21). It is therefore interesting to examine whether the mannose-6-phosphate specific receptor behaves in an analogous manner. Thus, in order to determine which oligosaccharide(s) mediate the intracellular transport of lysosomal enzymes and to assess the relative contribution of oligosaccharide and protein for recognition and uptake, we investigated the relative susceptibilities of phosphorylated high mannose-type oligosaccharides to endocytosis by human fibroblasts.

[1] The abbreviations used are: Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Glc-6-P, glucose-6-phosphate; HBSS, Hanks' buffered salt solution; Man, mannose; Man-1-P, mannose-1-phosphate; and Man-6-P, mannose-6-phosphate.

## MATERIALS AND METHODS

*Materials:* Most reagents were purchased from Sigma Chemical Co. (St. Louis, MO), including glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase; EC 1.1.1.49) and yeast phosphomannose isomerase (D-mannose-6-phosphate ketolisomerase; EC 5.3.1.8). Yeast phosphoglucoisomerase (D-glucose-6-phosphate ketolisomerase; EC 5.3.1.9) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

*Protein Determination:* Protein was measured colorimetrically (22) with bovine serum albumin as the standard.

*Man-6-P Assay:* Aliquots of phosphorylated oligosaccharides (3–6 × $10^3$ cpm) were analyzed for Man-6-P as described previously (1). All assays were conducted in duplicate both with the complete reagent and with reagent from which phosphomannose isomerase had been omitted. Man-6-P standards (8 and 16 pmol) were carried through the entire procedure to correct for the loss of Man-6-P (~20%) that occurs during hydrolysis. As before (10), oligosaccharides A-3 (see below) were found to contain twice as much Man-6-P as oligosaccharides A-1. The specific activity of all of the oligosaccharides, 814 cpm/pmol, was identical.

*Oligosaccharide Probes:* Oligosaccharides isolated from homogeneous human spleen β-glucuronidase (1) by endoglycosidase H hydrolysis were labeled with $NaB[^3H]_4$ (9.6 Ci/mmol) at their reducing termini, fractionated, and characterized as described previously (10). The oligosaccharides in A-1 contain one mole of Man-6-P that is in diester linkage, and the oligosaccharides in A-3 have two moles of Man-6-P both in diester linkage (10). The phosphate moieties are heterogeneous in location as delineated previously (10 and Fig. 1). Before use in these studies, these oligosaccharides were subjected to mild acid hydrolysis (pH 2.0, 100°C, 60 min) in order to release the α-linked N-acetylglucosamine residues and convert the phosphodiesters to phosphomonoesters (7, 8, 10). These oligosaccharides are therefore referred to in the text as deblocked A-1 and deblocked A-3, respectively, and their structures shown in Fig. 1. The neutral oligosaccharides, which were also derived from β-glucuronidase by endoglycosidase H digestion, are of the same specific activity as the phosphorylated oligosaccharides and have been previously characterized (10).

*Endocytosis Measurements:* Receptor-mediated endocytosis of oligosaccharides was measured using subconfluent normal human fibroblasts in 60-mm petri dishes. The oligosaccharides were dissolved in Hanks' buffered salt solution (HBSS) (23), and 1.4 ml was added per dish. After exposure to the oligosaccharides for 6 h at 37°C, the medium was removed and saved and the cells were washed 3× with 5 ml of HBSS at 4°C. The cells then received 2 ml of HBSS containing 10 mM Man-6-P for 30 min at 4°C. This medium was then removed and used to determine cell surface binding of the oligosaccharides. The cells were then solubilized with 1.1 ml of 2% sodium deoxycholate; 1.0 ml was used for [$^3$H] determination and the remainder used for protein determinations. Samples for scintillation analysis received 10 ml of 3a70 cocktail (Research Products International Corp., Mt. Prospect, IL) and were counted in triplicate. Data were analyzed for both specific and nonspecific uptake and only the former used in all calculations and graphs. Specific uptake is operationally defined as the amount of uptake that is inhibitable by 10 mM Man-6-P.

## RESULTS

### *Phosphomannosyl Receptor Recognition of Phosphorylated Oligosaccharides*

To determine whether phosphorylated oligosaccharides could be internalized by the phosphomannosyl receptor system, we added deblocked A-3 (Fig. 1) to cultured fibroblasts in the absence or presence of various monosaccharide inhibitors of endocytosis. Tables I and II present data showing that deblocked A-3 is internalized at a rate many times greater than the rate of nonspecific endocytosis (24). In addition, several sugars are potent inhibitors of this process, with the rank order of inhibitory potency being mannose-6-phosphate > glucose-6-phosphate > mannose-1-phosphate > mannose (Man-6-P > Glc-6-P > Man-1-P > Man) regardless of whether the inhibitors were present at a concentration of 0.5 or 10 mM (Table I). This inhibition profile qualitatively and quantitatively agrees with previous observations regarding the relative potency of monosaccharides on inhibiting endocytosis of lysosomal enzymes by fibroblasts (6, 11, 12, 25) and provides strong evidence that the phosphorylated oligosaccharides are internalized by the phosphomannosyl receptor. Finally, the insignificant spe-

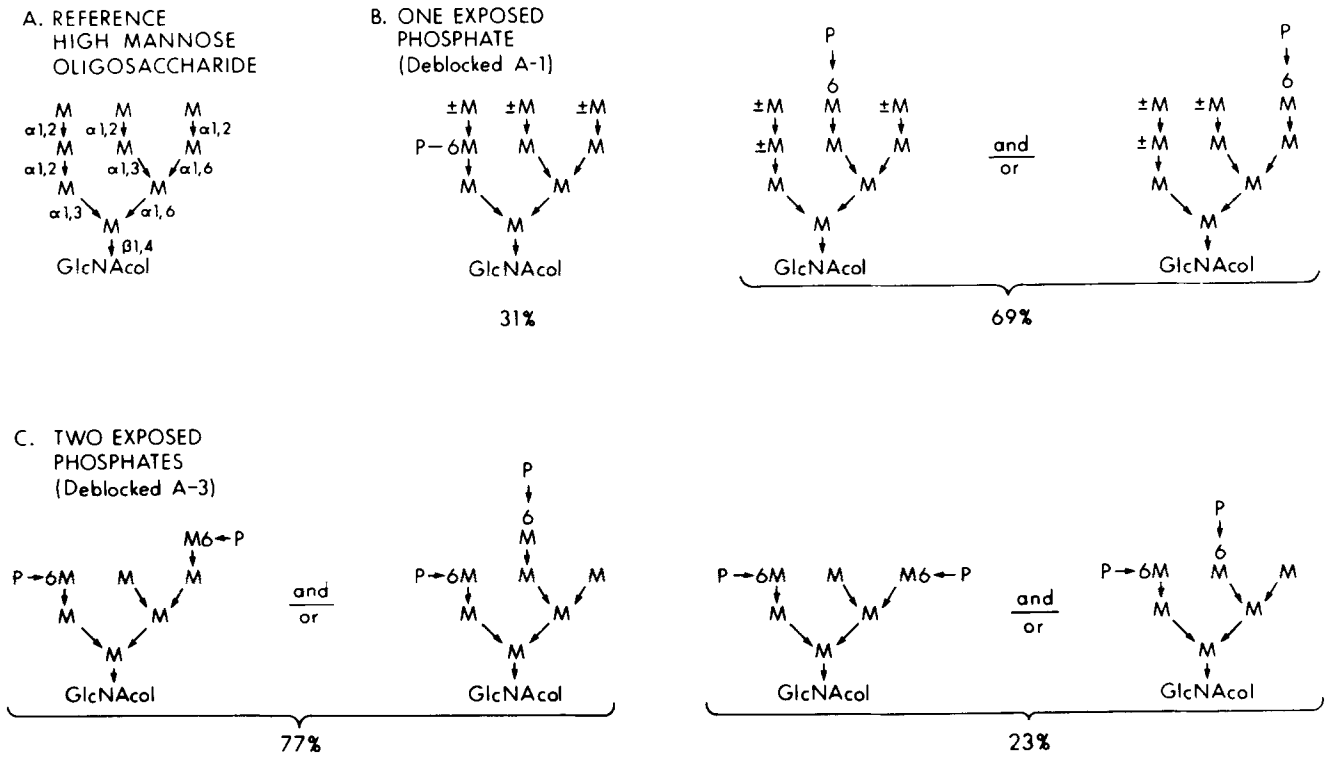


FIGURE 1 Structures of the phosphorylated oligosaccharides. Panel *A* shows a complete high mannose-type oligosaccharide. The glycosyl linkages not shown in Panels *B* and *C* are identical with those in this structure. Panel *B* shows the structure for the phosphorylated oligosaccharides containing one unblocked phosphate (i.e., deblocked A-1). Panel *C* shows the structure for deblocked A-3.

TABLE I

*Effect of Monosaccharides on Endocytosis of Phosphorylated Oligosaccharides* *

| Inhibitor, mM | % Inhibition |
|---|---|
| None | 0‡ |
| Man-6-P, | |
| 10.0 | 98 |
| 5.0 | 98 |
| 0.5 | 92 |
| 0.08 | 74 |
| 0.04 | 59 |
| Glc-6-P, | |
| 10.0 | 90 |
| 0.5 | 28 |
| Man-1-P, | |
| 10.0 | 64 |
| 0.5 | 0 |
| Man, | |
| 10.0 | 39 |
| 0.5 | 0 |

* The ligand used in these studies was deblocked A-3 at a concentration of $8.2 \times 10^{-9}$ M.
‡ Control uptake in this experiment was 0.322 pmol/mg cell protein/6 h.

TABLE II

*Relative Rates of Endocytosis of Phosphorylated and Neutral Oligosaccharides*

| Ligand, nM | Inhibitor, mM | Uptake, pmol/mg/6 h |
|---|---|---|
| Deblocked A-1, | | |
| 42.7 | None | 0.208 |
| 42.7 | Man-6-P, 10.0 | 0.043 |
| 135.4 | None | 0.564 |
| 135.4 | Man-6-P, 10.0 | 0.163 |
| Deblocked A-3, | | |
| 41.7 | None | 1.217 |
| 41.7 | Man-6-P, 10.0 | 0.150 |
| 132.1 | None | 2.662 |
| 132.1 | Man-6-P, 10.0 | 0.521 |
| Neutral oligosaccharides, | | |
| 165.2 | None | 0.102 |
| 165.2 | Man, 50.0 | 0.079 |
| 548.7 | None | 0.321 |
| 548.7 | Man, 50.0 | 0.254 |

cific uptake of neutral high mannose-type oligosaccharides, even in the presence of high concentrations of ligand, provides further evidence that the uptake of the phosphorylated oligosaccharides is mediated by the phosphomannosyl receptor (Table II) and not by a receptor analogous to the previously described reticuloendothelial Man/GlcNAc binding protein (21, 26).

## Kinetic Analysis of the Phosphorylated Oligosaccharide Uptake

Fig. 2 presents data comparing the specific uptake of the oligosaccharides having one or two phosphates in monoester linkage. The uptake of deblocked A-1 is linear over the 20-fold concentration range employed. In contrast, the uptake of deblocked A-3 is much greater than that of deblocked A-1 and approaches saturation in the same concentration range (Fig. 2*A*). This pattern was seen in three separate experiments. Furthermore, both ligands displayed time-dependent uptake (data not shown). Double reciprocal plots of the data shown in Fig. 2*A* and from two similar experiments establishes that the apparent $K_{uptake}$ obtained for deblocked A-1 is $3.2 \times 10^{-7}$ M and that for deblocked A-3 is $3.9 \times 10^{-8}$ M (Fig. 2*B* and Table III). Thus, the apparent $K_{uptake}$ of deblocked A-1 is a hundred-fold less than the $K_i$ for mannose-6-phosphate (11, 12, 27), whereas the apparent $K_{uptake}$ for the diphosphomonoester-containing ligand is a thousand-fold lower.

## DISCUSSION

Three important conclusions can be derived from the data presented in this study: (*a*) lysosomal enzyme-derived phosphorylated oligosaccharides contain all of the information necessary for binding and internalization via the phosphomannosyl receptor; (*b*) features of oligosaccharide other than terminal mannose-6-phosphate contribute to binding and internalization indicating that the phosphomannosyl receptor contains an extended binding site that recognizes subterminal hexose residues; and (*c*) the $K_{uptake}$ for oligosaccharides containing two phosphates in monoester linkage is 10-fold lower than that of structurally similar ligands having one phosphomonoester moiety, suggesting that oligosaccharides bearing two mannose-6-$PO_4$ moieties are likely to be the physiologic recognition marker.

It is important to emphasize that sufficient information for internalization via the phosphomannosyl receptor pathway is

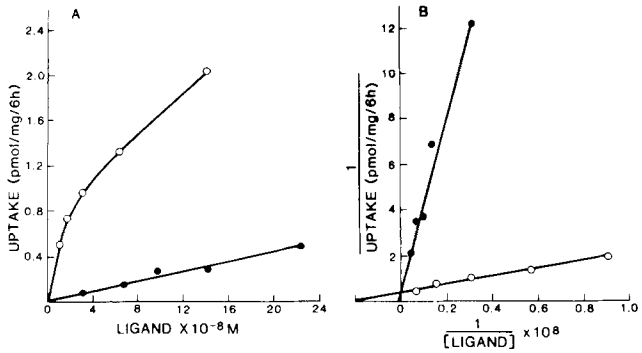


FIGURE 2 Rate of endocytosis of phosphorylated oligosaccharides as a function of enzyme concentration in the medium. The specific high affinity uptake of deblocked A-1 and A-3 was determined as described in Materials and Methods and is expressed as the pmoles of ligand internalized per mg of cellular protein per 6 h of incubation with the ligand. Deblocked A-1 is indicated by the filled circles and deblocked A-3 is indicated by the open circles. Panel *B* shows double reciprocal plots of the data in Panel *A*.

TABLE III

*Kinetic Constants for the Internalization of Phosphorylated Oligosaccharides and Lysosomal Enzymes by Cultured Human Fibroblasts*

| Ligand | $K_{uptake}$ | Reference |
|---|---|---|
| Deblocked A-1 | $3.2 \times 10^{-7}$ M | This study |
| Deblocked A-3 | $3.9 \times 10^{-8}$ M | This study |
| Human platelet β-glucuronidase | $6 \times 10^{-9}$ M | (11) |
| Human urinary α-iduronidase | $3 \times 10^{-9}$ M | (12) |
| *D. discoideum* β-glucosidase | $2 \times 10^{-9}$ M | (6) |

contained within the structure of the phosphorylated oligosaccharides derived from a high uptake lysosomal enzyme. The criteria used to establish that internalization has occurred include the fact that the cells were assayed for cell-associated oligosaccharides subsequent to stringent incubation conditions that have been previously shown to displace cell surface-associated ligand (28–30). In addition to demonstrating that internalization of the ligands occurred, this approach also revealed cell surface binding of deblocked A-3 with ~30,000 molecules of deblocked A-3 bound per cell at 6 h in the presence of ~1 $\times 10^{-7}$ M ligand. No cell surface binding of deblocked A-1 was detected.

The fact that receptor-mediated internalization of the phosphorylated oligosaccharides occurs also establishes that the behavior of the fibroblast cell surface phosphomannosyl receptor is similar to that of the Gal/GalNAc receptor of hepatocytes (20) and the Man/GlcNAc receptor of hepatic reticuloendothelial cells (21) in two respects. First, in each instance, specific oligosaccharide structures contain all of the information required for specific recognition and uptake by the receptor. The specificity of each of these receptors is directed at unique features of the individual oligosaccharide moieties in either the presence or absence of an additional peptide. Second, in view of the 100-fold difference in the $K_i$ for Man-6-P, $6 \times 10^{-5}$ M, and the $K_{uptake}$ for an oligosaccharide having one phosphate in monoester linkage, $3.2 \times 10^{-7}$ M, it appears likely that the phosphomannosyl receptor recognizes an extended oligosaccharide structure, analogous to the above-mentioned lectins.

Comparison of the $K_{uptake}$ values for deblocked A-3 and lysosomal enzymes indicates that they are within 10-fold of each other (Table III). This suggests that there may be an additional contribution to recognition made either by the peptide backbone or by polyvalent interactions between the phosphorylated oligosaccharides of lysosomal enzymes and the receptor. The former possibility is interesting in light of the contribution of the peptide of lysosomal hydrolases to recognition by the N-acetyl-glucosaminyl phosphotransferase (31, 32). Nevertheless, our experiments clearly demonstrate that the contribution of the protein is not essential for efficient recognition by the phosphomannosyl receptor. A similar conclusion was recently reached by Creek et al. (33) utilizing metabolically labeled phosphorylated oligosaccharides.

Polyvalent ligand:receptor interactions could also explain the 10-fold greater affinity of lysosomal enzymes as compared to diphosphomonoester oligosaccharides for the phosphomannosyl receptor. The efficient internalization of the ligands used in these studies definitively establishes that the presence of multiple phosphorylated oligosaccharides is not essential for recognition, although these data do not exclude the possibility that polyvalent interactions would not be of higher affinity.

Previous analyses of the specificity and affinity of the phosphomannosyl receptor (6, 11, 12, 33) have utilized probes that were not completely characterized in terms of their oligosaccharide structures. Despite the use of completely defined oligosaccharide probes in this study (10), some difficulty remains in evaluating the $K_{uptake}$ values for phosphorylated oligosaccharides due to the structural heterogeneity of the oligosaccharides present in both A-1 and A-3. It is not possible to be sure that all species contribute equally to binding and uptake, and the $K_{uptake}$ values we have obtained must, therefore, be considered minimum estimates. Regardless, it is clear that any contribution made by the peptide itself is not essential for efficient recognition by the phosphomannosyl receptor, nor is a multivalent interaction a necessary requirement for ligand:receptor interaction which can result in endocytosis.

It was also our intent to perform binding and internalization studies using oligosaccharides containing one and two phosphate moieties in diester linkage and thus determine the role, if any, of the blocking sugar in receptor:ligand interactions. Although significant uptake of A-3 was observed, 30–35% of the extracellular ligand had become deblocked over the course of the 6-h experiment. This amount of deblocked A-3 could account for all of the observed uptake. The presence of deblocking activity at the cell surface or in the medium was unexpected and must be considered in any evaluation of the functional role of the blocking moieties.

In summary, we have demonstrated that the minimum structures required for recognition and internalization by the Man-6-P specific receptor of fibroblasts resides in high mannose-type oligosaccharides bearing one or two exposed phosphates. The 10-fold lower $K_{uptake}$ for oligosaccharides with two phosphates indicates that in all probability they represent the physiologically important recognition marker for transport to lysosomes, either from the cell surface or from the site of synthesis.

## NOTE

Subsequent to the completion of these studies, Creek and Sly (34) reported similar results with I-cell fibroblasts and metabolically labeled mono- and di-phosphorylated oligosaccharides.

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