Altered Ligand Binding by Insulin-like Growth Factor II/Mannose 6-Phosphate Receptors Bearing Missense Mutations in Human Cancers


ABSTRACT

The M6P/IGF2R gene, encoding the insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor (IGF2R), is frequently inactivated during carcinogenesis. M6P/IGF2R is postulated to be a tumor suppressor gene due to its ability to bind and degrade the mitogen IGF-II, promote activation of the growth inhibitor transforming growth factor β, and regulate the targeting of lysosomal enzymes. In this study, we determined the effects of four M6P/IGF2R missense mutations associated with loss of heterozygosity in hepatocellular and breast cancers on the ligand binding properties of full-length membrane-bound receptors. Site-directed mutagenesis was used to prepare COOH-terminal, c-myc epitope-tagged human IGF2R cDNA expression constructs bearing point mutations that are located in the receptor’s extracytoplasmic domain. Ligand binding was measured in plasma membranes from 293T cells expressing full-length receptors. No binding of 125I-IGF-II to I1572T mutant receptors was observed. Binding to G1449V mutant receptors was decreased by 50% relative to wild-type (WT). However, IGF-II binding to the I1464E and Q1445H mutant receptors was equivalent to WT when plasma membranes were assayed immediately after preparation. The phosphomannosylated pseudoglycoprotein pentamannose 6-phosphate-BSA (PMP-BSA) was synthesized as a ligand for the M6P binding site. Binding of 125I-PMP-BSA was equivalent to WT for the I1572T, G1464E, and Q1445H mutations, but there was a 60% reduction in PMP-BSA binding to the G1449V mutant receptor. Thus, several missense mutations in M6P/IGF2R disrupt the ligand binding functions of the intact IGF2R, lending further support to the hypothesis that the M6P/IGF2R is a tumor suppressor gene.

INTRODUCTION

HCC ranks as the seventh most common cancer worldwide, with risk factors that include infection by hepatitis virus, alcohol consumption, and dietary exposure to aflatoxin B1. The treatments available include hepatic resection and transplantation, which are of limited efficacy (1), and therefore, a better understanding of the molecular mechanisms of hepatocarcinogenesis may lead to improved approaches for both diagnosis and treatment.

The M6P/IGF2R gene encoding the IGF2R is often mutated early in oncogenesis of liver (2–4) and breast (5). The IGF2R is a M, 300,000 transmembrane glycoprotein that has binding sites for IGF-II as well as for M6P-bearing glycoproteins (6, 7). The IGF2R is composed of a short COOH-terminal cytoplasmic domain connected via a single transmembrane domain to the extracytoplasmic region consisting of 15 homologous repeats (6, 7). In mammals, this bivalent M6P binding domain of the IGF2R has been localized to repeats 1–3 and 7–9 (7, 8), whereas IGF-II binds independently to its primary binding site located in repeat 11 (9–11), the function of which is aided by an affinity-enhancing domain located in repeat 13 (12). The receptor transports lysosomal enzymes and other phosphomannosylated glycoproteins from the lumen of the Golgi to an acidic prelysosomal compartment (6, 7). Gene knockout experiments and analysis of IGF2R expression in human cancers suggest that IGF-II binding by the IGF2R followed by internalization and degradation of the ligand constitutes a key regulatory mechanism for modulation of IGF-II levels, particularly during mammalian development (13, 14).

High levels of IGF-II expression have been observed in HCCs as a result of gene reactivation arising from relaxation of imprinting at the IGF2 locus (15–20). If IGF-II is mitogenic or even tumorigenic when it is expressed at high levels by tumor cells, then it follows that the IGF2R could serve as a suppressor of these IGF-II-dependent tumors. LOH, a hallmark of tumor suppressor involvement in many cancers, at the M6P/IGF2R locus (6q26-q27) is frequently observed in premalignant dysplastic lesions as well as in the phenotypically normal hepatocytes adjacent to dysplastic lesions and HCCs, indicating that this is an early, perhaps initiating, event in liver carcinogenesis (2, 3, 21). Recent evidence demonstrates that inactivation of M6P/IGF2R in liver carcinogenesis results from several types of mutations, including a splice junction alteration that leads to synthesis of a truncated receptor (3) and a frameshift caused by deletion of a G at a poly(G) region exhibiting microsatellite instability (21). Several missense mutations in M6P/IGF2R also have been detected, but it is unclear whether the predicted amino acid substitutions affect receptor function (4). A G:C→T:A transversion at nt 4493, resulting in a G1449V substitution, has been identified as a site of frequent mutation of M6P/IGF2R in human liver cancers (3, 4, 21). This missense mutation is located in repeat 10 of the receptor, which corresponds to exon 31 of the mouse gene (22) and lies between the M6P and IGF-II binding sites. A G:C→A:T transition resulting in a G1464E substitution (3) and a G:C→T:A transversion causing a Q1445H mutation (5) have been identified to be associated with LOH in HCC and breast cancer, respectively. In addition, an I1572T substitution arising from a T:A→C:G transition at nt 4862 in repeat 11 of the IGF2R has been observed in a single HCC. This mutation is located within the primary IGF-II binding site (9, 10, 23). We had shown previously that the presence of the I1572T mutation in a mini-receptor construct comprised of only repeat 11 caused complete loss of IGF-II binding (23). Glutamine 1445, glycine 1449, and isoleucine 1572 are conserved residues in the human, bovine, rat, and mouse receptors. To determine whether these missense mutations alter ligand binding by the IGF2R, we prepared WT and mutant full-length IGF2R constructs, transfected them into 293T cells, and analyzed them for their abilities to bind IGF-II and M6P. Here, we demonstrate that these IGF2R
missense mutations found in human cancers result in selective loss of or alterations in receptor ligand binding functions. These findings further support the hypothesis that the M6P/IGF2R is a tumor suppressor gene.

**MATERIALS AND METHODS**

**Materials**

Recombinant human IGFs were provided by M. H. Niedenthal (Lilly Research Laboratories, Indianapolis, IN). IGF-II was radioiodinated to specific activities between 40 and 85 Ci/g using carrier-free Na125I (Amersham, Arlington Heights, IL) by Enzymobead reagent (Bio-Rad, Hercules, CA). The native Y-2448 O-phosphophoman of *Hansenula holstii* was a gift from Dr. M. E. Slodki (Midwest Area Northern Regional Research Center, Peoria, IL, retired). The pCMV5 vector (24) was provided by Dr. David. W. Russell (University of Texas Southwestern Medical Center, Dallas, TX). The 8.6-kb human IGF2R cDNA (25) was given by Dr. William S. Sly (St. Louis University Medical Center, St. Louis, MO). Other reagents were from standard sources as indicated.

**Methods**

Preparation of Full-Length Mutant IGF2R Constructs. Point mutations in the human full-length IGF2R construct were generated by a cassette strategy using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with *Pfu* polymerase and a cassette containing a *PflM*I-*PflM*I fragment (nt 3847–6315) of the human IGF2R cDNA in pCRII (Invitrogen, San Diego, CA) as template. Complementary primer pairs corresponded to (a) nt 4840–4874 substituting T→C at nt 4862, creating the 1e1572Thr (I1572T) mutation; (b) nt 4530–4557 substituting G→C at nt 4493, creating the Gly1446Glu (G1446E) mutation; (c) nt 4478–4522 substituting G→T at nt 4493, creating the Gly1449Val (G1449V) mutation; and (d) nt 4470–4494 substituting G→T at nt 4482, creating the Gln1445His (Q1445H) mutation. The resultant products were first subcloned by *PflM*I digestion and ligation into a shuttle vector containing the IGF2R cDNA. Subsequently, each construct was subjected to an EcoRI digestion, and a 5.2-kb fragment (nt 162–5319) encoding the point mutation was subcloned into the pCMV5 vector containing a COOH-terminal human c-myc epitope-tagged WT IGF2R cDNA (12). The four mutant IGF2R cDNA expression constructs were subjected to sequence analysis to confirm the presence of the mutations.

**Transient Expression of WT and Mutant IGF2Rs in 293T Cells.** 293T cells were cultured in DMEM supplemented with 5% FCS plus 50 μg/ml gentamicin at 37°C in 50% CO2, 95% air. Cells were grown to ~40–50% confluence in 100-mm dishes. The transfection of plasmids containing the WT and mutant IGF2R cDNA constructs was carried out by a modification of the calcium phosphate method described previously (12, 26). Plasma membranes and lysates were isolated on the sixth day after transfection, as described previously (12, 27). Protein concentrations in the plasma membrane suspensions were measured using the bicinchoninic acid assay (Pierce, Rockford, IL).

**Immunoblot Detection of WT and Mutant IGF2Rs.** Immunoblot detection of the full-length receptors was performed with aliquots containing 200 μg of plasma membrane protein, which were reduced and alkylated under denaturing conditions and then electrophoresed on 6% SDS-PAGE, as described previously (28). After electroblotting to BA85 nitrocellulose, the immunoblots were blocked with 3% nonfat dry milk and probed with anti-13D antireceptor antibody (17-fold) and the G1449V mutant (30-fold), compared with WT IGF2R detected in the CMV5 control (Fig. 1C). In this transfection series, the degree of overexpression was highest for the WT cell IGF2R detected in the CMV5 control (Fig. 1A). Protein concentrations in the plasma membrane suspensions were measured using the bicinchoninic acid assay (Pierce, Rockford, IL).

**Analysis of IGF-II Binding and Affinity Cross-Linking.** Plasma membranes (50–100 μg of protein) were incubated with 2 nM 125I-IGF-II with or without 1 μM unlabeled IGF-II in binding buffer [25 mM HEPES (pH 7.5), 0.15 mM NaCl, and 1% BSA] on an end-over-end mixer for 16–18 h at 4°C. Direct binding assays were completed by washing with 1 ml of binding buffer, centrifugation of the suspensions for 7 min, and aspiration of the supernatant fractions. The tips of the tubes containing the pellets were cut and counted in a gamma counter. The cpm bound in the presence of 1 μM IGF-II were taken as nonspecific binding and were subtracted from the total bound cpm to calculate the specific binding. Cross-linking was performed by incubation of the membrane suspensions, labeled overnight, with 0.25 mM disuccinimidyl suberate, as described previously (23). Radioactivity levels in the individual bands were directly quantified by PhosphorImage analysis (Molecular Dynamics, Sunnyvale, CA).

**Expression of Full-Length IGF2Rs.** The Q1445H, G1449V, G1464E, and I1572T point-mutated IGF2Rs were generated by site-directed mutagenesis of a receptor cDNA construct encompassing the region between two *PflM*I sites (3847–6315) in the human receptor cDNA, followed by two-step subcloning of the various fragments encoding the missense mutations to create full-length IGF2R constructs that bear a c-myc epitope tag at the COOH terminus. All mutated cDNAs and the WT human IGF2R cDNA were transfected into 293T cells. Plasma membranes prepared from transfected cells were immunoblotted with anti-13D anti-IGF2R antibody to confirm expression over the relatively low background of endogenous 293T cell IGF2R detected in the CMV5 control (Fig. 1A). In this transfection series, the degree of overexpression was highest for the WT receptor (17-fold) and the G1449V mutant (30-fold), compared with the other three mutants (6–8-fold). The decreased receptor content in these membranes was derived from the transfected cDNAs was confirmed by immunoblotting with anti-myc antibody (Fig. 1B), which reinforced the quantitative differences in expression of the myc-tagged receptors. Cells transfected with vector only (CMV5) showed no detectable signal by blotting with anti-myc antibody. This pattern of relative expression of the different receptor constructs was observed in several transfections.

**Analysis of IGF-II Binding by WT and Mutant Full-Length IGF2Rs.** To determine whether these mutant receptors contained a functional IGF-II binding/cross-linking site, plasma membranes were incubated with 125I-IGF-II, affinity cross-linked with disuccinimidyl suberate, and analyzed by SDS-PAGE. The data in Fig. 2A demonstrate intense cross-linking of 125I-IGF-II to the WT IGF2R, which was increased ~10-fold over that of the endogenous 293T cell receptors (CMV5). The cross-linking is specific because the major radio-
active band disappears on incubation with excess unlabeled IGF-II during affinity labeling. Affinity cross-linking of $^{125}$I-IGF-II to the Q1445H, G1449V, and G1464E mutant IGF2Rs was also evident, although the intensities of those bands were substantially lower than that of the WT IGF2R (1.3–6-fold over the CMV5 control), which may suggest possible impairment of either binding or cross-linking relative to WT IGF2R. The low level of IGF-II affinity cross-linking to the Q1445H receptor preparation in this experiment was not a reproducible finding; this mutant receptor could be efficiently affinity-labeled in other experiments (see below). Finally, the I1572T mutant located within the primary IGF-II binding site did not cross-link $^{125}$I-IGF-II to a detectable extent above the CMV5 background. Failure of some of these mutants to cross-link $^{125}$I-IGF-II could arise from the absence of IGF-II binding or because of a conformational change in the receptor that interfered with or restricted cross-linking efficiency. To distinguish between these possibilities, we measured IGF-II binding by the WT and mutant receptors in a direct assay using a single concentration (2 nM) of radiolabeled IGF-II (Fig. 2B). The binding data were normalized to account for differences in expression of the exogenous receptor relative to the endogenous 293T cell IGF2R in each membrane preparation, as determined by antireceptor immunoblotting. The normalized data were expressed as a percentage of the WT expression, which was designated 100%. Thus, within the limitation of assay sensitivity, our results show that the Q1445H and G1464E mutant receptors bound IGF-II to approximately the same extent as WT. As expected from the affinity cross-linking data (Fig. 2A) and from our previous work with mini-receptor constructs (23), the I1572T mutant IGF2R showed no detectable IGF-II binding (Fig. 2B). Interestingly, the G1449V mutant showed an ~60% (range of 30–60% in three separate experiments) decrease in IGF-II binding under these assay conditions. It should be noted that all these assays were conducted with freshly prepared membranes.

**Analysis of Phosphomannosyl Ligand Binding.** PMP was purified and conjugated to BSA, and the resultant conjugate was iodinated to produce $^{125}$I-PMP-BSA for use as a ligand to assess the functionality of the M6P binding domain of the WT and mutant receptors. Assay of membranes bearing the WT and mutant IGF2Rs by $^{125}$I-PMP-BSA ligand blotting revealed detectable binding of the ligand in all cases (Fig. 3A). A direct $^{125}$I-PMP-BSA binding assay, in which binding was normalized for receptor expression and expressed relative
to WT as described above, confirmed binding of the phosphomannosylosyl ligand equivalent to the WT receptor for the Q1445H, G1464E, and I1572T mutants (Fig. 3B). Only the G1449V mutant IGF2R exhibited a reduction in 125I-PMP-BSA binding, which was ~55% in the experiment depicted in Fig. 3B. Thus, the G1449V substitution in extracytoplasmic repeat 10 of the IGF2R showed impairment in binding both types of ligands.

**DISCUSSION**

This is the first demonstration of the functional effects of M6P/IGF2R missense mutations that occur in HCCs and breast tumors using full-length IGF2R constructs. The G1449V mutation, which arises from a G:C→T:A transversion at nt 4493 and is present in ~15% of liver tumors (3), causes partial loss of both IGF-II and M6P binding functions. Glycine 1449 is a conserved residue among human, bovine, rat, and mouse IGF2Rs (Fig. 4). This residue is located in repeat 10 of the receptor’s extracytoplasmic domain, a region that is flanked by the binding sites for both M6P (repeats 3 and 9) and IGF-II (repeats 11 and 13). The substitution of valine, a bulky, hydrophobic residue, for glycine, a small, neutral amino acid with high conformational flexibility, may perturb the polypeptide backbone or disrupt side chain packing interactions (32). Although it is possible that this may lead to structural distortion or altered folding of the receptor, the G1449V mutant receptor has sufficient stability to be expressed in 293T cells. Whatever the changes that arise from this substitution, they lead remarkably to decreased binding at both the downstream IGF-II binding site and the upstream M6P binding domain. Additional experiments are needed to determine whether the impairment of ligand binding is due to reduced affinity or a decrease in available binding sites. It will also be important to assay effects of the G1449V mutation on the binding of a natural phosphomannosyl ligand in addition to the pseudoglycoprotein PMP-BSA.

Substitution of threonine, a polar residue, for the bulky, hydrophobic isoleucine at position 1572 may also induce local effects on folding and side chain burial (23). However, the IGF2R functional (and perhaps structural) change is confined to the primary IGF-II binding site of the receptor for the I1572T substitution, which is located in the heart of the primary IGF-II binding domain in repeat 11, whereas the effects of the G1449V mutation are long-range.

A G:C→A:T transition resulting in a G1464E substitution within repeat 10 of the IGF2R has also been observed to occur in HCCs with LOH (3). The substitution of glutamic acid, a bulky, acidic residue, for glycine would be predicted to have an effect on protein conformation; however, glycine at position 1464 is not conserved among human, rat, mouse, and bovine IGF2Rs like the other substitutions studied (Fig. 4). This missense mutation did not show any detectable change in ligand binding functions in our assays in comparison to the

![Fig. 3](image1.png)

**Fig. 3.** Analysis of PMP-BSA binding by the WT and mutant IGF2Rs. A, PMP-BSA ligand blot. Plasma membranes (100 μg of protein) were electrophoresed in duplicate on 6% SDS-PAGE under nonreducing conditions and then incubated with 1 nM 125I-PMP-BSA with or without 5 mM M6P for 18 h at 3°C. The membranes were washed, and the radioactivity was counted in a gamma counter. Columns, mean PMP-BSA cpm specifically bound (n = 3 replicates); bars, SE. The experiment shown is representative of those done with three sets of membranes from transfected 293T cells. Specific binding was calculated by subtracting the radioactivity bound in the presence of M6P from that in its absence, and the data have been expressed as a percentage of WT binding.

![Fig. 4](image2.png)

**Fig. 4.** Alignment of amino acid sequences from extracytoplasmic repeat 10 and repeat 11 regions of M6P/IGF2R of different species. Relevant regions of amino acid sequences deduced from cDNAs encoding the IGF2R or cation-independent MPR have been aligned to illustrate conservation of the amino acid residues investigated in mutant IGF2Rs. Sequences corresponding to the human (25, 35), bovine (34), mouse (22, 46), rat (47), and chicken (48) species have been analyzed; numbering is according to Oshima et al. (25).
WT IGF2R. It is possible that this allelic variant is merely a polymorphism with no altered characteristics as far as ligand binding to the receptor in vitro is concerned. However, further studies are under way to determine the effects of the WT versus mutant IGF2Rs on cellular responses to IGF-II, especially proliferation, and on uptake and degradation of IGF-II in IGF-II-responsive cells, to complete the phenotypic characterization of the IGF2Rs bearing the missense mutations. In addition, it is possible that the assays we have used in this study may not be sufficiently sensitive to detect small changes in the ligand binding properties of the receptor.

Another G:C-T:A transversion has been identified in a comedo breast carcinoma in situ with LOH resulting in the substitution of histidine for glutamine at residue 1445, located in repeat 10 of the IGF2R (5). Ligand binding analysis revealed that, when membranes were assayed for IGF-II binding very soon after preparation of the membranes, the IGF-II binding and cross-linking functions were equivalent to WT IGF2R. Interestingly, during storage at −80°C, a progressive loss of detectable IGF-II binding was observed (data not shown). Perhaps the most remarkable observation concerning this phenomenon was the lack of effect of storage at −80°C on the phosphomannosyl ligand binding, as radiolabeled PMP-BSA binding function of this mutant was comparable with the WT IGF2R in both fresh and frozen samples. However, the physiological significance of this unusual effect is not known.

Each of the extracellular repeat units of the IGF2R has been inferred to be capable of folding independently of each other, based on analogy with the CD-MPR, in which the entire extracellular domain is structurally homologous (14–28% identity) with each repeat of the IGF2R. There are eight conserved cysteinyl residues present in each repeat unit, with a few exceptions. Roberts et al. (33) have recently determined the three-dimensional structure of a truncated form of the CD-MPR encompassing the extracellular domain. The CD-MPR is composed primarily of β-strands organized into two β-sheets that form a flattened β-barrel. On the basis of the patterns of conservation of various cysteinyl residues in the bovine CD-MPR and IGF2R, Lobel et al. (34) have predicted the disulfide pattern to be: 1+2, 3+4, 5+7, and 6+8. This arrangement was essentially confirmed by the CD-MPR structure. It is interesting that three of the four mutations in IGF2R (Q1445H, G1449V, and I1572T) that show effects on ligand binding are clustered between the fourth and fifth cysteinyl residues in repeats 10 and 11. However, the G1464E mutation, which shows no apparent change in either IGF-II or PMP-BSA ligand binding, is located just COOH-terminal to the fifth cysteine in repeat 10. By extension of the sequence alignment between the extracellular domain of the CD-MPR and repeats 3 and 9 of the IGF2R done by Roberts et al. (33), we infer that glutamine 1445 and glycine 1464 are both located in repeat 10 within short β-strands on opposite sides of the β-barrel. In contrast, glycine 1449 is located in a turn between β-strands 4 and 5, which forms an important hinge or connecting region between the two β-sheets. Even more striking is the high degree of conservation of this particular glycyl residue, not just in repeat 10 of the IGF2R of different species, but in 11 of the 15 extracytoplasmic repeats of the bovine IGF2R (34), 9 of 15 repeats in the human receptor (25, 35), and also the extracellular domain of the CD-MPR (33, 36). Substitution of a bulky hydrophobic residue in this region might distort the hinge, causing abnormal protein folding, with consequent effects on ligand binding and/or instability of the mutant receptors. Thus, the more severe changes in ligand binding functions caused by the G1449V substitution have a basis in the structure of the receptor.

Local IGF-II levels are maintained by a delicate balance between synthesis and degradation. The latter process is mediated by IGF-II binding to the IGF2R, with subsequent ligand internalization and hydrolysis in the lysosomes. The IGF2 gene is often overexpressed in HCCs (15–20), with observed elevations of 10–20-fold higher in patients with HCCs associated with hypoglycemia than in normal liver (37). IGF-II is among the most potent mitogens known for breast cancer epithelial cells (38). Local high expression of IGF-II in the mouse mammary gland seems to accelerate tumor formation (39). Activation of TGF-β1, a potent inhibitor of epithelial cell growth (40, 41), by plasmin is facilitated by binding to the IGF2R through phosphomannosylated oligosaccharide side chains located in the propeptide domain of latent TGF-β1 precursor (42–45). Therefore, inactivation or alteration of the ligand binding functions of the IGF-II could lead to an increased extracellular concentration of IGF-II, a decreased level of activated TGF-β1, and misrouting with increased secretion of lysosomal enzymes. In conjunction with elevated IGF-II expression in HCCs and breast tumors, this combination of effects would be expected to result in increased cell proliferation, protection from apoptosis and enhanced local invasion and metastasis, suggesting that the M6P/IGF2R functions as a tumor suppressor in the liver and breast tissues. The frequent occurrence of M6P/IGF2R missense mutations in human cancers that result in loss or change in receptor function further supports this postulate.

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