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# A region of the insulin receptor important for ligand binding (residues 450–601) is recognized by patients' autoimmune antibodies and inhibitory monoclonal antibodies

BEI ZHANG AND RICHARD A. ROTH

Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **Chimeric receptors containing different portions of the homologous human insulin receptor, insulin-like growth factor I receptor, and insulin receptor-related receptor were utilized to identify the epitopes recognized by various anti-insulin receptor antibodies. The antibodies studied included 12 monoclonal antibodies to the extracellular domain of the human insulin receptor as well as 15 patients' sera with autoimmune anti-insulin receptor antibodies. All of the patients' sera and all 8 monoclonal antibodies that inhibit insulin binding were found to recognize an epitope contained within residues 450–601 of the α subunit of the receptor. In contrast, 2 monoclonal antibodies that do not inhibit insulin binding were found to recognize the cysteine-rich region of the α subunit. Chimeric insulin receptors that had residues 450–601 replaced with the homologous residues of the insulin-like growth factor I receptor exhibited a decreased ability to bind insulin. In contrast, insulin-like growth factor I receptors that have had the comparable region replaced with that of the insulin receptor showed no decrease in their ability to bind ligand. These results indicate that residues 450–601 of the insulin receptor are important for insulin binding and include the major site for recognition by inhibitory monoclonal antibodies and patients' autoimmune anti-receptor antibodies.**

Insulin elicits its diverse biological responses by binding to a specific receptor (for reviews, see refs. 1–3). This protein has been extensively studied and it is known that it exists as a disulfide-linked heterotetrameric membrane glycoprotein consisting of two extracellular α (135 kDa) and two transmembrane β (95 kDa) subunits. Insulin primarily interacts with the α subunit since this subunit is predominantly labeled when $^{125}$I-labeled insulin ($^{125}$I-insulin) is cross-linked to the receptor (4–6). Recently a fragment of this subunit that was linked to insulin has been isolated and identified as containing residues 205–316 of the receptor (7, 8). These residues are within a region of the α subunit that is particularly high in cysteines (9, 10) and is encoded by exon 3 of the insulin receptor (IR) gene (11). These results have led to the proposal that this cysteine-rich region is responsible for high-affinity binding of insulin (7, 8).

However, linking of a biotinylated insulin to the receptor was shown to label a fragment (residues 21–120) at the amino terminus of the receptor (12). Moreover, mutant forms of the receptor with changes in either residue 15 or 89 in the amino terminus exhibited a decreased ability to bind insulin (13, 14). In complementary studies, chimeric receptors have been constructed between the human IR and either the homologous insulin-like growth factor I receptor (IGF-IR) or the insulin receptor-related receptor (IRR) (15–18). These studies have indicated that the cysteine-rich region of the IGF-IR can confer high-affinity binding of IGF-I and IGF-II to the IR (16–18). However, these studies indicated that the IR-specific residues in the cysteine-rich regions were not necessary for high-affinity insulin binding (17, 18). Instead, the amino-terminal 68 residues of the IR were found to confer high-affinity insulin binding to a chimeric IGF-IR (18). These results indicated that residues 1–68 were important in the high-affinity interactions of insulin with its receptor.

Numerous monoclonal antibodies to the extracellular domain of the human IR have been developed (19–22). Several of these antibodies are potent inhibitors of insulin binding. In addition, patients with extreme insulin resistance (type B) associated with acanthosis nigricans have autoantibodies that inhibit insulin binding to its receptor (23–26). In the present studies, we have utilized chimeric receptors to identify the epitope(s) recognized by the inhibitory monoclonal antibodies and the autoimmune antibodies. Eight different inhibitory monoclonal antibodies and 15 different patients' sera were all found to recognize an epitope contained within residues 450–601. In addition, chimeric IR with this region replaced with the comparable region of the IGF-IR were found to exhibit a decreased ability to bind insulin. These studies indicate that residues 450–601 constitute another region of the IR that is involved in the high-affinity binding of insulin to its receptor.

## MATERIALS AND METHODS

**Construction of cDNA Clones Encoding Chimeric Receptors.** Chimeric receptors were constructed using the gene splicing by overlap extension (gene SOEing) procedure (27). cDNA fragments encompassing exon 7 or 8 of IR and IGF-IR were obtained by PCR using cDNA clones for IR or IGF-IR as templates (17). 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 of bovine serum albumin per ml, 200 μM of each dNTP, and Vent DNA polymerase (New England Biolabs). The reaction 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. The forward/reverse primers are 5′-AGGAACCAAGGGGCGCCAAAG-CAAAGGGGA-3′/5′-GAGATTGGATCCAGGGGAATG-GAAGGAACTGAAG-3′ (containing a *Bam*HI recognition site) and 5′-TCCACAAGATGGAAGAAGTT-3′/5′-CTGAAAGAACGTCCAAGGGCACAGAGGGGTTGGT-3′, for amplification of exons 7 and 8 of IGF-IR or IR, respectively. In the forward primer, the underlined sequence is identical to the corresponding sequence of IR and the sequence shared by IR and IGF-IR is double underlined. The underlined sequences in the reverse primers are complementary to the corresponding sequences in IR or IGF-IR. To construct IGF-IR Ex7,8/IR, a 372-base-pair (bp) cDNA fragment 5′ to exons 7 and 8 of IR containing an *Esp* I site was amplified. This fragment was mixed

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Abbreviations: IR, insulin receptor; IGF, insulin-like growth factor; IRR, insulin receptor-related receptor.

with the IGF-IR exons 7 and 8 described above and 7 cycles of PCR were performed. The resulting fragment was digested with *Bam*HI and *Esp* I and subcloned into SR$\alpha$-IR (28) completely digested with *Esp* I and partially digested with *Bam*HI. For the construction of IR Ex7,8/IGF-IR chimera, a 512-bp fragment 3′ to exons 7 and 8 of IGF-IR that contained an *Eag* I site was amplified. This fragment was annealed to the fragment encoding exons 7 and 8 of IR (containing a 5′ *Nar* I site) and 7 cycles of PCR were performed. The product was digested with *Eag* I and *Nar* I and subcloned into compatibly digested SR$\alpha$-IGF-IR. To eliminate possible coding errors introduced by PCR, DNA sequences of the chimeric constructs were determined using the Sequenase II kit (United States Biochemical).

**Transient Expression of the Wild-Type and Chimeric Receptors in COS-7 Cell Lines.** COS-7 cells were maintained and transfected with constructs encoding wild-type or chimeric receptors using the calcium phosphate coprecipitation method as described (28).

**Western Blot of the Receptors.** Lysates of transiently transfected COS-7 cells were prepared 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 of bacitracin per ml. The proteins were analyzed on 10% SDS/PAGE followed by electroblotting to nitrocellulose filters. The blots were incubated with rabbit polyclonal antibodies against the $\beta$ subunit of either the IR (17) or the IGF-IR (a gift of Delong Liu and Lu-Hai Wang, Mount Sinai School of Medicine, New York). The bound antibodies were then detected by an alkaline phosphatase-conjugated anti-rabbit secondary antibody and a chromagenic substrate (Promega).

**Ligand-Binding Studies.** $^{125}$I-insulin, IGF-I, monoclonal antibody 29B4, or $\alpha$IR-3 was prepared by the Iodo-Gen (Pierce) method. The specific activities were 128, 276, 21, and 26 Ci/g (1 Ci = 37 GBq), respectively. Forty microliters of the appropriately diluted lysates was added to 96-well polyvinylchloride microtiter plates previously coated with monoclonal antibody 2G7 or 17A3. After 6 hr at 4°C, the wells were washed three times with buffer A (50 mM Hepes, pH 7.6/150 mM NaCl/0.1% Triton X-100/0.1% Tween 20/0.1% bovine serum albumin). The radioactive ligands (40,000 cpm/40 $\mu$l) were then added to the wells, and after 12–16 hr at 4°C, the wells were washed three times with buffer A and assayed for radioactivity. For the inhibition studies, unlabeled ligands at different concentrations were added along with the radioactive labeled ligands [30,000 cpm/100 $\mu$l for $^{125}$I-insulin or 40,000 cpm/40 $\mu$l for $^{125}$I-labeled IGF-I ($^{125}$I-IGF-I)] to each well. The amounts of adsorbed receptors in these and the following experiments were quantitated by measuring the binding of $^{125}$I-labeled 29B4 and $^{125}$I-labeled $\alpha$IR-3 ($^{125}$I-29B4 and $^{125}$I-$\alpha$IR-3) for insulin and IGF-I receptors, respectively.

**Determination of Monoclonal Antibody Binding Sites.** Microtiter plates were incubated with the different monoclonal antibodies against IR (50 $\mu$l per well of 10 $\mu$g of antibody per ml in 20 mM $NaHPO_3$, pH 9.6) for 12–14 hr at 4°C. After washing the wells three times with buffer A, 40 $\mu$l of lysates was added to the wells and the incubations were continued for 6 hr. The wells were then washed three times, and $^{125}$I-29B4 or $^{125}$I-17A3 (22 Ci/g) was added to the wells (100,000 cpm/40 $\mu$l). After 6 hr at 4°C, the wells were washed three times and assayed for radioactivity.

**Determination of Regions of IR Recognized by Autoantibodies from Diabetic Patients.** Lysates of COS-7 transiently expressing receptors were added to microtiter wells precoated with 17A3. After three washes with buffer A, dilutions of sera from the patients were added to the wells and they were further incubated at 4°C for 6 hr. The wells were then washed, and $^{125}$I-labeled goat anti-human IgG (50,000 cpm/40 $\mu$l) was added. After 6 hr at 4°C, the wells were washed three times and assayed for radioactivity.

## RESULTS

The five chimeric receptors utilized in the present study were (*i*) IRR Ex3/IR, an IR with residues 191–297 of the IR replaced with the comparable region of IRR (29); (*ii*) IGF-IR Ex3/IR, an IR with residues 191–297 replaced with the comparable region of the IGF-IR; (*iii*) IRR Ex2,3/IR, an IR with residues 12–297 replaced with the comparable region of IRR; (*iv*) IGF-IR Ex7,8/IR, an IR with residues 450–601 replaced with the comparable region of IGF-IR; and (*v*) IR Ex7,8/IGF-IR, an IGF-IR with residues 440–586 (the IGF-IR residues homologous to IR residues 450–601) replaced with the comparable region of the IR (Fig. 1). All five chimeric receptors and the native IR and IGF-IR were greatly overexpressed in comparison to the endogenous receptors in transient transfections of COS cells as detected by Western blots (Fig. 2 and ref. 17). Lysates of the COS cells overexpressing the different receptors were incubated in microtiter wells previously coated with the different monoclonal antibodies. After washing the wells, the bound receptors were detected by $^{125}$I-labeled monoclonal antibodies to epitopes in the cytoplasmic domains of the IR and IGF-IR. All of the monoclonal antibodies precipitated the overexpressed native IR but not the IGF-IR (data not shown). The amount of IR captured by the various antibodies differed due to their relative affinities for the receptor. For comparative purposes, the amount of chimeric receptor bound by each antibody is expressed as a percentage of native IR bound by that antibody.

Two monoclonal antibodies (83-7 and 3D-7) were found incapable of binding the two chimeric receptors that had residues encoded by exon 3 (191–297) of the IR replaced (Fig. 3, top two panels). These results indicate that these 2 antibodies are primarily directed against an epitope in the cysteine-rich region of the IR. As expected, these 2 antibodies also did not bind the chimeric IR with residues 12–297 replaced with those of IRR (Fig. 3, middle panel). However, the remaining 10 other monoclonal antibodies recognized this chimeric receptor. These results indicated that none of the monoclonal antibodies is directed against an epitope contained within residues 12–191.

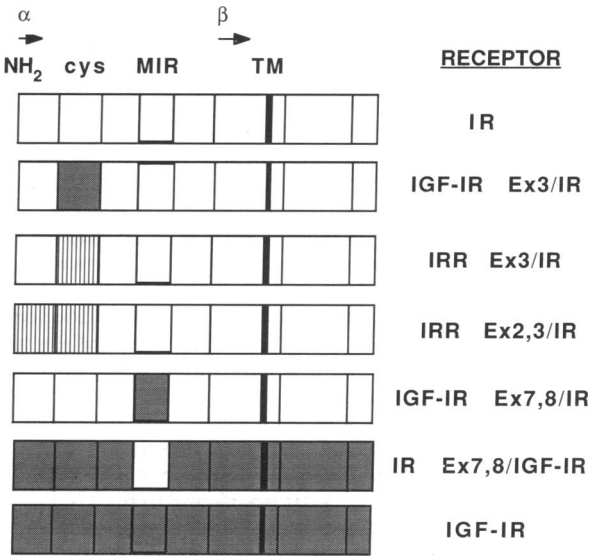


FIG. 1. Schematic of the chimeric and native receptors. The regions shown are the cysteine-rich region (cys), the major immunogenic region (MIR), which includes residues 450–601, and the transmembrane region (TM). The MIR is the region recognized by the eight inhibitory monoclonal antibodies and 15 patients' autoimmune antireceptor antibodies.

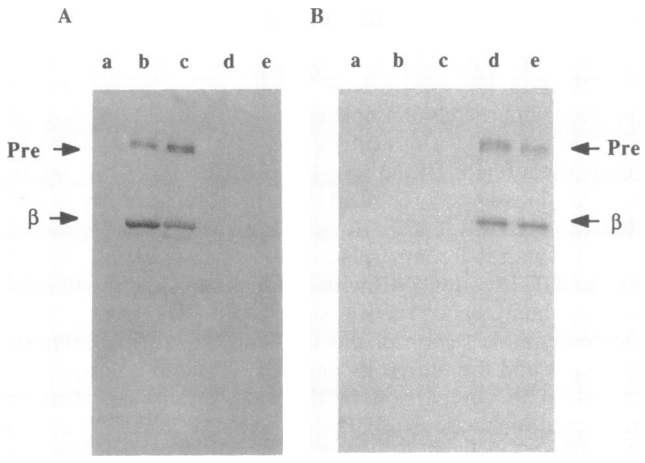


FIG. 2. Western blots of wild-type and chimeric receptors expressed in COS-7 cells. Lysates of the cells were analyzed on 10% SDS/PAGE followed by electroblotting to nitrocellulose filters. The filters were incubated with rabbit polyclonal antibodies to the β subunit of either the IR (*A*) or the IGF-IR (*B*). Arrows indicate protein bands corresponding to precursors (Pre) and mature β subunits. The samples are vector control (lanes a), IR (lanes b), IGF-IR Ex7,8/IR (lanes c), IR Ex7,8/IGF-IR (lanes d), and IGF-IR (lanes e).

Eight of the monoclonal antibodies (MA-5, MA-10, MA-20, 25-49, 83-14, 47-9, 5D9, and MC51) were found incapable of binding chimeric IR with residues 450–601 replaced with the comparable region of the IGF-IR (Fig. 3, fourth panel). These results suggested that these antibodies were directed against an epitope contained within these residues. Confirmation of this hypothesis was obtained by the finding that all of these antibodies did bind to a chimeric IGF-IR that had these residues of the IR in place of the comparable region of the IGF-IR (Fig. 3, bottom panel). In fact, these antibodies could bind to this chimeric receptor better than the native IR, possibly indicating that this region is more exposed in the chimera.

The remaining two monoclonal antibodies (18-44 and αIR-1) could bind the four chimeric IRs as well as the native IR (Fig. 3). These results indicate that these antibodies are not directed against any of the regions that have been changed in the present studies.

Fifteen different patients' autoimmune anti-IR antibodies were then tested for their ability to bind the different chimeric receptors (Fig. 4). All of the patients' antibodies could bind the chimeric receptors with residues 191–297 replaced (Fig. 4, top two panels). Several patients' antibodies (most notably, no. 13) showed a partial decrease in their ability to recognize the chimeric IR with residues 12–297 substituted with the IRR sequence (Fig. 4, middle panel). However, all 15 patients' antibodies showed a dramatic (>80%) decrease in their ability to bind chimeric IR lacking the IR-specific residues 450–601 (Fig. 4, fourth panel). Twelve of the antibodies could recognize the chimeric IGF-IR that contained residues 450–601 of the IR, although several of these only partially recognized this chimera (Fig. 4, bottom panel).

In our prior studies, IRR Ex3/IR and IGF-IR Ex3/IR were found to bind insulin with the same affinity as the native IR (17). In contrast, IRR Ex2,3/IR was found to bind only trace amounts of insulin, IGF-I, or IGF-II (data not shown). IGF-IR Ex7,8/IR was found to bind 19% ± 3% ($n$ = 7) as much insulin as wild-type IR when equal amounts of the two receptors (as detected by an $^{125}$I-labeled anti-receptor antibody) were immunoadsorbed on microtiter wells. Competitive binding studies (Fig. 5*A*) indicated that this chimeric receptor had ≈7-fold weaker affinity than native IR for insulin.

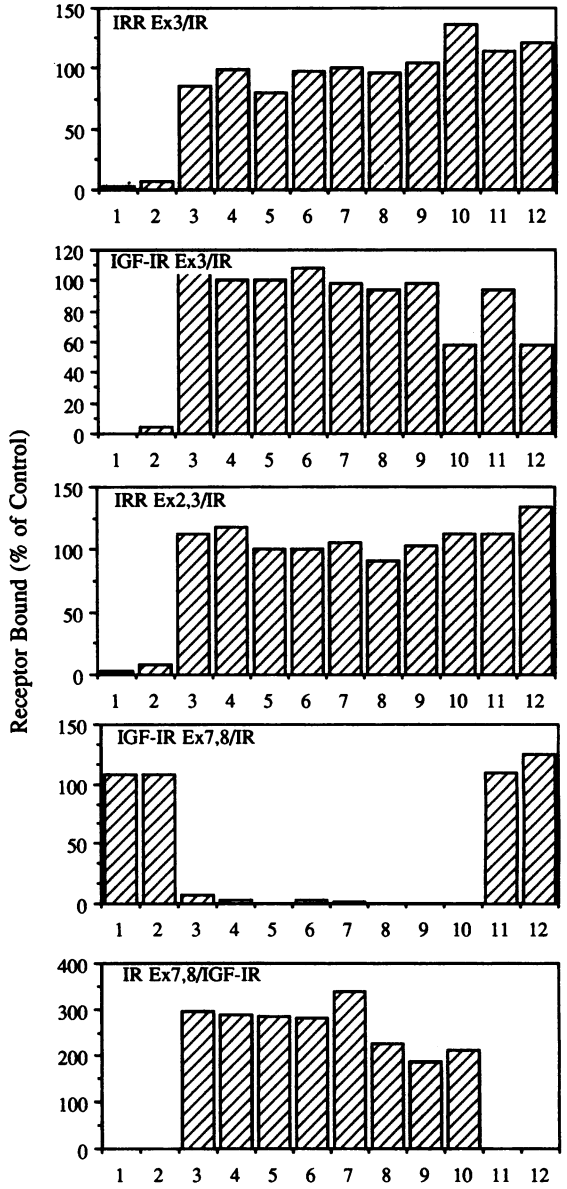


FIG. 3. Binding of the wild-type and chimeric receptors by the different monoclonal antibodies. Receptors transiently expressed in COS-7 cells were incubated with monoclonal antibodies adsorbed to microtiter wells. Bound receptors were detected with $^{125}$I-29B4 (top three panels) or $^{125}$I-17A3 (bottom two panels). The amount of chimeric receptor bound is expressed as a percentage of the wild-type IR bound by the same antibody. The monoclonal antibodies used are 83-7 (1), 3D-7 (2), MA-5 (3), MA-10 (4), MA-20 (5), 25-49 (6), 83-14 (7), 47-9 (8), 5D9 (9), MC51 (10), 18-44 (11), and αIR-1 (12). Results shown are means of triplicate determinations and are representatives of three independent experiments.

In contrast, the amount of $^{125}$I-IGF-I bound to IR Ex7,8/IGF-IR was equal to that found for the native IGF-IR. Competitive binding studies (Fig. 5*B*) indicated that this chimeric receptor had the same affinity as the native IGF-IR for IGF-I.

## DISCUSSION

In the present studies, we have examined the ability of 12 monoclonal antibodies to the extracellular domain of the human IR to recognize various chimeric IRs. Two of the monoclonal antibodies (83-7 and 3D-7) were found to recognize an epitope in residues 191–297, the cysteine-rich region (Fig. 3). This conclusion is consistent with the prior study of

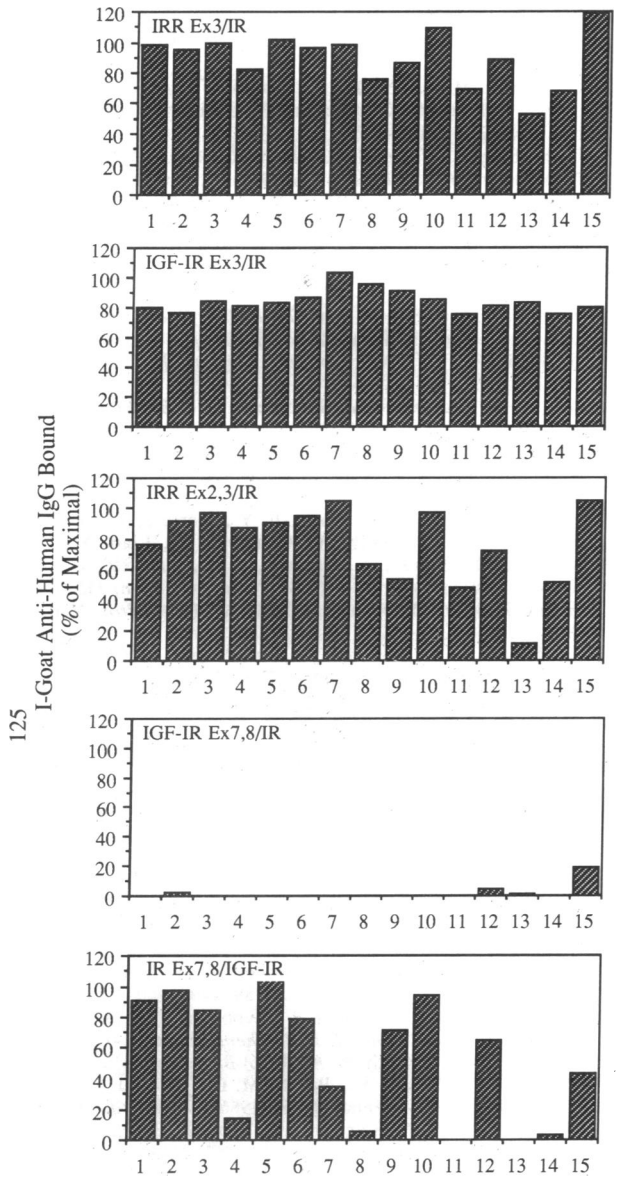


FIG. 4. Binding of the wild-type and chimeric receptors by the patients' autoantibodies. The indicated receptors were adsorbed to microtiter wells and then incubated with the patients' sera. The amount of bound human antibodies was quantitated with $^{125}$I-labeled goat anti-human IgG and expressed as the percentage of the amount bound to native IR. The patients' sera used (and their dilutions) are B3 (1:100) (1), B4 (1:500) (2), B5 (1:5000) (3), B6 (1:500) (4), B7 (1:4000) (5), B8 (1:4000) (6), B9 (1:500) (7), B10 (1:200) (8), B11 (1:100) (9), B14 (1:4000) (10), Ba (1:100) (11), Bb (1:5000) (12), Bc (1:100) (13), Bd (1:4000) (14), and Be (1:1000) (15). These dilutions were chosen as the minimum concentrations required to give at least a 2-fold increase in radioactivity bound over the vector control. Results shown are means of duplicate determinations and are representative of two independent experiments.

Schaefer *et al.* (30) showing that 83-7 recognizes an epitope in residues 1–324. Interestingly, neither of the antibodies to the cysteine-rich region inhibits insulin binding to the receptor (20, 21), although cross-linking studies have indicated that this region interacts with insulin (7, 8).

Two of the monoclonal antibodies (18-44 and αIR-1) recognized all four chimeric IRs tested, indicating that they did not recognize an epitope in residues 12–297 or 450–601 (Fig. 3). These findings are consistent with the prior study of Prigent *et al.* (31) indicating that 18-44 recognizes an epitope in residues 735–929 at the amino terminus of the β subunit.

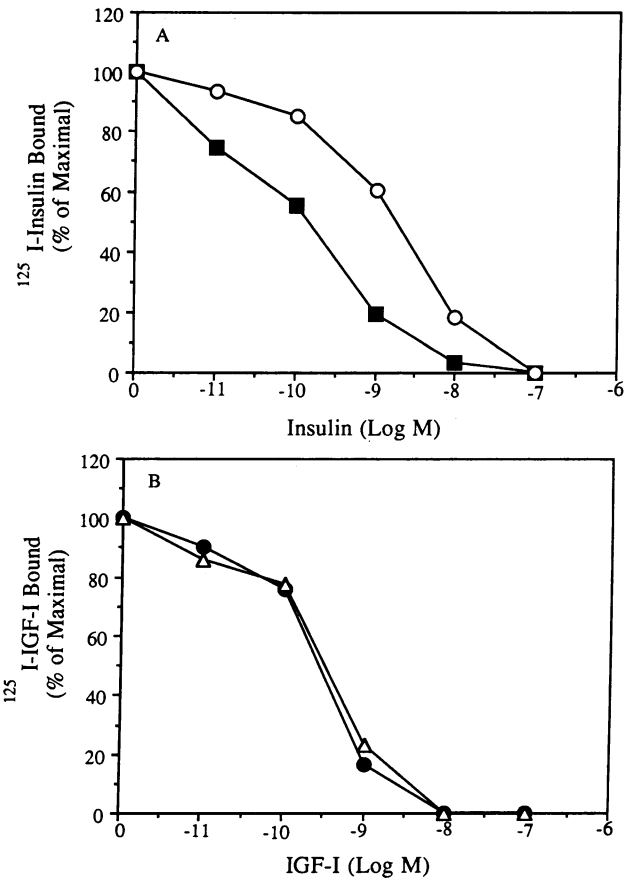


FIG. 5. Binding of $^{125}$I-labeled ligands to wild-type and chimeric receptors. Receptors were adsorbed to microtiter wells and then incubated with either $^{125}$I-insulin in the presence of increasing amounts of unlabeled insulin (*A*) or $^{125}$I-IGF-I in the presence of increasing amounts of unlabeled IGF-I (*B*). The receptors used were native IR (■), IGF-IR Ex7,8/IR (○), native IGF-IR (●), and IR Ex7,8/IGF-IR (△). Results shown are means of triplicate determinations and are representatives of three (*A*) or two (*B*) independent experiments.

Since this antibody does not inhibit insulin binding to its receptor, it may be that this region of the β subunit is not close to the insulin binding site.

The remaining 8 monoclonal antibodies (MA-5, MA-10, MA-20, 25-49, 83-14, 47-9, 5D9, and MC51) inhibit insulin binding to its receptor (20–22). All 8 of these antibodies were found to bind to an epitope in residues 450–601 of the IR (Fig. 3). These findings extend the prior study of Gustafson and Rutter (16) that showed that 3 of these antibodies (MA-20, 5D9, and MC51) recognize an epitope in residues 452–735. These 8 antibodies were produced in three different laboratories by procedures varying from injections of purified receptor to injections with intact cells (20–22). The properties of these 8 antibodies also differ considerably since several are potent mimickers of the biological actions of insulin, whereas others are antagonists of insulin (32–34). The finding that all 8 of these inhibitory antibodies recognize an epitope in the same region suggests that this region is close to an area that interacts with insulin.

Of the 12 monoclonal antibodies tested, none recognizes the mouse IR (19–22). A comparison of the sequences of the mouse (35) and human (9, 10) IRs helps to explain the present findings and to further define the epitopes recognized by these antibodies. The amino-terminal 191 residues of the two receptors exhibit only a single amino acid difference, possibly explaining the lack of antibodies generated to this region. In the region 450–601, all but one of the differences in amino acid

sequence are located in residues 538–550. These 13 amino acids are therefore likely to constitute at least part of the epitope recognized by the 8 inhibitory monoclonal antibodies.

Surprisingly, the anti-IR antibodies in 15 different patients also appeared to be primarily directed against epitope(s) in residues 450–601 (Fig. 4). At least one of these patients' antibodies (no. 13) also exhibited some reactivity with residues 12–191. It is possible that several others also contain lower titers of antibodies to other regions of the receptor. The recognition of residues 450–601 by several of the patients' antibodies (nos. 4, 8, 11, 13, and 14) also appeared more complex than that of the inhibitory monoclonal antibodies since these antisera weakly, or not at all, recognized chimeric IGF-IR containing residues 450–601 of the IR. These antibodies may recognize a specific conformation of IR residues 450–601 that is not duplicated in the chimera or they may recognize an epitope that is partly composed of residues 405–601 and partly of other regions of the IR. However, the finding that all 15 patients' autoimmune anti-IR antibodies do not recognize the IGF-IR Ex7,8/IR chimera indicates that residues 450–601 contain the epitope(s) for the majority of the anti-receptor antibodies in these patients' sera. Since these patients' anti-receptor antibodies are also capable of inhibiting the binding of insulin to its receptor (23–26), these results, like those described above for the monoclonal antibodies, are consistent with a role for residues 450–601 in binding insulin. It is also possible that these antibodies inhibit insulin binding indirectly by inducing a conformational change in the receptor.

Further evidence of a role for residues 450–601 in interacting with insulin came from binding studies with the chimeric receptors. Equivalent amounts of IGF-IR Ex7,8/IR only bound ≈20% as much insulin as native receptor. Competition studies indicated that this chimeric receptor also exhibited an ≈7-fold weaker affinity for insulin than the native IR (Fig. 5*A*). In contrast, IR Ex7,8/IGF-IR was found to bind IGF-I with the same affinity as native IGF-IR (Fig. 5*B*). These results indicate that the IGF-IR-specific residues in the region 440–586 are not required for the high-affinity interaction of IGF-I with its receptor. These results are consistent with the prior studies indicating that different regions of the insulin and IGF-I receptors confer high-affinity binding to their respective ligands (16–18). In addition, the conclusion that residues 450–601 of the IR are part of the insulin binding site is consistent with the prior studies of a mutant IR with a glutamic residue at 460 in place of the normal lysine (36). This mutation has been shown to cause an increase in affinity of the receptor for insulin (36, 37).

In summary, the present studies implicate an additional region of the IR (residues 450–601) in the high-affinity interaction of insulin with its receptor. It is therefore likely that multiple regions of the extracellular domain of the IR are involved in binding insulin. Residues 450–601 also appear to be a major immunogenic determinant for inhibitory monoclonal antibodies as well as patients' autoimmune anti-IR antibodies. We therefore propose that this region of the receptor be called the major immunogenic region (Fig. 1). This knowledge might be useful in screening for anti-receptor antibodies in other patients since it may be possible to genetically engineer bacteria to overproduce this region of the receptor and use this peptide in enzyme-linked immunoassays.

We are grateful to Dr. Simeon Taylor for the 15 patients' sera; Dr. Ken Siddle for monoclonal antibodies 83-7, 25-49, 83-14, 47-9, and 18-44; Dr. Ira Goldfine for antibodies MA-5, MA-10, and MA-20; Dr. Steven Jacobs for αIR-1 and αIR-3; Drs. Delong Liu and Lu-Hai Wang for the polyclonal antibody to the IGF-IR, and Dr. Naoki Arai for the SRα vector. This work was supported by National Institutes of Health Grants DK41765 and 34926 and a postdoctoral fellowship from the American Diabetes Association.

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