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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 stimulated the rate of internalization of ^{125}I-IGF-II to equal that of ^{125}I-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)[1] 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.

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[1] The abbreviations used are: M6P/IGF-II receptor, mannose 6-phosphate/insulin-like growth factor II receptor; IGF-II, insulin-like growth factor II; Man-6-P, mannose 6-phosphate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography.

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 purchased from Bachem California; IGF-II-(del 1–6) from Upstate Biotechnology, Inc., $Na^{125}I$ 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-D-Man-6-P(α1–2)α-D-Man]-Lys-(ABz)-Thr-[α-D-Man-6-P(α1–2)α-D-Man]-NH_2) 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 Cc2 and 344 cell lines, respectively, has been previously described (5, 24). The $Dom3^{ala}$ 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 described previously (24). This cell line, which has been engineered to secrete large amounts of β-glucuronidase, was kindly provided by Dr. William Sly (St. Louis University). For iodination, 30 μg of human β-glucuronidase was brought to 500 μl in PBS (pH 7.4); 2.5 μl of 1.86 μM lactoperoxidase, 1 mCi of $Na^{125}I$, and 2 μl of 0.5 nM hydrogen peroxide were added to start the reaction. After 3 min at room temperature, another 2 μl of 0.5 nM hydrogen peroxide was added, and after an additional 3 min, the reaction was stopped by adding 200 μl of quench solution (1 M NaCl, 100 mM NaI, 50 mM $NaPO_4$ (pH 7.5), 1 mM NaN_3, 2 mg/ml BSA, and 1 mg/ml protamine sulfate). The quenched reaction mixture was loaded onto a 1-ml M6P/IGF-II receptor affinity column (26) equilibrated in PBS and 0.1% BSA. After extensive washing, the β-glucuronidase was eluted with PBS and 0.1% BSA containing 10 mM Man-6-P. The peak fractions were pooled and dialyzed against PBS to remove the Man-6-P, and the ^{125}I-β-glucuronidase was stored at 4 °C. The typical specific activity assuming complete protein recovery was 10^6 to 10^7 cpm/μg of protein.

Iodination of IGF-II—10 μg of IGF-II was iodinated using the lactoperoxidase method described for β-glucuronidase. After quenching, the reaction mixture was loaded onto a Sephadex G-25 column equilibrated in PBS and 0.1% BSA. Three peaks of radioactivity were observed and subjected to trichloroacetic acid precipitation. The second peak was found to be 98% trichloroacetic acid-precipitable and contained monomeric IGF-II. The iodinated ligand was stored at 4 °C. The typical specific activity assuming complete protein recovery was 10^7 cpm/μg of protein.

Iodination of IGF-II-(del 1–6)—IGF-II-(del 1–6) was iodinated by coating an Eppendorf tube with 50 μg of IODO-GEN (Pierce) and adding 5 μg of IGF-II-(del 1–6) and 1 mCi of $Na^{125}I$. The mixture was incubated at room temperature for 3 min and then loaded onto a PD10 column (Amersham Pharmacia Biotech) pre-equilibrated in PBS plus 1% BSA. Fractions containing the first peak were pooled and stored at 4 °C. The typical activity assuming complete protein recovery was 10^8 cpm/μg of protein.

Rapid Endocytosis Assays—Cells were grown to confluence in 12-well plates. The cells were rinsed twice in ice-cold PBS and 1% BSA, and ligand was added in cold α-minimal Eagle's medium and 2% BSA (0.5 ml/well). ^{125}I-IGF-II was added to a 2 nM final concentration, and ^{125}I-β-glucuronidase was typically added to a final concentration of 0.12 nM. The plates were then floated on an ice water bath for 30 min. Unbound ligand was removed, and the wells were rapidly washed five times with 1 ml of ice-cold PBS and 1% BSA. To the three wells used for the 0 time point was added 1 ml of ice-cold stop/strip solution (SSS; 0.2 M acetic acid (pH 3.5) and 0.5 M NaCl). The plate was then floated in a 37 °C water bath, and 0.5 ml of α-minimal Eagle's medium prewarmed to 37 °C was quickly added to the remaining wells to initiate internalization. At each stopping point, the α-minimal Eagle's medium in the well was removed to a tube for counting, and 1 ml of cold SSS was added. At the end of the experiment (usually 3 min), the plate was removed from the water bath, and the surface-bound ligand was stripped from each well by incubation for a total of 10 min in ice-cold SSS (1 ml for 5 min, twice) and counted. The cells were then solubilized in 0.1 N NaOH (1 ml, twice) and counted. The M6P/IGF-II receptor-negative cell line was used as a control for non-receptor binding of ^{125}I-IGF-II and ^{125}I-β-glucuronidase. The sum of the labeled ligand remaining on the cell surface at the end of the internalization experiment (receptor-bound ligand) and the internalized ligand was used as a measure of the maximum potential internalization. The fraction of this value internalized at each time point was calculated and plotted. This calculation method was used to exclude the contributions of a specific, non-M6P/IGF-II receptor binding site for IGF-II observed in all the cell lines, including the receptor-negative cells. This site has a lower affinity for IGF-II than the M6P/IGF-II receptor, and with incubation at 37 °C, the ligand is released into the medium rather than internalized. This calculation method was used for both IGF-II and β-glucuronidase binding studies.

Preparation of the M6P/IGF-II Receptor—110 g of fresh bovine liver was minced and blended in a Waring blender in 200 ml of ice-cold extraction buffer (50 mM imidazole (pH 7), 150 mM NaCl, 5 mM sodium β-glycerophosphate, 2% Triton X-100, 0.25% deoxycholate, 10 mM EDTA, 50 μg/ml leupeptin, 50 μg/ml aprotinin, 50 μg/ml trypsin inhibitor, and 50 μg/ml phenylmethylsulfonyl fluoride) for ~10 s, four times. The homogenate was centrifuged at 30,000 × *g* for 30 min, and the supernatant was poured through cheesecloth. A ~15-ml packed volume of phosphopentamannose-agarose beads (27) was washed with extraction buffer without protease inhibitors and added to the supernatant. Receptor binding was allowed to occur for 30 min at 4 °C while rocking. The beads were collected by filtering over a coarse Buchner funnel, and the agarose beads were washed with 1 liter of extraction buffer followed by 500 ml of wash buffer (50 mM imidazole (pH 7), 150 mM NaCl, 5 mM sodium β-glycerophosphate, and 0.05% Triton X-100). The washed agarose 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 K_d, defined as $(V_e - V_o)/(V_t - V_o)$, was determined, and the $Erf^{-1}(1 - K_d)$ was plotted *versus* the known Stokes radius of the protein standards (28). The V_e 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. The V_e of complexes containing β-glucuronidase was determined by β-glucuronidase assays.

Determination of Sedimentation Coefficients—Continuous 6–21% sucrose gradients (4.8 ml) were prepared in wash buffer and allowed to equilibrate at 4 °C for 1 h. Samples were loaded onto the gradients, and the gradients were centrifuged for 4 h at 237,000 × g_{av} in an SW 55Ti rotor. Fractions (240 or 120 μl) were collected from the top of the gradient. Refractive indices of each fraction were measured to determine linearity of the gradients. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis following chloroform/methanol precipitation or by β-glucuronidase assays as described below. The sedimentation coefficients of the proteins and complexes were determined using protein standards as markers (29).

β-Glucuronidase Assays—A sample of each fraction to be tested (2–10 μl) was incubated with 100 μl of 10 mM 4-methylumbelliferyl β-D-glucuronide (Sigma) suspended in 0.1 M sodium acetate (pH 5.0) at 37 °C for 10–60 min. Following incubation, 3 ml of 0.25 M glycine (pH 10.3) was added to stop the reaction, and the fluorescence was determined in a Turner fluorometer.

RESULTS

IGF-II and β-Glucuronidase Are Internalized at Different Rates—The internalization rates of IGF-II and β-glucuronidase were compared using ^{125}I-IGF-II and ^{125}I-β-glucuronidase in an adaptation of the endocytosis assay developed by Jadot *et al.* (24). Following an initial lag of 15–20 s, ^{125}I-IGF-II was internalized in a nearly linear fashion, with a $t_{1/2}$ of 2–3 min (Fig. 1*B*). 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 assay. The internalization of IGF-II occurred exclusively via 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 $t_{1/2}$ of 30–45 s (Fig. 1*A*). 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,

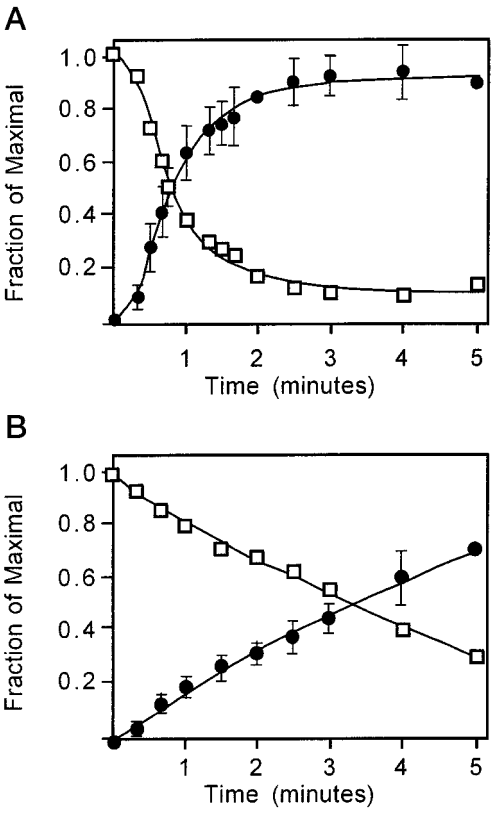

FIG. 1. **Internalization of IGF-II and β-glucuronidase occurs at different rates.** Cells expressing the wild-type receptor were incubated with ^{125}I-labeled ligand at 4 °C for 30 min, washed on ice to remove unbound ligand, and then shifted to 37 °C for the indicated times. The amount of ligand internalized is plotted as a fraction of the maximum possible internalized counts, which is the sum of the internalized 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-β-glucuronidase; *B*, the ligand added was ^{125}I-IGF-II.

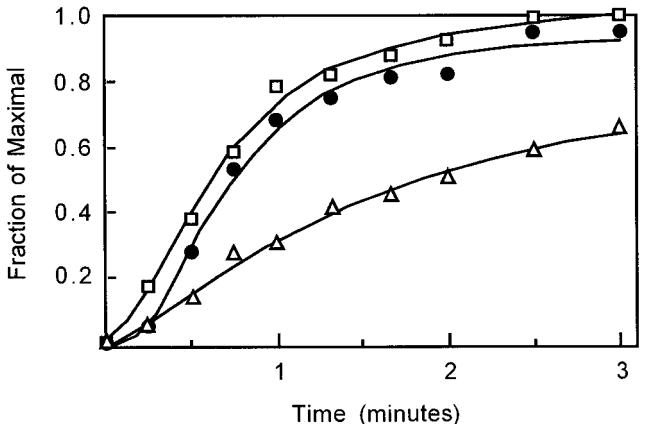

FIG. 2. **Unlabeled β-glucuronidase enhances the rate of internalization of ^{125}I-IGF-II.** Cells expressing the wild-type receptor were assayed for internalization of ^{125}I-β-glucuronidase (□), ^{125}I-IGF-II in the presence of 10 mM Man-6-P (△), or ^{125}I-IGF-II in the presence of unlabeled β-glucuronidase (●) as described under "Experimental Procedures." Values are the average of three independent experiments.

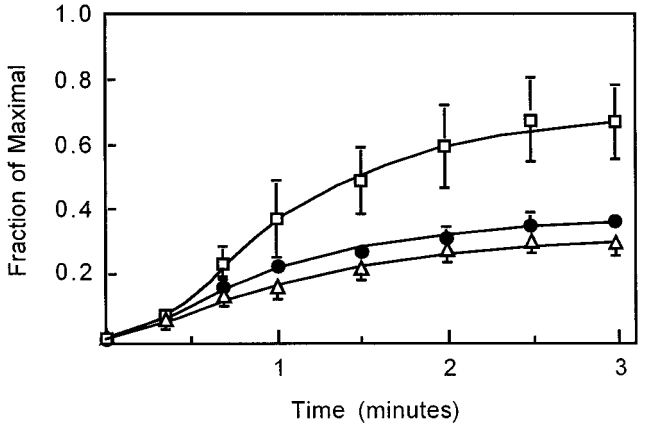

FIG. 3. **Intramolecular cross-linking does not enhance internalization of ^{125}I-IGF-II.** The 344 cell line was assayed for internalization of ^{125}I-IGF-II in the presence of 10 mM Man-6-P (△), 5 μM tripeptide containing two Man-6-P residues (●), or unlabeled β-glucuronidase (□) as described under "Experimental Procedures." Values are the average of three independent experiments. The 344 cell line expresses a M6P/IGF-II receptor that has a 29-amino acid cytoplasmic tail ending in the sequence AKYSKV. This receptor internalizes β-glucuronidase at the same rate as the wild-type receptor, but expresses 4-fold more receptor molecules at the cell surface, making it easier to perform the assay (24).

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

β-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 β-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 saturated 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 β-glucuronidase on the internalization of ^{125}I-IGF-II was next determined. In this experiment, the simultaneous binding of ^{125}I-IGF-II and β-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 β-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 β-glucuronidase stimulated the rate of endocytosis of ^{125}I-IGF-II to that observed with ^{125}I-β-glucuronidase alone (Fig. 2). This indicates that the unlabeled β-glucuronidase bound to a significant fraction of the receptors that had already bound ^{125}I-IGF-II, resulting in an increase in internalization rate that cannot be merely due to the receptor binding to Man-6-P residues.

Intermolecular Cross-linking of M6P/IGF-II Receptors Is Responsible for the Ligand-induced Increase in the Internalization Rate—β-Glucuronidase could enhance the rate of internalization of the M6P/IGF-II receptor by promoting either intramolecular 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 oligosaccharide could induce a conformational change in the extracellular 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 internalization 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

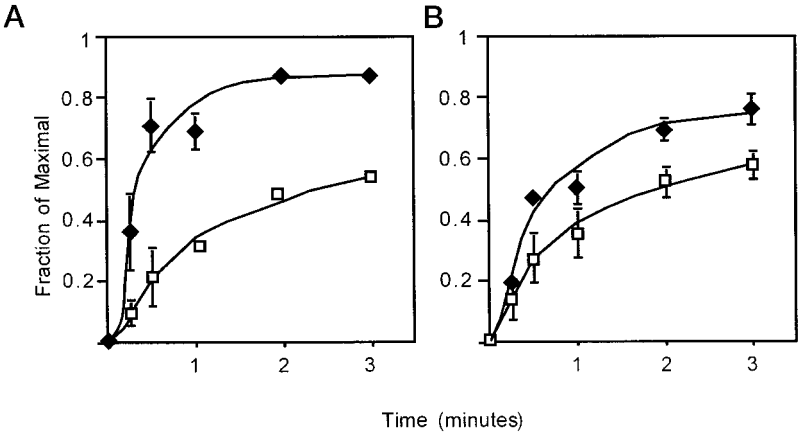

FIG. 4. **Intermolecular cross-linking is necessary for stimulation of ^{125}I-IGF-II internalization.** The rate of ^{125}I-IGF-II-(del 1–6) internalization was determined as described under "Experimental Procedures" in the absence (□) and presence (♦) of unlabeled β-glucuronidase. Values are the average of three independent experiments. 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.

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(α1–2)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 ^{125}I-IGF-II internalization, whereas unlabeled β-glucuronidase accelerated the rate of ^{125}I-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 Dom3^{ala} 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 Dom3^{ala} cells to internalize β-glucuronidase and IGF-II was determined (Fig. 4). In preliminary experiments, internalization of ^{125}I-IGF-II by Dom3^{ala} 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 that prevents binding to the IGF-II-binding proteins while maintaining internalization by the M6P/IGF-II receptor (32). IGF-II-(del 1–6) was internalized by the wild-type M6P/IGF-II receptor at a rate similar to that observed with native IGF-II (Fig. 4*A*), and the addition of unlabeled β-glucuronidase increased the rate of receptor internalization to that seen earlier (Fig. 2*A*). The rate of ^{125}I-IGF-II-(del 1–6) uptake by the mutant receptor in the Dom3^{ala} cells was similar to that of the wild-type receptor (Fig. 4*B*). This was expected since neither the IGF-II-binding site nor the internalization signal in the cytoplasmic domain is different. As with wild-type receptors, the addition of unlabeled β-glucuronidase increased the rate of internalization of the mutant receptor in Dom3^{ala} cells, as measured by ^{125}I-IGF-II-(del 1–6) uptake (Fig. 4*B*). This strengthens the conclusion that intramolecular cross-linking is not responsible for the increased rate of internalization and suggests that intermolecular cross-linking mediates the enhanced internalization. The internalization rate of ^{125}I-IGF-II-(del 1–6) by the Dom3^{ala} cells in the presence of β-glucuronidase was not as rapid as that seen with cells expressing the wild-type receptors. This may be because the single Man-6-P-binding site on the mutant receptor results in lower binding affinity for β-glucuronidase and consequently less efficient cross-linking of receptor molecules.

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 H_2O and D_2O 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. 5*B*) and by Western blotting (data not shown). It is apparent that the complex eluted significantly

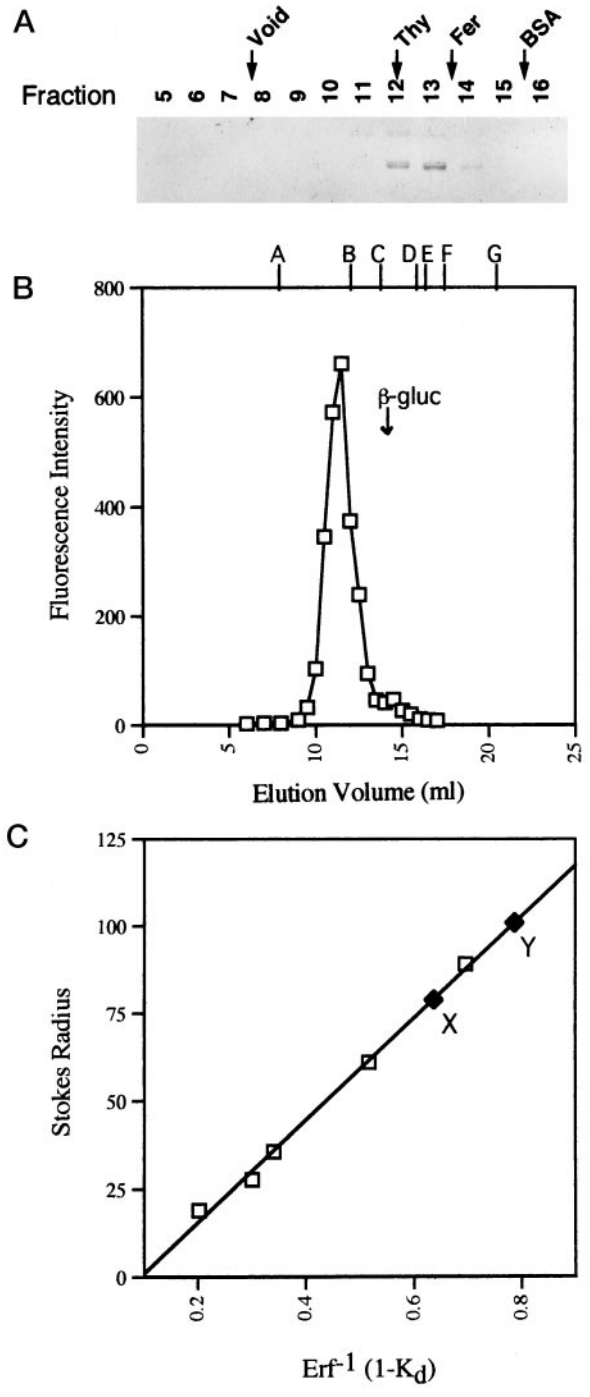

FIG. 5. **Determination of the Stokes radii of the M6P/IGF-II receptor and the complex containing both the receptor and β-glucuronidase.** *A*, 50 μg of the M6P/IGF-II receptor was applied to a Superose 6 FPLC column (10 mm, inner diameter, × 28.5 cm), and 1-ml fractions were collected. The protein was precipitated and analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining. The void volume and the elution positions of the protein standards are indicated at the top. The M6P/IGF-II receptor eluted in fraction 13. *B*, the M6P/IGF-II receptor was incubated with β-glucuronidase (*β-gluc*) at a molar ratio of 10 receptor molecules to 1 enzyme molecule for 30 min on ice. The reaction mixture was then loaded onto the Superose 6 FPLC column, and 0.5-ml fractions were collected. Aliquots (10 μl) from each fraction were analyzed for β-glucuronidase activity. The V_e of free β-glucuronidase at 14.5 ml is indicated, as are the elution positions of the protein standards. Complexed β-glucuronidase eluted at 11.5 ml. *C*, the Stokes radii of the protein standards are plotted against the inverse error coefficient of $1 - K_d$. These data were used to determine the Stokes radii of the M6P/IGF-II receptor (*X*) and the complex (*Y*). The protein standards and their Stokes radii are as follows: *B*, thyroglobulin (*Thy*), 89 (41); *C*, ferritin (*Fer*), 61 (42); *D*, BSA, 35.5 (42); *E*, ovalbumin, 27.5 (43); and *F*, myoglobin, 18.9 (44). The void volume is indicated by *A*, and the total volume of the column, as determined by the V_e of cyanocobalamin, is indicated by *G*.

ahead of free β-glucuronidase. Using this elution value, the Stokes radius was calculated to be 101 Å. The migration of the M6P/IGF-II receptor-β-glucuronidase complex in the sucrose gradient was determined in a similar manner (Fig. 7*A*). The sedimentation value of the complex was determined to be 21.6×10^{13} s (Fig. 7*B*).

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 β-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 β-glucuronidase molecules (expected molecular weight of 854,000), this is unlikely because incubation of the proteins at a ratio of 10 β-glucuronidase molecules to 1 receptor molecule gives rise to a complex with a lower molecular weight, indicative of a complex of one β-glucuronidase molecule and one M6P/IGF-II receptor (25) (data not shown). Thus, purified M6P/IGF-II receptor associates with β-glucuronidase in a complex composed of two receptors and a single β-glucuronidase molecule, consistent with the conclusion that β-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 β-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 β-glucuronidase is a multivalent ligand with multiple phosphorylated oligosaccharide units, whereas IGF-II is a monovalent ligand. This raised the possibility that the β-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 $Dom3^{ala}$ mutant receptor with a single Man-6-P-binding site retained the ability to respond to β-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 *in vitro* binding studies with the purified receptor established that one molecule of β-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 β-glucuronidase increase the rate of internalization? One po-

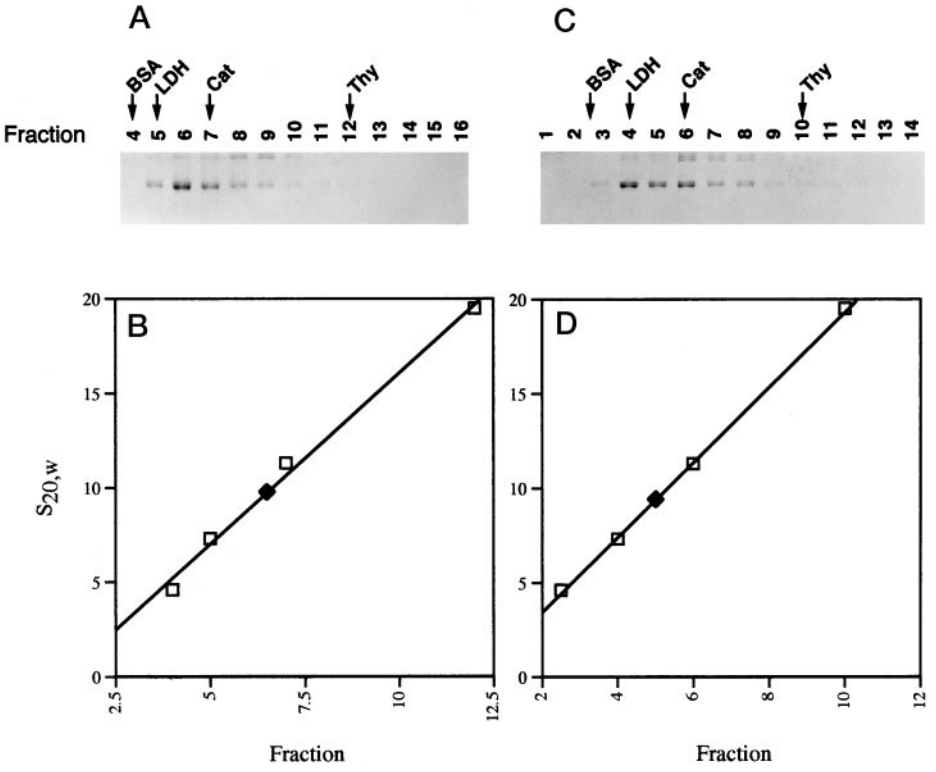

FIG. 6. **Determination of the sedimentation coefficient of the M6P/IGF-II receptor.** *A* and *C*, purified M6P/IGF-II receptor (10 μg) was loaded onto a continuous 6–21% sucrose gradient and centrifuged for 4 h at 50,000 rpm in an SW 55Ti rotor at 4 °C. Twenty fractions of 240 μl were collected, and protein was precipitated and analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining. Gradients were made in H_2O (*A*) or D_2O (*C*). *B* and *D*, the $s_{20,w}$ values of the protein standards were plotted against the fraction they peaked in, and these data were used to determine the $s_{20,w}$ of the M6P/IGF-II receptor (♦). The marker gradients were run in H_2O (*B*) or D_2O (*D*). The protein standards were as follows: BSA, $s_{20,w}$ = 4.6; lactate dehydrogenase (*LDH*), $s_{20,w}$ = 7.3 (45); catalase (*Cat*), $s_{20,w}$ = 11.3 (45); and thyroglobulin (*Thy*), $s_{20,w}$ = 19.5 (46).

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 $t_{1/2}$ values of 1 min or more, 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 contains three internalization signals, and its rate of internalization is slowed when individual signals are mutated (37, 38).

Dimerization of receptor tails, in a simplistic sense, would lead to a doubling of signals, which presumably would interact more effectively with the adaptor proteins localized in coated pits. Although the analysis of the solubilized receptor indicates that it is a monomer, it is not possible to directly extrapolate this finding to the membrane-bound form of the receptor since a loosely associated dimer in the membrane might fall apart upon solubilization. This is relevant to the rapid internalization assays. In these experiments, β-glucuronidase is added at saturation levels to cells kept at 4 °C, and the excess ligand is washed away before warming. Assuming that the incubation on ice completely immobilizes receptors in the plasma membrane, any intermolecular cross-linking of receptors must be occurring in a rigid membrane. The implication is that the receptor monomers are proximal to one another, perhaps already oligomerized, before the addition of β-glucuronidase. If that is the case, the enhanced internalization must be due to a more optimal presentation of the internalization signal upon cross-linking with the multivalent ligand.

Recently, the three-dimensional structure of the extracellular domain of the cation-dependent Man-6-P receptor has been solved by Roberts *et al.* (39). The domain crystallized as a dimer, with the single Man-6-P-binding site of each monomer being oriented in the same direction. The authors modeled the interaction of 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,

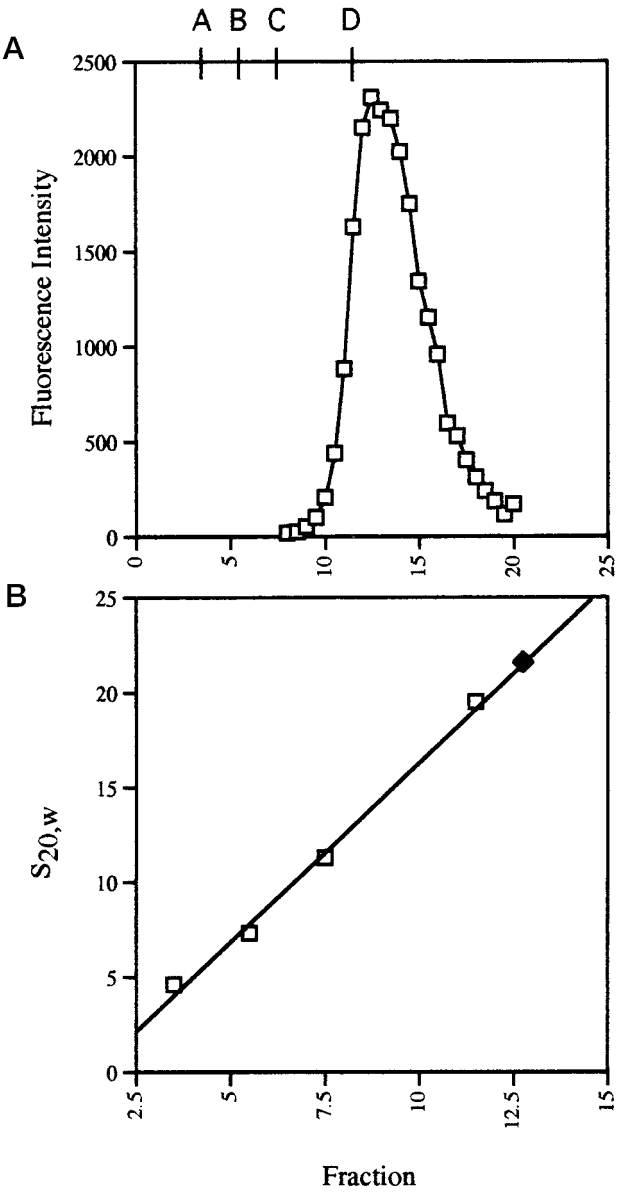

FIG. 7. **Determination of the sedimentation coefficient of the complex containing the M6P/IGF-II receptor and β-glucuronidase.** *A*, the M6P/IGF-II receptor and β-glucuronidase were incubated together on ice for 30 min at a ratio of 10 receptor molecules to 1 enzyme molecule and then loaded onto a 6–21% sucrose gradient made in H_2O and centrifuged for 4 h at 50,000 rpm in an SW 55Ti rotor. Fractions (120 μl) were collected and analyzed for β-glucuronidase activity. *B*, the $s_{20,w}$ values of the protein standards were plotted against the fraction they peaked in, and these data were used to determine the $s_{20,w}$ of the complex (■). The standard proteins are indicated at the top of *A* as follows: *A*, BSA, $s_{20,w} = 4.6$; *B*, lactate dehydrogenase (*LDH*), $s_{20,w} = 7.3$; *C*, catalase, $s_{20,w} = 11.3$; and *D*, thyroglobulin, $s_{20,w} = 19.5$.

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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