Disruption of Ligand Binding to the Insulin-like Growth Factor II/Mannose 6-Phosphate Receptor by Cancer-associated Missense Mutations*

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The insulin-like growth factor II/mannose 6-phosphate receptor (IGF2R) carries out multiple regulatory and transport functions, and disruption of IGF2R function has been implicated as a mechanism to increase cell proliferation. Several missense IGF2R mutations have been identified in human cancers, including the following amino acid substitutions occurring in the extracytoplasmic domain of the receptor: Cys-1262 → Ser, Gln-1445 → His, Gly-1449 → Val, Gly-1464 → Glu, and Ile-1572 → Thr. To determine what effects these mutations have on IGF2R function, mutant and wild-type FLAG epitope-tagged IGF2R constructs lacking the transmembrane and cytoplasmic domains were characterized for binding of insulin-like growth factor (IGF)-II and a mannose 6-phosphate-bearing pseudoglycoprotein termed PMP-BSA (where PMP is pentamannose phosphate and BSA is bovine serum albumin). The Ile-1572 → Thr mutation eliminated IGF-II binding while not affecting PMP-BSA binding. Gly-1449 → Val and Cys-1262 → Ser each showed 30–60% decreases in the number of sites available to bind both 125I-IGF-II and 125I-PMP-BSA. In addition, the Gln-1445 → His mutant underwent a time-dependent loss of IGF-II binding, but not PMP-BSA binding, that was not observed for wild type. In all, four of the five cancer-associated mutants analyzed demonstrated altered ligand binding, providing further evidence that loss of IGF2R function is characteristic of certain cancers.

The insulin-like growth factor II/mannose 6-phosphate receptor (IGF2R)† has evolved in mammals to carry out multiple functions. Distinct regions in its extracytoplasmic domain interact with two classes of ligands, namely the mitogenic growth factor, insulin-like growth factor II (IGF-II), and proteins that bear a mannose 6-phosphate (Man-6-P) marker as a result of post-translational modification in the Golgi (1). The IGF2R is a 300-kDa type I transmembrane receptor that is comprised of a 40-residue NH₂-terminal signal sequence, followed by 15 homologous repeats made up of 124–192 amino acid residues, a 23-residue transmembrane domain, and a 167-residue cytoplasmic domain (2, 3). Functional mapping studies of the extracytoplasmic domain have revealed the location of distinct binding sites for both Man-6-P (4–6) and IGF-II (7–11).

In addition to being localized to unique regions of the receptor, the two binding functions are thought to serve different physiologic roles. Along with the cation-dependent mannose 6-phosphate receptor (CD-MPR), the IGF2R carries out lysosomal enzyme targeting through its Man-6-P binding activity (for review see Refs. 1, 12, and 13). This same Man-6-P binding function of the IGF2R has also been shown to be necessary for the activation of transforming growth factor-β (14), which bears Man-6-P residues in its secreted, prohormone form (15). After activation, transforming growth factor-β exerts effects on cellular proliferation by interacting with its own serine/threonine kinase receptors, usually resulting in growth inhibition (16–19). In contrast, IGF-II binding to the IGF2R at the cell surface is thought to result in internalization and degradation of the ligand, thereby down-regulating the level of this mitogenic factor (20, 21).

The multiple functions of the IGF2R suggest a role for this receptor as a growth inhibitor. In addition, several observations support the hypothesis that Man-6-P/IGF2R acts as a tumor suppressor gene. Microsatellite instability has been observed at the Man-6-P/IGF2R locus in tumors of the gastrointestinal tract (22, 23) and endometrium (22). Increased secretion of cathepsin D and other Man-6-P-bearing proteins has been observed in association with cancer of both the prostate and breast (24–26). Furthermore, loss of heterozygosity (LOH) at the Man-6-P/IGF2R locus has been correlated with poorly differentiated states in early breast carcinomas (27). IGF2R has also been found at decreased levels in hepatocellular carcinomas (28, 29), which may be explained by LOH at the Man-6-P/IGF2R locus in these tumor types (30–32).

Whereas the observation of LOH in tumor samples suggests that loss of receptor function may be involved in the progression to a transformed phenotype in these cancers, another hallmark of a tumor suppressor is the presence of loss-of-function mutations in copies of the gene remaining in the tumor cells. The screening of tumors that exhibit LOH has led to the discovery of several mutations (31–33), which could serve...
to strengthen the hypothesis that the IGFR2 is a tumor suppressor if these mutations somehow alter normal receptor function. Many of the identified mutations are frameshift or nonsense mutations that would prevent the translation of the complete, mature IGFR2 (33, 34). However, several missense mutations, many of which are located in the extracytoplasmic domain of the receptor, have also been identified (33, 34).

To address the question whether the five cancer-associated missense mutations affect the function of the IGFR2, receptor constructs bearing these mutations in the extracytoplasmic domain were assayed for their ability to interact with both IGF-II and a Man-6-P-bearing protein. The Cys-1262 → Ser, Gly-1449 → Val, Gly-1464 → Glu, and Ile-1572 → Thr mutations that were observed in hepatocellular carcinoma and the breast cancer-associated Gln-1445 → His mutation were expressed as soluble receptor constructs. Ligand binding analysis revealed that four of the mutants, all but Gly-1464 → Glu, altered either Man-6-P binding, IGF-II binding, or both. These alterations in ligand binding to the IGFR2 mutants suggest a mechanism for loss of receptor function that is consistent with the Man-6-P/IGF2R as a tumor suppressor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human IGFRs were provided by M. H. Niedenthal, Lilly. Carrier-free Na2125I (Amersham Pharmacia Biotech) was used for radioiodination of IGFR2 to specific activities between 40 and 85 Ci/mg by Enzymobead reagent (Bio-Rad). The native Y-2448 O-phosphomannan of *Hansenula holstii* was a gift from Dr. M. E. Slodki (retired). The pCMV5 vector (35) was kindly provided by Dr. David W. Russell (University of Texas Southwestern Medical Center). The 8.6-kilobase pair human IGFR2 cDNA (2) was a gift of Dr. William S. Sly (St. Louis University Medical Center). Other reagents and supplies were obtained from sources as indicated.

**Primer-Tagged Soluble Receptor Constructs**—A cDNA encoding the human IGFR2 (2) was cloned into pCMV5 using the 5’ Sall site and the 3’ XbaI site. The 5,157-nt fragment from nt 162 to 5319 of the receptor cDNA was removed by digesting with EagI followed by re-ligation. This smaller insert allowed for the addition of the 24-nucleotide FLAG epitope followed by two stop codons using amplification with Vent polymerase and two primers. The 5’ primer contained an Sall restriction site preceding the sequence corresponding to nt 94–113 of the receptor cDNA. The 3’ primer represented sequence complementary to nt 7602–7620 at the carboxyl terminus of repeat 15 in the receptor cDNA followed by 24 nt encoding the FLAG epitope, (DYKDDDDK), two stop codons, and an EagI site and the 3’ restriction site preceding the sequence corresponding to nt 3847–6315 in pCRII (Invitrogen). Complementary primer MI (Q1445H) mutation; (3) nt 4840–4874 substituting T to C at nt 4862

**Expression of the Wild-type and Mutant Receptor Constructs in 293T Cells**—Transient expression of the constructs was carried out in 293T human embryonic kidney cells cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum plus 5 μg/ml gentamycin at 37 °C in 5% CO2. The transfections were carried out by a modification of the calcium phosphate method described previously (36). The major changes to the published protocol were that the cells were grown in the presence of 5 μg/ml gentamycin, and the chloroquine shock was not applied. Conditioned medium was prepared by replacing the transfection media with serum-free Dulbecco’s modified Eagle’s medium on day 3 and was collected on day 6 following transfection. Freeze/thaw cell lysates were prepared on day 6 by suspending the cells in 0.5 ml of 150 mM NaCl, 10 mM HEPES, pH 7.4, and freezing and thawing the cells 4 times at −80 °C, followed by centrifugation and collection of the supernatant. Finally, Triton X-100 cell extracts were prepared on day 5 or day 6 following transfection with a solution containing Triton X-100 (final conc 10 mM HEPES, pH 7.4) as described earlier (10). Protein concentrations of the crude cell lysates and extracts were determined using the bicinchoninic acid assay (Sigma).

**Immunoblot Analysis of Cell Lysates for Quantification of Receptor Construct Expression**—Immunoblot analysis was conducted using the M2 anti-FLAG antibody (VWR Scientific, Chicago, IL) on 0.2 ng of cell lysate protein. The cell lysate aliquots were electrophoresed on 6% reducing SDS-PAGE gels and transferred to BA85 nitrocellulose paper. The blots were blocked with 3% nonfat milk in 15 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1%Tween 20 and probed with the M2 anti-FLAG antibody (1:1000 dilution) followed by a secondary rabbit anti-mouse IgG (Dako). The resultant antibody complex was detected with 125I-labeled goat IgG (Amersham). Molecular weight markers were used to determine the molecular mass of the epitope-tagged proteins. The resultant antibody complex was developed with 125I-labeled goat IgG (Amersham). Molecular weight markers were used to determine the molecular mass of the epitope-tagged proteins. The data were fit to a model for one-site competitive binding using GraphPad Prism software.

**Analysis of Mannose 6-Phosphate Binding**—Mannose 6-phosphate (PMP) was hydrolyzed and purified from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The PMP-BSA was collected from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37).

**Analysis of Mannose 6-Phosphate Binding**—Pentamannose phosphophosphate (PMP) was hydrolyzed and purified from a yeast cell wall phosphomannan following the procedure of Murray and Neville (37). The product of the hydrolysis was conjugated to BSA following the procedure of Brawulke et al. (38). Briefly, 15 mg/ml BSA was incubated in the presence of 0.2 μCi PMP and 160 μCi Na2125I at 37 °C for 4–5 days. The resultant product was purified on a 30-ml G-50 Sephadex column in phosphate-buffered saline. The flow-through fractions were collected, pooled, and stored at −20 °C. Aliquots of the protein (25 μg) were subjected to a specific activity of 30 μCi/μg by incubation in 0.5 mM phosphate buffer, pH 7.4, with 2 μCi of Na2125I using pre-coated IODOGEN tubes (Fisher) for 25 min. The product was separated from free iodine on a G-50 column. The iodinated PMP-BSA was collected from the flow-through fractions and stored at −20 °C until use. Binding
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RESULTS

Expression of Wild-type (WT) and Mutant IGF2R Constructs—To address the question of how the cancer-associated missense mutations, occurring in the extracytoplasmic domain of the IGF2R (Fig. 1), affect the ligand binding functions of the receptor, the mutations were incorporated individually into truncated receptor constructs that contain all 15 repeats of the extracytoplasmic domain of the IGF2R followed by an 8-residue FLAG epitope tag. These constructs and the empty pCMV5 vector, as a control, were transiently expressed in 293T human embryonic kidney cells. Conditioned medium, freeze/thaw lysates, and Triton X-100 cell extracts were analyzed for the presence of the constructs. Surprisingly, none of the WT 15F constructs were secreted into the media but were found at high levels in both the freeze/thaw and Triton X-100 cell extracts (data not shown). Because they contained the highest levels of transfected construct, the Triton X-100 cell extracts were analyzed for relative expression levels by an M2 anti-FLAG immunoblot (Fig. 2) and were used as a source of the constructs in the remaining experiments. The WT 15F IGF2R construct and all of the mutant cDNA constructs were capable of making proteins. The levels of expression for each construct were quantified by PhosphorImager analysis and were found to be nearly equivalent, depending on the transfection. For example, although in Fig. 2 the I1572T mutant appears to be expressed to about half the level as the other constructs, this difference was not apparent in lysates from two other transfections (data not shown).

IGF-II Binding Analysis—Based on the PhosphorImager data, equal amounts of receptor constructs were immunoadsorbed to M2 anti-FLAG resin so that detailed analysis of IGF-II binding could be made by affinity cross-linking. Initially, the immunoadsorbed receptors were incubated in the presence of 125I-IGF-II, cross-linked with 0.25 mM DSS, and resolved on a 6% SDS-PAGE gel followed by autoradiography (Fig. 3A). Quantification of the amounts of displaceable 125I-IGF-II present in the 250-kDa cross-linked bands was carried out by PhosphorImager analysis. Both the Q1445H and G1464E mutant receptors showed the same amount of affinity labeling as the WT 15F, whereas the I1572T mutant demonstrated a complete loss of IGF-II affinity labeling under these conditions. The C1262S mutation caused an approximately 90–95% reduction in the intensity of the receptor/ligand band, and the G1449V mutation diminished the intensity of the cross-linked band by approximately 60%.

To determine if the changes in affinity labeling among the C1262S, G1449V, and I1572T mutant receptors were due to decreased ligand binding, a more direct assay of IGF-II binding was used. The immunoadsorbed receptor constructs were incubated with 2 nM 125I-IGF-II for 3–4 h at 3 °C. The amount of bound ligand was determined by washing the resin pellet and

analysis with this ligand was conducted in much the same way as for IGF-II. Typically, aliquots of immunoadsorbed receptor constructs were incubated at 3 °C for 5–4 h in HBST plus 1% BSA in the presence of 1 nM 125I-PMP-BSA, with or without 5 mM Man-6-P. The resin pellets were then washed with HBST and counted in a gamma counter to determine the amount of binding. Western ligand blotting was performed on cell lysates with 125I-PMP-BSA following a modified procedure published earlier for 125I-IGF-II detection of IGF-binding proteins (IGFBPs) (39). Cell lysates (0.2 mg) were electrophoresed on 6% SDS-PAGE and electrotblotted to BA85 nitrocellulose. The proteins were renatured, and the blots were blocked with 1% BSA. Affinity for PMP-BSA was detected by probing the blots with 1.5 × 106 cpm 125I-PMP-BSA in 8 ml of blocking solution for 16–24 h at 3 °C. The blots were then washed and exposed to x-ray film. Competitive binding analysis of receptor constructs using PMP-BSA was conducted as described for IGF-II. Briefly, equal amounts of immunoadsorbed receptor construct were incubated with 1 nM 125I-PMP-BSA in the presence of increasing amounts of unlabeled ligand. The amount of bound ligand was determined, and regressions for single-site competitive binding were calculated.

IGF-II and PMP Affinity Depletion Analysis—Equal amounts of wild-type, C1262S, and G1449V receptor constructs (approximately 5 mg of transfected 293T cell lysates) were immunoadsorbed to 0.5 ml of anti-FLAG M2 resin (Sigma) in the presence of 5 mM Man-6-P for 4 h at 3 °C. The resin was then collected and washed 4 times with 1 ml of HBST. The receptor constructs were then eluted with 0.5 ml of 1 mg/ml FLAG M2 resin (Sigma) in the presence of 5 mM Man-6-P for 4 h at 3 °C.

FIG. 1. Schematic illustration of the IGF2R showing the cancer-associated missense mutations. The overall structure of the human IGF2R is shown, with emphasis on the extracytoplasmic repeats thought to be involved in ligand binding and the position of the cancer-associated missense mutations. The extracytoplasmic repeats are represented by rectangles and are numbered from the amino terminus according to Lobel et al. (3). The arginine residues thought to coordinate interactions with Man-6-P (8) are also indicated.

FIG. 2. Expression of WT and mutant IGF2R constructs in 293T cells. The control vector (CMV5) and each of the IGF2R constructs were transfected into 293T cells. Cell lysates (0.2 mg of protein) prepared on days 5 or 6 after transfection were resolved by SDS-PAGE, and the G1464E mutant receptors showed the same amount of affinity labeling as the WT 15F, whereas the I1572T mutant demonstrated a complete loss of IGF-II affinity labeling under these conditions. The C1262S mutation caused an approximately 90–95% reduction in the intensity of the receptor/ligand band, and the G1449V mutation diminished the intensity of the cross-linked band by approximately 60%.

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Radioactivity retained in the presence of 1 M IGF-II was subtracted from each concentration of unlabeled IGF-II, and IC50 values were calculated. The WT receptor had an IC50 of 5.3 nM, whereas C1262S and G1449V showed no significant difference from WT (Fig. 4A). However, the C1262S and G1449V mutant constructs demonstrated decreases in Bmax of 43 and 61% relative to WT. The WT receptor had an IC50 of 5.3 nM, whereas C1262S and G1449V showed no significant difference from WT with IC50 values of 3.4 and 4.7 nM, respectively.

The competitive binding data for IGF-II were also analyzed by Scatchard plot analysis (Fig. 4B). The WT 15F receptor construct demonstrated a knockout of IGF-II binding, whereas G1449V caused a 36.5 ± 8.2% reduction in binding. The C1262S mutation, which caused a nearly complete obliteration of affinity labeling, demonstrated only a 51.5 ± 5.9% abrogation of IGF-II binding, suggesting a change in the IGF-II cross-linking efficiency for this mutated receptor construct.

To determine if these alterations in IGF-II binding caused by C1262S and G1449V were due to changes in either the affinity or the number of available binding sites (Bmax), competitive binding analysis was carried out (Fig. 4A). Equal amounts of WT and mutant 15F constructs were immunoadsorbed to M2 resin and incubated with 2 nM 125I-IGF-II in the presence of increasing concentrations of unlabeled IGF-II. The amount of radiolabeled IGF-II bound at equilibrium was determined for each concentration of unlabeled IGF-II. The data were fit to a single binding site model using GraphPad Prism software, which is represented as a line. Scatchard plots were prepared by replotted the data in A as Bound/Free versus Bound. Linear regression analysis showed that in each case, the major change in binding is due to a decrease in Bmax.

### Analysis of Man-6-P Binding Function

Two approaches were employed to probe the Man-6-P binding function of the receptor constructs. First, a Western ligand blotting procedure.
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A. Western ligand blotting was conducted to reveal affinity for Man-6-P-bearing ligands. Cell lysates (0.2 mg of protein) were transblotted to nitrocellulose following SDS-PAGE. The endogenous receptor and transfected receptor constructs were renatured and probed with 1.5 × 10^6 cpm of 125I-PMP-BSA. The autoradiogram shows both the higher molecular weight endogenous IGF2Rs and the lower molecular weight 15F IGF2R constructs (arrow). B, PMP-BSA binding was measured by incubating equal amounts of receptor constructs immunoadsorbed to anti-FLAG resin or resin exposed to 0.2 mg of pCMV5-transfected 293T cells; subsequent direct binding reactions on stored cell lysates revealed a loss of IGF-II binding ability that was not observed for the WT receptor construct (Fig. 7A) or any of the other mutant proteins (data not shown). This loss was specific to the IGF-II binding function, as PMP-BSA binding was unaltered upon extended storage (Fig. 7B). To characterize this phenomenon further, IGF-II binding was assayed on Q1445H lysates frozen at −80 °C for different times (Fig. 7C). These results indicated that the Q1445H mutant underwent a sharp transition in its ability to bind IGF-II after about 10 days at −80 °C. Competitive binding and Scatchard plot analyses, using Q1445H receptor constructs that demonstrated about a 50% reduction in IGF-II binding, revealed that the loss of the IGF-II binding function was due to a change in B_max (Fig. 7D). This loss of binding function during storage at −80 °C was specific to the IGF-II binding function of only the Q1445H mutant, as the other mutant constructs demonstrated no difference from the WT over a 2-month period (data not shown).

IGF-II and PMP Affinity Depletion Experiments—One possible explanation for the decrease in B_max for binding IGF-II or PMP-BSA observed for some of the mutant IGF2R constructs is loss of high affinity ligand binding of a subpopulation of the receptors. To test this prediction, the existence of subpopulations of C1262S and G1449V mutant constructs differing in ligand affinity was investigated by chromatography on immobilized IGF-II or PMP. The constructs were first purified on anti-FLAG resin in the presence of 5 mM Man-6-P to remove endogenous phosphomannosylated ligands. They were then subjected to two to three serial exposures to either PMP-Sepharose (Fig. 8, A and B) or IGF-II-Sepharose (Fig. 8, C and D). The amount of unbound receptor construct remaining in solution after each exposure was determined by anti-FLAG immunoblot analysis of the supernatant. Surprisingly, the C1262S and G1449V mutants were able to bind the immobilized ligands to the same extent as WT upon successive exposures to each resin. The C1262S mutant bound to both PMP- and IGF-II-Sepharose resins to the same extent as the WT construct; about 90–95% bound after only one exposure to either resin. The G1449V mutant, however, demonstrated a slower association with both complexes.
resins in comparison to the WT and C1262S constructs, with 52% of the receptor still present after one exposure to PMP-Sepharose and about 26% remaining after one exposure to IGFIISepharose. Whereas the G1449V mutant was less able to bind the immobilized ligands after one round of the depletion when compared with both the WT and C1262S constructs, it demonstrated almost complete binding after 2 exposures to PMP-Sepharose or 3 exposures to IGFIISepharose (Fig. 8, A and B). No evidence for binding-incompetent subpopulations of either mutant construct could be detected in this assay.

DISCUSSION

The observation of several missense mutations occurring in the extracytoplasmic domain of the IGFI2R in specific tumors raises the question of whether and how these mutations might alter the function of this protein. Sequence analysis of the positions corresponding to the C1262S, Q1445H, G1449V, G1464E, and I1572T mutations in the human, rat, and bovine receptors revealed that all but G1464E are conserved without exception, suggesting that they are important in the overall structure and function of their respective repeats. In order to determine how these five Man-6-P/IGFI2R missense mutations affect the ligand binding functions of the receptor, the mutants have been tested as IGFI2R cDNA constructs comprised of the signal sequence and the 15 extracytoplasmic repeats, followed by an 8-residue FLAG epitope tag at the carboxyl terminus. This 15F construct allows isolation and separation of the mutants from the endogenous receptor background found in most cell types, by virtue of its unique epitope tag.

When expressed transiently in 293T cells, the 15F construct was not secreted into the culture medium but was found at high levels in cell lysates prepared by either Triton X-100 extraction or freeze/thaw procedures. This finding was unexpected, as a detergent-free solution by freeze/thaw indicates that it is water-soluble. Retention of the 15F construct within the cell may also indicate the presence of an independent intracellular retention signal in the extracytoplasmic domain. Such a signal has been proposed to exist by Dintzis et al. (42), who found that the IGFI2R extracytoplasmic domain was required for a predominantly intracellular localization of epidermal growth factor receptor/IGFI2R chimeras. Further experiments are under way to determine the significance and molecular basis of 15F retention within the transfected 293T cells.

Triton X-100 cell lysates were used for the remainder of the current study, because they contained the highest levels of transfected construct. Immunoblotting of these lysates revealed that mRNAs derived from all of the mutant cDNAs were predominantly intracellular localization of epidermal growth factor receptor/IGFI2R chimeras. Further experiments are under way to determine the significance and molecular basis of 15F retention within the transfected 293T cells.

To test whether the mutations affect ligand binding to the IGFI2R, the mutant receptor constructs were analyzed for the ability to interact with IGFI-II and PMP-BSA, a Man-6-P-bearing ligand. It has been shown previously that the two Man-6-P-binding sites localize to repeats 1–3 and 7–9 of the extracytoplasmic domain (5, 6). In particular, Arg-435 and Arg-1334 in repeats 3 and 9 of the bovine receptor are thought to be involved in coordinating interactions with the phosphate groups of Man-6-P-bearing proteins (6, 43). Previous studies have shown repeats 11 and 13 to be important for high affinity IGFI-II binding (8–11). With binding functions mapping to such an extensive portion of the receptor, mutations occurring in the extracytoplasmic domain could disrupt one or both of its binding activities.
the IGF-II binding domain. These mutations are located in repeats 9, 10, and 11, respectively. The hepatocellular carcinoma-associated mutant, I1572T, substituting a polar residue, Thr, for a bulky, hydrophobic residue, Ile, in the heart of the IGF-II binding domain, causes a complete knockout of IGF-II binding. This mutation had previously been shown to knock out IGF-II binding in a truncated receptor construct comprising the first half of repeat 1 fused to repeat 11 (10). It is less obvious how C1262S and G1449V, which reside outside the IGF-II binding domain, are capable of affecting IGF-II binding. Several possibilities could account for this effect. Destabilization of a single repeat may foster interdomain effects. Also, disruption of the conserved disulfide bonding pattern of the repeats may have global effects, which may account for the observed ligand binding disruption caused by the C1262S mutation. This cysteinyI residue is thought to be involved in the conserved disulfide bonding pattern of the 9th repeat (3). The disulfide bond pattern appears to be disrupted to a degree that when electrophoresed on a non-reducing SDS-PAGE gel, the mobility of the C1262S mutant is altered relative to WT (data not shown).

In addition to disrupting IGF-II binding, C1262S causes a decrease in IGF-II cross-linking efficiency when compared with WT 15F. One possible interpretation of these data is that C1262S causes the displacement of a lysyl residue away from radiolabeled IGF-II in the bound state. The homobifunctional cross-linking agent employed in these studies, DSS, utilizes lysyl side chains for its cross-linking chemistry. With a spacer arm length of 11.4 Å, small alterations of the protein backbone could be responsible for decreased cross-linking efficiency. The G1449V mutation results in the substitution of a bulky, hydrophobic residue, Val, for a small, neutral residue, Gly, that would be predicted to have a high degree of conformational freedom (44). This mutation, also located in repeat 10, outside the major IGF-II- or Man-6-P-binding regions of the receptor, is capable of decreasing the binding interactions of the 15F construct. However, in contrast to the C1262S mutation, the G1449V mutation does not seem to affect the efficiency of IGF-II/IGF2R cross-linking.

The C1262S and G1449V mutations also affect interactions with the Man-6-P-bearing ligand, PMP-BSA. C1262S failed to show any detectable binding to PMP-BSA in the Western ligand blot, whereas G1449V showed variable ability to bind PMP-BSA in this assay. The ligand blotting protocol involves denaturing the proteins with subsequent renaturation after non-reducing SDS-PAGE. Because of this property of the assay, not only are inherent binding interactions detected, but the ability of these mutant receptors to renature on the nitrocellulose membrane also contributes to the measured end point. It was necessary to determine if the mutant receptors were capable of binding PMP-BSA in an assay that did not involve a cycle of denaturation-renaturation. Direct binding analysis confirmed that both C1262S and G1449V have reduced PMP-BSA binding when compared with WT. These mutations did not completely eliminate PMP-BSA binding, as the ligand blot would suggest, but only reduced binding, as reminiscent of the IGF-II binding data. Thus, failure to detect PMP-BSA binding to these mutant IGF2Rs in the ligand blot suggests the possibility of a potential defect in in vitro refolding caused by these mutations.

Competitive binding analysis with both IGF-II and PMP-BSA revealed that the C1262S and G1449V mutations reduce binding by decreasing the number of binding sites (Bmax) in the population of receptors, while having no apparent effect on the relative affinity toward IGF-II or PMP-BSA. In addition, affinity depletion with either IGF-II or PMP-BSA covalently attached to Sepharose beads demonstrated that nearly all the C1262S and G1449V receptor molecules are eventually capable of interacting with ligand, even though the G1449V mutant showed a decrease in the rate of depletion with both immobi-

**FIG. 8.** PMP- and IGF-II-Sepharose depletion analysis. Aliquots (0.2 ml) of purified WT (●), C1262S (○), and G1449V (□) 15F IGF2R constructs were subjected to serial incubations with either PMP-Sepharose (A) or IGF-II-Sepharose (C) for 3-h periods at 3 °C. The amount of unbound construct at the end of each 3-h period was determined by anti-FLAG immunoblot analysis of each supernatant, followed by PhosphorImager analysis for both PMP-Sepharose (B) and IGF-II-Sepharose (D). The amount prior to any exposure to resin was set as 100%.
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It is interesting to note the location of each of the mutations examined in this study relative to the conserved cysteinyl residues within each repeat. Most of the repeats contain 8 conserved cysteinyl residues, and based on the patterns of conservation in the bovine receptor, these have been predicted to form disulfide bonds in the pattern: $1 + 2, 3 + 4, 5 + 7, 6 + 8$ (3). The CD-MPR is a 46-kDa membrane protein that shares sequence homology to each repeating unit of the IGF2R (3, 48). Recent analysis of the crystal structure of the CD-MPR revealed that the extracytoplasmic domain is made up of $\beta$-strands that comprise two $\beta$-sheets (49). By assuming that the repeating units in the extracytoplasmic domain of IGF2R are similar, then each of the extracytoplasmic repeats is comprised of these two $\beta$-sheet half-domains connected by a coil between the 4th and 5th cysteinyl residues. The I1572T mutation occurs near this putative linking region in the 4th $\beta$-strand. It is possible that this mutation alters the folded conformation of the repeat so that the final compact structure is not formed. Substituting a polar residue in place of the Ile at position 1572 could interfere with the van der Waals interactions between hydrophobic residues that are proposed to hold the two $\beta$-sheets together. Based on the homology to the CD-MPR, the substitution of Val for Gly at position 1449, which decreases binding to both IGF-II and PMP-BSA, may limit the conformational flexibility of the peptide backbone in a turn between the 5th and 6th $\beta$-strands. The Q1445H mutation also occurs near this turn within the 5th $\beta$-strand. Interestingly, the G1464E mutation, which shows no measurable change in its ligand binding characteristics, occurs just after the 5th conserved cysteinyl residue. The disulfide bond that occurs at this position may stabilize the overall structure of the 10th repeat containing the G1464E mutation.

If the observed mutations are capable of disrupting the conformation of the repeat in which they occur, the question still remains as to how disruption of a single repeat can obliterate functions that occur in other parts of the IGF2R. It has been postulated that each of the extracytoplasmic repeats is capable of folding independently of the others based on the analogy to the CD-MPR. This hypothesis has been supported by the fact that truncated receptor constructs are capable of binding IGF-II and Man-6-P ligands, suggesting that the two binding functions act independently of each other (6, 7, 10, 11). In addition, reciprocal inhibition of Man-6-P and IGF-II binding has been observed for the IGF2R suggesting some interaction between the binding sites for these two classes of ligands (50–52). The observation that the C1262S and G1449V mutations, which reside outside of the minimal IGF-II and Man-6-P binding domains, reduce ligand interactions implies that the domains do not act independently of each other.

In summary, we have demonstrated that four of five mutations found in association with LOH in human cancers have altered ligand-binding properties. Both the C1262S and G1449V mutations decreased the IGF-II and Man-6-P binding functions of the IGF2R. The I1572T mutation caused a complete loss of detectable binding to IGF-II, while leaving the Man-6-P binding function intact. Unlike these mutations, the Q1445H mutation caused a loss of IGF-II binding capability but only on extended storage at low temperature. These decreases in ligand interaction occurred as a result of changes in the number of binding sites without changing the apparent affinity of ligand-receptor complex formation. Overall, the observation that these cancer-associated mutations affect the normal function of the IGF2R supports the hypothesis that this receptor is involved in the progression of tumorigenesis.

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