DOMAIN INTERACTIONS OF THE MANNOSE 6-PHOSPHATE/INSULIN-LIKE GROWTH FACTOR II RECEPTOR

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Short Title: Domain Interactions of the Man6P/IGF2R

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SUMMARY

The mannose 6-phosphate/insulin-like growth factor II receptor (Man6P/IGF2R) forms oligomeric structures important for optimal function in binding and internalization of Man6P-bearing extracellular ligands as well as lysosomal biogenesis and growth regulation. However, neither the mechanism of inter-receptor interaction nor the dimerization domain has yet been identified. We hypothesized that areas near the receptor’s ligand binding domains would contribute preferentially to oligomerization. Two panels of minireceptors were constructed that involved truncations of either the N- or C-terminal regions of the Man6P/IGF2R encompassing deletions of various ligand binding domains. α-FLAG or α-Myc-based immunoprecipitation assays showed that all of the minireceptors tested were able to associate with a full-length, Myc-tagged Man6P/IGF2R (WT-M). In the α-FLAG but not α-Myc immunoprecipitation assays, the degree of association of a series of C-terminally truncated minireceptors with WT-M showed a positive trend with length of the minireceptor. In contrast, length did not seem to affect the association of the N-terminally truncated minireceptors with WT-M, except that the 12th extracytoplasmic repeat appeared exceptionally important in dimerization in the α-FLAG assays. The presence of mutations in the ligand binding sites of the minireceptors had no effect on their ability to associate with WT-M. Thus, association within the heterodimers was not dependent on the presence of functional ligand binding domains. Heterodimers formed between WT-M and the minireceptors demonstrated high-affinity IGF-II and Man6P-ligand binding, suggesting a functional association. We conclude that there is no finite Man6P/IGF2R dimerization domain, but rather that interactions between dimeric partners occur all along the receptor’s extracytoplasmic region.
INTRODUCTION

The mannose 6-phosphate/insulin-like growth factor II receptor (Man6P/IGF2R) is a multi-functional member of the p-lectin family and is a type-1 integral membrane glycoprotein of approximately 300 kDa (1,2). This receptor comprises a large extracytoplasmic (EC) domain, a single membrane-spanning region, and a short cytoplasmic tail. The EC domain is the principal ligand-binding region of the receptor, consisting of 15 homologous repeats of ~145 amino acids each (1,3,4). The Man6P/IGF2R has been shown to bind at least two classes of ligands, the Man6P-containing and the non-Man6P-containing polypeptide ligands, all of which bind to sites within the EC region (5-7). Newly synthesized Man6P-containing ligands such as lysosomal acid hydrolases bind to the Man6P/IGF2R in the trans-Golgi network through Man6P residues on their N-linked oligosaccharides, whereas other Man6P-containing ligands such as latent transforming growth factor-β1 (TGF-β1), proliferin, and granzyme B, bind at the cell surface (1,8). Through binding of this large class of ligands, the Man6P/IGF2R mediates several important cellular functions, such as the endocytosis and/or targeting of acid hydrolases to lysosomes (9), the proteolytic activation of latent TGF-β1 (10-12), mediation of the migration and angiogenesis induced by proliferin (13), and the internalization of granzyme B (14). Two distinct high-affinity binding sites and one, recently discovered, low-affinity binding site for the Man6P-containing ligands map to specific residues that are common to repeats 3 and 9, and repeat 5 of the EC domain, respectively (15-19). The two high-affinity binding sites are not functionally equivalent with respect to ligand preference, having distinct dissociation constants for the multivalent Man6P-ligand β-glucuronidase (2.0 vs. 4.3 nM for repeats 3 and 9 respectively) (20). The pH optimum for carbohydrate binding is also more acidic for repeat 9 than repeat 3 (pH 6.4 vs. pH 6.9, respectively), and the two sites differ in their ability to
recognize distinctive modifications found on *Dictyostelium discoideum* glycoproteins, such as mannose 6-sulfate and Man6P methyl esters (21). Additionally, repeat 9 alone can fold into a high-affinity ligand-binding domain, whereas repeat 3 depends on residues in adjacent repeats 1 and/or 2 for optimal ligand binding (22). Although it exhibits significant sequence homology with repeats 3 and 9, as well as sharing four conserved residues key for Man6P binding, repeat 5 has an ~300-fold lower affinity for Man6P than repeat 9 or repeats 1-3, possibly due to the absence of two half-cystines that form a stabilizing disulfide bond in repeats 3 and 9 (19).

The non-Man6P-containing class of ligands includes the polypeptide mitogen, insulin-like growth factor II (IGF-II). The IGF-II binding site has been mapped to repeat 11 of the EC region, with high-affinity binding being conferred by residues contributed by the 13th repeat (23-26). Repeat 13 is thought to act as an enhancer of IGF-II affinity by slowing the rate of IGF-II dissociation (27). Structural analyses of repeat 11 identified the putative IGF-II binding site in a hydrophobic pocket at the end of a β-barrel structure (28). Another member of this class is retinoic acid, a unique ligand for the Man6P/IGF2R in that it binds the cytoplasmic region and is thought to function by altering intracellular trafficking of the Man6P/IGF2R and its cargo (29). The other members of the non-Man6P-containing ligands are urokinase-type plasminogen activator receptor (uPAR) and plasminogen, whose binding sites have been mapped to a peptide region within EC repeat 1 (30-32). The proposed function for the interactions between the Man6P/IGF2R and these ligands is involvement in the complex responsible for the activation of TGF-β1 at the cell surface, as well as endocytosis and targeting of uPAR for degradation (31,32). The uPAR-Man6P/IGF2R interaction appears to be weak, low-affinity and confined to a small subpopulation of uPAR molecules (33), which calls into question the physiological relevance of this interaction.
Recent crystal structure data have given insight into structural features of the Man6P/IGF2R (28,34). The crystal structures for repeat 11 by Brown et al. and repeats 1-3 by Olson et al. have allowed these groups to propose different models for the overall structure of the EC domain of the Man6P/IGF2R (28,34,35). The EC domain of the receptor shows considerable homology among repeats and the cation-dependent Man6P-receptor (16-38% identity) (4). This high level of sequence identity accounts for structural similarities among domains, including conserved disulfide bond organization, random coil linker regions connecting the domains, and an overall core flattened β-barrel structure. The 1-3 triple-repeat crystal revealed a structure in which repeat 3 sits on top of repeats 1 and 2 (34). Olson et al. have proposed that the Man6P/IGF2R forms distinct structural units for every three repeats of the EC region, producing five tri-repeat units that stack in a back-to-front manner (34). In this model, the IGF-II binding site is located on the opposite face of the structure relative to the Man6P binding sites.

Traditionally thought to function as a monomer (36), the Man6P/IGF2R is now considered to operate optimally in the membrane as an oligomer for high-affinity Man6P binding and efficient internalization of ligands (37-39). Intermolecular cross-linking of two Man6P/IGF2R partners was shown to occur upon binding of the multivalent ligand, β-glucuronidase, resulting in increased rate of ligand internalization (37). The initial rate of internalization of β-glucuronidase was faster than for the monovalent ligand, IGF-II, which showed that multivalent ligands enhance the rate of receptor movement, likely due to clustering of the Man6P/IGF2R for improved interaction with the endocytic machinery in the formation of clathrin-coated pits (37). Further studies demonstrated that alignment of the Man6P binding domains of monomeric partners of a receptor dimer is responsible for bivalent, high-affinity binding, also supporting the importance of receptor oligomerization (38).
In order to determine the interrelationship between dimer formation and the function of the receptor’s ligand binding domains, we co-expressed full-length receptors with truncated receptors from which either N- or C-terminal EC repeats were deleted, and that lacked functional ligand-binding domains and/or transmembrane and cytoplasmic domains. One of the main goals of this project was to map the dimerization domain(s) of the Man6P/IGF2R. We hypothesized that one or more dimer-interaction domains would be located at or near ligand-binding domains in the EC region of the receptor, and that these regions would contribute preferentially to receptor dimerization. A panel of Man6P/IGF2R mini-receptors was constructed to test for association with a full-length version of the receptor. It was observed that all of the truncated receptors were able to associate with the full-length receptor, suggesting that dimerization domains or contacts occur all along the EC region of the receptor, not just near regions of ligand interaction. However, repeat 12 seems to be particularly important to association, as N-terminally truncated minireceptors lacking this repeat gave distinctive results in the immunoprecipitation assays. We conclude that a distinct dimerization domain for the Man6P/IGF2R does not exist per se, but instead, interactions between monomeric receptor partners apparently occur all along the EC region of the receptor with special contribution made by repeat 12.
EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) or the University of Nebraska Medical Center Molecular Biology Core Facility (Omaha, NE). D-Man6P, disodium salt, anti-(α)-FLAG M2 antibody, α-FLAG M2-agarose affinity gel, and the bicinechonic acid kit for protein determination were purchased from Sigma (St. Louis, MO). The α-Myc 9E10 antibody was purchased from Upstate Biotechnology, Inc. (Hercules, CA) or the University of Nebraska Medical Center Monoclonal Antibody Facility (Omaha, NE). The polyclonal α-13D antibody (referred to as α-Man6P/IGF2R throughout this report) that recognizes a peptide domain in repeat 4 of the Man6P/IGF2R has been previously described (40). Rabbit α-mouse IgG was from DAKO (Carpinteria, CA). Carrier-free Na\(^{125}\)I and \(^{125}\)I-protein A were from PerkinElmer Life Sciences (Boston, MA). Recombinant human IGFs were provided by M. H. Niedenthal (Lilly Research Laboratories, Indianapolis, IN). Radiolabeled IGF-II and unlabeled and radiolabeled pentamannose phosphate-bovine serum albumin (PMP-BSA) were prepared as described previously (41). The pCMV5 vector was provided by Dr. David W. Russell (University of Texas Southwestern Medical Center, Dallas, TX) (42). The 8.6-kilobase-pair human Man6P/IGF2R cDNA and affinity-purified human β-glucuronidase (hGUS) were provided by Dr. William S. Sly (St. Louis University Medical Center, St. Louis, MO) (3). Radiolabeled hGUS was prepared by iodination using precoated IODOGEN tubes (Pierce, Rockford, IL) according to the manufacturer’s specifications to a specific activity of 26-40 Ci/g. Other reagents and supplies were obtained from sources as indicated.

Preparation, Expression and Analysis of Epitope-tagged Mini-receptors—The truncated Man6P/IGF2R minireceptors 1-8F, 1-9F, 1-9F-R/A, 1-11F, and 1-15F were tagged with the
eight-residue FLAG epitope (DYKDDDDK) followed by a stop codon and an Xba I restriction site at the C-terminus and cloned into the pCMV5 vector as previously described (Fig. 1A) (38). Using the full-length Man6P/IGF2R cDNA as the template, the following mini-receptors, containing the EC repeats starting with the repeat indicated in the name of the receptor and ending with the 15\textsuperscript{th} repeat, followed by the transmembrane and cytoplasmic regions of the Man6P/IGF2R, were synthesized by amplification with \textit{Vent}\textsuperscript{TM} polymerase (New England Biolabs, Beverly, MA): 10-15CF (nt 4239-7620), 11-15CF (nt 4675-7620), 12-15CF (nt 5092-7620), 13-15CF (nt 5542-7620) (Fig. 5A). To ensure consistent translation, the signal sequence containing the N-terminal 71 residues of repeat 1 was fused to the beginning of each construct as described previously (43). All of these minireceptors were C-terminally tagged with the FLAG-epitope followed by a stop codon and Xba I restriction site for cloning purposes.

The cDNA plasmids in the vector pCMV5R1X encoding the 10-15CF-I/T and 11-15CF-I/T minireceptors, bearing the isoleucine to threonine mutation that has been previously shown to prevent IGF-II binding to the Man6P/IGF2R, were synthesized from the 10-15CF and 11-15CF constructs as well as a 1-15F construct containing the I1572T mutation (1-15F-I/T) (39). Briefly, the 10-15CF or 11-15CF and 1-15F-I/T cDNAs were digested with \textit{Bst}E II (Man6P/IGF2R nt 4698) and \textit{Bst}B I (Man6P/IGF2R nt 5507) serially and the resulting fragments of interest [809 base-pair fragment for 1-15F-I/T, corresponding to the region containing the I\rightarrow T mutation, and the larger fragment (> 7000 base pairs) for 10-15CF and 11-15CF, which also encompasses the pCMV5 plasmid] were gel-purified (Qiagen, Valencia, CA). The purified fragments were then ligated together using T4 DNA Ligase (Invitrogen, Carlsbad, CA) and used to transform XL-10 Gold competent cells (Stratagene, La Jolla, CA).
The full-length Myc-tagged Man6P/IGF2R was prepared as follows: full-length Man6P/IGF2R cDNA that had been digested with Eag I and re-ligated (lacking nt 162-5319) was used as template for amplification with a 5’-primer containing an Xho I restriction site preceding the sequence corresponding to nt 94-113 of the receptor cDNA and a 3’-primer with sequence complementary to nt 7602-7620 at the C-terminal end of the expressed Man6P/IGF2R followed by the 36-nt sequence encoding the Myc epitope, MEQKLISEEDLN (44), followed by two stop codons and an Xba I site. The products from these amplifications were digested with Xba I and Xho I and subcloned into pBKCMV (Invitrogen). These plasmids were then digested with Hind III and Xba I and subcloned into the target vector, pCMV5. Finally, wild-type Eag I fragments were subcloned into the construct, reconstituting the completed Man6P/IGF2R-Myc (WT-M) cDNA construct.

A full-length, Myc-tagged Man6P/IGF2R triple mutant (R2AxI/T-M) for residues in the repeats 3 and 9 Man6P binding domains (R426A and R1325A, respectively) and the repeat 11 IGF-II binding domain (I1572T) was prepared as follows: nt 100-3242 of the Man6P/IGF2R cDNA served as template for amplification using a 5’-primer containing an EcoR I restriction site preceding the sequence corresponding to nt 1112-1129 of the receptor cDNA and a 3’-primer with sequence complementary to nt 2474-2487 of the Man6P/IGF2R cDNA, followed by an Xho I site. The products from these amplifications were digested with EcoR I and Xho I and subcloned into pBluescript SK II+ (pBSKII+) (Stratagene). This construct was subjected to two rounds of amplification with primers designed to incorporate the R426A mutation responsible for altering the EC repeat 3 Man6P binding site using the Megaprimer approach (45). The first round of amplification involved producing the mutation, by amplifying from that site (nt 1425) to the 3’ end of the minireceptor (nt 2487). This “megaprimer” was then used in a second round of
amplification with the 5’-primer used above. The repeat 3 mutant amplification product was
digested with EcoRI and XhoI and subcloned back into pBSKII+. An ~1 kb BsmI-BsmI I
fragment (sites at Man6P/IGF2R nt 1408 and 2449) containing the mutation was removed from
the megaprimer and subcloned into the corresponding positions of pBSKII+/KpnI, which
contained a 2.2 kb KpnI-KpnI fragment derived from Man6P/IGF2R nt 100-3242, creating a
pBSKII+/KpnI-R426A minireceptor with the 3rd repeat Man6P-binding mutation. The KpnI-KpnI
fragment from this construct was then subcloned into pCMV5/R1325A-M, a full-length Myc-
tagged receptor containing the 9th repeat Man6P-binding mutation synthesized as previously
described (39), that had also been digested with KpnI and XmnI, creating a full-length
Man6P/IGF2R bearing Man6P binding-site mutations at both repeats 3 and 9 (R2A-M). The
R2A-M cDNA was digested with AflII (Man6P/IGF2R nt 4740) and MluI (pCMV5 nt 933) and
this ~5-kb fragment was subcloned into pCMV5/I1572T-M, a full-length Myc-tagged receptor
containing the 11th repeat IGF-II binding mutation at nt 4862 (24), which had also been digested
with AflII and MluI. The final product resulted in the binding-defective triple-mutant, R2Axl/T-
M, in the vector pCMV5.

Transient expression of the minireceptors by calcium phosphate-mediated transfection
into 293T human embryonic kidney cells and immunoblot analysis of cell lysates to measure
expression of the truncated and full-length receptors were performed as previously described
(39,46). Additionally, expression was tested by the use of the α-Man6P/IGF2R polyclonal
antibody (40). Aliquots (25 μl) of Triton X-100 extracts were resolved by electrophoresis on 6%
reducing SDS-PAGE gels in sample buffer (50 mM Tris-HCl, pH 6.8, 2.5% SDS, 5% sucrose,
and 0.01% bromophenol blue) plus 50 mM dithiothreitol (DTT), and then transferred to BA85
nitrocellulose paper (Schleicher and Schuell, Keene, NH) at 0.35 Amp for 3 h at 22°C in
immunoblotting buffer (0.4 M Tris, pH 7.5, 3 M glycine, 20% methanol). The blots were incubated with blocking buffer (4% nonfat dry milk in 50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Tween-20, 0.02% sodium azide) for 1 h at 22°C and probed with the α-Man6P/IGF2R antibody (1:500 dilution). The blots were then developed with 125I-protein A, and detected by autoradiography.

**Western Ligand Blot Analysis**—Aliquots (25-50 µl) of 293T whole-cell lysates were electrophoresed on 6% SDS-PAGE in sample buffer and transferred to BA85 nitrocellulose paper at 0.35 Amp for 3 h at 20°C in ligand transfer buffer (15 mM Tris-HCl, pH 8.3, 120 mM glycine, 20% methanol), according to the method of Hossenlopp et al. (47). The blots were probed overnight with 1 x 10^6 cpm of 125I-IGF-II or 125I-PMP-BSA or 0.5 x 10^6 cpm of 125I-hGUS. The blots were subsequently washed three times for 10 min at 4°C and developed by autoradiography.

**Dimer Formation Assay—α-FLAG Immunoprecipitation**: Equal volumes (20 or 50 µl) of whole cell lysates prepared from 293T cells co-transfected with the various cDNAs for the FLAG-tagged minireceptors and the Myc-tagged Man6P/IGF2R were incubated with 8 µl of packed M2 resin in 25 mM HEPES, pH 7.4, 150 mM NaCl (HBS) plus 1.0% BSA at 4°C for 3 h. The resin pellets were collected by centrifugation at 13,000 x g for 30 s and washed twice with 1 ml of HBS plus 0.05% Triton X-100 (HBST). Immunoblot analysis was performed by subjecting the resin pellets to treatment with sample buffer plus DTT, electrophoresis on 6% reducing SDS-PAGE gels, and transfer to BA85 nitrocellulose paper. The blots were incubated in blocking buffer and probed with either α-FLAG or α-Myc monoclonal antibodies (1:2000 or 1:500 dilution, respectively). The blots were then incubated with rabbit α-mouse IgG secondary antibody (1:1000), developed with 125I-protein A, and detected by autoradiography. Levels of
FLAG- and Myc-tagged proteins immunoprecipitated with the M2 resin were quantified using Storm or Typhoon PhosphorImager (American Biosciences) analysis of the immunoblots. 

**α-Myc Immunoprecipitation:** Equal volumes (20 µl) of whole-cell lysates prepared from 293T cells co-transfected with the various cDNAs for the FLAG-tagged minireceptors and the Myc-tagged Man6P/IGF2R were incubated with 1 µl of 9E10 α-Myc monoclonal antibody in 79 µl of HBST at 4°C for 16 h. Protein G-Sepharose aliquots (stored as a 50% (v:v) slurry in 20% EtOH buffer) were washed four times with excess HBS plus 1.0% BSA to remove EtOH and to block the resin finally aspirated to a 50% slurry (resin:buffer). Aliquots (25 µl) of resin slurry were added to the overnight incubations along with 75 µl of HBS plus 1.0% BSA and 5 mM Man6P (200 µl total reaction volume) and then incubated at 4°C for 5 h. The resin pellets were collected by centrifugation at 13,000 x g for 30 s and washed three times with 1 ml of HBST. Immunoblot analysis and quantification were performed as described above for the α-FLAG immunoprecipitation assays.

**Radioligand Binding Assays—** Four aliquots of whole-cell lysates prepared from 293T cells co-transfected with cDNAs encoding the various FLAG-tagged minireceptors and the Myc-tagged Man6P/IGF2Rs were incubated with 8 µl of packed M2 affinity resin in HBS + 1% BSA buffer at 4°C for 6 h in a total volume of 200 µl. Lysate volumes were calculated by quantification of immunoblots of these lysates by Typhoon PhosphorImager using ImageQuant 5.0 software to achieve equivalent loading of WT-M. Addition of 5 mM Man6P at this point prevented the co-precipitation of endogenous Man6P-containing ligands. The resin pellets were collected by centrifugation at 13,000 x g for 30 sec, washed three times with 1 ml of HBST, and aspirated to a final volume of 100 µl after the final wash. The ability of the immunoprecipitated receptors to bind $^{125}$I-IGF-II was measured by incubating the resin pellets with 2 nM $^{125}$I-IGF-II.
plus 100 nM unlabeled IGF-I in HBST for 16 h at 4°C. The addition of IGF-I to the binding reaction prevented interference from IGF-binding proteins in the cell lysates. The resin pellets were washed twice with 1 ml of HBST to remove unbound ligand, collected by centrifugation, and counted in a Wizard 1000 γ-counter (Perkin-Elmer). Three of the replicate aliquots were used to assess total binding, and specific $^{125}$I-IGF-II binding was determined by subtracting counts per minute radioligand bound in the fourth replicate, which was incubated in the presence of 1 µM IGF-II. Binding of $^{125}$I-PMP-BSA was measured under similar conditions using 1 nM $^{125}$I-PMP-BSA in the presence or absence of 5 mM Man6P to compensate for nonspecific binding. The data were graphed and analyzed using GraphPad Prism™ software.
RESULTS

Transient Expression of C-terminally Truncated Man6P/IGF2R Minireceptors—In an attempt to map specific regions of the EC domain that would contribute to Man6P/IGF2R intersubunit interaction, a series of C-terminally truncated, soluble minireceptors was synthesized that encompassed various regions of the EC domain followed by a FLAG epitope tag (Fig. 1A). The numbers in the name of each construct indicate the most N-terminal and C-terminal EC repeats expressed by that construct. All of the minireceptors are FLAG epitope-tagged at the C-terminal end, as denoted by the “F” in the construct name. Additionally, all of the minireceptors encode the first half of repeat number one plus the signal sequence for proper translation and localization (43). The truncated receptors were expressed in 293T cells either singly or by co-transfection with the full-length Myc-tagged Man6P/IGF2R (WT-M). As we have previously reported, the exogenous proteins produced from the various truncation constructs were not secreted into the medium, but rather were soluble in detergent cell lysates (33,41). Cell extracts were prepared using Triton X-100 for the analysis of interactions with WT-M. Immunoblotting with the α-FLAG and α-Myc antibodies was employed to quantify expression of the proteins (Fig. 1B).

C-terminally truncated Man6P/IGF2R Minireceptors Bind Ligands According to their Available Binding Sites—To ensure that the prepared minireceptors were able to fold and function properly, i.e., bind ligands according to the available binding sites within the minireceptors, ligand blots were performed. The panel of C-terminally truncated minireceptors was transfected singly into 293T cells and whole-cell lysates were prepared. Aliquots of the lysates were electrophoresed on polyacrylamide gels and transferred to nitrocellulose. Radiolabeled ligands were then used to probe the membrane to test for receptor binding
capability. The ligands used were $^{125}$I-IGF-II, which binds to the 11th and 13th EC repeats of the Man6P/IGF2R, $^{125}$I-PMP-BSA, a pseudoglycoprotein that binds the receptor in a Man6P-dependent manner to the two high-affinity sites within repeats 1-3 and 9, and finally, $^{125}$I-hGUS, a naturally occurring Man6P-dependent ligand for the Man6P/IGF2R. IGF-II was able to bind to the C-terminally truncated minireceptors that contained all or part of the IGF-II binding site, namely 1-11F and 1-15F, but not to those that excluded this region: 1-8F and 1-9F (Fig. 2A). The two different Man6P-dependent ligands showed similar results. As predicted by the available Man6P-binding domains, both PMP-BSA and hGUS bound to all of the minireceptors (Fig. 2B & C). However, both ligands showed a preference for binding the 1-9F minireceptor over 1-8F or 1-11F (Fig. 2B & C, compare lanes 5-6 with lanes 3-4 and 7-8). The decrease in binding of the Man6P ligands to the 1-8F minireceptor was anticipated because deletion of the 9th repeat leaves it with only one Man6P binding site (repeats 1-3). The reduced ability of the 1-11F minireceptor to bind PMP-BSA and hGUS in these ligand blots was unexpected but may be due to the absence of repeat 12 from the 10-12 tri-repeat unit of this protein. Lysates prepared from cells transfected with the CMV5 vector alone showed ligand binding only to the endogenous 293T cell receptor (Fig. 2).

The Man6P/IGF2R Interacts with All of the C-terminally Truncated Minireceptors—In order to investigate the potential dimerization domains in the Man6P/IGF2R’s EC domain, immunoprecipitation experiments were performed using lysates from cells co-transfected with FLAG-tagged Man6P/IGF2R minireceptors and WT-M. By immunoprecipititating with an α-FLAG affinity resin, any full-length Myc-tagged receptors retained on the resin would indicate an association between the receptors. Equal volumes of each singly- or co-transfected lysate were immunoadsorbed to aliquots of α-FLAG M2 resin. An α-Myc immunoblot probed with a
monoclonal Myc antibody was used to assess which C-terminally truncated minireceptors were able to form stable interactions with full-length WT-M receptor. All of the FLAG-tagged minireceptors were immunoadsorbed to the resin (Fig. 3A, upper panel, lanes 2-6). Lysates from cells transfected with the WT-M expression plasmid alone or in a 2:1 transfection with empty pCMV5 vector were used as controls, to establish that WT-M would not be immunoadsorbed to the α-FLAG affinity resin in the absence of a FLAG-tagged partner (Fig. 3A, upper and lower panels, lanes 1 and 7). However, the WT-M receptor was able to immunoprecipitate along with all of the C-terminally truncated minireceptors tested (Fig. 3A, lower panel, lanes 2-6). In other experiments (data not shown), even smaller C-terminally truncated minireceptors were able to immunoprecipitate WT-M, including 1F and 1-3F, as well as the C- and N-terminally truncated minireceptor 7-9F, constructs that contain only one or three EC repeats, as indicated by their names.

To make certain that the observed associations were not merely a result of the use of the α-FLAG affinity resin, immunoprecipitation experiments were also performed using the α-Myc antibody followed by incubation with Protein G-Sepharose. In this reciprocal assay, retention of FLAG-tagged minireceptors with WT-M in the α-Myc immunoprecipitation would be indicative of association between the receptor species. Equal lysate volumes were incubated with aliquots of α-Myc antibody and subsequently precipitated by Protein G-Sepharose. An α-FLAG immunoblot was used to assess association of the C-terminally truncated minireceptors with WT-M. WT-M was immunoadsorbed to the α-Myc matrix as expected (Fig. 3C, lower panel, lanes 1-7). Additionally, all of the FLAG-tagged minireceptors were co-immunoprecipitated with WT-M (Fig. 3C, upper panel, lanes 2-6). Lysates from cells transfected with empty pCMV5 vector or 1-15F alone were used as negative controls, to verify that FLAG-tagged minireceptors were not
immunoprecipitated by Protein G-Sepharose in the absence of a Myc-tagged partner (data not shown).

Semi-quantitative analysis of the interactions between WT-M and the C-terminally truncated minireceptors revealed a trend that indicated a dependence of association on the length of the truncated receptor in the α-FLAG association assays. In these α-FLAG immunoprecipitation assays, when adjusted for the amount of immunoprecipitated FLAG-tagged receptor, the minireceptors comprising a greater number of EC repeats retained progressively more WT-M receptor molecules on the α-FLAG affinity resin. The resulting immunoblots from the immunoprecipitation experiments summarized above were quantified using Typhoon PhosphorImager and ImageQuant software. Values for co-immunoprecipitation of WT-M were adjusted for the amount of FLAG-tagged receptor immunoprecipitated in each assay based on PhosphorImager data (Fig. 3B). The analysis reveals a trend in the amount of WT-M interaction with the C-terminally truncated receptors as a function of increasing minireceptor length, with 1-8F being the weakest partner and 1-15F being the strongest. The only break in this trend occurred in the assay of WT-M co-immunoprecipitation with the 1-9F-R1325A (R/A) mutant minireceptor relative to its wild-type counterpart, 1-9F (Fig. 3B, compare lanes 4 and 3). These data suggest that the interaction with WT-M is not dependent on function of the Man6P ligand-binding domains.

The same analyses were performed for the reciprocal α-Myc immunoprecipitation experiments (Fig. 3D). These data were adjusted for the amount of WT-M receptor immunoprecipitated and quantified as above. The length-dependence trend was not evident for these assays (compare Fig. 3B & D). Furthermore, the 1-15F minireceptor was not as efficiently co-immunoprecipitated with WT-M as the other C-terminally truncated minireceptors, as ~30%
of 1-15F was immunoprecipitated in comparison to ~70-80% immunoprecipitation of the other minireceptors (data not shown); quantification therefore showed a significant decrease in WT-M association with 1-15F in the α-Myc immunoprecipitation experiments (Fig. 3D, last bar).

**Transient Expression of N-terminally Truncated Man6P/IGF2R Minireceptors**—Another panel of minireceptors, which lacked repeats from the N-terminus of the EC domain but contained the transmembrane and cytoplasmic regions, was designed to provide further insight into the dimerization domain of the Man6P/IG2R (Fig. 4A). None of the minireceptors in this panel contains any Man6P binding sites, and four of the minireceptors lack a functional IGF-II binding site as well (10-15CF-I/T, 11-15CF-I/T, 12-15CF, and 13-15CF). In order to test whether the full-length Man6P/IGF2R was able to associate with these receptors, 293T cells were co-transfected with the N-terminally truncated minireceptors plus the full-length WT-M receptor. Additionally, 1-15F was used as a positive control because it contains the full EC domain with all of the ligand binding domains intact. Whole-cell lysates were prepared and tested for protein expression by α-FLAG and α-Myc immunoblotting (Fig. 4B). All of the transfected proteins were expressed and appeared at the expected molecular masses.

**N-terminally truncated Man6P/IGF2R Minireceptors Bind Ligands According to their Available Binding Sites**—Similar to the series of C-terminally truncated minireceptors, lysates from this panel of truncated receptors were subjected to ligand blot analysis to determine whether the truncated receptors were able to fold and function properly (Fig. 5). As predicted by the presence or absence of the various ligand binding domains, only 1-15F, 10-15CF, and 11-15CF were able to bind radiolabeled IGF-II (Fig. 5A, lanes 2, 3, and 5). None of the other minireceptors were able to bind IGF-II, likely due to the absence of repeat 11 (CMV5, 12-15CF, and 13-15CF) or due to mutation of isoleucine 1572 to threonine, which abrogates IGF-II
binding (10-15CF-I/T and 11-15CF-I/T) (Fig. 5A, lanes 1, 4, 6-8). None of the N-terminally truncated minireceptors were able to bind \(^{125}\)I-PMP-BSA, except for the 1-15F positive control, as this is the only member of this panel that contains functional Man6P binding sites (Fig. 5B).

**The Man6P/IGF2R Interacts with the N-terminally Truncated Minireceptors in a Length-Independent Manner**—To further investigate the potential dimerization sites in the Man6P/IGF2R’s EC domain, immunoprecipitation experiments were performed using the N-terminally truncated Man6P/IGF2R minireceptors. As with the C-terminal truncation panel, immunoprecipitation/immunoblotting experiments with the \(\alpha\)-FLAG affinity resin followed by expression analysis with \(\alpha\)-Myc antibody indicated association of the truncated receptors with the Myc-tagged full-length receptor, WT-M. Equal volumes of transfected whole-cell lysates were again immunoadsorbed to aliquots of \(\alpha\)-FLAG M2 resin. All of the FLAG-tagged truncated receptors were able to immunoadsorb to the \(\alpha\)-FLAG resin (Fig. 6A, upper panel, lanes 2-8). Immunoblotting with \(\alpha\)-Myc revealed that all of the N-terminally truncated minireceptors tested were able to associate with WT-M (Fig. 6A, lower panel, lanes 2-8). As before, lysates from cells transfected with WT-M and the vector pCMV5 showed no immunoprecipitation to the \(\alpha\)-FLAG resin on either the FLAG or Myc immunoblots due to the lack of a FLAG-tagged partner (Fig. 6A, upper and lower panels, lane 1).

Semi-quantitative analysis of the association between WT-M and the N-terminally truncated receptors was performed with normalization for the amount of input Myc-tagged receptor (Fig. 6B). Unlike the C-terminally truncated receptors, the N-terminally truncated minireceptors did not show consistent differences as a function of the number of EC repeats in the amount of association between the different truncated receptors with WT-M (Fig. 6B, lanes 2-7). The lone exception to this finding was 13-15CF, which did show a sharp, consistent
decrease in WT-M association over that of the other minireceptors in this series (Fig. 6B). Additionally, the presence or absence of the IGF-II binding domain does not seem to affect the association with WT-M at all (Fig. 6B, compare WT to I/T mutants for 10-15CF and 11-15CF).

This panel of minireceptors was also tested for association with the WT-M receptor by a reciprocal immunoprecipitation strategy using α-Myc antibody (Fig. 6C & D). These experiments again showed that WT-M was immunoprecipitated by α-Myc antibody-Protein G-Sepharose (Fig. 6C, lower panel). Further, all of the N-terminally truncated minireceptors were able to co-immunoprecipitate with WT-M (Fig. 6C, upper panel, lanes 3-8). As before, vector alone or singly transfected 1-15F were not immunoprecipitated (data not shown). Quantification was performed as above with normalization for the amount of immunoprecipitated Myc-tagged receptors (Fig. 6D). As with the α-FLAG immunoprecipitation experiments, no length dependence was evident for the N-terminally truncated minireceptors associating with WT-M. However, the sharp drop-off of association between 13-15CF and WT-M observed in the α-FLAG immunoprecipitation assays was reversed to a substantial increase in association in the α-Myc immunoprecipitation experiments (Fig. 6B & D, compare last bars).

Association of M6P/IGF2R Minireceptors with a Ligand-Binding Mutant Receptor--Both the α-FLAG and α-Myc immunoprecipitation experiments using the C-terminally and N-terminally truncated minireceptors were repeated with the triple-mutant form of the Man6P/IGF2R, R2AxI/T-M, which is unable to bind IGF-II or Man6P-containing glycoproteins. All of the minireceptors were able to co-immunoprecipitate R2AxI/T (data not shown). In addition, the pattern of association for both the C- and N-terminally truncated receptors with the mutated Man6P/IGF2R was similar to that seen with the wild-type receptor as in Fig. 3B &D and Fig. 6B & D, respectively (data not shown).
WT-M Forms Functional Associations with the N-terminally Truncated Minireceptors—

To determine if the associations between the Man6P/IGF2R N-terminally truncated minireceptors and WT-M were functional, binding of radiolabeled ligands to coimmunoprecipitated receptors was assayed. The full panel of N-terminally truncated minireceptors co-transfected with WT-M was immunoabsorbed to M2 affinity resin and subsequently assayed for binding of either $^{125}$I-IGF-II or $^{125}$I-PMP-BSA (Fig. 7A & B). A parallel set of immunoprecipitations was used to confirm expression of both the FLAG-tagged and Myc-tagged partners by immunoblot analysis (data not shown).

In this case, the pattern of IGF-II binding was highly dependent on the IGF-II binding capability of the FLAG-tagged N-terminally truncated minireceptors. The 1-15F/WT-M, 10-15CF/WT-M, and 11-15CF/WT-M pairs were able to bind labeled IGF-II to a greater extent than the other minireceptor/WT-M combinations tested (Fig. 7A, asterisk-labeled combinations). These three groups have the only FLAG-tagged receptors tested in this panel that contain a wild-type IGF-II binding site. The contribution of WT-M to IGF-II binding in the combinations is evident, however, when the combinations containing FLAG-tagged partners that are unable to bind IGF-II are compared with the CMV5/WT-M and WT-M alone (not shown) controls, in which the WT-M receptor fails to be immunoprecipitated by the M2 resin because of the absence of any FLAG-tagged partners (Fig. 7A).

PMP-BSA binding assays were done to determine whether the WT-M partners could bind Man6P-containing ligands in dimeric structures with the N-terminally truncated minireceptors that have no Man6P binding-competent domains. The 1-15F minireceptor used as a control in these experiments contains both complete Man6P binding domains. As expected, the 1-15F/WT-M combination was able to bind PMP-BSA to a greater extent than any of the others tested (Fig.
The CMV5/WT-M and WT-M controls (not shown) had no detectable PMP-BSA binding because WT-M is not immunoadsorbed to the M2 resin in these combinations (Fig. 7B, control, C). Interestingly, all of the N-terminally truncated minireceptors associated with WT-M displayed measurable PMP-BSA binding (Fig. 7B, unlabeled combinations). This observation provides evidence that N-terminally truncated minireceptors and the full-length Man6P/IGF2R can form functional dimers, even though the only PMP-BSA binding-competent species in these combinations is WT-M.

**R2AxI/T-M Reduces the Amount of Radioligand Bound by Heterodimeric Man6P/IGF2Rs**—To establish the validity of the radioligand binding detected using the co-immunoprecipitated N-terminally truncated minireceptors and WT-M, further ligand binding studies were performed using the panel of N-terminally truncated minireceptors with the mutant Man6P/IGF2R, R2AxI/T-M. N-terminally truncated minireceptors co-expressed with R2AxI/T-M were assayed as above for binding of $^{125}$I-IGF-II or $^{125}$I-PMP-BSA. This series showed IGF-II binding to combinations in which the partner minireceptor contained an intact IGF-II binding domain (Fig. 7A, asterisk-labeled), but there was little to no detectable binding to the combinations in which FLAG-tagged receptors did not contain an intact IGF-II binding domain (Fig. 7A, unlabeled combinations). This was a marked decrease from the amount of IGF-II binding demonstrated with WT-M (Fig. 7A). For PMP-BSA, none of the N-terminally truncated minireceptors were able to bind $^{125}$I-PMP-BSA when associated with R2AxI/T-M (Fig. 7B, unlabeled combinations). Only the control 1-15F minireceptor demonstrated PMP-BSA binding in combination with R2AxI/T-M, which is not surprising since this is the only minireceptor in this panel that still contains Man6P binding-competent sites (Fig. 7B, asterisk-labeled). PMP-BSA binding to the N-terminally truncated minireceptors was significantly reduced from that.
observed in combination with WT-M (*compare WT-M and R2AxI/T-M*, Fig. 7B). Thus, studies using this triple-mutant, ligand-binding defective Man6P/IGF2R further support the notion that the associations formed between full-length Man6P/IGF2R and the minireceptors are functional with respect to contributions from both partners in the oligomeric structure.
DISCUSSION

The original objective of this project was to map a dimerization domain within the EC region of the Man6P/IGF2R and to test the hypothesis that the ligand binding domains of the receptor are important in the formation of dimeric receptors. By using two series of minireceptors based on C-terminal and N-terminal truncations, we intended to delimit potential dimerization domains to one or more repeats within the EC region, paying particular attention to truncation, deletion, and missense mutations that would allow insight into the importance of the ligand binding regions of the receptor. Overall, our association assays revealed that a finite dimerization domain does not really exist, but rather that interactions between receptors occurred all along the EC region of the Man6P/IGF2R. To test the hypothesis that ligand binding domains are important to the formation of interacting receptor partners, each set of minireceptors was designed to test the contribution made by such domains.

For the panel of C-terminally truncated receptors, the α-FLAG-based association assays revealed overall that longer truncated receptors immunoprecipitated WT-M to a greater degree than the shorter receptors. This increasing avidity for interaction with WT-M does not seem to be related to the presence or absence of ligand binding domains, but rather depends simply on the length of the truncated receptors themselves. Minireceptors containing even a single repeat (1F) or those containing both N- and C-terminal truncations (7-9F) were still able to associate with WT-M. The possibility that increasing association with WT-M depends simply on the length of the EC region of the minireceptor implies an organization of the receptor dimer in which the receptors’ intermolecular association is mediated by a number of contact points between receptors all along the Man6P/IGF2R EC domain, rather than contact points being made only between EC repeats that are involved in providing functionality for the Man6P/IGF2R, such as
repeats involved in ligand binding. All of the N-terminally truncated minireceptors tested were able to interact with WT-M in both α-FLAG association assays. However, the degree of association between the N-terminally truncated minireceptors and the Man6P/IGF2R in the immunoprecipitation was not dependent on the length of the minireceptor molecules. For the α-FLAG immunoprecipitations, 13-15CF was the exception to this finding in that this minireceptor did not associate with WT-M as well as the other constructs, suggesting special importance to the presence of the 12th repeat in the EC region.

In contrast, α-Myc association-based assays with these same panels did not show a trend toward length dependence for either C- or N-terminally truncated minireceptors. The major differences between the data sets from the two assays were observed in the degree of association between 1-15F and WT-M, and between 13-15CF and WT-M. These apparently conflicting data may be reconciled if we consider that the association assays measure the net outcome of several competing processes that affect: 1) binding affinity between Myc- and FLAG-tagged partners in heterodimer formation; 2) the tendency for formation of homodimers, which likely affects longer minireceptors to a greater degree than shorter minireceptors; and 3) the physical demands of immunoprecipitation between different-sized partners. If we accept the seemingly logical premise that binding affinity increases with greater number of EC repeats, then the disparities between the FLAG- and Myc-based immunoprecipitation assays, which are expressed most markedly in the largest minireceptor, 1-15F, and the smallest, 13-15CF, can be resolved. If by virtue of its length and physical constraints in the immunoprecipitation assay, 1-15F forms homodimers with a preference over heterodimer formation with WT-M, this would cause underestimation of its ability to interact with WT-M in the α-Myc assay. By contrast, due to its small size and the absence of repeat 12, all dimers formed between 13-15CF and its partners...
would be of relatively low affinity. This would reduce its effectiveness in immunoprecipitations where 13-15CF acts as the bait and WT-M is the prey, i.e. in the α-FLAG immunoprecipitations. In contrast, 13-15CF makes a much better prey when WT-M serves as the bait in the α-Myc assays, due to increased degrees of freedom and decreased physical constraints on the complex, which would lead to overestimation of its association with WT-M. Although neither assay presents a complete representation of the association between partners in heterodimer formation, the composite value of using both α-FLAG and α-Myc assays leads to important insights into dimer interactions of the Man6P/IGF2R.

The presence of repeat 12 seems to be important for dimer formation in the α-FLAG assays, as can be observed in the differences between 12-15CF vs. 13-15CF and also 1-11F vs. 1-15F, as well as by some of the α-Myc results. If repeat 12 were important for Man6P/IGF2R complex formation, it would be important in both homo- and heterodimer formation, not overly affecting the results of the α-FLAG assays, but affecting receptors containing repeat 12 for the α-Myc assays due to the tendency of the repeat 12-containing minireceptors to homodimerize. In the C-terminally truncated minireceptor panel, 1-15F is the only minireceptor containing repeat 12 and its ability to associate with WT-M is likely underrepresented in the α-Myc assays. In the N-terminally truncated minireceptor series, 13-15CF is the only minireceptor lacking repeat 12 and its ability to associate with WT-M is likely overrepresented in the α-Myc assay. The α-FLAG immunoprecipitation data in which 13-15CF did not associate with WT-M to the same extent as the other N-terminally truncated minireceptors are further supported by previous experiments from Byrd et al. that used a Man6P/IGF2R-epidermal growth factor receptor (EGFR) chimera containing EC repeats 11-15 or 13-15 plus the transmembrane region of the Man6P/IGF2R and the cytoplasmic domain of the EGFR (39). Immunoprecipitation and
phosphorylation experiments showed that, although the 13-15 chimeric receptor was able to immunoprecipitate with a full-length Man6P/IGF2R-EGFR chimera and participate in autophosphorylation, it did so at approximately 30% of the activity of the 11-15 chimera (39). These assays also used α-FLAG immunoprecipitation as the method of receptor immobilization, but in these assays both receptor species were FLAG epitope-tagged. Repeat 12 may contribute to stabilization of the Man6P/IGF2R dimer by: 1) serving as the anchoring repeat to stabilize the structure of a tri-repeat mini-domain as proposed by the recent report of the repeat 1-3 crystal structure; 2) serving as a stabilizer of an important functional feature of the receptor, such as cooperation in IGF-II binding between repeats 11 and 13; or 3) by direct intermolecular association with the corresponding repeat 12 from its interacting dimeric partner.

Studies with these panels of truncated receptors also permitted analysis of the potential contributions of the distal Man6P- and IGF-II-binding domains to receptor association. The minireceptors in these panels containing the 9th repeat R/A substitution that eliminates Man6P binding or the 11th repeat I/T mutation that eliminates IGF-II binding did not show any differences in immunoprecipitation with WT-M from that of their wild-type counterparts. Even when using the triple ligand-binding mutant, R2AxI/T-M, in conjunction with these mutated minireceptors, we still did not observe an effect on the ability of the receptors to form heterodimeric complexes, supporting the overall notion that ligand binding sites do not seem to affect oligomeric complex formation by the M6P/IGF2R.

Based on the finding that the minireceptors were capable of binding to full-length Man6P/IGF2R molecules, we sought to determine if these intermolecular associations were functional with respect to ligand binding. Binding assays showed that the N-terminally truncated minireceptors are able to form functional oligomers with the full-length Man6P/IGF2R
molecules. In this assay, the FLAG-tagged minireceptors, having no functional Man6P binding domains, mediated immunoprecipitation of either WT-M or R2AxI/T-M. To our surprise, WT-M associated with ligand binding-deficient N-terminally truncated minireceptors showed substantial high-affinity PMP-BSA binding. Previous data have shown that a single Man6P binding site is not capable of high-affinity Man6P binding, but rather that two competent sites are required (38). The high-affinity binding observed in these experiments could be explained by three possible mechanisms. First, intramolecular binding between the Man6P binding sites on WT-M may cooperate to bind Man6P ligands, which agrees with the traditional view of how the Man6P/IGF2R functions (6,37). Second, pairs of dimers attached to the α-FLAG affinity resin could be coming into close proximity to one another and forcing the formation of higher-ordered oligomeric structures. These larger structures may force two wild-type Man6P/IGF2R molecules on different heterodimers to approach closely enough to facilitate high-affinity, bivalent Man6P binding. Finally, the most intriguing possibility is that the receptor molecules are not naturally forming just dimers, but rather, oligomers of higher-order structure or mixed clusters. This model would allow for the intermolecular interaction of two Man6P binding sites between WT-M receptors and explain the high-affinity binding observed in combinations involving the N-terminally truncated minireceptors with WT-M. Definitive evidence that this binding was contributed by the WT-M partners in such heterodimers was provided by experiments in which PMP-BSA binding was not detectable in dimers formed from N-terminally truncated minireceptors in combination with the triple ligand-binding mutant, R2AxI/T-M.

Similarly, the N-terminally truncated panel transfected with WT-M showed binding to labeled IGF-II in the presence or absence of intact IGF-II binding sites on the minireceptors. However, co-expression with R2AxI/T-M eliminated IGF-II binding for all combinations
involving minireceptors not containing a competent IGF-II binding site. We also attempted this same series of experiments with the C-terminally truncated receptors, but the results indicated that binding of the radioligands was dependent solely on the ligand-binding ability of the FLAG-tagged minireceptors with little binding contribution made by WT-M. The large disparity in length of the C-terminal ends of the C-terminally truncated minireceptors and WT-M may have caused strain on full-length WT-M, possibly forcing WT-M to fold back on itself in these interactions to alleviate interference by the resin beads. This may have led to blocking of or failure to form competent binding sites in our assays.

Our results raise the question whether the Man6P/IGF2R dimerizes naturally or as a result of ligand binding. York et al. showed by gel filtration and sucrose gradient sedimentation that the Man6P/IGF2R in the presence of the multivalent Man6P ligand, β-glucuronidase, resulted in a Stokes radius and sedimentation coefficient consistent with the presence of two Man6P/IGF2Rs and one β-glucuronidase molecule, whereas upon incubation with IGF-II, the receptor appeared to exist as a monomer (37). Further, experiments testing the internalization of 125I-IGF-II in the presence of β-glucuronidase revealed that this ligand accelerated the rate of IGF-II uptake, suggesting that intermolecular crosslinking of receptor molecules could affect internalization (37). On the other hand, Byrd et al. reported, by mutational analysis, that receptors with one functional Man6P binding domain were capable of forming high affinity interactions with Man6P ligands, suggesting that oligomerization of the Man6P/IGF2R contributes to high-affinity binding (39). Furthermore, native gel electrophoresis experiments demonstrated that the Man6P/IGF2R could be separated into dimeric and monomeric forms in the presence and/or absence of Man6P ligands, and that the two forms of the receptor displayed differing Man6P ligand binding characteristics (39). Tong et al. showed that only 1 mol of an
oligosaccharide with two phosphomonoesters bound per mole of Man6P/IGF2R monomer at saturation, providing the first evidence that the Man6P/IGF2R has two high-affinity Man6P-binding sites (48). The structural data of Olson et al. have also provided evidence relating to the bivalent binding of Man6P-containing ligands by the Man6P/IGF2R (34). Their overall proposed structure does not allow for a diphosphorylated oligosaccharide to bind to repeats 3 and 9 within a single receptor due to steric considerations, but rather that high-affinity Man6P binding must be due to the diphosphorylated oligosaccharide spanning binding sites contributed by two adjacent Man6P/IGF2R molecules (34).

The ligand binding data from our N-terminally truncated minireceptors favors a mechanism of Man6P/IGF2R association in which the receptor can form oligomeric structures in the absence of ligands, but that oligomerization does promote high-affinity ligand binding (39). In our studies, the N-terminally truncated minireceptors were able to associate with full-length receptors in the absence of ligands according to immunoprecipitation assays. These associated receptors were able to bind PMP-BSA even though none of the N-terminally truncated minireceptors contain a Man6P binding domain, suggesting that the receptors did not require the presence of Man6P ligands to associate. This ligand binding was not evident in associations between the N-terminally truncated minireceptors and the triple-mutant R2AxI/T-M receptor molecules. Our immunoprecipitation data with the R2AxI/T-M receptor suggest that the Man6P/IGF2R forms oligomeric complexes regardless of the ligand binding state of the receptor. The oligomeric state of the receptor likely influences the affinity of bivalent Man6P-containing ligands for the receptor to a greater degree than bivalent Man6P binding promotes oligomerization.
In conclusion, we report, for the first time, that severely truncated minireceptors can associate with the full-length Man6P/IGF2R regardless of the presence of ligand binding domains. Additionally, we have shown that associations between the N-terminally truncated minireceptors and WT-M are functional by the criterion of ligand binding. Future studies should focus on how mutated and/or truncated Man6P/IGF2Rs influence the receptor concerning the possibility of how “oligomer interference” would affect functional properties of the receptor. Additionally, further studies are needed to definitively address the oligomerization state of the receptor, \textit{i.e.} to determine whether the receptor forms dimers or high-order structures and to yield a better understanding of the mechanisms by which the oligomeric state of the Man6P/IGF2R contributes to its functional properties.
REFERENCES


FOOTNOTES

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ABBREVIATIONS

1The abbreviations used are: Man6P/IGF2R, mannose 6-phosphate/insulin-like growth factor II receptor; Man6P, mannose 6-phosphate; EC, extracytoplasmic; TGF-β1, transforming growth factor-β1; IGF-II, insulin-like growth factor II; uPAR, urokinase-type plasminogen activator receptor; α-, anti-; PMP-BSA, pentamannose phosphate-bovine serum albumin; hGUS, human β-glucuronidase; WT-M, full-length wild-type Man6P/IGF2R-Myc; R2AxI/T-M, full-length triple-mutant Man6P/IGF2R-Myc; nt, nucleotide(s); pBSKII+, pBluescript SK II+; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; HBS, HEPES-buffered saline; HBST, HEPES-buffered saline plus 0.1% Triton X-100; EGFR, epidermal growth factor receptor.
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Fig. 1. **Schematic diagram and expression of C-terminally truncated and full-length Man6P/IGF2R minireceptors.** A, the minireceptors are diagrammed, with *rectangles* representing the repeating units of the EC domain of the Man6P/IGF2R. The *light gray boxes* represent the locations of the high-affinity Man6P binding sites, and the *dark gray boxes* indicate the location of the principal IGF-II binding site. The *broken hatched lines* denote the addition of a FLAG epitope tag, whereas the *gray cross-hatched pattern* shows the addition of the Myc epitope tag, both to the C-terminus. The *solid black bar* represents the transmembrane domain of the receptor and the *vertical lines* indicate the presence of the cytoplasmic region. B, expression of the Man6P/IGF2R minireceptors. Aliquots (25 µl) of Triton X-100 extracts of 293T cells transfected with the indicated construct(s) were resolved by SDS-PAGE on 6% gels, immunoblotted with α-FLAG, α-Myc, or α-Man6P/IGF2R antibodies, and developed by autoradiography. *Lane 7* corresponds to the expression of WT-M on the α-Myc immunoblot. This receptor was not probed on the α-FLAG or α-Man6P/IGF2R immunoblots. A blot representative of four transfections is shown.

Fig. 2. **Ligand blot analysis of the C-terminally truncated Man6P/IGF2R minireceptors.** Proteins from aliquots (25 µl) of Triton X-100 extracts from 293T cells transfected with the C-terminally truncated minireceptors were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and ligand blotted with the following probes: *A*, 1 x 10^6 cpm ^125_I-IGF-II, *B*, 1 x 10^6 cpm ^125_I-PMP-BSA, or *C*, 0.5 x 10^6 cpm ^125_I-hGUS. After washing, the blots were exposed to film by autoradiography. The *arrows* indicate radioligand binding to the endogenous 293T cell Man6P/IGF2R.
Fig. 3. Immunoprecipitation of C-terminally truncated minireceptors and analysis of association between minireceptors and full-length Man6P/IGF2R. A, combination immunoprecipitation/immunoblot of C-terminally truncated FLAG-tagged minireceptors and full-length Myc-tagged Man6P/IGF2R. Equal volumes (50 µl) of Triton X-100 extracts from 293T cells co-transfected with the minireceptors and full-length Man6P/IGF2R (10 µg/20µg DNA transfected, respectively) were immunoprecipitated with 8 µl of α-FLAG M2 resin in a volume of 0.2 ml. The resin pellets were collected by centrifugation, washed, and heated in sample buffer with DTT. The proteins were resolved by SDS-PAGE on 6% gels, subjected to immunoblot analysis with α-FLAG or α-Myc, and developed by 125I-protein A. An autoradiogram of a representative blot is shown. B, Quantitative analysis of association between the truncated minireceptors and full-length receptors was performed using Storm or Typhoon PhosphorImager analysis. The ordinate represents the relative density units of association between the tagged receptors as Myc/FLAG ratios, whereas the abscissa indicates the receptors transfected. The experiment was repeated for three sets of transfections in either duplicate or triplicate. These data represent the mean ± S.E.M. of three independent experiments. C, α-Myc immunoprecipitation and subsequent immunoblot analysis of C-terminally truncated FLAG-tagged minireceptors and WT-M. Equal volumes (20 µl) of detergent extracts from co-transfected 293T cells (10 µg minireceptor DNA/20 µg WT-M DNA) were incubated with 1 µl of α-Myc 9E10 antibody in a volume of 0.1 ml. Subsequently, aliquots (25 µl) of washed/block Protein G-Sepharose were added to the reactions in a total volume of 0.2 ml and further incubated for immunoprecipitation. The pellets were processed and analyzed as in A. D, Quantitative analysis of the α-Myc association assays was performed as in B.
Fig. 4. **Schematic diagram and expression of N-terminally truncated and full-length Man6P/IGF2R minireceptors.** *A,* the minireceptors are diagrammed similar to Fig. 1, representing the various structures of the N-terminally truncated receptors. *B,* expression of the Man6P/IGF2R minireceptors. Aliquots (25 µl) of Triton X-100 extracts of 293T cells transfected with the construct(s) indicated were resolved by SDS-PAGE on 6% gels, immunoblotted with α-FLAG, α-Myc, or α-Man6P/IGF2R antibodies as indicated, and developed by autoradiography. A blot representative of four replicated experiments is shown.

Fig. 5. **Ligand blot analysis of the N-terminally truncated Man6P/IGF2R minireceptors.** Proteins from aliquots (25 µl) of Triton X-100 extracts from 293T cells transfected with the N-terminally truncated minireceptors were treated as in the legend to Fig. 2. The blots were exposed to film by autoradiography. The arrows indicate endogenous Man6P/IGF2R binding to the radioligands.

Fig. 6. **Immunoprecipitation of N-terminally truncated minireceptors and analysis of association between minireceptors and full-length Man6P/IGF2R.** *A,* combination immunoprecipitation/immunoblot of N-terminally truncated FLAG-tagged minireceptors and full-length Myc-tagged Man6P/IGF2R. Equal volumes (50 µl) of Triton X-100 extracts from 293T cells co-transfected with the minireceptors and full-length Man6P/IGF2R were immunoprecipitated and resolved as in the legend to Fig. 3. *B,* Quantitative analysis of association between the truncated minireceptors and full-length receptors was performed using Storm or Typhoon PhosphorImager analysis as in the legend to Fig. 3. This experiment was repeated for three sets of transfections and these data represent the mean ± S.E.M. of three
independent experiments. C, α-Myc immunoprecipitation and subsequent immunoblot analysis of N-terminally truncated FLAG-tagged minireceptors and WT-M. α-Myc immunoprecipitation experiments and quantification were performed as in the legend to Fig.3. D, Quantitative analysis of the α-Myc immunoprecipitation reactions was performed as in the legend to Fig. 3.

Fig. 7. Radioligand binding analysis of N-terminally truncated minireceptors co-transfected with WT-M or R2AxI/T-M. A, 125I-IGF-II binding. Four aliquots of Triton X-100 extracts from 293T cells co-transfected with the N-terminally truncated minireceptors plus WT-M or R2AxI/T-M were immunoadsorbed to M2 affinity resin for 4-6 h at 4°C. After incubation, the resins were washed, and incubated with 2 nM 125I-IGF-II in the presence or absence of 1 μM IGF-II overnight at 4°C. The resins were washed and the pellets counted. The results of the binding assays were analyzed using Prism™ and graphed according to 125I-IGF-II binding on the y-axis vs. receptor combinations on the x-axis. B, 125I-PMP-BSA binding. The binding assay was performed as above, except using 1 nM 125I-PMP-BSA in the presence or absence of 5 mM unlabeled Man6P. Radioactivity retained in the presence of either 1 μM IGF-II or 5 mM Man6P was subtracted from each binding reaction to determine the specific binding for 125I-IGF-II and 125I-PMP-BSA, respectively. The lighter bars represent the amount of radioligand bound by the indicated minireceptor co-transfected with WT-M, whereas the darker bars show binding by the minireceptors transfected with R2AxI/T-M. Asterisks denote minireceptors that contain an intact ligand binding domain for the radioligand tested. The control transfections are denoted by a C. Values represent the mean ± SEM of three replicate measurements for each condition. These data are representative of three replicate experiments for each of the radioligands.
Figure 1

Construct Name:

1-8F 5’
1 2 3 4 5 6 7 8 Flag

1-9F or 1-9F-R/A 5’
1 2 3 4 5 6 7 8 9 Flag

1-11F 5’
1 2 3 4 5 6 7 8 9 10 11 Flag

1-15F 5’
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Flag

WT-M or R2Axl/T-M 5’
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Flag

Myc
Figure 1

B

IB: α-FLAG

IB: α-Myc

IB: α-Man6P/IGF2R
Figure 2

A

CMV5  1-8F  1-9F  1-11F  1-15F

kDa

250

160

IGF-II

B

PMP-BSA

C

hGUS

1 2 3 4 5 6 7 8 9 10
Figure 3

A

+ WT-M

CMV5 1-8F 1-9F 1-9F-R/A 1-11F 1-15F WT-M

IB: α-FLAG
IB: α-FLAG
IP: α-FLAG
IB: α-Myc

kDa

1 2 3 4 5 6 7

B

WT-M Association

Relative Density Units

CMV5 1-9F 1-9F 1-9F-R/A 1-11F 1-15F WT-M
**Figure 3**

C

[Image of gel electrophoresis with labeled bands and molecular weight markers.]

- **IP:** α-Myc
- **IB:** α-FLAG

D

[Graph showing relative density units for various samples.]
Figure 4

A

Construct Name:

- **10-15CF or 10-15CF-I/T**
  - 5' * 10 11 12 13 14 15 Flag

- **11-15CF or 11-15CF-I/T**
  - 5' * 11 12 13 14 15 Flag

- **12-15CF**
  - 5' * 12 13 14 15 Flag

- **13-15CF**
  - 5' * 13 14 15 Flag

- **WT-M or R2AxI/T-M**
  - 5' 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Myc
Figure 4

B

+ WT-M


IB: α-FLAG

IB: α-Myc

kDa

250
160
105

1 2 3 4 5 6 7 8
Figure 5

A

B
**Figure 6**

### C

- **CMV5**
- **1-15F**
- **10-15CF**
- **10-15CF-I/T**
- **11-15CF**
- **11-15CF-I/T**
- **12-15CF**
- **13-15CF**
- **WT-M**

**IB:**
- **α-FLAG**
- **α-Myc**

**IP:**
- **α-Myc**

### D

- **WT-M Association**
- **Relative Density Units**

- **1-15F**
- **10-15CF**
- **10-15CF-I/T**
- **11-15CF**
- **11-15CF-I/T**
- **12-15CF**
- **13-15CF**
Figure 7

A

![Graph A showing binding of \(^{125\text{I}}\)-IGF-II to different constructs](image)

B

![Graph B showing binding of \(^{125\text{I}}\)-PMP-BSA to different constructs](image)