Mechanisms for High Affinity Mannose 6-Phosphate Ligand Binding to the Insulin-like Growth Factor II/Mannose 6-Phosphate Receptor

NEGATIVE COOPERATIVITY AND RECEPTOR OLIGOMERIZATION*

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The two mannosine 6-phosphate (Man-6-P) binding domains of the insulin-like growth factor II/mannose 6-phosphate receptor (Man-6-P/IGF2R), located in extracytoplasmic repeats 1–3 and 7–9, are capable of binding Man-6-P with low affinity and glycoproteins that contain more than one Man-6-P residue with high affinity. High affinity multivalent ligand binding sites could be formed through two possible mechanisms: the interaction of two Man-6-P binding domains within one Man-6-P/IGF2R molecule or by receptor oligomerization. To discriminate between these mechanisms, truncated FLAG epitope-tagged Man-6-P/IGF2R constructs, containing one or both of the Man-6-P binding domains, were expressed in 293T cells, and characterized for binding of pentamannosyl bovine serum albumin (PMP-BSA), a pseudoglycoprotein bearing multiple Man-6-P residues. A construct containing all 15 repeats of the Man-6-P/IGF2R extracytoplasmic domain bound PMP-BSA with the same affinity as the full-length receptor (Kd = 0.54 nM) with a curvilinear Scatchard plot. The presence of excess unlabeled PMP-BSA increased the dissociation rate of pre-formed 125I-PMP-BSA/receptor complexes, suggesting negative cooperativity in multivalent ligand binding and affirming the role of multiple Man-6-P/IGF2R binding domains in forming high affinity binding sites. Truncated receptors containing only one Man-6-P binding domain and mutant receptor constructs, containing an Arg1325 → Ala mutation that eliminates binding to the repeats 7–9 binding domain, formed high affinity PMP-BSA binding, but with reduced stoichiometries. Collectively, these observations suggest that alignment of Man-6-P binding domains of separate Man-6-P/IGF2R molecules is responsible for the formation of high affinity Man-6-P binding sites and provide functional evidence for Man-6-P/IGF2R oligomerization.

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The sorting of newly synthesized lysosomal enzymes from the trans-Golgi network to pre-lysosomal acidic vesicles occurs through the recognition of mannosine 6-phosphate (Man-6-P)1 markers by two membrane-bound mannosine 6-phosphate receptors (MPRs), the cation-dependent Man-6-P receptor (CD- MPR), and the insulin-like growth factor II/Man-6-P receptor (Man-6-P/IGF2R). Most lysosomal enzymes are modified in the Golgi to contain multiple Man-6-P groups as a part of their glycosylation repertoire (for review see Ref. 1). Although multiple pathways may participate in lysosomal targeting of proteins, disruption of the Man-6-P sorting pathway has been found in several human diseases, including lysosomal enzyme storage diseases such as Pseudo-Hurler polydystrophy and I-cell disease (2–4). In addition, increased secretion of Man-6-P-bearing lysosomal enzymes has been associated with cancers of the breast and prostate (5–8), which may be due to disruption of targeting carried out by the CD-MPR, the Man-6-P/IGF2R, or both.

It is not yet fully understood why mammals produce two different MPRs. Whereas both the CD-MPR and Man-6-P/ IGF2R can recognize proteins containing Man-6-P residues, they have overlapping but distinct functions in the sorting of specific hydrolases. Many studies have shown that the expression of only one or the other MPR is insufficient for targeting all Man-6-P-bearing hydrolases to the lysosomal compartment (9–14). Furthermore, studies using antibodies against the ligand binding domains of the CD-MPR and Man-6-P/IGF2R have shown that the Man-6-P/IGF2R participates in the endocytosis of lysosomal enzymes from the cell surface, whereas the CD-MPR does not (15). Although the two receptors appear to have distinct functions, the CD-MPR and Man-6-P/IGF2R are likely to have shared a similar ancestral origin, as each of the 15 extracytoplasmic repeats of the Man-6-P/IGF2R shares 14 to 28% sequence identity with the entire extracytoplasmic domain of the CD-MPR (16).

The CD-MPR is a 46-kDa membrane glycoprotein that binds monophosphomannosylated ligands with low affinity (Kd = 6–8 μM) and forms a high affinity bivalent Man-6-P-binding site (Kd = 200 nM) through receptor oligomerization (17–19). The Man-6-P/IGF2R is a much larger (300-kDa) type I transmembrane glycoprotein that comprises a short NH2-terminal signal sequence, followed by 15 homologous repeats, a transmembrane domain, and a 167-residue cytoplasmic domain (16, 20).

1 The abbreviations used are: Man-6-P, mannose 6-phosphate; IGF, insulin-like growth factor; Man-6-P/IGF2R, mannosine 6-phosphate/insulin-like growth factor II receptor; PMP, pentamannosylate bovine serum albumin; CD-MPR, cation-dependent mannosine 6-phosphate receptor; MPR, mannosine 6-phosphate receptor; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; HBST, HEPES-buffered saline containing Triton X-100.

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The Man-6-P/IGF2R binds at least three classes of ligands through unique sites in the extracytoplasmic domain. The Man-6-P/IGF2R binds IGF-II at the cell surface, resulting in the internalization and degradation of this mitogenic growth factor in the lysosomal compartment (21–24). The receptor also binds urokinase-type plasminogen activator receptor, which may be involved in the activation of latent transforming growth factor-β (25–27). Finally, the receptor interacts with proteins that bear the Man-6-P marker, resulting in sorting to the lysosomal compartment. Functional mapping studies of the extracytoplasmic domain of the Man-6-P/IGF2R have revealed the location of two distinct binding domains for Man-6-P (28, 29), which may have distinct specificities for sorting lysosomal enzymes (30). Arg426 in repeat 3 and Arg1325 in repeat 9 of the human receptor are thought to interact non-covalently with the Man-6-P moiety during ligand binding, as mutation of these residues in the bovine receptor eliminates Man-6-P binding to their respective binding domains (28).

Like the CD-MPR, the Man-6-P/IGF2R binds monovalent Man-6-P ligands with low affinity (Kₐ = 6–7 μM) and divalent phosphomannosylated ligands with high affinity (Kₐ = 1–20 nM) (31), but the mechanism for the formation of two unique high affinity sites is not understood. Two models have been proposed to explain the Man-6-P/IGF2R’s ligand binding characteristics. Intramolecular interactions between the two extracytoplasmic Man-6-P binding domains could account for high affinity binding (31). On the other hand, receptor oligomerization could result in the formation of two unique high affinity sites, which may explain observations of functional differences between the repeat 3 and repeat 9 Man-6-P binding domains with respect to their preference for sorting specific hydrolases (30). Studies of the oligomeric state of the receptor, however, have led to paradoxical results. Sucrose gradient centrifugation and gel filtration chromatography of purified bovine receptors in the absence of exogenous ligands suggest that the receptor exists in solution in the monomeric state (32, 33), whereas the presence of receptor dimers as determined by gel filtration chromatography (33). Studies of the oligomeric state of the receptor, however, have led to paradoxical results. Sucrose gradient centrifugation and gel filtration chromatography of purified bovine receptors in the absence of exogenous ligands suggest that the receptor exists in solution in the monomeric state (32, 33), whereas the presence of receptor dimers as determined by gel filtration chromatography (33).

In order to discriminate between the intramolecular and intermolecular models of high affinity multivalent phosphomannosylated ligand binding to the Man-6-P/IGF2R, we have characterized wild-type and mutant receptor constructs for the ability to interact with a multivalent Man-6-P ligand. Our data indicate that the Man-6-P/IGF2R displays negative cooperativity in binding the multivalent ligand, and that receptors containing only one functional Man-6-P binding domain, either repeat 3 or repeat 9, are capable of forming a high affinity Man-6-P binding site. Truncation of the Man-6-P/IGF2R, however, revealed that multiple regions outside of the minimal Man-6-P binding domains in repeats 3 and 9 play a role in both the formation of bivalent Man-6-P binding sites and the resultant affinity of these sites toward PMP-BSA. These observations suggest that receptor oligomerization is the mechanism for the formation of high affinity Man-6-P binding in the extracytoplasmic domain of this receptor.

**EXPERIMENTAL PROCEDURES**

**Materials—**Oligonucleotides were synthesized by Integrated DNA Technologies (Corvalle, IA) or the University of Nebraska Medical Center Molecular Biology Core Facility (Omaha, NE). Recombinant human IGFs were provided by M. H. Niedenthal (Lilly Research Laboratories, Indianapolis, IN). The native Y-2448 O-phosphomannan of Hansenula holstii was a gift from Dr. M. E. Slodki (Midwest Area Regional Northern Research Center, Peoria, IL, retired). Carrier-free Na¹²⁵I (Amersham Pharmacia Biotech) was used for radiodination of IGF-II and pentamannose phosphate-bovine serum albumin (PMP-BSA) to specific activities between 40 and 80 Ci/g by Enzymobead Fractionating Insulin Gel (BIF) and IODOSep™ reagent (t1/2), respectively. The pCMV5 vector (34) was provided by Dr. Daniel W. Rubel (University of Texas Southwestern Medical Center, Dallas, TX), and the 8.6-kilobase pair human Man-6-P/IGF2R cDNA (20) was a gift of Dr. William S. Sly (St. Louis University Medical Center, St. Louis, MO). Other reagents and supplies were obtained from sources as indicated.

**Synthesis and Expression of Truncated FLAG Epitope-tagged Receptor Constructs—**A minireceptor construct, termed 7–15F, encompassing all 15 repeats of the extracytoplasmic domain of the Man-6-P/IGF2R followed by a FLAG epitope tag, was created from a full-length human Man-6-P/IGF2R cDNA (20) as described previously (35). The R1325A mutant was created using the QuikChange™ mutagenesis kit (Stratagene) and a two-step cassette strategy described previously (35). Complementary mutagenic primers were designed for that procedure corresponding to nt 4105–4140, changing CCC to GCG at nt 4120–4122 to create the R1325A missense mutation and a C to G silent mutation at nt 4125, which incorporated a novel SaI site for diagnostic purposes. The presence of the mutation was confirmed by sequencing across the mutated region.

A set of eight carboxyl-terminal FLAG-tagged minireceptor constructs was engineered in a pCMV5RIN expression vector using a strategy developed previously in our laboratory (36). Using the full-length Man-6-P/IGF2R and 15F(R1325A) cDNAs as templates, the following constructs encompassing truncated forms of the extracytoplasmic domain of the Man-6-P/IGF2R were made by amplification with Vent polymerase (New England Biolabs): repeats 1–3F (nt 148–1554, corresponding to residues 1–469), 1–8F (nt 148–3807, corresponding to residues 1–1220), 1–9F (nt 148–4242, corresponding to residues 1–1365), 1–11F (nt 148–5100, corresponding to residues 1–1651), 7–9F (nt 2926–4242, corresponding to residues 927–1365), 7–15F (nt 2926–7002, corresponding to residues 927–2285), 1–9F with the R1325A mutation, and 1–11F with the R1325A mutation. To ensure consistent translation, the signal sequence containing the amino-terminal 71 residues of the first repeat was fused to the beginning of the 7–9F and 7–15F constructs as described (36). All of the constructs contained a 3′-24 nt sequence encoding an eight-residue FLAG tag, DYYDDDDK, followed by a stop codon and an XbaI restriction site. The nucleotide sequences of the Man-6-P/IGF2R cDNA constructs that passed through mutagenesis or amplification procedures were verified by sequence analysis. In addition, the constructs that demonstrated unexpected or incorrect ligand binding properties were re-amplified and sequenced to ensure accuracy. All of the constructs were expressed in 293T cells.

**Transient expression of the constructs was carried out in 293T human embryonic kidney cells cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum plus 5 μg/ml gentamicin. The transfactions were performed by a modification of the calcium phosphate method described previously (37).** Cells were fed with medium supplemented with 5% fetal bovine serum plus 5 μg/ml gentamicin 24 h after transfection, and cell lysates were prepared on the 5th to 6th day after transfection by solubilization with 1% Triton X-100, 1 mM MgCl₂, 10 mM HEPES, pH 7.4, as described earlier (38). Once the lysates were collected, 20 μl aliquots were electrophoresed on an 8–18% gradient SDS-PAGE gel under reducing conditions and electroblotted to BA85 nitrocellulose. The blots were blocked with 3% nonfat milk in 15 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 and probed with the M2 anti-FLAG antibody (Dako, Carpinteria, CA). The resultant antibody complexes were developed with 125I-protein A (NEN Life Science Products) and anti-mouse IgG (Dako, Carpinteria, CA). The resultant antibody complexes were developed with 125I-protein A (NEN Life Science Products) and anti-mouse IgG (Dako, Carpinteria, CA). The resultant antibody complexes were developed with 125I-protein A (NEN Life Science Products) and anti-mouse IgG (Dako, Carpinteria, CA). The resultant antibody complexes were developed with 125I-protein A (NEN Life Science Products) and anti-mouse IgG (Dako, Carpinteria, CA).
IODogen tubes for 25 min. The product was separated from free iodine on a G-50 Sephadex column. The iodinated PMP-BSA was collected from the flow-through fractions and stored at −20 °C until use.

**Ligand Binding Analysis**—The minireceptor constructs were routinely immunoabsorbed to anti-FLAG M2 resin (Sigma) to separate them from other proteins, especially the endogenous 293T Man-6-P/IGF2R, present in the cell lysates. Aliquots (20–30 μl) of Triton X-100 cell lysates containing equal amounts of FLAG-tagged construct, based on PhosphorImager analysis of anti-FLAG immunoblots, were incubated with 12 μl of packed M2 resin in HEPES-buffered saline, pH 7.4, with 1% BSA and 5 mM Man-6-P at 3 °C for 14–16 h. Addition of 5 mM Man-6-P at this stage prevented co-precipitation of endogenous phosphomannosylated ligands. The construct-laden resin was collected by centrifugation at 14,000 × g for approximately 10–12 s. Finally, the resin pellets were washed twice with 0.75 ml of HEPES-buffered saline, pH 7.4, containing 0.05% Triton X-100 (HBST) and then subjected to the studies described below.

The ability of the constructs to bind 125I-PMP-BSA or 125I-IGF-II was first measured by incubating the immunoabsorbed constructs, or resin exposed to CMV-transfected control lysates, with 1 nM 125I-PMP-BSA or 2 nM 125I-IGF-II in binding buffer (HBST + 0.5% BSA) at 3 °C for 3–5 h. Three hours of incubation was adequate for the constructs to reach binding equilibrium, as determined by affinity chromatography experiments (35) and by replicate binding reactions incubated for 16 h, in which the amount of binding was identical to reactions incubated for only 3 h (data not shown). Following the binding reaction, the resin pellets were washed twice with 0.75 ml of HBST to remove unbound ligand, collected by centrifugation, and counted in a gamma counter. Specific binding was determined by subtracting the counts/min ligand bound in replicate reactions carried out in the presence of 5 mM Man-6-P or 1 μM IGF-II. Affinity measurements were carried out by competitive binding analysis. Equal amounts of the receptor constructs were immunoabsorbed to M2 resin and incubated with 1 nM 125I-PMP-BSA in the presence of increasing concentrations of unlabeled PMP-BSA (from 0 to 500 nM) at 3 °C for 4–5 h. The resins were washed and counted as described above. The data were then fit to a model for one-site competitive binding using GraphPad Prism™ software. In addition to PMP-BSA binding, the ability of 1–5F, 7–9F, 1–9F, 15F, and 15FR1325A receptor constructs to interact with immobilized Man-6-P was determined using a PMP-Sepharose affinity depletion assay developed previously (35).

**Test for Negative Cooperativity**—To determine if the Man-6-P/IGF2R displays negative cooperativity in binding PMP-BSA, the rate of 125I-PMP-BSA dissociation from immunoabsorbed 15F construct was measured in the presence of 25 nM unlabeled PMP-BSA or Man-6-P. Equal amounts of immunoabsorbed 15F were incubated in the presence of 250 pM 125I-PMP-BSA in binding buffer. Following a 3-h incubation at 3 °C, the resin pellets were washed twice with 0.75 ml of HBST at 3 °C, and then diluted 75-fold with HBST only, HBST with 25 mM PMP-BSA, or HBST with 25 mM Man-6-P and incubated at 3 °C. At time intervals (from 0 to 100 min), the amount of remaining bound ligand was determined by collecting and counting the resin pellets. The increase in dissociation rate was also assayed in the presence of increasing concentrations of Man-6-P (0–50 mM) or PMP-BSA (0–150 nM) in a similar fashion. Equal amounts of the pre-formed 125I-PMP-BSA-15F complexes were diluted with 1 ml of HBST containing increasing concentrations of either Man-6-P or PMP-BSA, and dissociation was allowed to proceed for 20 min at 3 °C. The resin pellets were collected and counted to determine the amount of bound radioligand remaining.

**RESULTS**

**IGF-II and PMP-BSA Binding Analysis of the Extracytoplasmic Domain of the Man-6-P/IGF2R**—To study the ability of the Man-6-P/IGF2R to interact with multivalent Man-6-P-bearing proteins, a wild-type soluble receptor construct, called 15F, was engineered and expressed in human embryonic kidney cells (293T cells). This construct comprised the entire extracytoplasmic domain of the Man-6-P/IGF2R—mic Domain of the Man-6-P/IGF2R—playing Negative Cooperativity in Binding PMP-BSA—To determine if the curvilinear Scatchard plot for PMP-BSA binding observed for the wild-type 15F construct was due to negative cooperativity, we used an experimental approach developed by De Meyts and colleagues, who addressed the rate of dissociation of radiolabeled insulin from the insulin receptor in the presence of unlabeled ligand (36). If the Man-6-P/IGF2R displays negative cooperativity in binding PMP-BSA, we would expect that the dissociation rate of PMP-BSA would increase with increasing ligand occupancy of the receptor. First, the rate of 125I-PMP-BSA dissociation from a pre-formed 125I-PMP-BSA-15F complex was measured in the presence or absence of 25 mM Man-6-P or PMP-BSA (Fig. 1A). The presence of 25 nM PMP-BSA dramatically increased the rate of dissociation in comparison to the control and 25 mM Man-6-P reactions, causing 50% of the radioligand/receptor complex to dissociate after only 25 min. To eliminate the possibility that receptor rebinding accounts for this observation, the effect of increasing dilution of radioligand on the dissociation of the pre-formed 125I-PMP-BSA-15F complex was measured in the presence or absence of 25 μM Man-6-P and was found to be constant over a dilution range of 30–180-fold (data not shown).

To further characterize this phenomenon, the ability of PMP-BSA and Man-6-P to increase the dissociation rate of radiolabeled PMP-BSA from 15F was measured over a wide range of unlabeled ligand concentrations. Aliquots of resin bearing the immunoabsorbed 125I-PMP-BSA-15F complexes were incubated with increasing concentrations of Man-6-P (0–50 mM) or PMP-BSA (0–150 nM). Dissociation of radioligand from the receptor complex was allowed to proceed for 20 min at 3 °C, after which the amount of bound radioligand remaining was determined. An enhancement of 125I-PMP-BSA dissociation from 15F with the addition of unlabeled ligands resulted in dose-response curves with EC50 values of 8.2 nM for PMP-BSA and 28.4 μM for Man-6-P (Fig. 1C). A concentration of Man-6-P over 1000-fold higher than that of PMP-BSA was necessary to cause the same increase in dissociation, which correlates with the lower affinity displayed by the Man-6-P/IGF2R in binding free Man-6-P relative to the multivalent ligand, PMP-BSA.

**Only One Intact Man-6-P Binding Domain Is Necessary for the Formation of High Affinity PMP-BSA Binding**—To determine if both the repeat 3 and repeat 9 Man-6-P binding domains are required for the formation of high affinity binding, a 15F Man-6-P/IGF2R construct bearing an R1325A missense mutation was generated by site-directed mutagenesis. Mutation of the residue corresponding to the human Arg1325 to an...
alanine in the bovine receptor has been shown previously to destroy the function of the repeat 9 Man-6-P binding domain (28). The wild-type 15F and 15F(R1325A) constructs were transiently expressed in 293T cells, and Triton X-100 cell lysates were subjected to immunoblot analysis with anti-FLAG M2 antibody. The blots revealed that both of the constructs were transiently expressed in 293T cells, and Triton X-100 cell lysates were subjected to immunoblot analysis with anti-FLAG M2 antibody. The blots revealed that both of the constructs were expressed as homogeneous 250-kDa proteins (Fig. 2A).

In comparison to the wild-type 15F construct, the 15F(R1325A) mutant demonstrated very similar IGF-II binding characteristics in a direct binding assay (Fig. 2B). But when analyzed for its ability to bind [125I]PMP-BSA, the R1325A mutant demonstrated a 48.8 ± 4.1% (n = 5) reduction in binding compared with wild type (Fig. 2B). When subjected to competitive binding analysis, the R1325A mutant demonstrated high affinity for PMP-BSA (Kd = 2.29 ± 0.78 nM, number of transfections = 5). This overall affinity is 4.2-fold lower than observed for the wild-type 15F. However, the affinity of PMP-BSA binding to the R1325A mutant was somewhat variable when comparing lysates prepared from different transfections, suggesting that conditions present at the time of transfection or cell lysis contributed to the formation of the Man-6-P binding affinity. Scatchard plot analysis of the R1325A mutant from a transfection that demonstrated affinity identical to the wild-type 15F showed parallel plots that retained curvilinear nature (Fig. 2C).

Because there was an observed decrease in the number of binding sites for PMP-BSA in the R1325A mutant samples, the ability of this construct to bind immobilized PMP was measured using a PMP-Sepharose resin affinity depletion assay. Purified 15F and 15F(R1325A) were exposed to sequential rounds of incubation with PMP-Sepharose, after which the amount of unbound receptor construct was determined by M2 immunoblot analysis of the supernatant fractions (Fig. 3). The affinity chromatography revealed that 15F(R1325A) is capable of binding PMP-Sepharose to the same extent as wild type, with about 80% binding after only one exposure. Within the limitations of this assay, no evidence was found for the presence of subpopulations of Man-6-P/IGF2R constructs differing in affinity for PMP-Sepharose.

Man-6-P Binding Characteristics of Truncated Minireceptor Constructs—A series of truncated receptor constructs was designed that encompassed the repeat 3, repeat 9, or both Man-6-P binding domains. These constructs were expressed in 293T cells to determine the minimum portions of the extracytoplasmic domain required for the formation of high affinity Man-6-P binding sites (Fig. 4A). Aliquots of Triton X-100 cell lysates from cells transiently transfected with the constructs, or transfected with the empty pCMV5 vector, were analyzed by anti-FLAG immunoblots to verify and quantify construct expression. All eight constructs were expressed as homogeneous proteins consistent with their predicted Mf, (Fig. 4B). The expression of the truncated constructs was quantified by PhosphorImager analysis so that equimolar amounts of all the constructs could be immunoadsorbed to anti-FLAG resin for analysis of ligand binding.

The minimal domains of the Man-6-P/IGF2R required for Man-6-P binding have been previously localized to repeats 1–3 and 7–9 (28, 29). To determine the relative affinities of truncated receptors containing only these minimal binding domains for PMP-BSA, the 1–3F and 7–9F Man-6-P/IGF2R constructs...
FIG. 2. Comparison of the Man-6-P and IGF-II binding characteristics of 15F and 15F(R1325A). A, expression of 15F wild-type (WT) and 15F mutant (R1325A) constructs was determined using equal volumes (20 μl) of Triton X-100 cell extracts from cells transiently transfected with each construct, or the empty CMV5 vector. Proteins were resolved on 6% reducing SDS-PAGE, subjected to anti-FLAG M2 immunoblot analysis, and developed with 125I-protein A. Band intensities were quantified by PhosphorImager analysis. B, direct ligand binding assays. Equal amounts of immunoadsorbed 15F and 15F(R1325A) constructs were incubated in the presence of 2 nM 125I-IGF-II or 1 nM 125I-PMP-BSA for 3–4 h at 3 °C. Bound ligand was determined by centrifuging the resin pellets, washing, and counting in a γ counter. Radioactivity retained in the presence of either 1 μM IGF-II or 5 mM Man-6-P was subtracted from each binding reaction to determine the specific binding for 15F and 15F(R1325A). C, Scatchard plot analysis of PMP-BSA binding was conducted by incubating equimolar amounts of immunoadsorbed 15F and 15F(R1325A) in the presence of increasing concentrations of 125I-PMP-BSA from 0.05 to 2.5 nM for 3 h at 3 °C. The amounts of bound and unbound ligand were determined as described above, and nonspecific binding was calculated by including 5 mM Man-6-P in a parallel set of binding reactions for each concentration of labeled ligand.

were transiently expressed in 293T cells. Whereas both of these constructs were capable of binding PMP-Sepharose (data not shown), they showed little ability to interact with 1 nM 125I-PMP-BSA in the direct binding assay (Fig. 5), suggesting that they bind with a much lower affinity than the 15F construct. Another construct containing repeats 1–9 followed by a FLAG epitope tag, 1–9F, was capable of binding 125I-PMP-BSA under these same assay conditions (Fig. 5). Even though 1–9F contains both Man-6-P-binding domains, it bears a large deletion of the extracytoplasmic domain. We therefore compared the ability of 1–9F and 15F to bind PMP-BSA using a direct binding assay. Equimolar amounts of 1–9F and 15F were immunoadsorbed to M2 resin and subjected to direct binding analysis or immunoblot analysis (Fig. 6). From three separate sets of transfections, 1–9F bound only 42.5 ± 6.1% (n = 4) of the PMP-BSA as compared with 15F. In addition, we tested the ability of the 1–9F and 15F constructs to bind PMP-Sepharose to determine if subpopulations of the 1–9F constructs were present in a form that could not bind ligand. Whereas 1–9F demonstrated an approximately 50% decrease in the number of high affinity binding sites for PMP-BSA, no differences were observed in its ability to interact with PMP-Sepharose as compared with the wild-type 15F construct (data not shown).

To rule out the possibility that the observed decrease in PMP-BSA binding to the 1–9F construct was due to a loss of binding to the repeat 9 Man-6-P binding domain, two experimental approaches were employed. First, in view of the possibility that the addition of a FLAG epitope next to repeat 9 may disrupt ligand binding to this region, we generated a 1–11F construct that contained two additional repeats carboxyl-terminal to the repeat 9 binding site. Surprisingly, this construct demonstrated the same binding characteristics as the 1–9F construct in the direct binding assay (Fig. 5). Second, we generated 1–9F and 1–11F constructs bearing the R1325A mutation to eliminate binding to the 9th repeat. If the failure to form available high affinity binding sites displayed by the 1–9F and 1–11F constructs was due to an inability to form an intact repeat 9 binding domain, we would predict that these mutants, 1–9F(R1325A) and 1–11F(R1325A) would demonstrate the same PMP-BSA binding characteristics as their wild-type counterparts. However, when assayed for the ability to bind 125I-PMP-BSA, the mutants demonstrated a 52 ± 3% (n = 6) reduction in 125I-PMP-BSA binding relative to wild-type 1–9F, which is analogous to the observed loss of binding for the 15F(R1325A) relative to the wild-type 15F construct (Fig. 5). Competitive binding and Scatchard plot analyses of 1–9F and
1–9F(R1325A) revealed that both constructs had high affinity for PMP-BSA, equivalent to the wild-type 15F, but the mutation caused a reduction in the number of apparent binding sites measurable in this assay (Fig. 7, panels A and B). These data suggest a role for the carboxyl-terminal region of the Man-6-P/IGF2R extracytoplasmic domain in the formation of the number of competent high affinity Man-6-P binding sites.

To determine what other regions of the Man-6-P/IGF2R extracytoplasmic domain play a role in the formation of the Man-6-P binding sites, constructs containing repeats 1–8 and 7–15, with only one Man-6-P binding domain each, were analyzed. The 1–8F and 7–15F constructs were capable of binding when incubated with 1 nM 125I-PMP-BSA, but the overall binding was substantially reduced when compared with equimolar amounts of the 1–9F construct, with a 71.2 ± 1.8% (n = 3) reduction for 1–8F and a 71.8 ± 1.9% (n = 3) decrease for 7–15F in comparison to 1–9F (Fig. 5). Affinity analyses of 1–8F and 7–15F revealed an approximately 50% decrease in B_max in comparison to 1–9F, and a 1.8–2.0-fold reduction in affinity, as shown by competitive binding analysis and Scatchard plot (Fig. 7, panels A and C).

**DISCUSSION**

In order to determine the portions of the Man-6-P/IGF2R extracytoplasmic domain that contribute to the formation of high affinity multivalent Man-6-P ligand binding, we have characterized the binding properties of several truncated receptor constructs. First, a construct encoding the extracytoplasmic domain, followed by a FLAG epitope tag, 15F, was assayed for its ability to interact with a synthetic multivalent ligand, PMP-BSA. As previously reported, transient expression of the 15F construct in 293T cells revealed that it was not secreted into the medium, but was found at high levels in Triton X-100 and freeze/thaw cell extracts (35). This construct bound IGF-II and PMP-BSA with the same affinities as reported for the full-length rat Man-6-P/IGF2R. In addition, like other multivalent phosphomannosylated ligands binding to the full-length Man-6-P/IGF2R, both naturally occurring and synthetic (30, 31), PMP-BSA binding to the 15F construct exhibited a curvilinear Scatchard plot.

Two models have been previously proposed to explain the difference in affinity of the Man-6-P/IGF2R toward monovalent Man-6-P and multivalent phosphomannosylated ligands. First, the simultaneous interaction of a single multivalent ligand with each of the Man-6-P binding domains in the extracyto-
plasmic domain of the receptor could account for the observed increase in affinity (31). In such a model, the intramolecular interconnection between the two domains would account for the increase in affinity for multivalent ligand binding over the monovalent ligand. The second model accounts for the increased affinity through intermolecular alignment of two Man-6-P binding domains on separate receptor molecules through receptor oligomerization (33). However, in order for this type of intermolecular interaction to account for increased affinity, an interaction between two receptor molecules would have to exist outside of the bridging effect caused by the presence of a multivalent ligand. Tethering the receptors to the membrane could account for high affinity in the full-length Man-6-P/IGF2R, but it seems that another type of receptor-receptor interaction must occur to explain the ability of soluble receptor constructs containing only one of these Man-6-P binding domains. Flag-tagged constructs encoding the extracytoplasmic domain of the receptor.

The second approach employed to determine if the presence of only the repeat 3 or the repeat 9 binding domain is sufficient for formation of high affinity Man-6-P binding was the study of truncated receptor constructs containing only one of these Man-6-P binding domains. Flag-tagged constructs encoding the minimal binding domains for Man-6-P, repeats 1–3 and 7–9, did not bind PMP-BSA with high affinity even though they were capable of binding PMP-Sepharose (data not shown). This observation suggests that regions outside repeats 1–3 and 7–9 are necessary for the formation, or stabilization, of high affinity binding sites. In agreement with this assessment, constructs containing only the repeat 7–9 Man-6-P binding domain (28) were capable of binding PMP-BSA with high affinity in the nanomolar range ($K_d = 2.85$ nM), but demonstrated a $\sim 50\%$ reduction in the number of available binding sites. This result suggests that the formation of high affinity occurs through an intermolecular mechanism, with little contribution from intramolecular binding. As previously reported, if the intramolecular model accounted for high affinity binding, the cooperative contribution of each binding site would be multiplicative rather than additive (31). In addition, the overall affinity of the R1325A mutant was variable when measured in lysates prepared from separate transfections in 293T cells. While this study was ongoing, Marron-Terada et al. (30) reported that the full-length bovine receptor bearing the Arg$_{1324}^{1324}$ $\rightarrow$ Ala mutation sorted lysosomal enzymes with about 50% of the wild-type receptor's efficiency. They also reported that the mutant bound $\beta$-glucuronidase, a heavily glycosylated, phosphomannosylated lysosomal enzyme, with an affinity about one-half that of the wild-type receptor. This phenomenon of variable affinity could be explained if mutation of this Arg residue destabilized the quaternary or tertiary structure of the extracytoplasmic domain of the receptor.

The existence of Man-6-P/IGF2R oligomers has been previously reported. Chemical cross-linking studies of the Man-6-P/IGF2R in intact U937 monocytes were the first to suggest that Man-6-P/IGF2R oligomers may exist in the cell membrane (43). However, studies of purified Man-6-P/IGF2R in solution using both gel filtration chromatography and sucrose gradients have determined that the Man-6-P/IGF2R exists in a monomeric form under these conditions (32). While this report was in preparation, York et al. (33) confirmed those studies and further reported that the addition of a lysosomal enzyme to purified Man-6-P/IGF2R preparations allows the detection of a protein complex with a Stokes radius consistent with two molecules of receptor bound to one molecule of enzyme as measured by gel filtration chromatography. The existence of receptor oligomers alone, however, does not exclude the possibility that the Man-6-P/IGF2R binds multivalent phosphomannosylated ligands through an intramolecular interaction.

Two approaches were undertaken in this study to discern between the intramolecular and intermolecular models for multivalent Man-6-P ligand binding. First, the Man-6-P binding domain of repeat 9 in 15F was mutated to eliminate its binding function. The mutation of Arg$_{1325}^{1325}$ in the bovine receptor, corresponding to Arg$_{1325}^{1325}$ in the human receptor (16), to either alanine or lysine, was shown to cause a loss of binding to the repeat 7–9 Man-6-P binding domain (28). If the intramolecular model accounts entirely for the formation of high affinity sites, we would predict that mutation of the 9th repeat binding domain in an intact receptor would affect the affinity, while having no effect on the overall stoichiometry of PMP-BSA binding. However, the 15F(R1325A) mutant Man-6-P/IGF2R bound with high affinity in the nanomolar range ($K_d = 2.85$ nM), but demonstrated a $\sim 50\%$ reduction in the number of available binding sites. This result suggests that the formation of high affinity occurs through an intermolecular mechanism, with little contribution from intramolecular binding. As previously reported, if the intramolecular model accounted for high affinity binding, the cooperative contribution of each binding site would be multiplicative rather than additive (31). In addition, the overall affinity of the R1325A mutant was variable when measured in lysates prepared from separate transfections in 293T cells. While this study was ongoing, Marron-Terada et al. (30) reported that the full-length bovine receptor bearing the Arg$_{1324}^{1324}$ $\rightarrow$ Ala mutation sorted lysosomal enzymes with about 50% of the wild-type receptor's efficiency. They also reported that the mutant bound $\beta$-glucuronidase, a heavily glycosylated, phosphomannosylated lysosomal enzyme, with an affinity about one-half that of the wild-type receptor. This phenomenon of variable affinity could be explained if mutation of this Arg residue destabilized the quaternary or tertiary structure of the extracytoplasmic domain of the receptor.

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In addition to forming fewer binding sites per receptor molecule, the 1–8F and 7–15F constructs bound PMP-BSA with
half the affinity of the 15F construct, indicating that regions in both halves of the Man-6-P/IGF2R’s extracytoplasmic region contribute to the affinity of the resultant multivalent ligand binding sites. Whereas the 1–8F construct bound with a reduced $B_{\text{max}}$ and lower affinity than the 15F construct, constructs containing repeats 1–9 and 1–11 bearing the R1325A mutation, 1–9F(R1325A) and 1–11F(R1325A), showed dramatic increases in the number and affinity of sites formed. However, these constructs still formed fewer high affinity Man-6-P binding sites than the 15F(R1325A) construct. In the context of Man-6-P/IGF2R oligomers, these observations may be explained if multiple interactions along the extracytoplasmic domains between monomers of a dimeric receptor are necessary for the formation of high affinity Man-6-P binding. Currently available data are consistent with the possibility that development of a binding site of proper conformation requires other portions of the receptor, either within a monomeric structure or contributed by the partner subunit of a dimeric species.

Further evidence that regions outside of the minimal Man-6-P binding domains are necessary for the formation of high affinity binding comes from studies using the wild-type 1–9F and 1–11F constructs, which contain both Man-6-P binding domains. These constructs bind PMP-BSA with very similar characteristics. Both display high affinity toward the multivalent ligand, with affinities identical to the 15F construct. However, when compared with the number of binding sites formed by equimolar amounts of 15F, the 1–9F and 1–11F constructs demonstrate ~50% fewer sites. In these constructs, both the repeat 3 and repeat 9 Man-6-P binding domains appear to contribute equally to the overall number of binding sites, as the 1–9F(R1325A) and 1–11F(R1325A) mutant constructs both demonstrate a further 50% reduction in PMP-BSA binding, without affecting the overall affinity, compared with their wild-type counterparts. We infer from these data that repeats 12–15 likely contain necessary components for the formation of binding-competent receptors. We anticipated that receptor subspecies with only low affinity for multivalent ligand would exist in preparations of the constructs that show reduced numbers of PMP-BSA binding sites as compared with 15F. The 1–9F construct, however, demonstrated no alteration in its ability to interact with PMP-Sepharose as compared with 15F (data not shown). As the 1–3F and 7–9F constructs were also capable of interacting with PMP-Sepharose, while showing almost no binding to PMP-BSA in the direct binding assay, it seems likely that this affinity chromatography is capable of detecting both low and high affinity binding sites. Resolution of this question will have to await better techniques directed at discriminating between low and high affinity binding displayed by the Man-6-P/IGF2R.

Scatchard plot analyses of all the constructs characterized in this study revealed curvilinear plots for $^{125}$I-PMP-BSA binding. The curvilinear nature of the Scatchard plot for PMP-BSA binding to the Man-6-P/IGF2R could be accounted for by any one of three explanations (for review see Ref. 44). First, the presence of more than one binding site with differing affinities toward PMP-BSA could account for a curvilinear Scatchard plot. Second, the presence of subpopulations of the Man-6-P/IGF2R with interconverting affinities, between low and high affinity could account for this observation. Finally, the affinity of the Man-6-P/IGF2R toward PMP-BSA could depend on the ligand occupancy of the receptor resulting in negative cooperativity between more than one binding domain. In order to address the possibility that the Man-6-P/IGF2R displays negative cooperativity in binding PMP-BSA, we used an experimental approach developed by De Meyts and colleagues (40) to identify negatively cooperative interactions of insulin with its receptor. In studying the effect of the presence of unlabeled ligand concentration on the dissociation rate of a pre-formed radioligand-receptor complex, negative cooperativity would manifest as an increase in the rate of dissociation of radioligand from the receptor as a function of increasing unlabeled ligand concentration. In other words, $k_{\text{off}}$ would be dependent on the ligand occupancy of the receptor, as has been observed for insulin binding to its receptor (40, 45–47).

The observation that the presence of unlabeled PMP-BSA can accelerate the dissociation rate of radioligand from a pre-formed $^{125}$I-PMP-BSA:15F complex reported in this study supports a negative cooperativity model for ligand interaction with the 15F construct. In addition, constructs containing only one Man-6-P binding domain demonstrate a curvilinear Scatchard plot in binding PMP-BSA. Therefore, the observed negative cooperativity is not likely due to intramolecular interaction between the repeats 3 and 9 Man-6-P binding domains of a monomeric receptor, but likely due to alignment of corresponding binding domains between two receptor molecules as shown in Fig. 8. A similar mechanism for negative cooperativity can be found in the example of insulin binding to its receptor. Recent electron cryomicroscopy studies of the insulin receptor have revealed that an insulin receptor dimer forms a single binding site in which the L1 Cys-rich domain of one $\alpha$-subunit and the L2 domain of the other $\alpha$-subunit interact with distinct residues of insulin (48). The same study also revealed that an insulin binding site from a single dimer was capable of simultaneously binding two insulin molecules, explaining the negative cooperativity of binding at high concentrations of insulin.

Negatively cooperative Man-6-P binding interactions may play a role in the rate of trafficking of the Man-6-P/IGF2R. The reported finding that the addition of up to 10 nM $\beta$-glucuronidase to cells in culture increased the internalization of the Man-6-P/IGF2R, whereas the addition of 5000 nM of a small
multivalent Man-6-P bearing peptide did not (33), is of particular interest. One possible interpretation of those findings is that increased internalization of the Man-6-P/IGF2R may only occur under conditions where one molecule of ligand is bound per receptor, and not at concentrations of ligand that result in higher stoichiometries of ligand binding. The binding of a single bivalent ligand to the Man-6-P/IGF2R in a high affinity state may result in a conformational change in a receptor oligomer, or complex, such that the tyrosine-based internalization signal of the cytoplasmic domain more effectively interacts with adaptor complexes such as AP-1 and AP-2. It will be interesting to determine the internalization rate of the Man-6-P/IGF2R at various concentrations of bivalent ligand to determine if high concentrations of ligand can interfere with the increase in Man-6-P/IGF2R internalization. It will also be important to test whether the CD-MPR is capable of displaying a similar phenomenon in binding multivalent lysosomal enzymes. An important consideration for lysosome biogenesis is that the specificity of hydrolase targeting displayed by the Man-6-P/IGF2R and the CD-MPR may result from differences in their affinity toward lysosomal enzymes at different concentrations of ligand that are present in specific environments of the cell.

In summary, this study of truncated receptor constructs has led to several important observations about the Man-6-P/IGF2R with respect to its ability to form high affinity Man-6-P binding sites. First, only one of the two extracytoplasmic Man-6-P binding domains is required for the formation of high affinity, strongly supporting the hypothesis that receptor oligomerization is responsible for the ability of multivalent ligands to interact with the receptor with high affinity. In addition, our laboratory has recently found that the IGF2R is capable of forming dimers (49). Second, regions of the IGF2R outside of the minimal Man-6-P binding domain play a role in the formation of high affinity sites. Finally, the high affinity sites display negative cooperativity in their ability to bind multivalent ligands. These observations may not only be important in the context of the specificity of lysosomal enzyme targeting by this receptor, but may lay a foundation for understanding how this protein functions in the context of the cell.

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Mechanisms for High Affinity Mannose 6-Phosphate Ligand Binding to the Insulin-like Growth Factor II/Mannose 6-Phosphate Receptor: NEGATIVE COOPERATIVITY AND RECEPTOR OLIGOMERIZATION
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