The Rate of Internalization of the Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor Is Enhanced by Multivalent Ligand Binding*

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The cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF-II receptor) undergoes constitutive endocytosis, mediating the internalization of two unrelated classes of ligands, mannose 6-phosphate (Man-6-P)-containing acid hydrolases and insulin-like growth factor II (IGF-II). To determine the role of ligand valency in M6P/IGF-II receptor-mediated endocytosis, we measured the internalization rates of two ligands, β-glucuronidase (a homotetramer bearing multiple Man-6-P moieties) and IGF-II. We found that β-glucuronidase entered the cell 3–4-fold faster than IGF-II. Unlabeled β-glucuronidase simulated the rate of internalization of I25I-IGF-II to equal that of 125I-β-glucuronidase, but a bivalent synthetic tripeptide capable of occupying both Man-6-P-binding sites on the M6P/IGF-II receptor simultaneously did not. A mutant receptor with one of the two Man-6-P-binding sites inactivated retained the ability to internalize β-glucuronidase faster than IGF-II. Thus, the increased rate of internalization required a multivalent ligand and a single Man-6-P-binding site on the receptor. M6P/IGF-II receptor solubilized and purified in Triton X-100 was present as a monomer, but association with β-glucuronidase generated a complex composed of two receptors and one β-glucuronidase. Neither IGF-II nor the synthetic peptide induced receptor dimerization. These results indicate that intermolecular cross-linking of the M6P/IGF-II receptor occurs upon binding of a multivalent ligand, resulting in an increased rate of internalization.

The mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF-II receptor) is a type I transmembrane glycoprotein that cycles through the Golgi, endosomes, and the plasma membrane to carry out its role in the biogenesis of lysosomes and in the clearance of the polypeptide insulin-like growth factor II (IGF-II) (1, 2). In the Golgi, the receptor binds newly synthesized acid hydrolases modified with mannose 6-phosphate (Man-6-P) residues on their asparagine-linked oligosaccharides and transports them to endosomes via clathrin-coated vesicles (3–5). The acid hydrolases are released in the acidified endosome and then packaged into lysosomes while the receptor either returns to the Golgi to bind another ligand or moves to the plasma membrane (6, 7). At the plasma membrane, the M6P/IGF-II receptor mediates internalization of Man-6-P-containing ligands and IGF-II (3, 5, 8).

The interactions of IGF-II and Man-6-P-containing ligands with the M6P/IGF-II receptor have been characterized in several studies (8–12). The extracellular portion of the M6P/IGF-II receptor contains 15 homologous repeating domains of ~147 amino acids each (13). Domains 3 and 9 (numbering from the amino terminus) each bind 1 mol of Man-6-P, and the single IGF-II-binding site has been mapped to domain 11 in the extracellular region (14–16). Man-6-P residues do not inhibit binding of IGF-II to the receptor, verifying that the two ligand-binding sites are distinct. However, proteins containing Man-6-P residues do compete with IGF-II for receptor binding, and IGF-II can inhibit binding of lysosomal enzymes to the receptor (8–10, 17). In neither case is the competition complete, and the most plausible explanation is that the inhibition is due to steric hindrance.

Although the M6P/IGF-II receptor has been shown to be constitutively internalized from the cell surface, it is not clear whether ligand binding influences the trafficking of the receptor. It has been reported that in the absence of ligand, the M6P/IGF-II receptor accumulates in the Golgi, whereas the addition of lysosomotropic agents that prevent the release of ligand from the receptor in endosomes results in an accumulation of the receptor in these organelles (18–20). Other investigators have found that constitutive trafficking of the M6P/IGF-II receptor continues under these conditions (21, 22). Together, these data are consistent with the concept that ligand binding modulates the rate of receptor trafficking. Thus, the absence or presence of bound ligand may regulate the trafficking from specific compartments, resulting in a shift in the steady-state distribution of the receptor. However, none of these studies have actually determined the kinetics of receptor trafficking.

In this study, we have compared the internalization of β-glucuronidase, a homotetramer with multiple phosphorylated oligosaccharides, with that of IGF-II. We found that the initial rate of internalization of β-glucuronidase is much more rapid than that of IGF-II, providing direct evidence that a multivalent ligand enhances the rate of movement of the receptor.
Furthermore, we present data that the mechanism of this effect is due to dimerization of the receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human IGF-II was produced from Bachem California; IGF-II-(del 1–6) from Upstate Biotechnology, Inc., Na125I from Amersham Pharmacia Biotech; lactoperoxidase from Calbiochem; Man-6-P from Sigma; and Lipofectin and G418 from Life Technologies, Inc. Other reagent-grade chemicals were from standard suppliers. The bivalent ligand (Ac-Thr-[α-thr-Man-6-P-[α-thr-Man]-Lys-[α-thr-Man]-Thr-[α-thr-Man-6-P-[α-thr-Man]-Lys-[α-thr-Man]-NH2]) was kindly provided by Dr. K. Bock (Carlsberg Laboratory, Copenhagen, Denmark) (23).

**Transfection of Receptor-deficient Cells**—The transfection of the M6P/IGF-II receptor-negative mouse L cell line (L(Rec−)) with constructs encoding the wild-type receptor and a mutant receptor with a 29-amino acid cytoplasmic tail to give the Ce2 and 344 cell lines, respectively, has been previously described (5, 24). The Dom3Δα cell line expressing a receptor with an R435A mutation was generated as described (25).

**Purification and Iodination of β-Glucuronidase**—Human β-glucuronidase was purified from the secretions of 13.2.1 mouse L cells as previously described (24) by the following protocol. The quenched reaction was poured into a column, and the M6P/IGF-II receptor was eluted with wash buffer containing 10 mM Man-6-P. Fractions containing the receptor were pooled, and protein concentration was determined by the Bradford assay (47). The recovery of the receptor was 660 μg.

**Determination of the Stokes Radius**—A Superose 6 FPLC column was equilibrated in filtered and degassed wash buffer. Protein standards were run and detected by absorbance at 280 nm. The Ksv, defined as (Vc − Vv)/(Vc − Vv), was determined, and the Eff* (1 − Ksv) was plotted versus the known Stokes radius of the protein standards (28). The Vc of the membrane form of the M6P/IGF-II receptor was determined by collecting 1-ml fractions and analyzing the contents by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining of the gel. 

**RESULTS**

**IGF-II and β-Glucuronidase Are Internalized at Different Rates**—The internalization rates of IGF-II and β-glucuronidase were compared using 125I-IGF-II and 125I-β-glucuronidase in an adaptation of the endocytosis assay developed by Jadot et al. (24). Following an initial lag of 15–20 s, 125I-IGF-II was internalized in a nearly linear fashion, with a t1/2 of 2–3 min (Fig. 1B). No plateau was observed during the 5-min incubation because very little of the IGF-II was released from the M6P/IGF-II receptor during the course of this experiment. Internalization of IGF-II occurred exclusively from the M6P/IGF-II receptor since the untransfected parent cell line did not take up any IGF-II under these conditions (data not shown).

By contrast, β-glucuronidase was internalized ~3–4-fold more rapidly, with a t1/2 of 20–45 s (Fig. 1A). A plateau was reached when essentially all of the ligand originally present on the cell had been either internalized or released from the receptor into the medium, where it was greatly diluted. Together,
binding.

rate of receptor internalization over that observed upon IGF-II
 already bound, resulting in an increase in internal-
dase bound to a significant fraction of the receptors that had
the ligand added was $^{125}$I-IGF-II. This indicates that the unlabeled
binding to two Man-6-P residues on a phosphorylated oligosac-
were washed, and the uptake of $^{125}$I-IGF-II was determined.
The presence of $\text{Man-6-P}$ Does Not—To test whether the increased rate
were incubated with $^{125}$I-labeled ligand at 4 °C for 30 min, washed on ice to
remove unbound ligand, and then shifted to 37 °C for the indicated
times. The amount of ligand internalized is plotted as a fraction of the
maximum possible internalized counts, which is the sum of the internal-
ized ligand plus the ligand remaining on the cell surface at the end
of the assay (see “Experimental Procedures” for further explanation).
Values are the average of eight independent experiments, and the S.D.
is indicated by the error bars; ●, internalized ligand (radioactivity that
is resistant to stripping with pH 3.5 for 10 min); □, surface ligand. A,
the ligand added was $^{125}$I-$\beta$-glucuronidase; B, the ligand added was
$^{125}$I-IGF-II.

these data show that $\beta$-glucuronidase binding stimulates the rate of receptor internalization over that observed upon IGF-II
binding.

$\beta$-Glucuronidase Stimulates Internalization of $^{125}$I-IGF-II,
whereas Man-6-P Does Not—To test whether the increased rate
of internalization of the receptor with bound $\beta$-glucuronidase
was a result of ligand occupation of the two Man-6-P-binding
sites, the effect of 10 mM Man-6-P on the rate of $^{125}$I-IGF-II
uptake was determined. This concentration of Man-6-P satu-
rated the Man-6-P-binding sites on the receptor. Although Man-6-P caused a small increase in total $^{125}$I-IGF-II binding, it
had no effect on the rate of $^{125}$I-IGF-II internalization (data not
shown), indicating that the increase in internalization rate was
not solely due to Man-6-P binding. The effect of $\beta$-glucuronidi-
dase on the internalization of $^{125}$I-IGF-II was next determined.
In this experiment, the simultaneous binding of $^{125}$I-IGF-II and
$\beta$-glucuronidase was maximized by first incubating cells on ice
with $^{125}$I-IGF-II for 5 min to allow maximum binding of this
ligand. Excess unlabeled $\beta$-glucuronidase (10 nM) was then
added to each well for an additional 25 min on ice. The cells
were washed, and the uptake of $^{125}$I-IGF-II was determined.
The presence of $\beta$-glucuronidase stimulated the rate of endo-
cytosis of $^{125}$I-IGF-II to that observed with $^{125}$I-$\beta$-glucuronidase
alone (Fig. 2). This indicates that the unlabeled $\beta$-glucuronidi-
dase bound to a significant fraction of the receptors that had
already bound $^{125}$I-IGF-II, resulting in an increase in internal-
ization rate that cannot be merely due to the receptor binding
to Man-6-P residues.

Intermolecular Cross-linking of M6P/IGF-II Receptors Is Re-
ponsible for the Ligand-induced Increase in the Internalization Rate—$\beta$-Glucuronidase could enhance the rate of internaliza-
tion of the M6P/IGF-II receptor by promoting either intramo-
lecular or intermolecular cross-linking. Since each monomer of
the receptor contains two Man-6-P-binding sites, simultaneous
binding to two Man-6-P residues on a phosphorylated oligosac-
charide could induce a conformational change in the extracel-
lar domain of the receptor that is transmitted to the cytosolic
domain, where the internalization signal is located. This could
result in a more favorable presentation of the internalization
signal. Alternatively, the ligand could cross-link two receptor
molecules, resulting in an increased density of the internaliza-
tion signals. This could enhance the likelihood of the receptors
being retained in a forming clathrin-coated pit, thus increasing
the probability of internalization and consequently the rate. To
distinguish between these possibilities, two approaches were
used. First, the effect of a small bivalent Man-6-P-containing peptide on the rate of IGF-II uptake was determined. The peptide, a Thr-Lys-Thr tripeptide with a Man-6-P-(α-2-Man disaccharide attached to each threonine, has an affinity for the M6P/IGF-II receptor that is similar to that of an oligosaccharide with two Man-6-P residues and over 1000-fold higher than that of Man-6-P (23, 30, 31). This high binding affinity indicates that the ligand is interacting with two binding sites on the M6P/IGF-II receptor. As shown below, this peptide does not mediate intermolecular cross-linking of the receptor. In control experiments, the peptide competed with β-glucuronidase for the Man-6-P-binding site on the receptor, but did not interfere with binding of IGF-II to the receptor (data not shown). A saturating concentration of the peptide (5 μM) did not significantly alter the rate of 125I-IGF-II internalization, whereas unlabeled β-glucuronidase accelerated the rate of 125I-IGF-II uptake considerably (Fig. 3). These results suggest that intramolecular cross-linking of extracellular domains 3 and 9 of the M6P/IGF-II receptor does not alter the rate of internalization.

The second approach to distinguish intra- from intermolecular cross-linking utilized cells expressing a mutant receptor that has only a single functional Man-6-P-binding site. The Man-6-P binding of domain 3 was abolished by substituting an alanine for Arg-435 (14, 25). The full-length receptor containing this mutation was transfected into L(Rec) cells, creating the Dom3ala cell line (25). The M6P/IGF-II receptors in this cell line are incapable of intramolecular cross-linking due to the presence of only a single functional Man-6-P-binding site per receptor, but could potentially undergo intermolecular cross-linking.

The ability of Dom3ala cells to internalize β-glucuronidase and IGF-II was determined (Fig. 4). In preliminary experiments, internalization of 125I-IGF-II by Dom3ala cells was partially obscured by a high background resulting from IGF-II binding to other proteins (32). This technical problem was resolved by using IGF-II-(del 1–6), which contains a deletion in the amino acid composition and expected carbohydrate additions. The absence of an error bar indicates that the S.D. was <0.02. A, internalization by the wild-type M6P/IGF-II receptor; B, internalization by the M6P/IGF-II receptor with a mutation in domain 3 that abolishes its Man-6-P binding. Consequently, this receptor can bind only one Man-6-P residue via domain 9.

Characterization of the Oligomeric State of the Receptor—We next determined the state of oligomerization of purified M6P/IGF-II receptor in both the presence and absence of β-glucuronidase. Perdue et al. (33) have reported that the M6P/IGF-II receptor is a monomer when solubilized, whereas Stein et al. (34) suggested that it may exist as a dimer in the plasma membrane, as determined by cross-linking studies. The M6P/IGF-II receptor was solubilized and purified from fresh bovine liver. The purified receptor was analyzed by FPLC gel filtration to determine its Stokes radius (Fig. 5, A and C) and by sedimentation in a continuous 6–21% sucrose gradient to determine its sedimentation coefficient (Fig. 6). The Stokes radius of the receptor was calculated to be 79 Å, which is somewhat greater than the previously reported value of 72 Å (33). The sedimentation coefficient was determined to be 10.1 × 10^-13 s, in close accordance with the previously published value (33). The partial specific volume was calculated to be 0.73, based on the amino acid composition and expected carbohydrate additions. No corrections for detergent were applied due to the negligible amount of bound detergent found by us (compare migration relative to protein standards in H2O and D2O gradients in Fig. 6) and others (33). Using these values, the calculated molecular weight for the receptor was 334,000. This value is somewhat higher than previously published (290,000) due to the slight difference in the Stokes radius. Nevertheless, the calculated molecular weight indicates that the purified M6P/IGF-II receptor solubilized in Triton X-100 is a monomer. Similar results were obtained when the receptor was solubilized in digitonin (data not shown).

To determine the effect of β-glucuronidase on the oligomeric state of the receptor, the ligand was incubated with the receptor for 30 min on ice at a ratio of 10 receptor molecules to 1 β-glucuronidase molecule. The resulting complex was then analyzed by gel filtration and sucrose gradient sedimentation to allow calculation of the molecular weight. The elution position of the receptor-β-glucuronidase complex in the gel filtration column was determined by analyzing the fractions for β-glucuronidase activity (Fig. 5B) and by Western blotting (data not shown). It is apparent that the complex eluted significantly
the complex (\(Y\)) used to determine the Stokes radii of the M6P/IGF-II receptor (\(X\)) follows:

\[B\]

Aliquots (10 μl) of the Superose 6 FPLC column, and 0.5-ml fractions were collected. The reaction mixture was then loaded onto a Superose 6 FPLC column (10 mm, inner diameter, 3.2 cm, 28.5 cm), and 1-ml fractions were collected. The protein was precipitated and analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining. The void volume and the elution positions of the protein standards are as follows: A, thyroglobulin (Thy), 89 (41); B, ferritin (Fer), 61 (42); C, ovalbumin, 27.5 (43); and D, myoglobin, 18.9 (44). The void volume is indicated by \(A\), the total volume of the column, as determined by the \(V_r\) of cyanocobalamin, is indicated by \(G\).

Using these values for the Stokes radius and sedimentation coefficient, along with a calculated partial specific volume of 0.73, the molecular weight for the complex was determined to be 912,000. This value is within 2% of the predicted value of 928,000 for a complex composed of two M6P/IGF-II receptors and one \(\beta\)-glucuronidase molecule, based on the empirically determined values for the molecular weights of these two proteins. Although the molecular weight of the complex could also be consistent with the presence of one receptor and two \(\beta\)-glucuronidase molecules (expected molecular weight of 854,000), this is unlikely because incubation of the proteins at a ratio of 10 \(\beta\)-glucuronidase molecules to 1 receptor molecule gives rise to a complex with a lower molecular weight, indicative of a complex of one \(\beta\)-glucuronidase molecule and one M6P/IGF-II receptor (data not shown). Thus, purified M6P/IGF-II receptor associates with \(\beta\)-glucuronidase in a complex composed of two receptors and a single \(\beta\)-glucuronidase molecule, consistent with the conclusion that \(\beta\)-glucuronidase can cross-link two M6P/IGF-II receptor molecules.

In other experiments, the receptor was incubated with saturating concentrations of either IGF-II or the Man-6-P-containing peptide and then subjected to FPLC gel filtration. In both instances, the elution positions of the receptor were identical to that of the receptor alone, indicating that neither compound induced dimerization of the receptor (data not shown).

### DISCUSSION

The data presented in this study show that the M6P/IGF-II receptor internalizes \(\beta\)-glucuronidase three to four times more quickly than the monovalent ligand IGF-II. This finding provides strong evidence that ligand binding can modulate the rate of trafficking of the receptor, a point that has been open to debate in the literature (18–22). A key question is the mechanism of this effect. A clue came from the fact that \(\beta\)-glucuronidase is a multivalent ligand with multiple phosphorylated oligosaccharide units, whereas IGF-II is a monovalent ligand. This raised the possibility that the \(\beta\)-glucuronidase effect arises from intermolecular cross-linking of receptor molecules or by intramolecular cross-linking of the two Man-6-P-binding sites located in domains 3 and 9 of the extracellular domain. The latter possibility was excluded by the finding that the Dom3\(^{\text{AL}}\) mutant receptor with a single Man-6-P-binding site retained the ability to respond to \(\beta\)-glucuronidase binding with an increased rate of internalization, despite being incapable of undergoing intramolecular cross-linking. The observation that a small bivalent glycopeptide that binds to the receptor with high affinity fails to enhance the rate of internalization is also consistent with this conclusion. This glycopeptide does not induce intermolecular cross-linking of receptor molecules due to its small size. On the other hand, the \textit{in vitro} binding studies with the purified receptor established that one molecule of \(\beta\)-glucuronidase cross-links two molecules of receptor, whereas IGF-II does not cross-link the receptor.

How, then, does cross-linking of the M6P/IGF-II receptor by \(\beta\)-glucuronidase increase the rate of internalization? One po-
tential mechanism is that the cross-linking increases the efficiency of the interaction between the tyrosine-based internalization signal present in the cytoplasmic domain of the receptor and the AP-2 adaptor complex at the site of clathrin-coated pit formation at the plasma membrane. Fire et al. (35) have reported that productive interactions with coated pits may be one of the rate-limiting steps for rapid endocytosis of receptors. These investigators used fluorescence photobleaching recovery measurements to determine the lateral diffusion coefficient of wild-type influenza virus hemagglutinin, which is slowly internalized, and a mutant hemagglutinin (Tyr-543) that is internalized at a more rapid rate. Using these values and the size and number of coated pits at the cell surface, they estimated that all the hemagglutinin molecules encounter a coated pit every 3.7 s. They concluded that the Tyr-543 mutant hemagglutinin enters and exits coated pits many times before a productive interaction occurs since its internalization rate is only 4%/min. In fact, most receptors judged to undergo rapid endocytosis into clathrin-coated pits are internalized with 4%/min, not seconds. This implies either that their lateral diffusion is limited or that many of the entries into coated pits fail to result in the trapping and subsequent internalization of the protein. Collawn et al. (36) found that adding a second internalization signal to the cytoplasmic tail of the transferrin receptor increased its internalization rate above that of the wild-type receptor. This suggested that two internalization signals may be better than one. Similarly, the cation-dependent Man-6-P receptor dimer with β-glucuronidase, orienting the receptor with respect to the membrane such that the Man-6-P-binding sites faced away from the membrane, allowing docking of Man-6-P residues present on the oligosaccharides of β-glucuronidase. The large Stokes radius of the M6P/IGF-II receptor indicates that it is not globular in shape, but rather protrudes from the membrane as a cigar-shaped molecule. Analysis of the oligomerization of the soluble receptor in association with β-glucuronidase revealed that binding of receptor molecules to this multivalent ligand did not result in the formation of large multimers despite the potential presence of up to 16 phosphorylated oligosaccharides on the β-glucuronidase tetramer (40). Rather, a discrete complex with a stoichiometry of two receptor molecules to one enzyme formed, suggesting that steric hindrance may prevent more receptors from binding. Taken together with the Stokes radius information, this suggests a simple model for receptor-enzyme association in which two M6P/IGF-II receptors cradle a single β-glucuronidase molecule between their extracellular domains, covering most of the enzyme’s surface. Functionally, this interaction would prevent a single β-glucuronidase molecule from interacting with M6P/IGF-II receptors on two cells.

Fig. 6. Determination of the sedimentation coefficient of the M6P/IGF-II receptor. A and C, purified M6P/IGF-II receptor (10 μg) was loaded onto a continuous 6–21% sucrose gradient and centrifuged for 4 h at 50,000 rpm in an SW 55Ti rotor at 4 °C. Twenty fractions of 240 μl were collected, and protein was precipitated and analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining. Gradients were made in H2O (A) or D2O (C). B and D, the s20,w values of the protein standards were plotted against the fraction they peaked in, and these data were used to determine the s20,w of the M6P/IGF-II receptor (♀). The marker gradients were run in H2O (B) or D2O (D). The protein standards were as follows: BSA, s20,w = 4.6; lactate dehydrogenase (LDH), s20,w = 7.3 (45); catalase (Cat), s20,w = 11.3 (45); and thyroglobulin (Thy), s20,w = 19.5 (46).
possibly preventing undesirable intercellular adhesion from occurring.

Ligand-induced internalization of plasma membrane signaling receptors, such as the epidermal growth factor receptor, provides a mechanism for the down-regulation of these receptors and the termination of the signaling. However, it seems unlikely that this would be the physiologic function of this process in the case of the M6P/IGF-II receptor, which cycles constitutively between the plasma membrane, endosomes, and the Golgi. The major role of the receptor at the cell surface is to bind and internalize IGF-II, and the receptor probably does not encounter significant amounts of acid hydrolases at this location. On the other hand, this mechanism could impact on the kinetics of sorting of the M6P/IGF-II receptor in the trans-Golgi network. If the AP-1 adaptor complex of the Golgi clathrin-coated pits interacts preferentially with receptor associated with an acid hydrolase ligand, then free receptor would be present in the trans-Golgi network for a somewhat longer period of time and have more opportunity to bind ligand. As a consequence, sorting efficiency would be enhanced, particularly if the amount of the M6P/IGF-II receptor in the trans-Golgi network is limiting. Conversely, sorting at the endosome would be most efficient if the receptor exited this compartment faster after ligand release. In this case, the sorting signal for targeting from the endosome to the trans-Golgi network would be exposed optimally upon release of ligand. It has been reported that cells devoid of acid hydrolase ligands exhibit an accumulation of M6P/IGF-II receptors in the Golgi, whereas cells in which ligand dissociation is blocked accumulate M6P/IGF-II receptors in endosome-like structures (18–20). Although several studies have shown that constitutive trafficking of the receptor continues under these circumstances (21, 22), these experiments did not exclude the possibility that ligand occupancy modulates the rate of receptor movement between compartments and therefore the steady-state level at each station. Our results show that receptor dimerization induced by multivalent ligands does alter the kinetics of internalization of the M6P/IGF-II receptor at the plasma membrane, and this process could potentially influence receptor movement at other sites as well.

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Regulation of M6P/IGF-II Receptor Internalization

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