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Binding Properties of Chimeric Insulin Receptors Containing the Cysteine-Rich Domain of either the Insulin-like Growth Factor I Receptor or the Insulin Receptor Related Receptor†

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ABSTRACT: We constructed and expressed chimeric receptor cDNAs with insulin receptor exon 3 (residues 191–297 of the cysteine-rich region) replaced with either the comparable region of the insulin-like growth factor I receptor (IGF-IR) or the insulin receptor related receptor (IRR). Both chimeric receptors still could bind insulin with as high affinity as the wild-type receptor. In addition, chimeric receptors containing exon 3 of the IGF-IR could also bind with high affinity both IGF-I and IGF-II. In contrast, chimeric receptors containing exon 3 of IRR did not bind either IGF-I, IGF-II, or relaxin. These results indicate that (1) the high affinity of binding of insulin to its receptor can occur in the absence of insulin receptor specific residues encoded by exon 3, the cysteine-rich region; (2) the cysteine-rich region of the IGF-I receptor can confer high-affinity binding to both IGF-I and IGF-II; and (3) the IRR is unlikely to be a receptor for either IGF-I, IGF-II, or relaxin.

The insulin receptor (IR)[1] is a disulfide-linked heterotetrameric membrane glycoprotein consisting of two extracellular α (M_r = 135 000) and two transmembrane β (M_r = 95 000) subunits [for review, see Roth (1990)]. The predominant labeling of the α subunit by ^{125}I-insulin in affinity cross-linking and photoaffinity labeling studies indicates that insulin primarily interacts with the α chain (Pilch & Czech, 1979; Jacobs et al., 1979; Yip et al., 1980). Subsequent studies by Waugh et al. (1989) and Yip et al. (1988, 1991) identified a proteolytic fragment of the α subunit that was linked to insulin as including amino acids 1–312 and 205–316, respectively.[2] The latter residues of the receptor are contained within a region of the α subunit that is particularly high in cysteines (Ebina et al., 1985; Ullrich et al., 1985) and is encoded by exon 3 of the IR gene (Seino et al., 1989).

In complementary studies, Andersen et al. (1990) and Gustafson and Rutter (1990) analyzed chimeric receptors containing various portions of the α subunit of IR and the homologous insulin-like growth factor I receptor (IGF-IR). The latter studies also implicated residues in the cysteine-rich region of the IR (residues 230–285) in determining hormone-binding specificity. We, therefore, set out to produce and characterize chimeric IR that contained the cysteine-rich region of IGF-IR (Ullrich et al., 1986) and the insulin receptor related receptor (IRR) (Shier & Watt, 1989) in place of the comparable region of the IR. To minimize disruptions in the structure of the IR, we chose to replace exon 3 (which encodes residues 191–297, the bulk of the cysteine-rich region) (Seino et al., 1989) of the IR with the comparable sequences of the other receptors since discrete functional and structural domains are often encoded by specific exons. The goals of the present studies were to (1) test whether the cysteine-rich region of the IGF-IR conferred high-affinity binding to both IGF-I and -II

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[1] Abbreviations: IR, insulin receptor; IGF, insulin-like growth factor; IRR, insulin receptor related receptor; IGF-I Ex3/IR, chimeric insulin receptor with exon 3 of the insulin receptor replaced with the comparable sequence of the IGF-I receptor; IRR Ex3/IR, chimeric insulin receptor with exon 3 of the insulin receptor replaced with the comparable sequence of the insulin receptor related receptor.

[2] B. Zhang and R. A. Roth, unpublished studies.

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since some studies suggested that these two ligands interact with different regions of the IGF-IR (Casella et al., 1986) and (2) determine whether the cysteine-rich region of IRR could confer high-affinity binding to either IGFs or relaxin since the ligand recognized by this new member of the IR family has not yet been identified (Shier & Watt, 1989).

Experimental Procedures

Construction of cDNA Clones Encoding Chimeric Receptors. A cDNA encompassing exon 3 of IRR (encoding amino acids 188–288) was obtained by amplification of human genomic DNA by polymerase chain reaction (PCR). The corresponding cDNA fragment that encodes amino acids 184–286 of IGF-IR was obtained by amplification of human IGF-IR cDNA (Steele-Perkins et al., 1988). For receptor chimera constructions, the gene splicing by overlap extension (gene SOEing) procedure (Horton et al., 1990) was performed. Briefly, a 191-bp cDNA fragment 5′ to exon 3 of IR was amplified by PCR. The forward/reverse primers for the amplification were 5′-TTGGCCACTATCGACTGGTC-3′/5′-CATGGGGGCAGGGGCAAACTTTCTGGCAGTGAC-3′ and 5′-TTGGCCACTATCGACTGGTC-3′/5′-ACACGTGCTTGGGCAAACTTTCTGGCAGTGACTA-3′ for IRR Ex3/IR and IGF-IR Ex3/IR chimeras, respectively. The underlined sequences in the reverse primers are complementary to the corresponding 5′ sequences of exon 3 of IRR and IGF-IR. The fragment contained a unique *Kpn*I restriction site at the 5′ end. Meanwhile, a 200-bp fragment 3′ to the exon 3 of IR was amplified by using 5′-CACCCGTAATAGCAGCAACTTGCTGTGCACCCC-3′/5′-TTTCTTCAATGAGGCCGA-3′ (for IRR Ex3/IR) and 5′-ACTCGCAACGGCAGCAACTTGCTGTGCAC-CCCA-3′/5′-TTTCTTCAATGAGGCCGA-3′ (for IGF-IR Ex3/IR) as primers. The underlined sequences correspond to the respective 3′ sequences of exon 3 of IRR and IGF-IR. This fragment contained a unique *Pvu*II site at the 3′ end. PCR was carried out in a buffer containing 10 mM KCl, 20 mM Tris-HCl, pH 8.8, 10 mM ammonium sulfate, 2 mM magnesium sulfate, 0.1% Triton X-100, 100 μg/mL bovine serum albumin (BSA), 200 μM each dNTP, and Vent DNA polymerase (New England Biolabs). The reactions consisted of 25 cycles of denaturation at 94 °C for 1 min, annealing at 55–62 °C for 1 min, and polymerization at 72 °C for 2 min. Amplified cDNA fragments were gel purified. Exons 3 of IRR or IGF-IR were first mixed with the 5′ IR fragments with the overlapping sequences, and 7–10 cycles of PCR were performed. The resulting DNA strands were then mixed with the 3′ IR fragments, and another 7–10 cycles of PCR were performed. The final products of the SOEing procedure were then digested with *Kpn*I and *Pvu*II and ligated into the compatibly digested SRα-IR (Zhang et al., 1991). The correct junctions and sequences of the chimeras were confirmed by DNA sequencing using the Sequenase II kit (United States Biochemicals).

Expression of the Wild-Type and Chimeric Receptors in COS-7 Cell Lines. COS-7 cells were maintained and transfected by calcium phosphate coprecipitation as described (Zhang et al., 1991).

Western Blot of the Receptors. Transiently transfected COS-7 cells were lysed in a buffer containing 50 mM Hepes, pH 7.6, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 150 mM NaCl, 20 mM sodium pyrophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate, 20 mM sodium fluoride, and 1 mg/mL bacitracin. The cell lysates were mixed with protein G–agarose beads (Genex) previously coated with monoclonal antibody 5D9 (Morgan & Roth, 1986) and rotated at 4 °C for 16 h. The beads were then washed three times with buffer A (20 mM sodium phosphate, pH 8.6, 150 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 0.02% sodium azide). The adsorbed proteins were analyzed on a 10% SDS–PAGE followed by electroblotting to a nitrocellulose filter. The precursor and the mature β subunit were detected with a rabbit polyclonal antibody directed against a peptide (residues 1300–1309) in the tyrosine kinase domain of the IR followed by an alkaline phosphatase conjugated anti-rabbit secondary antibody and a chromagenic substrate (Promega).

Ligand-Binding Studies. ^{125}I-Labeled insulin, IGF-I, IGF-II, relaxin, and monoclonal antibody 29B4 were prepared by the Iodo-Gen (Pierce) method, and their specific activities were 200, 204, 224, and 350 Ci/g, respectively. Forty microliters of the lysates of transfected COS-7 cells was added to 96-well poly(vinyl chloride) plates previously coated with monoclonal antibody 17A3 (Morgan & Roth, 1986). After overnight incubation at 4 °C, the wells were washed three times with buffer B (50 mM Hepes, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.1% Tween 20, 0.1% BSA). The radioactive ligands (40000 cpm/40 μL) were then added to each well, and after 12–16 h at 4 °C, the wells were washed three times with buffer B and counted. For the inhibition studies, unlabeled ligand was added along with the radioactive ligands (20000 cpm/40 μL) to each well.

Results

Expression vectors containing the cDNAs encoding either the wild-type IR or the IR containing exon 3 of the IGF-IR (IGF-IR Ex3/IR) or IRR (IRR Ex3/IR) were transiently expressed in COS-7 cells. The expressed proteins were first analyzed by immunoprecipitation with conformation-specific antibodies to either the extracellular domain of IR (5D9) (Morgan et al., 1986) or IGF-IR (αIR-3) (Kull et al., 1983). The immunoprecipitates were electrophoresed on SDS gels, transferred to nitrocellulose filters, and detected with an anti-peptide antibody directed against a sequence in the kinase domain of IR. Comparable amounts of wild-type and chimeric IR were precipitated with antibody 5D9 although IRR Ex3/IR was less completely processed to mature α and β subunits than native IR (Figure 1A). Chimeric IGF-IR Ex3/IR was equally precipitated with αIR-3 and 5D9 (Figure 1B), consistent with the prior studies indicating that αIR3 recognizes residues in the cysteine-rich region of the IGF-IR (Gustafson & Rutter, 1990). Control cells transfected with vector alone showed barely detectable levels of receptor precipitated with either antibody (Figure 1), confirming that in this system we are mainly detecting the expressed receptors (Zhang et al., 1991).

The expressed receptors were then captured for binding studies on microtiter wells coated with a monoclonal antibody (17A3) that binds equally the cytoplasmic domains of IR and IGF-IR (Morgan & Roth, 1986). To quantitate the amount of receptor captured, we utilized ^{125}I-labeled monoclonal antibody (29B4) to a distinct antigenic epitope of IR (Morgan et al., 1986). This assay demonstrated that comparable levels of the wild-type and chimeric IR were bound to the wells (Figure 2A). (The amounts of wild-type IGF-I receptor captured on the wells could not be quantitated since the labeled antibody poorly cross-reacts with the IGF-IR.) The captured receptors were then tested for their ability to bind labeled insulin, IGF-I, and IGF-II. IGF-IR Ex3/IR was found to bind much more ^{125}I-IGF-I and IGF-II than the wild-type IR (Figure 2C,D). Surprisingly, this chimeric receptor also bound

Table I: Displacement Data for the Different Receptors[a]

	IC_{50} (M)								
	^{125}I-insulin			^{125}I-IGF-I			^{125}I-IGF-II		
receptor	insulin	IGF-I	IGF-II	insulin	IGF-I	IGF-II	insulin	IGF-I	IGF-II
IR	$3.3 \pm 1.5 \times 10^{-10}$	$>10^{-7}$	$1.5 \pm 0.5 \times 10^{-8}$	ND	ND	ND	ND	ND	ND
IRR Ex3/IR	$3.2 \pm 0.9 \times 10^{-10}$	$>10^{-7}$	$3.3 \pm 0.1 \times 10^{-9}$	ND	ND	ND	ND	ND	ND
IGF-IR Ex3/IR	$1.6 \pm 0.7 \times 10^{-10}$	$5.0 \pm 2.0 \times 10^{-9}$	$7.2 \pm 0.4 \times 10^{-10}$	$8.0 \pm 0.4 \times 10^{-11}$	$2.1 \pm 0.6 \times 10^{-10}$	$7.5 \pm 2.2 \times 10^{-11}$	$6.7 \pm 0.4 \times 10^{-11}$	$1.2 \pm 0.4 \times 10^{-9}$	$3.6 \pm 1.1 \times 10^{-10}$
IGF-IR	ND	ND	ND	$>10^{-8}$	$1.8 \pm 0.2 \times 10^{-10}$	$4.1 \pm 0.2 \times 10^{-10}$	$>10^{-8}$	$1.6 \pm 0.2 \times 10^{-10}$	$1.8 \pm 0.3 \times 10^{-10}$

[a] Presented are the concentrations of unlabeled ligand that inhibited 50% of the specific binding in displacement studies. Results shown are means ± SE of two or three experiments all performed in triplicate. ND, not determined.

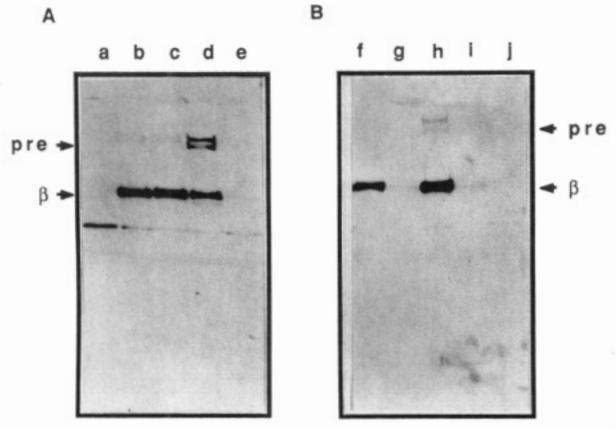

FIGURE 1: Immunoprecipitation and immunoblot of wild-type and chimeric receptors expressed in COS-7 cells. The expressed receptors were immunoprecipitated with a monoclonal antibody to either IR, 5D9 (A), or IGF-IR, αIR-3 (B), and detected by immunoblotting. The immunoblot bands correspond to the precursor (pre) and β subunit of the receptors (indicated by arrows). Since the antibody used in the blot only partially cross-reacts with the IGF-IR, the amounts of wild-type IGF-IR observed (lanes e and f) are an underestimate of the amount precipitated. The samples are vector control (a, j), IR (b, i), IGF-IR Ex3/IR (c, h), IRR Ex3/IR (d, g), and IGF-IR (e, f).

as much insulin as native IR (Figure 2B). In contrast, IRR Ex3/IR did not bind significantly more IGF-I or -II than receptor captured from control cells transfected with vector alone (Figure 2C,D). However, this chimeric receptor did bind significantly more insulin than the control cells (about one-third the amount of wild-type IR) (Figure 2B).

To further study the affinity of the different receptors for the three ligands, we performed competition studies. ^{125}I-Insulin was almost equally displaced by unlabeled insulin from the wild-type receptor and two chimeric receptors (Figure 3A). Concentrations of insulin that inhibited 50% of the binding (IC_{50}) were 0.33, 0.32, and 0.16 nM for IR, IRR Ex3/IR, and IGF-IR Ex3/IR, respectively (Table I). In addition, IGF-I was also a very potent inhibitor of insulin binding to IGF-IR Ex3/IR (IC_{50} = 5.0 nM) but not to IRR Ex3/IR and wild-type IR (Table I). Relaxin at concentrations as high as 1 μM also did not displace the binding of insulin from IRR Ex3/IR.

The binding of ^{125}I-IGF-I was also almost equally displaced by IGF-I from IGF-IR Ex3/IR and the native IGF-IR (Figure 3B). However, insulin potently displaced labeled IGF-I from the chimeric receptor (IC_{50} = 0.08 nM) but not from IGF-IR (Table I). Another difference between these two receptors was that monoclonal antibody αIR-3 inhibited the binding of labeled IGF-I to IGF-IR but not to IGF-IR Ex3/IR (Figure 3B). Finally, the binding of ^{125}I-IGF-II was almost equally displaced by IGF-II from IGF-IR Ex3/IR and IGF-IR (Figure 3C) but was readily displaced by insulin from only the chimeric receptor (Table I).

Discussion

Prior cross-linking and chimeric receptor studies have implicated the cysteine-rich region of the insulin receptor family in binding ligand (Yip et al., 1988, 1991; Gustafson & Rutter, 1990). Thus, replacement of exon 3 of the IR (which encodes residues 191–297 of the cysteine-rich region) with the comparable region of the IGF-IR would be expected to yield a chimeric receptor with high affinity for IGF-I. The present studies show exactly this; IGF-IR Ex3/IR was found to bind IGF-I with affinity comparable to that of native IGF-IR (Figure 3B). The finding that this chimeric receptor also binds IGF-II with high affinity (Figure 3C) implicates the same region of the IGF-IR in interacting with this ligand. Prior studies had suggested that these two ligands bind to distinct regions of the IGF-IR since a monoclonal antibody (αIR-3) inhibited the binding of IGF-I but not IGF-II to this receptor (Casella et al., 1986). However, the mechanism whereby this antibody inhibits IGF-I binding does not appear to be via a simple steric blockage since this antibody still can bind to the chimeric IGF-IR Ex3/IR (Figure 1B) but does not inhibit the binding of IGF-I to this receptor (Figure 3B). These results are best explained by proposing that this antibody inhibits IGF-I binding to the native IGF-I receptor through an indirect mechanism, possibly via an antibody-induced conformational change (Steele-Perkins & Roth, 1990).

The present studies of IRR Ex3/IR demonstrate that the cysteine-rich region of the IRR identified by Shier and Watt (1989) does not confer high-affinity binding to IGF-I or -II. This conclusion is based on both the inability of this chimeric receptor to bind these two labeled ligands (Figure 2) and the relatively poor inhibition of labeled insulin binding to the chimeric receptor by unlabeled IGF-I or -II in comparison to IGF-IR Ex3/IR (Table I). In addition, this chimeric receptor did not appear to bind labeled relaxin nor did unlabeled relaxin inhibit insulin binding to it.[2] The extracellular domain of this chimeric receptor did appear to at least be partially folded correctly since a conformation-specific antibody (5D9) was capable of recognizing this protein (Figure 1) and since this chimeric receptor could still bind insulin with high affinity (Figure 3A). This binding of insulin was potently inhibited by antibody 5D9, indicating that the insulin was binding to IR-specific residues.[2] Thus, these results suggest that IRR is not a receptor for either IGF-I or -II or relaxin. However, until one can identify a ligand that binds to IRR, these results must be considered tentative since it is possible that the IRR requires more than the cysteine-rich region to confer high-affinity ligand binding.

Most surprising was the finding that both chimeric receptors were still capable of binding insulin with an affinity compa-

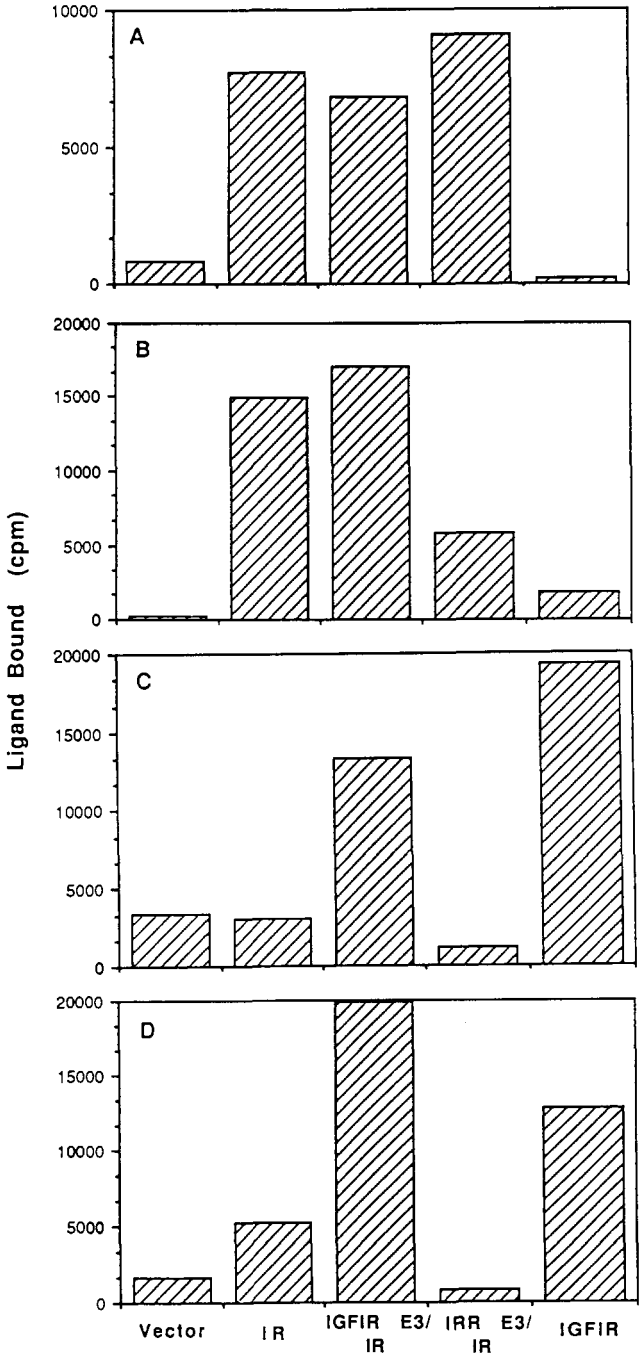

FIGURE 2: Binding of ^{125}I-labeled ligand to wild-type and chimeric receptors. Receptors from COS-7 cells transfected with either vector alone or expression vectors encoding IR, IGF-IR Ex3/IR, IRR Ex3/IR, or IGF-IR were adsorbed to microtiter wells and tested for the binding of ^{125}I-labeled antibody to IR, 29B4 (A), insulin (B), IGF-I (C), or IGF-II (D). The level of IGF-IR detected by 29B4 underestimates the amount present since this antibody only poorly recognizes the IGF-IR (Morgan et al., 1986). Results shown are means of triplicate determinations and are representative of three independent experiments.

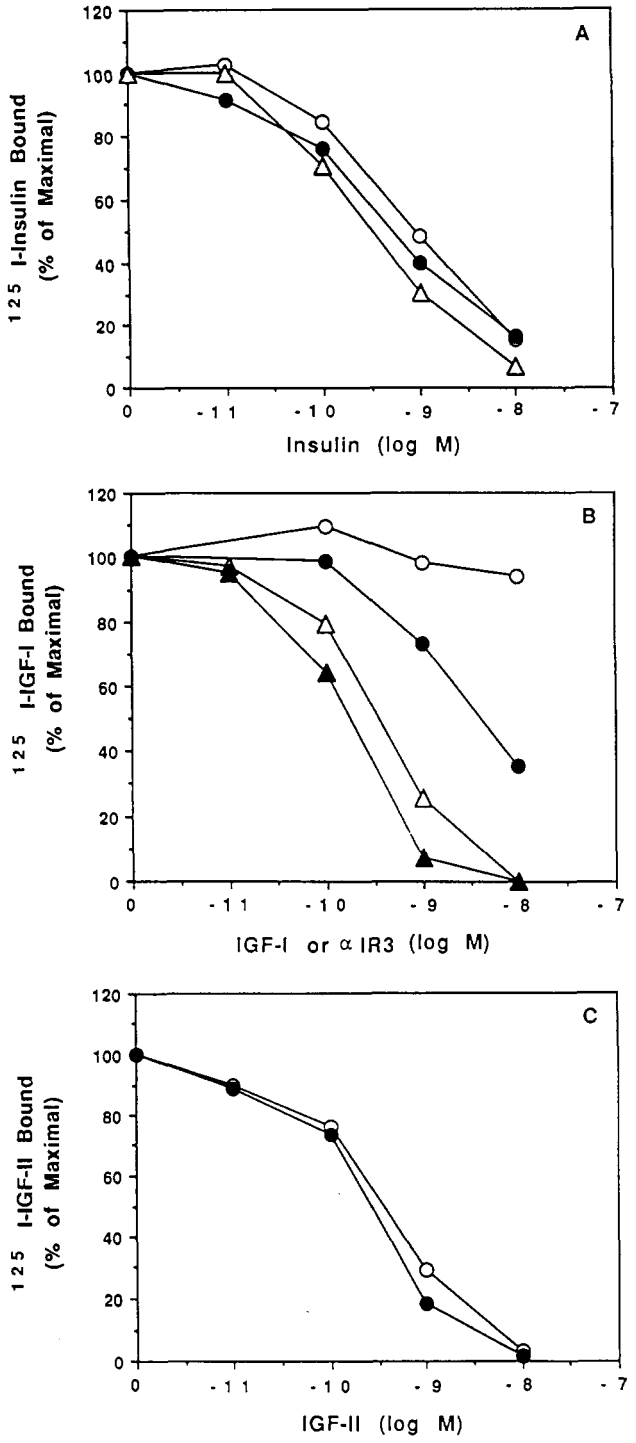

FIGURE 3: Inhibition of binding of ^{125}I-labeled ligands to wild-type and chimeric receptors: (A) competition of ^{125}I-insulin binding to IR (○), IRR Ex3/IR (●), and IGF-IR Ex3/IR (△) with insulin; (B) competition of ^{125}I-IGF-I binding to either IGF-IR (●, ▲) or IGF-IR Ex3/IR (○, △) by either αIR-3 (●, ○) or IGF-I (▲, △); (C) competition of ^{125}I-IGF-II binding to IGF-IR Ex3/IR (○) and wild-type IGF-IR (●) with IGF-II. Results shown are means of triplicate determinations and are representative of three independent experiments.

rable to that of the native IR (Figure 3A). The dissociation constants for IR, IRR Ex3/IR, and IGF-IR Ex3/IR were calculated to be 0.45 ± 0.1, 0.48 ± 0.1, and 0.41 ± 0.1 nM, respectively (means ± SE of three experiments). These results indicate that the IR-specific residues in the cysteine-rich region of the IR are not required for conferring high-affinity binding of insulin. It is, therefore, likely that insulin interacts with residues in other regions of the α subunit in addition to the cysteine-rich region. Evidence in support of this hypothesis was also obtained in the studies of Gustafson and Rutter (1990), since they found that monoclonal antibodies which inhibit insulin binding recognize a region carboxy to the cysteine-rich region (Gustafson & Rutter, 1990). In another study, biotinylated insulin was found to be linked to a peptide outside of the cysteine-rich region (including residues 20–120 of the α subunit) (Wedekind et al., 1989). Moreover, various mutations in the IR in residues outside of the cysteine-rich region have also been found to affect insulin binding (Kadowaki, H., et al., 1990; Kadowaki, T., et al., 1990; De Meyts

et al., 1990). Finally, recent preliminary reports by Andersen et al. (1991) of additional chimeric receptors of IR and IGF-IR also implicated other regions in insulin binding. Thus, it is most likely that ligand binding to the IR family involves interactions with a number of residues in the cysteine-rich region as well as outside of this region. In addition, it is possible that flexibility in the insulin and IGF molecules as well as in their receptors allows for a greater affinity of these ligands for the chimeric receptors than one might have expected on the basis of the interactions of these ligands with the native receptors.

Added in Proof

Since submission of this manuscript, we have produced a chimeric receptor containing exons 2 and 3 of IRR in place of the comparable region of the IR. This chimeric receptor was found not to bind either insulin, IGF-I, IGF-II, or relaxin. These results further support the conclusion that IRR is not the receptor for these hormones.

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Registry No. IGF-I, 67763-96-6; IGF-II, 67763-97-7; insulin, 9004-10-8; cysteine, 52-90-4; relaxin, 9002-69-1.

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