Binding of Urokinase-Type Plasminogen Activator Receptor (uPAR) to the Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor: Contrasting Interactions of Full-length and Soluble forms of uPAR.

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Short title: Interactions of uPAR with Man-6-P/IGF2R

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SUMMARY

Urokinase-type plasminogen activator receptor (uPAR) binding by the mannose 6-phosphate/insulin-like growth factor II receptor (Man-6-P/IGF2R) is considered important to the Man-6-P/IGF2R’s tumor suppressor function via regulation of cell-surface proteolytic activity. Our goal was to map the uPAR binding site of the Man-6-P/IGF2R by analyzing the uPAR binding characteristics of a panel of mini-receptors containing different regions of the Man-6-P/IGF2R’s extracytoplasmic domain. Coimmunoprecipitation assays revealed that soluble recombinant uPAR (suPAR) bound the Man-6-P/IGF2R at two distinct sites, one localized to the amino-terminal end of the Man-6-P/IGF2R’s extracytoplasmic domain (repeats 1-3) and the other to the more carboxyl-terminal end (repeats 7-9). These sites correspond with the positions of the Man-6-P/IGF2R’s two Man-6-P binding domains. Indeed, the suPAR:Man-6-P/IGF2R interaction was inhibited by Man-6-P, and binding-competent suPAR species represented a minor percentage (8-30%) of the suPAR present. In contrast, Man-6-P/IGF2R binding of endogenous, full-length uPAR solubilized from plasma membranes of the prostate cancer cell line, PC-3, was not inhibited by Man-6-P. Further studies showed that very little (< 5%) endogenous uPAR was Man-6-P/IGF2R binding-competent. We conclude that, contrary to previous reports, the interaction between uPAR and Man-6-P/IGF2R is a low-percentage binding event and that suPAR and full-length uPAR bind the Man-6-P/IGF2R by different mechanisms.
INTRODUCTION

The mannose 6-phosphate/insulin-like growth factor II receptor (Man-6-P/ IGF2R)\(^1\) is a 300-kDa transmembrane receptor protein composed of four domains: the amino-terminal signal sequence, a short carboxyl-terminal cytoplasmic tail, a transmembrane domain, and a large extracytoplasmic domain. The extracytoplasmic region consists of 15 homologous repeats approximately 147 residues in length (1). Separate regions in the receptor’s extracytoplasmic domain have been shown to interact with two classes of ligands, insulin-like growth factor II (IGF-II), and proteins that contain a Man-6-P marker as a result of post-translational modification (2,3). Mapping studies have shown that these ligands bind to distinct regions of the extracytoplasmic domain. The IGF-II binding site has been localized to repeat 11, with higher affinity conferred by cooperation with repeat 13 (4-8), whereas the receptor contains two Man-6-P binding sites in repeats 1-3 and 7-9, with residues critical for binding located in repeats 3 and 9, respectively (9-11). These separate binding functions contribute to the various cellular functions ascribed to the Man-6-P/IGF2R, which controls the local concentration of IGF-II at the cell surface by internalizing IGF-II and targeting it for degradation in lysosomes (3). The Man-6-P binding function of the receptor is involved in the transport of lysosomal enzymes from the Golgi to the pre-lysosomal compartment and in the endocytosis of extracellular phosphomannosyl ligands from the cell surface (2,3). Man-6-P binding also contributes to the activation of Man-6-P-containing precursor forms of transforming growth factor-\(\beta\) (TGF-\(\beta\)) by binding pro-TGF-\(\beta\), which is subsequently cleaved to allow release of the active growth factor (3,12-14).

The Man-6-P/IGF2R has also been implicated as a growth and tumor suppressor. Experiments with knockout mice have shown that the Man-6-P/IGF2R is paternally imprinted
and is important for normal fetal development (15-18). Loss of heterozygosity at the Man-6-P/IGF2R locus has been observed for several cancers (19-21), and missense mutations cause altered ligand binding to the Man-6-P/IGF2R (22,23). Down-regulation of the Man-6-P/IGF2R mediated by transfection of antisense Man-6-P/IGF2R cDNA has been shown to increase cell growth rate \textit{in vitro} and increased tumor growth \textit{in vivo} (24,25), whereas increased expression of the Man-6-P/IGF2R gene suppresses growth and induces apoptosis in SW48 colorectal carcinoma cells (26).

Several years ago, the Man-6-P/IGF2R was reported to bind uPAR via a novel interaction that was not inhibited by either Man-6-P or IGF-II (27,28). Binding of uPAR to the Man-6-P/IGF2R was found to be a low-affinity interaction ($K_D \sim 11 \, \mu M$) with a binding stoichiometry of ~1 mole of uPAR per mole of Man-6-P/IGF2R (27). Recently, the binding site on the Man-6-P/IGF2R for uPAR was mapped to the first half of repeat 1 in the extracytoplasmic domain (29). uPAR is a heavily glycosylated, 40- to 60-kDa protein containing three internally homologous domains of ~90 residues each (30,31). A glycosylphosphatidylinositol (GPI) anchor attaches the carboxyl-terminal hydrophobic domain of uPAR to the cell membrane (32). Upon binding of uPAR to its ligand, urokinase-type plasminogen activator (uPA), inactive pro-uPA is proteolytically cleaved and thereby activated (33). The function of active uPAR-bound uPA, a serine protease, is proteolytic conversion of plasminogen to plasmin, another serine protease (34). Thus, by binding uPA, uPAR regulates cell migration and tissue remodeling processes as well as pericellular proteolysis (31,35,36). Currently, the functional significance of the binding between uPAR and the Man-6-P/IGF2R is unclear. Three possible roles have been proposed involving control of cell-surface uPAR expression, TGF-β activation or uPAR-mediated functions such as fibrinolysis, cell adhesion, and migration (27-29).
The goal of this project was to map the uPAR binding site of the Man-6-P/IGF2R. To this end, a panel of Man-6-P/IGF2R mini-receptors containing portions of the extracytoplasmic domain was used in coimmunoprecipitation with soluble uPAR. Studies with soluble recombinant uPAR, truncated after Arg$^{281}$ to delete the GPI-linkage site, revealed that suPAR binds the receptor in a bivalent manner corresponding to the same repeat regions as the Man-6-P binding sites. Subsequent studies showed that suPAR binds the Man-6-P/IGF2R at two separate sites in the extracytoplasmic domain and that the interaction is sensitive to Man-6-P under all conditions tested. Further, we investigated the interaction between the Man-6-P/IGF2R and full-length endogenous uPAR extracted from plasma membrane preparations of PC-3 human prostate cancer cells. Pull-down assays of membrane extract proteins and immobilized Man-6-P/IGF2R demonstrated that only a small percentage of endogenous uPAR bound the Man-6-P/IGF2R and that the binding was not inhibited by Man-6-P. Attempts to coimmunoprecipitate endogenous uPAR with the Man-6-P/IGF2R mini-receptors were unsuccessful. We conclude that the interactions between full-length uPAR and suPAR with the Man-6-P/IGF2R are of low abundance and they bind the Man-6-P/IGF2R by differing mechanisms.
EXPERIMENTAL PROCEDURES

Materials—D-Man-6-P disodium salt, D-glucose 6-phosphate (Glc-6-P), the α-FLAG M2 antibody, and α-FLAG M2-agarose affinity gel reagents were purchased from Sigma (St. Louis, MO). Radiolabeled pentamannose phosphate-bovine serum albumin (PMP-BSA) and unlabeled PMP-BSA were prepared as described previously (22,37). PMP-Sepharose was prepared as previously described (5). Radiolabeled suPAR was prepared by iodination using pre-coated IODOGEN® tubes (Pierce, Rockford, IL) according to manufacturer’s specifications to a specific activity of 2.82 Ci/µg. Purified full-length and soluble forms of bovine Man-6-P/IGF2R were prepared as previously described (38). The pCMV5 vector was provided by Dr. David W. Russell (University of Texas Southwestern Medical Center, Dallas, TX) (39). The 8.6-kilobase pair human Man-6-P/IGF2R cDNA was a gift of Dr. William S. Sly (St. Louis University Medical Center, St. Louis, MO) (40). Purified suPAR and the α-uPAR polyclonal antibodies were prepared as described before (41). The recombinant soluble uPAR was affinity-purified from the culture medium of human embryonic kidney (HEK) 293 cells transfected with suPAR cDNA. The uPAR was purified at a concentration of 1.13 mg/ml with Sepharose conjugated to the amino-terminal fragment of uPA. The rabbit polyclonal antibodies (clones 2 and 3) were purified by immunoaffinity chromatography. The uPAR monoclonal antibody clone 3 was obtained from Monozyme (Copenhagen, Denmark). Other reagents and supplies were obtained from sources as indicated.

Preparation, Expression, and Analysis of Epitope-tagged Soluble Mini-Receptors—The truncated Man-6-P/IGF2R mini-receptors 1-3F, 1-8F, 1-15F, 7-9F, and 7-15F were tagged with the 8-residue FLAG epitope (DYKDDDDK) at the carboxyl terminus and cloned into the pCMV5 vector as previously described (37). The construct 1-15F, formerly called 15F (22), will
be identified as 1-15F throughout this manuscript to clarify that this construct contains all 15 extracytoplasmic repeats, but lacks the transmembrane and cytoplasmic domains. The 11F construct was engineered in the pCMV5RIX expression vector using a strategy previously described (42). The 1F construct was synthesized in a similar manner. Using the full-length Man-6-P/IGF2R cDNA as a template, 1F was amplified with Vent™ polymerase (New England Biolabs, Beverly, MA) by incorporating the FLAG epitope and using primer pairs targeted to the region of interest, extracytoplasmic repeat 1 [nucleotides (nt) 148-639, corresponding to residues 1-164].

To make the 11Fsp construct lacking the first half of repeat one but containing the signal sequence, a cDNA fragment encompassing nt 94-267 of the Man-6-P/IGF2R cDNA was cloned into pCMV5. This region includes sequences encoding the signal sequence and 63 nt of the 5’ untranslated region followed by an EcoR I site. This vector, pCMV5sp, was generated by Vent™ polymerase amplification of the human Man-6-P/IGF2R cDNA with a 5’-primer containing an Xho I site followed by sequence corresponding to nt 94-113 and a 3’-primer complementary to nt 277-291 followed by an Xba I site. This construct was digested with Xho I and Xba I, subcloned into pBKCMV (Invitrogen, Carlsbad, CA), and subsequently digested with Hind III and Xba I. This fragment was ligated into the pCMV5RIX vector generating the pCMV5sp vector. The 11F construct (42) was excised from the pCMV5 vector with EcoR I and Xba I digestion and ligated into pCMV5sp, generating the 11Fsp construct.

Transient expression of the mini-receptors by calcium phosphate-mediated transfection into 293T human embryonic kidney cells and immunoblot analysis of cell lysates to measure expression of each construct were also performed as described (37,43).
Coimmunoprecipitation of suPAR and truncated Man-6-P/IGF2R—Aliquots of cell lysates containing the expressed Man-6-P/IGF2R mini-receptors were incubated with 12 µl of packed M2 resin in 25 mM HEPES, pH 7.4, 150 mM NaCl (HBS) plus 0.1% BSA at 4°C for 3 h. The resin pellets were collected by centrifugation at 14,000 x g for 15 s and washed twice with 1 ml of HBS plus 0.05% Triton X-100 (HBST). Purified suPAR (1.67 µg) in HBST, ± Man-6-P (5 mM), was incubated with resin at 4°C overnight. The resin pellets were again collected and washed twice with HBST. Immunoblot analysis of the resultant pellets was performed using an α-uPAR antibody. The resin pellets were heated at 100°C for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The proteins released from the resin pellets were electrophoresed on 8-16% reducing SDS-PAGE gels and transferred to BA85 nitrocellulose paper (Schleicher & Schuell, Keene, NH) at 350 milliamps for 3 h at 20°C. The blots were blocked with 4% nonfat dry milk in 15 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20 and probed with α-uPAR polyclonal antibody #3 (1:300 dilution). The blot was then developed with 125I-protein A (Perkin Elmer Life Sciences, Boston, MA) and detected by autoradiography.

Native Gel/Ligand Blot Analysis—Aliquots (40 µg) of purified full-length bovine Man-6-P/IGF2R were electrophoresed on a 4-12% native PAGE containing 0.1% Triton X-100 and then transferred to BA85 nitrocellulose paper. The blots were washed, blocked, and probed as described previously (38), except using 125I-suPAR (~5 x 10⁷ cpm/8 ml) as the ligand and developed by autoradiography.

Man-6-P/IGF2R-Sepharose Affinity Adsorption—Man-6-P/IGF2R-Sepharose was prepared using purified, full-length bovine receptor based on the procedure of Brown and Farquhar (44) and similarly to the previously described procedure (5). Briefly, full-length bovine
Man-6-P/IGF2R (~520 µg) was coupled to 1 g (~3.5 ml) of cyanogen bromide-activated Sepharose 4B (Sigma) in 7.5 ml of 0.1 M NaHCO₃, 0.5 M NaCl, pH 8.0, overnight at 4°C. Ethanolamine (1 M) was added to the slurry (1:1, vol:vol) and incubated for 2 h at 20°C. The resin was then washed with HBST and stored as a 50% (w/v) suspension in HBST with 0.02% NaN₃ at 4°C.

suPAR Affinity Adsorption: Aliquots (25 µl packed) of Man-6-P/IGF2R-Sepharose were incubated with ¹²⁵I-suPAR in HBST, with or without additives at 4°C, overnight. The resin pellets were collected by centrifugation and washed three times with 1 ml of HBST. The resin pellets incubated in HBST alone were then eluted with HBST with or without additives at 4°C for 3 h. The resin pellets were again collected and washed three times with 1 ml of HBS plus 0.1% BSA. The pellets were heated at 100°C for 5 min in sample buffer containing dithiothreitol (DTT, 50 mM) and the proteins were resolved by SDS-PAGE on 6-16% gradient gels. The gels were stained with Coomassie blue and destained. The dried gels were then developed by autoradiography.

Endogenous uPAR Affinity Adsorption: PC-3 cells were cultured in RPMI 1640 medium plus 7% fetal bovine serum and 0.1% gentamycin. Plasma membranes were prepared as previously described (45). Protein concentration was determined using the bicinchoninic acid method (Sigma). PC-3 plasma membrane preparations were extracted using 1:1 (vol:vol) extraction buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, with antiproteases: 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, 10 µg/ml antipain, 80 µg/ml benzamidine, 10 µg/ml leupeptin, and with antiphosphatases: 5 mM NaF and 5 mM β-glycerophosphate) with mixing for 1 h at 4°C. The suspension was centrifuged for 10 min at 14,000 x g at 4°C, and the resulting supernatant fractions were collected and retained for SDS-PAGE.
Aliquots (~500 µg) of PC-3 plasma membrane extract proteins were incubated with aliquots (20-40 µl) of packed Man-6-P/IGF2R-Sepharose or ethanolamine-blocked cyanogen bromide-activated Sepharose for 16 h at 4°C in the presence or absence of the additives 5 mM Man-6-P, 5 mM Glc-6-P, or 1 µM IGF-II. Extraction buffer (500 µl) or suPAR (500 ng) was incubated with Man-6-P/IGF2R-Sepharose as controls. The reaction mixtures were centrifuged to collect the resin pellets. The supernatants were transferred to new tubes and the resin pellets washed twice with 1 ml of HBST. The resin pellets and aliquots (100 µl, 20%) of the supernatant fractions were heated at 100°C for 5 min with sample buffer without DTT, and the released proteins were resolved by 6-16% gradient SDS-PAGE. The gels were immunoblotted with α-uPAR monoclonal antibody (16h, 4°C, 1:1000) followed by a secondary rabbit anti-mouse IgG (30 min, 20°C, 1:1000), and then developed with 125I-protein A (30 min, 20°C, 30 µl).

Alkaline Phosphatase Treatment of suPAR—Either 125I-suPAR (~150,000 cpm) or 125I-PMP-BSA (~50,000 cpm) was incubated in phosphatase buffer (1 mM ZnCl₂, 1 mM MgCl₂, 10 mM Tris-HCl, pH 8.3) plus 0.1% BSA ± 1-5 U E. coli alkaline phosphatase (Sigma) for 16 h at 37°C. Man-6-P/IGF2R-Sepharose in low pH-HBST (HBST buffer at pH 6.8 rather than the standard pH 7.4) plus either 5 mM Glc-6P or 5 mM Man-6-P were added as indicated to the reaction mixtures to achieve a final pH of ~7.4 and incubated for 8 h at 4°C. The resin pellets were collected by centrifugation, washed three times with 1 ml of HBST, then heated for 5 min at 100°C in sample buffer with 50 mM DTT. The eluted proteins were subjected to SDS-PAGE on 6-16% gradient gels, stained with Coomassie blue, and destained. The dried gels were developed by autoradiography. Expression levels of bound uPAR were quantified using Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analysis of the dried gels.
Coimmunoprecipitation of endogenous uPAR and truncated Man-6-P/IGF2R—Aliquots of 293T cell lysates containing equimolar amounts of the expressed Man-6-P/IGF2R mini-receptors were incubated with 15 µl of packed M2 resin in HBS plus 0.1% BSA at 4°C for 3 h. The resin pellets were collected and washed twice. Additionally, HT1080 cells, a human fibrosarcoma cell line, were cultured in DMEM plus 5% FBS and 0.05% gentamycin. HT1080 plasma membranes and extracts were prepared in a similar manner as the PC-3 membranes and extracts. The immunoprecipitated M2 resin pellets were incubated with aliquots of extract proteins (~300 µg of PC-3, ~500 µg of HT1080 in volumes of 0.3 or 0.5 ml, respectively), for 16 h at 4°C. The resin pellets were collected by centrifugation. The supernatant fractions were transferred to new tubes and the resin pellets washed twice with 1 ml of HBST. The resin pellets and aliquots (100 µl, representing 33% or 20%) of the supernatants were heated at 100°C for 5 min, and resolved by 6-16% SDS-PAGE. The gels were then immunoblotted with polyclonal #2 α-uPAR antibody (1:500, 4°C, 16 h) and developed with 125I-protein A.

Immunodepletion analysis of endogenous uPAR binding to the Man-6-P/IGF2R construct 1-15F—Aliquots (300 µl) of lysates from 293T cells transfected with 1-15F or the vector, pCMV5, were immunoprecipitated with 15 µl of α-FLAG M2 resin as described above. Aliquots of PC-3 plasma membrane extract proteins (~600 µg) were left untreated or were treated with polyclonal #2 α-uPAR antibody (1:250 dilution) or with non-immune rabbit IgG (60 µg) for 2 h at 4°C. Aliquots (10 µl packed resin) of protein A-Sepharose (Sigma) were added to the treated PC-3 plasma membrane extracts and incubated for an additional 1 h at 4°C. The protein A-Sepharose was collected by centrifugation and the supernatants were carefully transferred to new tubes. The immunoprecipitated M2 resin pellets were then coimmunoprecipitated with aliquots (500 µl) of untreated or treated PC-3 plasma membrane extracts for 16 h at 4°C. The resins were
collected by centrifugation at 14,000 x g for 15 s and the supernatants were transferred to new tubes. The resin pellets were washed twice with 1 ml of HBST. The pellets, aliquots (100 µl, 20%) of the supernatants, and aliquots (60 µl) of treated or untreated PC-3 plasma membrane extracts were heated at 100°C for 5 min in sample buffer without DTT and resolved by 6-16% SDS-PAGE. The gels were immunoblotted with α-uPAR polyclonal antibody #2 as described above.
RESULTS

Transient Expression of Truncated Man-6-P/IGF2R Mini-Receptors—In order to map the binding domain for uPAR on the Man-6-P/IGF2R, a series of truncated, soluble mini-receptors was engineered that encompasses various regions of the Man-6-P/IGF2R extracytoplasmic domain followed in each case by a FLAG epitope tag (Fig. 1A). The numbers in the name of each construct denote the most amino-terminal and carboxyl-terminal extracytoplasmic repeats expressed in that segment. All of the mini-receptors are FLAG epitope-tagged at the carboxyl-terminal end, denoted by F, and all except 11Fsp contain the first half of repeat number one. The truncated receptors were transiently expressed in 293T cells. As we had observed with other such truncated Man-6-P/IGF2R mini-receptors, the exogenous proteins were not secreted into conditioned medium, but rather were soluble in cell lysates (22). Cell extracts were prepared using Triton X-100 for the analysis of suPAR binding. Immunoblotting with the α-FLAG M2 antibody was employed to quantify expression of the proteins (Fig. 1B).

suPAR Binds the Man-6-P/IGF2R at Two Separate Extracytoplasmic Sites—Coimmunoprecipitation experiments were performed using the truncated Man-6-P/IGF2R mini-receptors. Equimolar amounts of each construct were immunoadsorbed to aliquots of α-FLAG M2 resin, which were subsequently incubated with suPAR. An α-uPAR immunoblot using polyclonal antibody #3 determined which FLAG mini-receptors were suPAR binding-competent (Fig. 2A). As expected, suPAR was capable of binding the 1-15F construct, which comprises the entire extracytoplasmic domain associated with the ligand-binding function of the receptor, followed by a FLAG epitope tag. In attempting to refine the binding map, we discovered that suPAR bound to the Man-6-P/IGF2R at two separate sites within the extracytoplasmic domain. Along with the 1-15F construct, the 1-3F, 1-8F, 7-9F and the 7-15F mini-receptors all were

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capable of binding suPAR, although with different avidities. Different efficiencies of immunoadsorption for each mini-receptor to the M2 resin confound quantitative analysis in this experiment. Thus, suPAR band intensity cannot be taken as a quantitative measure of affinity but rather a qualitative measure of suPAR binding. There was no detectable suPAR binding to the 1F and 11Fsp mini-receptors. The uPAR bands arising from binding to the 7-9F construct did not reproduce well from film to paper and are difficult to discern in the scanned films from standard coimmunoprecipitation experiments. In order to demonstrate the binding capability of 7-9F more clearly, coimmunoprecipitation experiments were performed using larger volumes of cell lysates. Overexposure of the blots to film showed that the 1-15F, 1-3F, and 7-9F mini-receptors still bound suPAR and that 11F was unable to bind (Fig. 2B).

To determine if suPAR was the only Man-6-P/IGF2R ligand that bound preferentially to the amino-terminal Man-6-P-binding site (repeats 1-3), binding of the mini-receptors to PMP-Sepharose was investigated. PMP-Sepharose contains multiple phosphomannosyl moieties and would therefore be expected to bind to both the 1-3 and 7-9 domains of the extracytoplasmic region. Lysates from 293T cells transfected with the 1-3F and 7-9F mini-receptors were incubated with PMP-Sepharose. The resin pellets were then collected and washed with buffer alone, then with buffer containing Glc-6-P, and finally eluted with buffer containing Man-6-P. Aliquots of the lysates, Glc-6-P washes, and Man-6-P elutions were immunoblotted with α-FLAG M2 antibody (Fig. 2C). Although both 1-3F and 7-9F showed equivalent expression on the immunoblot and were not eluted by Glc-6-P, a greater abundance of 1-3F bound to the PMP-Sepharose, as shown by the levels of 1-3F and 7-9F eluted by Man-6-P.

**Man-6-P Inhibits Coimmunoprecipitation of suPAR with the Man-6-P/IGF2R**—The apparent ability of suPAR to bind to two different sites on the Man-6-P/IGF2R, along with the similarities
in binding avidities between PMP-Sepharose and suPAR, suggested that suPAR binds to the Man-6-P binding sites of the Man-6-P/IGF2R receptor. We therefore tested the ability of Man-6-P to inhibit the coimmunoprecipitation of suPAR and the Man-6-P/IGF2R. Equal volumes of cell lysates were incubated with M2 resin and subsequently incubated with suPAR in the presence or absence of Man-6-P. An α-uPAR immunoblot using polyclonal antibody #3 showed that incubation with Man-6-P did inhibit binding of suPAR to the Man-6-P/IGF2R mini-receptors (Fig. 3A). The 1-3F, 1-15F, and 7-9F mini-receptors were capable of binding suPAR in the absence but not in the presence of 5 mM Man-6-P. The binding of suPAR to the 7-9F mini-receptor again did not reproduce well from film to the scanned image, but overexposure of the blot indicated that 7-9F mini-receptors were capable of binding suPAR and that this interaction was also inhibited by Man-6-P (data not shown). The 1F mini-receptor did not bind suPAR in the presence or absence of Man-6-P.

To confirm that the suPAR:Man-6-P/IGF2R interaction was indeed sensitive to Man-6-P inhibition, several approaches were used, including native gel/ligand blot analysis. Full-length Man-6-P/IGF2R, purified from bovine liver, was electrophoresed on polyacrylamide gels under native conditions and transferred to nitrocellulose. Radiolabeled suPAR was then used to probe the membrane either alone or in the presence of Man-6-P or IGF-II (Fig. 3B). suPAR alone was able to bind both the monomeric and dimeric forms of the Man-6-P/IGF2R. This binding was completely inhibited by incubation with 5 mM Man-6-P. Incubation of suPAR with 0.5 μM IGF-II also showed a slight reduction in the ability of suPAR to bind the Man-6-P/IGF2R, but the inhibition was incomplete. This is compatible with the known partial inhibition of binding of phosphomannosylated ligands to the Man-6-P/IGF2R by IGF-II (46,47). PhosphorImager analysis revealed a reduction in binding of ~34% compared to suPAR alone.
Another strategy used to test the Man-6-P sensitivity of the suPAR:Man-6-P/IGF2R interaction was affinity adsorption analysis. Man-6-P/IGF2R-Sepharose was incubated with radiolabeled suPAR alone or in conjunction with either Glc-6-P or Man-6-P. Additional samples of Man-6-P/IGF2R-Sepharose were incubated with radiolabeled suPAR alone and then eluted with either Glc-6-P or Man-6-P. The pellets were collected and the resin-bound proteins were eluted and analyzed by SDS-PAGE (Fig. 3C). The autoradiogram shows that suPAR was able to bind the immobilized Man-6-P/IGF2R and the interaction was inhibited by incubation with Man-6-P. However, binding was not disrupted by Glc-6-P, indicating that the inhibition by Man-6-P is specific. Elution with Man-6-P significantly reduced the binding, but a small amount of suPAR did remain on the resin after elution in one experiment (Fig. 3C).

The sensitivity of the suPAR:Man-6-P/IGF2R interaction to Man-6-P could be dependent on the presence of Man-6-P moieties on suPAR or simply due to competition for proximal binding domains on the Man-6-P/IGF2R. To determine which of these possibilities was responsible for the inhibition by Man-6-P, suPAR and the pseudoglycoprotein phosphomannosyl ligand, PMP-BSA, were tested for binding competency to the Man-6-P/IGF2R after treatment of the ligands with alkaline phosphatase. Radiolabeled samples of suPAR or PMP-BSA were incubated with either buffer alone or buffer plus *E. coli* alkaline phosphatase. After the initial incubation with enzyme at pH 8.3, Man-6-P/IGF2R-Sepharose ± Glc-6-P or Man-6-P were added to the reaction mixtures in the presence of a lower-pH buffer. The pH change was necessary to switch from an environment that the phosphatase preferred to one that is optimal for ligand binding by the Man-6-P/IGF2R. suPAR and PMP-BSA incubated with resin were both capable of binding the Man-6-P/IGF2R by itself or in the presence of Glc-6-P (Fig. 3D, lanes 1 and 2). But in the presence of Man-6-P, neither ligand was able to bind the receptor resin (Fig. 3D, lane 3). After treatment
with 1 U of alkaline phosphatase, only a small fraction (~10%) of the suPAR from our preparation was able to bind the Man-6-P/IGF2R resin (Fig. 3D, lane 4). Alkaline phosphatase treatment also reduced PMP-BSA binding [~62% decrease relative to untreated controls (Fig. 3D, compare lanes 1 and 4)], demonstrating that the presence of phosphate residues on both ligands is important in the binding. Treatment with 5 U of alkaline phosphatase inhibited suPAR from binding the Man-6-P/IGF2R resin [~96% decrease relative to untreated controls (Fig. 3D, compare lanes 1 and 5)]. Additionally, only a small percentage (~6%) of PMP-BSA was able to bind after treatment with 5 U of alkaline phosphatase (Fig. 3D, lane 5). In all cases, suPAR behaved in a manner similar to that of the positive control Man-6-P ligand, PMP-BSA. The ability of PMP-BSA to bind to the Man-6-P/IGF2R decreased progressively after treatment with the 1 U and 5 U of alkaline phosphatase, due to the larger number of Man-6-P moieties present on the pseudoglycoprotein ligand. On the other hand, suPAR would likely have very few Man-6-P moieties per unit protein. Thus, suPAR showed severely reduced binding ability after treatment with only 1U of enzyme.

*Only a Small Population of suPAR is Man-6-P/IGF2R Binding-Competent—*Because the suPAR:Man-6-P/IGF2R interaction is dependent on Man-6-P, an affinity depletion experiment was performed to determine what proportion of suPAR molecules in the preparation from HEK 293 cells was capable of such binding. Aliquots of Man-6-P/IGF2R-Sepharose were incubated with radiolabeled suPAR or PMP-BSA. As the resin pellets were collected, the supernatants were exposed to fresh aliquots of receptor resin. This process was repeated until the original radiolabeled ligands had been exposed to a total of four aliquots of receptor resin over a 3-day incubation period. The proteins from the resin pellets and aliquots of the remaining supernatants were analyzed by SDS-PAGE (Fig. 4A). As expected of a high-affinity ligand, the resulting
A Small Percentage of Endogenous uPAR Binds the Man-6-P/IGF2R in a Man-6-P-Independent Manner—To determine if endogenous uPAR that retains its GPI anchor after extraction from cell membranes would behave similarly to suPAR in binding Man-6-P/IGF2R, a series of experiments was performed using full-length uPAR from PC-3 prostate cancer cells. Triton X-100 extracts were prepared from PC-3 plasma membranes and immunoblotted with an anti-uPAR monoclonal antibody to determine the level of uPAR in the extracts. Endogenous uPAR was readily detectable in the PC-3 plasma membrane extracts (Fig. 5, lower panel, lane 1). In order to determine whether endogenous uPAR binds to the Man-6-P/IGF2R, an affinity adsorption experiment was performed. Aliquots of Man-6-P/IGF2R-Sepharose were incubated with PC-3 plasma membrane extracts with or without the additives Man-6-P, Glc-6-P, or IGF-II.
Only a small fraction of the uPAR population in the extracts was detectable in binding the Man-6-P/IGF2R-Sepharose (Fig. 5, upper panel, lane 3), but binding of this uPAR sub-population to the receptor was not inhibited in the presence of Man-6-P, Glc-6-P, or IGF-II (Fig. 5, upper panel, lanes 4-6). The supernatant fractions, which represent only 20% of the total supernatant, reveal that the amount of uPAR bound by the Man-6-P/IGF2R is small (<10%) compared to the total uPAR present [Fig. 5, lower panel, compare lanes 1-2 with lanes 3-6]. As negative controls, Man-6-P/IGF2R-Sepharose was also incubated with extraction buffer (B) alone (Fig. 5, lane 7) or ethanolamine-blocked Sepharose was incubated with PC-3 plasma membrane extracts (Fig. 5, lane 2). As a positive control, suPAR (su) was incubated with Man-6-P/IGF2R-Sepharose and did exhibit detectable binding to the Man-6-P/IGF2R (Fig. 5 upper panel, lane 8). Similar to previous experiments with suPAR, only a fraction of the suPAR was Man-6-P/IGF2R binding-competent (Fig. 5 lower panel, lane 8); a large percentage of the suPAR population was still detectable in the supernatant fraction.

**Only a Small Percentage of Full-Length uPAR is Man-6-P/IGF2R Binding-Competent**—An affinity depletion experiment was performed to determine the proportion of endogenous uPAR able to interact with the Man-6-P/IGF2R. Similar to the experiments performed with the soluble uPAR preparations, aliquots of Man-6-P/IGF2R-Sepharose were incubated with PC-3 plasma membrane extracts. As the resin pellets were collected, the supernatant fractions were re-exposed to new aliquots of the receptor-Sepharose. The PC-3 plasma membrane extracts were exposed to a total of four aliquots of Man-6-P/IGF2R-Sepharose. A very small percentage of the uPAR population was able to bind the Man-6-P/IGF2R-Sepharose and the binding was detectable upon each exposure of the extract to new resin aliquots (Fig. 6, lanes 1-4). The highest percentage of binding did occur in the first exposure of uPAR to the resin (~5%), and the binding-competent
molecules were depleted by the fourth exposure to resin. Most (~85%) of the endogenous uPAR molecules remained in the supernatant after four rounds of depletion, revealing that full-length uPAR binds the Man-6-P/IGF2R with low abundance and, seemingly, low affinity as well.

*Endogenous uPAR Does Not Bind the Man-6-P/IGF2R Mini-Receptors*—Coimmunoprecipitation experiments with soluble uPAR had shown that suPAR bound the Man-6-P/IGF2R in a bivalent manner and binding was inhibited by Man-6-P. Since endogenous uPAR did not bind in a Man-6-P-dependent manner, we hypothesized that the binding site for uPAR on the Man-6-P/IGF2R would be different from that for suPAR on the receptor. Attempts to map the binding site of full-length uPAR using the Man-6-P/IGF2R mini-receptors described earlier with endogenous uPAR in PC-3 plasma membrane extracts were unsuccessful (Fig. 7A).

Equimolar amounts of 293T cell lysates transiently expressing the Man-6-P/IGF2R mini-receptors (1F, 1-3F, 1-15F, 7-9F, and 7-15F) or a vector-only control (pCMV5) were bound to M2 resin and then incubated with PC-3 plasma membrane extracts. As a control, an aliquot of the extract was incubated with the M2 resin by itself (Fig 7A, lane 1). The polyclonal uPAR antibody #2 was used to detect uPAR binding to the Man-6-P/IGF2R mini-receptors and the amount of uPAR remaining unbound in the supernatant fractions was also measured. An aliquot of the extract alone was also immunoblotted to show uPAR expression (Fig. 7A top, lane 8). The coimmunoprecipitation experiments did not show any binding of endogenous uPAR to the Man-6-P/IGF2R mini-receptors, including the 1-15F mini-receptor consisting of the entire extracytoplasmic region of the receptor. The supernatant fractions also revealed that uPAR expression in the PC-3 plasma membrane extracts was abundant (Fig 7A, lower panel).

As a further test of uPAR’s ability to bind the Man-6-P/IGF2R mini-receptors, an immunodepletion experiment was performed. Equal volumes of 1-15F or a vector-only control
(pCMV5) were bound to M2 resin as described above and then subsequently incubated with PC-3 plasma membrane extracts that had been untreated, treated with the polyclonal #2 uPAR antibody, or a non-immune rabbit IgG as a control. The collected resin pellets, supernatant aliquots, and aliquots of the treated and untreated extracts were immunoblotted with the polyclonal #2 uPAR antibody to detect uPAR binding to 1-15F and to determine whether the uPAR population in the PC-3 plasma membrane extracts was immunodepleted by the antibody (Fig. 7B). Although the uPAR population was significantly depleted upon exposure to the antibody, no detectable uPAR binding was visible when uPAR was coimmunoprecipitated with the 1-15F/M2-resin complex.

The inability to coimmunoprecipitate uPAR to the Man-6-P/IGF2R mini-receptors caused us to consider the possibility that the uPAR population in PC-3 cells may not be representative of that present in other cell types. In order to test this hypothesis, we repeated the coimmunoprecipitation assays using plasma membrane extracts prepared from the HT1080 fibrosarcoma cell line. Similar to the PC-3 experiments, no detectable binding of full-length uPAR to the Man-6-P/IGF2R mini-receptors was detectable using the HT1080 plasma membrane extract preparations even though, according to immunoblotting, uPAR is expressed in this cell line (Fig. 8).
DISCUSSION

The original objective of this project was to map the uPAR binding site of the Man-6-P/IGF2R. By using the truncated receptors and a soluble uPAR preparation in pull-down assays, we had planned to localize the uPAR binding site, if possible, to a single repeat of the Man-6-P/IGF2R extracytoplasmic domain. Surprisingly, the early mapping studies revealed that suPAR bound the Man-6-P/IGF2R at two separate sites in the extracytoplasmic domain. Furthermore, these suPAR binding sites localized to the same regions of the Man-6-P/IGF2R as the Man-6-P binding sites, namely extracytoplasmic repeats 1-3 and 7-9. Experiments employing several different approaches, including coimmunoprecipitation, affinity adsorption, and native gel/ligand blot analysis, all supported our hypothesis that suPAR bound the Man-6-P/IGF2R in a Man-6-P-dependent manner. Additionally, affinity depletion and alkaline phosphatase experiments demonstrated that only a small subpopulation of the suPAR preparation used in these experiments is binding-competent and presumably bears the Man-6-P moieties necessary for binding. In our experiments, there was no evidence for a novel interaction between suPAR and the Man-6-P/IGF2R.

These results were surprising because the uPAR:Man-6-P/IGF2R interaction was previously reported to be independent or mutually exclusive of both Man-6-P and IGF-II binding (27,28). In those studies, several different assays, including affinity chromatography, microdialysis, cross-linking, and in vitro binding experiments, had been employed in the presence or absence of Man-6-P. Man-6-P did not affect the interaction between uPAR and the Man-6-P/IGF2R under those conditions. However, Ploug et al. also reported the interaction between suPAR and the Man-6-P/IGF2R to be sensitive to Man-6-P inhibition (48). As in the present study, their ligand-blotting experiments revealed that recombinant soluble uPAR expressed in Chinese hamster ovary cells
binds the Man-6-P/IGF2R, but this binding is inhibited by co-incubation of the receptors with Man-6-P. Thus, Ploug et al. hypothesized that the interaction between uPAR and the Man-6-P/IGF2R was due to a low-abundance glycosylation variant in their uPAR preparation, and they predicted that the percentage of uPAR molecules in a given population carrying phosphorylated high-mannose oligosaccharides would differ among preparations (48).

Our native gel/ligand blot data not only agree well with the previous report concerning Man-6-P inhibition of the suPAR:Man-6-P/IGF2R interaction (48), but also with previously published data concerning the decrease in uPAR binding to the Man-6-P/IGF2R in the presence of IGF-II (27). This reduction in suPAR binding is consistent with the notion that suPAR interacts with the Man-6-P/IGF2R in a Man-6-P-dependent manner, because binding of IGF-II has been shown to decrease binding of Man-6-P-bearing lysosomal enzymes to the Man-6-P/IGF2R (46,49). Reciprocally, binding of β-galactosidase, a lysosomal enzyme containing Man-6-P moieties has been shown to decrease the binding affinity of the Man-6-P/IGF2R for IGF-II (47). IGF-II binds to the repeats 11-13 region of the Man-6-P/IGF2R extracytoplasmic domain, by which it could cause steric hindrance of suPAR binding to the Man-6-P binding sites. Another possibility for reduction of suPAR binding in the presence of IGF-II is that IGF-II binding to the Man-6-P/IGF2R causes a conformational change in the receptor that partially shields one or both Man-6-P binding sites. The native gel/ligand blot experiments also indicate that suPAR is capable of interacting with both the monomeric and dimeric forms of the Man-6-P/IGF2R. This type of binding is typical of IGF-II as well (38). However, PMP-BSA, the control Man-6-P ligand, binds with high affinity only to the Man-6-P/IGF2R dimer due to the multiple Man-6-P residues on this ligand and the nature of the high-affinity interaction between PMP-BSA and the Man-6-P/IGF2R (38).
Throughout the mapping studies performed in this project, suPAR consistently bound the amino-terminal Man-6-P binding site (repeats 1-3) with higher avidity than the more carboxyl-terminal binding site (repeats 7-9) and the 1-15F construct, which contains the entire extracytoplasmic domain. If suPAR does bind the Man-6-P/IGF2R in a Man-6-P-dependent manner, what could account for the preferential binding of suPAR to one Man-6-P binding site over the other? We showed that PMP-Sepharose affinity resin also binds with higher avidity to the amino-terminal Man-6-P site. One possible explanation for this binding variation may be found in the conformational differences between 1-3F and 7-9F when expressed in various cell lines. The 7-9F mini-receptors may have a folding impairment when expressed in a mini-receptor form. An additional trivial explanation for the variations would be differences in binding of the truncated receptors to the M2 resin. Although equimolar amounts of the various mini-receptors are incubated with the M2 affinity resin, different amounts of the mini-receptors are actually bound to the resin. This variation would explain the differences in suPAR band intensity bound to 1-3F, 7-9F, 7-15F, and 1-15F in our pull-down assays. Additionally, the work of Dahms and colleagues has determined that the two Man-6-P-recognition domains on the Man-6-P/IGF2R are functionally distinct, with the amino-terminal Man-6-P domain possessing a higher pH optimum than the carboxyl-terminal site (pH 6.9 vs. pH 6.4-6.5, respectively) (50). Marron-Terada et al. showed that truncated Man-6-P/IGF2R receptors encompassing repeats 1-3 or repeats 7-9 displayed similar affinities for Man-6-P, but the two Man-6-P binding domains differed in the binding activity under acidic conditions and in their preferences for binding various lysosomal enzymes from Dictyostelium discoideum, which contain modified Man-6-P residues. Additionally, Hancock et al. demonstrated that constructs encoding repeat 3 or 9 alone expressed
in *Pichia pastoris* were each able to bind several Man-6-P ligands, but the repeat 9 receptor alone could bind the ligands with high affinity (11).

All of our data to this point had dealt with the binding of the Man-6-P/IGF2R with soluble, recombinant uPAR expressed by cDNA-transfected HEK 293 cells. One concern with using recombinant, suPAR is that soluble proteins may transit through the Golgi network differently from membrane-bound proteins. The difference in protein transit routes or rates can change post-translational modifications such as glycosylation or phosphorylation. Therefore, this may account for the difference in the percentage of binding-competent molecules in our preparation vis-à-vis those used in previous work (27,28). Another possibility, that dephosphorylation or deglycosylation of the suPAR preparation occurred during the radioiodination procedure, was ruled out by the coimmunoprecipitation experiments, which were done using unlabeled suPAR. The finding that a small population of the suPAR molecules in our preparation is Man-6-P/IGF2R binding-competent is entirely consistent with the observations of Ploug *et al.* (48) using a similar suPAR preparation.

However, due to the discrepancies between our data and that of recently published work, we decided to repeat many of the suPAR:Man-6-P/IGF2R experiments using an endogenous uPAR source. To obtain this full-length uPAR population, we chose to prepare Triton X-100 extracts of plasma membranes from the human prostate cancer cell line PC-3 because of the naturally abundant uPAR present in the cell line. Affinity adsorption experiments with full-length bovine Man-6-P/IGF2R-Sepharose revealed that full-length uPAR did bind the Man-6-P/IGF2R, and incubation with Man-6-P or IGF-II did not inhibit this binding. The data for full-length uPAR therefore agreed with the previously published work from Nykjær *et al.* and Godár *et al.* (27,28).

However, the binding in our assay was so low-abundance that it could only be visualized upon
long exposure of the blots to film, displaying a different type of interaction from the robust interactions described in previous work (27,28).

The affinity depletion experiment using full-length uPAR confirmed an interaction of very low abundance in our uPAR population and of seemingly very low affinity, calling into question the biological relevance of this interaction. With less than 15% of the total uPAR population able to bind the full-length Man-6-P/IGF2R upon four exposures to the affinity resin, the question remains as to whether the binding is real or non-specific in nature.

The coimmunoprecipitation experiments with full-length uPAR did not reveal any binding between uPAR and the Man-6-P/IGF2R mini-receptors. These data either support our previous findings as to the low abundance/low affinity nature of the interaction or indicate that the binding involves sites on the Man-6-P/IGF2R outside of the extracytoplasmic domain, such as the transmembrane or cytoplasmic regions of the receptor.

During preparation of this manuscript, Leksa et al. published a paper that indicated uPAR binds to the Man-6-P/IGF2R within the first-half of repeat 1 in the extracytoplasmic region of the receptor, actually mapping to a specific peptide sequence within this region (29). They utilized a similar mapping strategy to the one we had been using, namely, constructing a group of Man-6-P/IGF2R mini-receptors in which they used a modified ELISA procedure to bind the mini-receptors to immobilized uPAR. One major difference between the mini-receptors synthesized by that group and ours is that our mini-receptors do not contain the transmembrane or cytoplasmic regions of the Man-6-P/IGF2R. This difference in construct design offers one explanation as to the discrepancies between our results. We did use full-length Man-6-P/IGF2R in our affinity adsorption and affinity depletion experiments and were able to show only a very weak interaction between uPAR and the full-length receptor. The full-length Man-6-P/IGF2R
was purified from bovine liver and not human tissue. However, the sequence corresponding to the binding site on the Man-6-P/IGF2R that Leksa et al. reported is highly conserved (83% identical at the peptide level) between bovine and human and should not have posed a problem in our binding assays.

The first-half of repeat 1, including the proposed uPAR binding sequence, is included in all of our Man-6-P/IGF2R mini-receptors except for the 11Fsp construct used in the suPAR experiments. Therefore, according to the published data, all of the truncated receptors used in our endogenous uPAR coimmunoprecipitation experiments should have bound full-length uPAR, but we detected no binding of uPAR to any of the mini-receptors. Also, Leksa et al. used cell lysates from NIH 3T3 cells stably expressing full-length, tagged uPAR, whereas we performed experiments using uPAR in plasma membrane extracts from either PC-3 or HT1080 cells. Variations in post-translational modifications between cell lines offer one additional explanation as to the discrepancies between these studies, but the large difference in the binding analyses makes this possibility somewhat unlikely.

Several hypotheses have been proposed as to the binding function of uPAR and the Man-6-P/IGF2R. First, the Man-6-P/IGF2R is thought to regulate the cell-surface concentration of uPAR by directing uPAR to lysosomes as well as internalizing uPAR when it interacts with uPA (27). The uPAR/uPA/plasmin complex is capable of proteolytically cleaving several extracellular matrix components, thereby promoting invasiveness or cellular migration (51,52). The overexpression of uPAR also increases the invasiveness of several tumor types, whereas expression of antisense uPAR or uPAR inhibitors has been shown to decrease the rate of metastasis (53-55). The detection of uPAR in endosomes agrees well with the primary function of the Man-6-P/IGF2R (27), which is the transport of ligands from the Golgi or cell surface to
the endosome. The presence of uPAR in the lysosome, however, does not necessarily imply degradation of uPAR. Whether uPAR is degraded in the lysosomal compartment, recycled, or destined for some other subcellular location has not been addressed experimentally.

Another possible function for the binding of uPAR by the Man-6-P/IGF2R is that the complex may serve as a scaffold for the activation of TGF-β (28). In support of this proposed role, the Man-6-P/IGF2R is capable of simultaneously binding the latent pro-form of TGF-β, uPAR, and plasminogen (28). Finally, the interaction between uPAR and the Man-6-P/IGF2R is proposed to control uPAR-mediated functions including regulation of fibrinolysis, cell adhesion, and migration (29). The present study does not necessarily favor one of the above-mentioned proposals over the others. However, it may call into question the physiological relevance of the interaction between the two proteins. Our affinity depletion data show that only small percentages of our suPAR (≤30%) or uPAR (<15%) preparations were Man-6-P/IGF2R binding-competent, but these preparations do differ from those used in many of the experiments reported by Nykjær et al., Godár et al., and Leksa et al. (27-29).

In conclusion, we have shown that suPAR binds at two distinct locations in the extracytoplasmic domain of the Man-6-P/IGF2R, and that these sites correspond to the Man-6-P binding sites of the Man-6-P/IGF2R. Also, the interaction between suPAR and the Man-6-P/IGF2R is completely sensitive to inhibition by Man-6-P. Treatment of suPAR with alkaline phosphatase also inhibits binding between the two proteins, revealing that suPAR binds the Man-6-P/IGF2R in a phosphate-dependent manner. Affinity depletion analysis determined that only a small percentage of our suPAR preparation was binding-competent and, therefore, it seems likely that only a small fraction contained the Man-6-P moieties necessary for binding the Man-6-P/IGF2R. Our data show that the interaction between full-length uPAR and the Man-6-P/IGF2R
is not dependent on Man-6-P, but is also a low-affinity/low-abundance interaction. Affinity adsorption and depletion experiments showed a very weak interaction between the proteins, but this binding was undetectable when using the Man-6-P/IGF2R mini-receptors. Further studies will need to focus on analyzing the nature of the interaction and its affinity and abundance, in order to determine the biological relevance, if any, of the binding.
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FOOTNOTES

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ABBREVIATIONS

The abbreviations used are: Man-6-P/IGF2R, mannose 6-phosphate/insulin-like growth factor II receptor; Man-6-P, mannose 6-phosphate; IGF-II, insulin-like growth factor II; TGF-β, transforming growth factor beta; uPAR, urokinase-type plasminogen activator receptor; GPI, glycosylphosphatidylinositol; uPA, urokinase-type plasminogen activator; suPAR, soluble urokinase-type plasminogen activator receptor; Glc-6-P, glucose 6-phosphate; PMP, pentamannose phosphate; BSA, bovine serum albumin; nt, nucleotide(s); HBS, HEPES-buffered saline; HBST, HEPES-buffered saline with 0.1% Triton X-100; SDS-PAGE, sodium dodecyl phosphate-polyacrylamide gel electrophoresis; DTT, dithiothreitol.
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Fig. 1. Schematic diagram and expression of truncated Man-6-P/IGF2R mini-receptors. A, The receptors are diagrammed, with rectangles representing the repeating units of the extracytoplasmic domain of the Man-6-P/IGF2R. The asterisk denotes the first half of repeat one plus the signal sequence. The gray boxes represent the Man-6-P binding sites on the Man-6-P/IGF2R, and the stippled boxes denote the IGF-II binding site. The black boxes represent addition of the FLAG epitope tag to the carboxyl terminus. B, Expression of Man-6-P/IGF2R mini-receptors. Duplicate samples containing 25 µl of Triton X-100 extracts from cells transfected with each construct were resolved by SDS-PAGE on 8-16% polyacrylamide gels, subjected to α-FLAG M2 immunoblot analysis and developed with ^125^I-protein A. An autoradiogram of a representative blot is shown.

Fig. 2. Coimmunoprecipitation of suPAR bound to Man-6-P/IGF2R mini-receptors and binding analysis of 1-3F and 7-9F. A, Coimmunoprecipitation of suPAR and the Man-6-P/IGF2R. Equimolar amounts of FLAG-tagged mini-receptors from transfected cell lysates were immunoprecipitated with 15 µl of α-FLAG M2 resin in duplicate. The resin pellets were collected by centrifugation, washed, and incubated with purified suPAR (1.65 µg) in a volume of 0.2 ml. At the end of the incubation, the resin pellets were collected, washed, and heated in sample buffer. The proteins were resolved by SDS-PAGE on 8-16% gels, subjected to immunoblot analysis with α-uPAR (PC#3), and developed with ^125^I-protein A. An autoradiogram of a representative blot is shown. B, Coimmunoprecipitation of suPAR and the Man-6-P/IGF2R. Equal volumes of the indicated cell lysates (0.3 ml) were incubated with 15 µl of packed M2 resin, then suPAR, and analyzed by α-uPAR immunoblot as above. C, PMP-Sepharose affinity adsorption of 1-3F and 7-9F mini-receptor constructs. Aliquots of lysates (0.3
ml) from transfected cells were incubated with 0.1 ml of PMP-Sepharose. The resin pellets were collected and washed with HBST, then HBST containing 5 mM Glc-6-P, and finally eluted with HBST containing 5 mM Man-6-P. Aliquots of the lysates (60 µl, lanes 1), Glc-6-P wash (20 µl, lanes 2), and the Man-6-P eluates (20 µl, lanes 3) were heated with SDS sample buffer containing DTT. The proteins were resolved on 6-12% polyacrylamide gels, immunoblotted with α-FLAG M2 antibody followed by a secondary rabbit anti-mouse IgG, and then developed with 125I-protein A. A representative autoradiogram is shown.

Fig. 3. Determination of Man-6-P sensitivity of suPAR binding to the Man-6-P/IGF2R. A, Coimmunoprecipitation of suPAR bound to Man-6-P/IGF2R mini-receptors with or without Man-6-P. The indicated lysates (0.3 ml) were incubated with 15 µl of packed M2 resin as in Fig. 2. The resin pellets were collected by centrifugation, washed twice with HBST, and incubated with 1.65 µg suPAR in 0.2 ml of HBST ± 5 mM Man-6-P. The resin pellets were collected, washed and analyzed as described in Fig. 2. B, Ligand blot analysis of native Man-6-P/IGF2R species. Aliquots (40 µg) of purified full-length Man-6-P/IGF2R were electrophoresed in duplicate loadings on 4-12% native gels. The proteins were electroblotted to nitrocellulose, probed with 12.5 nM 125I-suPAR in the presence or absence of 5 mM Man-6-P or 0.5 µM IGF-II, and developed by autoradiography. C, Man-6-P/IGF2R-Sepharose affinity adsorption of suPAR. Aliquots (50 µl) of Man-6-P/IGF2R-Sepharose were incubated with 1.35 nM 125I-suPAR in 0.2 ml of HBST alone (lane 1), HBST with 10 mM Man-6-P (lane 2), or HBST with 10 mM Glc-6-P (lane 3). Two additional aliquots were incubated with 125I-suPAR and then eluted with solutions containing 10 mM Man-6-P (lane 4) or 10 mM Glc-6-P (lane 5). Resin pellets were collected by centrifugation, washed, and heated with sample buffer containing DTT. The proteins were
resolved by SDS-PAGE on 6-16% polyacrylamide gels, stained with Coomassie blue, and destained. The dried gels were developed by autoradiography. D, Effect of alkaline phosphatase treatment on the suPAR-Man-6-P/IGF2R interaction. Radiolabeled suPAR (~150,000 cpm) or PMP-BSA (~50,000 cpm) was incubated with phosphatase buffer alone (lane 1), buffer plus either 1 U of E. coli alkaline phosphatase (lane 4) or 5 U of E. coli alkaline phosphatase (lane 5) in pH 8.3 buffer for 16 h at 37°C. Aliquots (10 µl) of Man-6-P/IGF2R-Sepharose in low pH-HBST (pH 6.8) ± 5 mM Glc-6-P or Man-6-P (lanes 2 and 3) were added to all of the reaction mixtures and incubated for 8 h at 4°C. The resins were collected by centrifugation and washed three times with HBST (pH 7.4). The resins were heated in sample buffer with DTT and the released proteins were resolved by SDS-PAGE on 6-16% gradient gels. The gels were stained with Coomassie blue, destained, and the dried gels were exposed to film by autoradiography.

Fig. 4. Man-6-P/IGF2R-Sepharose affinity depletion analysis. Aliquots (20 µl) of Man-6-P/IGF2R-Sepharose were incubated with 0.2 ml ligand mixes that contained ~40,000 cpm of 125I-suPAR or 125I-PMP-BSA at 4°C for 4 h initially, and 16-24 h for each additional exposure. The resin pellets were collected by centrifugation; the supernatant fractions were transferred to new aliquots of Man-6-P/IGF2R-Sepharose, and the resin pellets were washed once with 1 ml of HBST. This process was repeated for a total of four exposures of supernatant fractions to fresh resin, corresponding to lanes 1-4 on the gel. The resin pellets and aliquots (50 µl, ~25%) of the supernatant fractions (supnt) were heated at 100°C for 5 min with sample buffer containing 50 mM DTT, and the released proteins were subjected to 6-16% SDS-PAGE. The gels were stained with Coomassie blue, destained, and exposed to x-ray film. B, Quantitative analysis of affinity depletion. Two dried gels were quantified by Storm PhosphorImager analysis. The y-axis
represents the percentage of bound ligand plotted on a logarithmic scale, whereas the x-axis represents the number of exposures of the ligands to Man-6-P/IGF2R-Sepharose. These data represent the means of two independent experiments.

Fig. 5. **Man-6-P/IGF2R-Sepharose: endogenous uPAR binding analysis.** PC-3 plasma membranes were collected by homogenization and differential centrifugation. Triton X-100 extracts of the PC-3 plasma membranes were prepared and aliquots (~500 µg protein) were incubated with aliquots (20 µl) of Man-6-P/IGF2R-Sepharose or ethanolamine-blocked Sepharose for 16 h at 4°C in the presence or absence of the indicated additives, 5 mM Man-6-P, 5 mM Glc-6-P, or 1 µM IGF-II. Additionally, extraction buffer (B, 500 µl) or suPAR (su, 500 ng) were incubated with Man-6-P/IGF2R-Sepharose as negative and positive controls, respectively. The resin pellets were collected by centrifugation and the supernatants transferred to new tubes. The resin pellets were washed twice with HBST. The resin pellets and aliquots (100 µl, 20%) of the supernatant fractions were heated with sample buffer without DTT, and the released proteins were subjected to 6-16% SDS-PAGE. The gels were immunoblotted with α-uPAR monoclonal antibody followed by a secondary rabbit anti-mouse IgG, and then developed with 125I-protein A. Representative autoradiograms are shown from five trials.

Fig. 6. **Man-6-P/IGF2R-Sepharose: endogenous uPAR affinity depletion analysis.** PC-3 plasma membranes were collected by homogenization and differential centrifugation. Triton X-100 extracts of the PC-3 plasma membranes were prepared and aliquots (~1 mg protein) were incubated with aliquots (25 µl) of Man-6-P/IGF2R-Sepharose in a volume of ~1 ml for 24 h at 4°C upon each exposure. The resin pellets were collected by centrifugation; the supernatant
fractions were transferred to new aliquots of Man-6-P/IGF2R-Sepharose, and the resin pellets were washed twice with 1 ml of HBST. This process was repeated for a total of four exposures of supernatant fractions to fresh resin, corresponding to lanes 1-4 on the gel. The resin pellets and aliquots (100 µl, 10%) of the supernatant fractions (supnt) were heated at 100°C for 5 min with sample buffer without DTT, and the released proteins were subjected to 6-16% SDS-PAGE. The gels were immunoblotted with α-uPAR monoclonal antibody followed by a secondary rabbit anti-mouse IgG, and then developed with 125I-protein A. A representative autoradiogram is shown from four trials.

Fig. 7. Coimmunoprecipitation of endogenous uPAR to Man-6-P/IGF2R mini-receptors and immunodepletion analysis. A, Coimmunoprecipitation of uPAR and the Man-6-P/IGF2R. Equimolar amounts of FLAG-tagged mini-receptors present in 293T cell lysates were immunoprecipitated with 15 µl of α-FLAG M2 resin. The resin pellets were collected by centrifugation, washed, and incubated with aliquots (~300 µg protein) of PC-3 plasma membrane Triton X-100 extracts in a volume of 0.3 ml. At the end of the incubation, the resin pellets were collected, washed, and heated in sample buffer, and the supernatants transferred to additional tubes. The resin proteins and aliquots (100 µl, 33%) of the supernatants were resolved by SDS-PAGE on 6-16% gels, subjected to immunoblot analysis with α-uPAR (PC#2), and developed with 125I-protein A. A representative blot is shown from three trials. B, Immunodepletion analysis of the interaction between uPAR and 1-15F. Aliquots (300 µl) of 1-15F or the vector, pCMV5, from transfected cell lysates were immunoprecipitated with 15 µl of α-FLAG M2 resin. PC-3 plasma membranes were extracted with Triton X-100 and left untreated or treated with polyclonal #2 α-uPAR antibody or with non-immune rabbit IgG. The treated extracts were then
incubated with protein A-Sepharose. At the end of the immunodepletion, the extract supernatants were collected by centrifugation. The immunoprecipitated M2 resin pellets were collected by centrifugation, washed and coimmunoprecipitated with aliquots (500 µl) of untreated or treated PC-3 plasma membrane extracts. After the incubation, the resin pellets were collected by centrifugation, washed and heated in sample buffer. The supernatants were collected and transferred to new tubes. Proteins from the resins, aliquots (100 µl, 20%) of the supernatants, and aliquots (60 µl) of treated or untreated PC-3 plasma membrane extracts were resolved by 6-16% SDS-PAGE, subjected to immunoblot analysis with polyclonal #2 α-uPAR antibody, and developed with 125I-protein A. A representative blot is shown from two trials.

Fig. 8. Coimmunoprecipitation of truncated Man-6-P/IGF2R receptors and endogenous uPAR from HT1080 plasma membranes. Equimolar amounts of FLAG-tagged mini-receptors in 293T cell lysates were immunoprecipitated with 15 µl of α-FLAG M2 resin. The resin pellets were collected by centrifugation, washed, and incubated with aliquots (~500 µg protein) of HT-1080 plasma membrane Triton X-100 extracts in a final volume of 0.5 ml. At the end of the incubation, the resin pellets were collected, washed, and heated in sample buffer and the supernatants were transferred to additional tubes. The resin proteins and aliquots (100 µl, 20%) of the supernatants were resolved by SDS-PAGE on 6-16% gels, subjected to immunoblot analysis with polyclonal #2 α-uPAR antibody, and developed with 125I-protein A. A representative blot is shown from two trials.
Figure 1

A

Construct Name:

1F NH$_2$\[\phantom{\text{FLAG}}\]

1-3F NH$_2$\[\phantom{\text{FLAG}}\]

1-8F NH$_2$\[\phantom{\text{FLAG}}\]

1-15F NH$_2$\[\phantom{\text{FLAG}}\]

7-9F NH$_2$\[\phantom{\text{FLAG}}\]

7-15F NH$_2$\[\phantom{\text{FLAG}}\]

11F NH$_2$\[\phantom{\text{FLAG}}\]

11Fsp NH$_2$\[\phantom{\text{FLAG}}\]
Figure 2

A

| 1F | 11Fsp | 1-15F | 1-8F | 7-15F | 1-3F | 7-9F | CMV5 |
|----|-------|-------|------|-------|------|------|------|

B

| 1-3F | 1-15F | 7-9F | 11F |

suPAR

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Figure 2

C

| 1-3F | 7-9F |
|------|------|
| 1    | 1    |
| 2    | 2    |
| 3    | 3    |

$M_r \times 10^{-3}$

46

66
Figure 3

A

|          | 1F | 1-3F | 1-15F | 7-9F |
|----------|----|------|-------|------|
| Man-6-P: | −  | +    | −     | +    |

Arrows indicate suPAR.
Figure 3

B

Man-6-P/IGF2R Dimer

Man-6-P/IGF2R Monomer

Control  + Man-6-P  + IGF-II

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Figure 3

C

1 2 3 4 5

suPAR
Figure 3

D

suPAR

PMP-BSA
Figure 4

A

suPAR

PMP-BSA

B

log % Bound

# of Exposures

Exposure

1 2 3 4 Supnt

Figure 4 Exposures

52
**Figure 5**

| Lanes: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|--------|---|---|---|---|---|---|---|---|
|        | + Extract                     | B | su |
| Blocked: | - | + | - | - | - | - | - | - |
| Man-6-P/IGF2R: | - | - | + | + | + | + | + | + |
| Resin: | ![Resin Image] |
| Supernatant: (20%) | ![Supernatant Image] |
| Additions: | None | None | None | M6P | G6P | IGF-II |
Figure 6
Figure 7

A

| Lanes:  | 1   | 2   | 3   | 4   | 5   | 6   | 7   |
|--------|-----|-----|-----|-----|-----|-----|-----|
| Input Construct: | None | CMV5 | 1F  | 1-3F | 1-15F | 7-9F | 7-15F |
| Resin:  |      |      |     |      |       |      |      |
| Supernatant: (33%) |      |      |     |      |       |      |      |

Extract Only

uPAR

Extract Only

uPAR

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**Figure 7 B**

**Input Construct:**

|             | CMV5 | 1-15F | 1-15F | 1-15F | Extract Only | Extract Only | Extract Only |
|-------------|------|-------|-------|-------|--------------|--------------|--------------|
| α-uPAR:     | -    | -     | +     | -     | -            | +            | -            |
| Non-immune IgG: | -    | -     | -     | +     | -            | -            | -            |

**Resin:**

Supernatant: (33%)

---

|         | 
|---------|
| uPAR    |

---
Figure 8

Input Construct: None, CMV5, 1F, 1-3F, 1-15F, 7-9F, 7-15F, Extract Only

Resin: Supernatant: (20%)
Binding of Urokinase-type plasminogen activator receptor (uPAR) to the mannose 6-phosphate/insulin-like growth factor II receptor: Contrasting interactions of full-length and soluble forms of uPAR
Jodi L. Kreiling, James C. Byrd, Robert J. Deisz, Ikuko F. Mizukami, Robert F. Todd III and Richard G. MacDonald

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