Expression and Characterization of a 70-kDa Fragment of the Insulin Receptor That Binds Insulin

MINIMIZING LIGAND BINDING DOMAIN OF THE INSULIN RECEPTOR

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In order to characterize regions of the insulin receptor that are essential for ligand binding and possibly identify a smaller insulin-binding fragment of the receptor, we have used site-directed mutagenesis to construct a series of insulin receptor deletion mutants. From 112 to 246 amino acids were deleted from the α-subunit region comprising amino acids 469–729. The receptor constructs were expressed as soluble insulin receptor IgG fusion proteins in baby hamster kidney cells and were characterized in binding assays by immunoblotting and chemical cross-linking with radiolabeled insulin. The shortest receptor fragment identified was a free monomeric α-subunit deleted of amino acids 469–703 and 718–729 (exon 11); the mass of this receptor fragment was found by mass spectrometry to be 70 kDa. This small insulin receptor fragment bound insulin with an affinity (K<sub>d</sub>) of 4.4 nM, which is similar to what was found for the full-length ectodomain of the insulin receptor (5.0 nM). Cross-linking experiments confirmed that the 70-kDa receptor fragment specifically bound insulin.

In summary we have minimized the insulin binding domain of the insulin receptor by identifying a 70-kDa fragment of the ectodomain that retains insulin binding affinity making this an interesting candidate for detailed structural analysis.

Insulin mediates its effects by binding to specific tyrosine kinase receptors in the plasma membrane of target cells. The structure of the insulin receptor has been investigated extensively and recently reviewed (1, 2); also a number of naturally occurring mutations in the insulin receptor gene that affects receptor function have been identified and reviewed by Taylor et al. (3, 4).

The insulin receptor is a glycoprotein of a relative molecular mass of 350–400 kDa, which is synthesized as a single chain polypeptide and proteolytically cleaved yielding a disulfide-linked α-β monomer insulin receptor. Two α-β monomers are linked by disulfide bonds between the α-subunits, resulting in the β-α-α-β subunit configuration. The exact disulfide pattern responsible for this receptor configuration was elusive for a long time until first an α-α contact was shown to connect Cys-524 of the two monomers (5), and more recently Sparrow et al. (6) described the disulfide pattern in the C terminus of the ectodomain. Along with the mutational data available (7–9), these reports suggest the IR<sup>1</sup> ectodomain is connected by the disulfide bonds shown in Fig. 1.

The intracellular part of the β-subunit includes the tyrosine kinase domain that acquire kinase activity upon binding of insulin to epitopes in the ectodomain. The x-ray crystal structure of the tyrosine kinase domain has been solved (10), whereas detailed three-dimensional structure of the insulin-binding site is not available, so only indirect information can be used to identify important structural features necessary for insulin binding. Predictions of the tertiary structure of the IR ectodomain have been based on alignment with epidermal growth factor receptor sequences (11, 12). The consensus from these alignments is that the insulin receptor α-subunits have two large homologous domains, L1 and L2, separated by a cysteine-rich region. The L1 and L2 domains are comprised of four repeats of α-helices followed by β-strand, turn, and β-strand. The best conserved feature in these repeats is a central glycine residue responsible for the turn (11). The L1 region spans amino acids 1–155 (nomenclature of Ebina <i>et al.</i> (13), and the cysteine-rich region comprises residues 155–312, and L2 comprises residues 313–468 (12). Thus, the first 468 amino acids of the α-subunit are predicted to be well defined domains with extensive homologies to the epidermal growth factor receptor. The corresponding domain of the IGF1 receptor (1–486) was expressed in C6 rat glioblastoma cells resulting in inhibited IGF1 receptor signaling and inhibition of growth (14), and recently McKern <i>et al.</i> (15) have reported crystallization of these first three domains of the IGF1 receptor (residues 1–462).

The major ligand binding determinants of the IR appear to reside in the α-subunit. Studies with chimeric receptors (16, 17), alanine scanning mutagenesis (18), and cross-linking studies (19) have suggested a binding epitope within the N-terminal 120 amino acids. Other cross-linking studies have identified hormone-receptor contact sites in the cysteine-rich region (20, 21) and just to the carboxyl side of the cysteine-rich domain around residue 390 (22). Finally in the C terminus of the α-subunit, cross-linking with photocytotoxic insulin derivatives (23) and alanine scanning mutagenesis (24) indicate that an important binding domain is found between amino acids 704 and 716. The insulin contact sites are apparently located exclusively in the α-subunit which was further verified by investigating expression of free α-subunit in COS cells (25). The α-subunit was secreted as a monomer that bound insulin with near wild-type affinity, but the expression level of free α-subunit was low allowing only limited characterization (25).

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1 The abbreviations used are: IR, insulin receptor; BHK, baby hamster kidney; DSS, disuccinimidyl suberate; GH, growth hormone; IGF1, insulin-like growth factor I; MALDI-TOF, matrix associated laser desorption ionization-time of flight; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; TBS, Tris-buffered saline; PNGase, peptide N-glycosidase.

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Affinities (K_d) of the receptor deletion constructs for insulin are shown. Each affinity is the average ± S.D. for at least three independent experiments. The data were determined from binding curves similar to those shown in Fig. 2, as described under “Materials and Methods.”

| Construct | Deletion (amino acids) | Affinity (K_d) Protein A assay | Affinity (K_d) FRG assay |
|-----------|------------------------|-------------------------------|-------------------------|
| IRwt      |                        | 1.5 ± 0.4^a                   | nM                      |
| IR659     | 447–599                | 3.9 ± 2.3                    | 2.3 ± 0.6               |
| IR613     | 447–613                | 1.7 ± 0.5                    | 2.5 ± 0.3               |
| IR629     | 447–629                | 1.6 ± 0.1                    | 1.8 ± 0.1               |
| IR649     | 447–649                | 1.9 ± 1.0                    | 1.3 ± 1.0               |
| IR673     | 469–673                | 1.1 ± 0.2                    | 1.3 ± 0.2               |
| IR685     | 469–685                | 6.4 ± 2.4                    |                         |
| IR685*    | 469–685, 718–729       | 7.3 ± 1.6                    |                         |
| IR703     | 469–703, 718–729       | 4.4 ± 0.8                    |                         |
| sIR       |                        | 5.0 ± 0.2                    |                         |

^a sIR is soluble IR ectodomain, all other constructs are expressed as IR ectodomain fused to Fc region of IgG. Δ denotes deletion and the number is the last amino acid deleted. IR659* and IR703 are exon 11—others are exon 11. + Affinities of IRwt are for low affinity site only.

The deletion mutant IR659 was made by PCR amplification using the sense primer 5’-CCTCTAGATCCCTTGATCCATCTCAGTG-3’ (BglII and BamHI sites underlined) and an antisense primer downstream from EagI site (amino acid 467–680). This fragment was digested with BglII and EagI and ligated into the corresponding site of the plasmid encoding IRwt, resulting in a cDNA sequence where amino acids 485–599 are deleted. IR613 and IR629 were made using the same strategy (BglII/EagI site) and the sense primers 5’-TGACAGATCTGTGAATGGAACACCCTCCG-3’ and 5’-TGACAGATCTGTGTTTTCGGAGAGCGAGAC-3’, respectively (BglII sites underlined). In the construct IR649, exons 7–9 were deleted by PCR amplification using a sense primer located upstream from Bsu36I site (amino acids 417–419) and the antisense primer 5’-AGTCCCTCGAGCCACCTCAAGCAGATGTTCCGCC-3’ (XhoI site underlined). This fragment was digested with Bsu36I and XhoI and ligated into corresponding site of the plasmid encoding IRwt, resulting in a cDNA sequence in which amino acids 469–649 are deleted. IR673 was constructed by overlap extension of fragments I and II. Fragment I was amplified using a sense primer upstream from Bsu36I site and the antisense primer 5’-CTCAACGTACGCCCTTGGCTTCCCCCAACTGGTCA-3’ (Nhel site underlined). Fragment II was amplified using sense primer 5’-GGGGAGAAGGCTTACGGTTGAGATATGAGAGGCGCCGC-3’ (Nhel site underlined) and an antisense primer downstream from the AorII site (amino acids 728–729 in exon 11). The overlap fragment was digested with Bsu36I and AorII and ligated into corresponding site of the plasmid encoding IRwt, resulting in a cDNA sequence deleted of amino acids 469–673, and a new silent Nhel site is introduced (amino acids 466–467). IR685 was made using the sense primer 5’-GACAAGGCTAGCTTGGCAGACATGACCTCAGTCC-3’ (Nhel site is underlined) and an antisense primer downstream from the AorII site (amino acids 728–729). This fragment was digested with NheI and AorII and ligated into corresponding site of the plasmid encoding IR673, resulting in a cDNA sequence deleted of amino acids 469–685.

Finally IR685* and IR703 were made by PCR amplification using a sense primer upstream from the NolI site (amino acids 450–452) and the antisense primers 5’-TTTTCTTGGGACAGAATCCCGG-3’ and 5’-TTTTCTTGGGCCAGACAAAACCATGTTGCTACATACCTCAAGACAGGTAGGTGCTGTTCCC (AorII site underlined). The fragments were digested with NolI and AorII and ligated into the corresponding site of the plasmid encoding IRwt, resulting in cDNA sequences deleted of amino acids 469–685 + 718–729 and 469–703 + 718–729, respectively. In these two constructs a new silent AorII site was introduced (amino acids 718–717).

Insulin Receptor Binding Assay—Two binding assays were used, a microtiter plate assay and a polystyrene glycol precipitation assay. For the plate assay receptor, IgG fusions were immobilized on protein A-coated microtiter plates, as described in detail (31). Briefly wells were coated with protein A, washed 3 times with binding buffer (100 mM Hepes, pH 8.0, 100 mM NaCl, 10 mM MgCl2, 0.05% (v/v) bovine serum albumin, 0.025% (w/v) Triton X-100) before a dilution of receptor fusion construct in binding buffer was added to each well. After incubation for...
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3 h at room temperature, the plates were washed 3 times with binding buffer. Binding experiments were performed by adding a total volume of 150 μl of binding buffer with A14-125I-insulin (5–10 pM) and varying concentrations of insulin. After 16 h at 4 °C unbound ligand was removed by aspirating the buffer and washing once with cold binding buffer, and the A14-125I-insulin bound in each well was counted in a γ-counter.

The precipitation assay was performed by incubating a suitable dilution of BHK medium containing receptor in a total volume of 200 μl with A14-125I-insulin (5–10 pM) and varying concentrations of unlabeled ligand in binding buffer for 16 h at 4 °C. Subsequently bound counts were recovered by precipitation with 2% polyethylene glycol and 500 μl of 25% (w/v) polyethylene glycol 8000. Bound A14-125I-insulin was counted in a γ-counter.

In both assays the concentration of receptor was adjusted to yield 10–15% binding of tracer when no competing ligand was added in the competition assay. The binding data were fitted using nonlinear regression algorithm in GraphPad Prism 2.01 (GraphPad Software Inc., San Diego, CA).

Immunoblotting—The expressed receptors were detected by immunoblotting using the monoclonal antibody mAb-F26. This antibody was raised against a peptide corresponding to amino acids 39–75, mapping at the N terminus of the insulin receptor α-subunit. The antibody was kindly donated by Jes Thorn Clausen, Novo Nordisk. For blotting, medium from BHK cells expressing receptor constructs was mixed with 0.33 volume of SDS-PAGE loading buffer (40% w/v sucrose, 563 mM Tris base, 423 mM Tris-HCl, 278 mM SDS, 2 mM EDTA, 0.88 mM Serva Blue G250, 0.7 mM phenol red), and 20 μl was run on a 6% SDS-polyacrylamide gel. Reduced samples were mixed with loading buffer containing 0.1 M dithiothreitol (DTT) and incubated at 70 °C for 10 min before loading 20 μl on SDS-polyacrylamide gel. After electrophoresis proteins were blotted onto Immobilon-P membrane (Millipore). The membrane was blocked by incubating with blocking buffer (5% defatted skim milk, 2% bovine serum albumin in TBS (150 mM NaCl, 10 mM Tris- HCl, pH 7.5)) for 16 h at 4 °C. The receptor antibody mAb-F26 was diluted in blocking buffer, and after incubating with receptor antibody the membrane was washed with TBS before incubating with peroxidase-conjugated anti-mouse immunoglobins antibody (P260 from DAKO, Denmark). Finally the blot was washed with TBS and immunoreactive protein was detected using ECL reagent from Amersham Pharmacia Biotech.

Cross-linking of 125I-X92 to Receptors—For chemical cross-linking the high affinity analogue X92 (A8H, B10D, B25Y-amine, des-B26–30 insulin) was used because the high affinity of this analogue allowed detection of receptors directly in BHK medium, and cross-linking was performed essentially as described (16, 32). Medium from BHK cells expressing receptor constructs was incubated for 60 min at room temperature with A14-125I-X92 (0.14 nM) in the presence or absence of unlabeled X92 (1 μM). DSS in dimethyl sulfoxide was added from a 10 mM stock solution to a final concentration of 0.1 mM. After 15 min on ice the reaction was stopped by adding 0.33 volume of SDS-PAGE loading buffer or 0.33 volume SDS-PAGE loading buffer with 0.1 M DTT. Reduced samples were incubated at 70 °C for 10 min before running on a 6% SDS-polyacrylamide gel. The gel was fixed in 10% acetic acid, 20% methanol and washed once with cold binding buffer or 0.33 volume SDS-PAGE loading buffer with 0.1 M DTT. Reduced samples were incubated at 70 °C for 10 min before running on an SDS-PAGE gel. The gel was fixed in 10% acetic acid, 20% methanol and washed once with cold binding buffer or 0.33 volume SDS-PAGE loading buffer with 0.1 M DTT.

Deglycosylation and Mass Spectrometry—The smallest receptor fragment, IR703 was purified by affinity chromatography using immobilized insulin as described previously (33). IR703 in Hepes, pH 8.0, 0.5% n-octyl glucosyponside, was treated with a deglycosylation mixture containing neuraminidase, endo-β-N-acetylgalactosaminidase H, and PNGaseF, either in the presence or absence of 0.1% SDS, for 18 h at 37 °C. MALDI-TOF mass spectra were recorded on a Voyager DE (Perceptive Biosystems) in sinapinic acid matrix. Calibration was performed on the MH+ and MH2+ [eq] ions of human serum albumin.

Results

Cloning and Expression of Receptor Deletion Constructs—We initially introduced the deletion described by Sung et al. (27) into our soluble IR-IgG fusion construct; this is the IRΔ599, deleted of amino acids 487–599. The constructs IRΔ613 and IRΔ629 were also deleted from amino acid 487, whereas the remaining five constructs were deleted from amino acid 469. The rationale for this is that amino acid Cys-468 is the last amino acid in exon 6 and also the last amino acid in the L2 domain; thus IR residues 1–468 are predicted to be a large domain with homology to the N-terminal domain of the epidermal growth factor receptor (11).

Receptor Deletion Constructs: Binding of Insulin—Insulin receptor secreted into BHK medium was analyzed in two binding assays. The receptor used here is fused to the Fc region of IgG, and therefore intact receptors could be immobilized using protein A. In this way binding curves for IRwt, IRΔ599, IRΔ613, and IRΔ629 receptors could be generated; the affinities of these receptors were 2–3 nM (Kd) (Table I). In contrast no binding was detectable in the protein A assay for all receptor constructs deleted of amino acids 630–649. The polyclonal glycol precipitation assay yielded binding curves for all the constructs expressed (Fig. 2 and Table I). In Fig. 2 are shown binding curves obtained when using insulin to displace A14-125I-insulin from IRwt, IRΔ599, IRΔ613, and IRΔ629 receptors could be generated; the affinities of these receptors were 2–3 nM (Kd) (Table I). In contrast no binding was detectable in the protein A assay for all receptor constructs deleted of amino acids 630–649. The polyclonal glycol precipitation assay yielded binding curves for all the constructs expressed (Fig. 2 and Table I). In Fig. 2 are shown binding curves obtained when using insulin to displace A14-125I-insulin from IRwt, IRΔ599, IRΔ613, and IRΔ703. The IRwt receptor displacement curve is biphasic fitting to a two-site binding model with binding affinities in the picomolar range (31) for the high affinity site and a Kd of 3.1 ± 0.9 nM for the low affinity site in the protein A assay. The binding curves for all deleted receptors were clearly one-sided (Fig. 2) with affinities ranging from 1 to 7 nM in the binding assays. The lowest Kd of 1.1 nM was observed for IRΔ673, and the shortest construct IRΔ703 gave a Kd of 4.4 ± 0.8 nM. For the full-length ectodomain the affinity for insulin was 5.0 ± 0.2 nM.

Two of the deletion constructs IRΔ685 and IRΔ685 only differ by the exon 11 region (amino acids 718–729); the affinity of IRΔ685 for insulin was 6.4 ± 2.4 nM, and when exon 11 is deleted in IRΔ685 the affinity was 7.3 ± 1.6 nM, so in these deletion receptors the exon 11 region does not influence binding of insulin significantly, in contrast to what has been reported for the IR holoreceptor (34) or the insulin proreceptor (35).

Detecting Receptor Fragments by Immunoblotting—The antibody used for immunoblotting was raised against an N-terminal epitope of the insulin receptor, and thus it would be expected to recognize the α-subunit of all receptors deletion constructs expressed. Immunoblotting was performed on non-reduced as well as reduced samples of medium from transfected BHK cells. The immunoblots are shown in Fig. 3. On the reduced gel (Fig. 3A) the antibody detects the full-length α-subunit of 130 kDa in the IRwt sample, whereas the deletion constructs show a gradual decrease in size of the α-subunit from apparent mass of 130 kDa to approximately 80 kDa for...
the smallest receptor construct IRΔ703. This receptor is also shown in its deglycosylated form after PNGaseF treatment, where it acquires an apparent mass of approximately 55 kDa comparable to what was predicted from the amino acid sequence. For immunoblotting similar volumes of BHK medium were loaded, so in addition to smaller size there seems to be better expression yields when expressing the smaller receptor fragments. Immunoblotting of the unreduced samples reveals three groups of immunoreactive bands (Fig. 3). The full-length receptor fusion IRwt has been reported to migrate as 380-kDa protein on SDS-PAGE (29) consistent with the high molecular mass band of more than 200 kDa observed on the blot (Fig. 3A). The blot shows bands that have not entered the separation gel indicating aggregation to very high molecular weight complexes, which is also consistent with the previous report on this receptor (29). IRΔ599, IRΔ613, and IRΔ629 have mobility corresponding to intact receptor IgG fusion so apparently deletion of Cys-524 is well tolerated in terms of disulfide linkage of the receptor subunits, indicating that Cys-647 is responsible for the only disulfide linkage between α- and β-subunits. There seems to be small amounts of monomeric α-subunit even in the IRΔ673 construct.
probably because the α-α dimer is not very stable when one of the α-α disulfides (Cys-524) has been removed. Possibly interactions between the Fe regions fused to the ectodomain also stabilize the disulfide between the two α-subunits. Finally, the constructs IRΔ685, IRA685*, and IRΔ703 all represent free monomeric α-subunit fragments only. This supports that Cys-682, Cys-683, or Cys-685 is involved in the second α-α disulfide bond.

Cross-linking of 125I-X92 to Receptors—Chemical cross-linking of A14,125I-X92 insulin analogue was performed using BHK medium directly. After cross-linking, samples were run under reduced as well as non-reduced conditions (Fig. 4). Both reduced and non-reduced gels show a cross-linking pattern similar to the immunoblotting pattern, demonstrating that all receptor fragments that are recognized by the antibody specific for IR α-subunit bind insulin. On the reduced gel (Fig. 4B) faint bands are visible, with apparent molecular mass of more than 200 kDa, and they appear in the first four constructs; this is probably due to intrareceptor cross-linking artifacts. For these cross-linking experiments similar volumes of medium were applied, and thus the intensity of the bands reflects affinity as well as amount of receptor so quantitative conclusions cannot be drawn directly. Nevertheless, considering the similar affinities found in the binding assay (Fig. 2 and Table I), the IRΔ703 receptor fragment appears to be very efficiently expressed in the BHK cells. The non-reduced gel (Fig. 4A) shows that in several of the deletion constructs a mixture of β-α-α-β, α-α dimers, and free α-subunits appear, but the binding data clearly suggest that there is a homogenous population of insulin-binding sites in all constructs (nm affinity). The structure of this nanomolar binding site appears very stable, as it is not influenced by the large deletions in the 469–703 region, nor does insulin binding depend on α-β or α-α-subunit contacts.

Deglycosylation and Mass Spectrometry—The mass of IRΔ703 was found by mass spectrometry to be 70.5 kDa (Fig. 5A) which is somewhat lower than the apparent molecular mass observed by SDS-PAGE (Fig. 3). The band was fairly broad, presumably due to glycosylation heterogeneity. Complete deglycosylation in the presence of 0.1% SDS gave a sharper peak at 54.5 kDa (Fig. 5C) which is in good agreement with the calculated value of 55.2 kDa. Partial deglycosylation gave a series of peaks with a spacing of 1.5–2.0 kDa corresponding to the core protein with 0–5 remaining carbohydrate chains (Fig. 5B). The difference in molecular weights between the native and the completely deglycosylated forms is consistent with extensive use of the 10 potential N-glycosylation sites.

FIG.5. MALDI-TOF mass spectra of IRΔ703. Mass spectra of native IRΔ703 protein (A) or IRΔ703 treated with a deglycosylation mixture containing neuraminidase, endo-β-N-acetylglucosaminidase H, and PNGaseF. Complete deglycosylation was obtained in the presence of 0.1% SDS (C), and in the absence of SDS only partial deglycosylation was obtained (B).

We wanted to characterize the domains of the IR that are essential for ligand binding. Characterizing the binding domains was approached indirectly by investigating which domains could be deleted from the IR without compromising insulin binding. The deletion constructs were expressed as fusion protein of IR ectodomain and Fe, the constant region of IgG heavy chain, which allows immobilization for binding assay. This construct (IRwt) has been described in detail by Bass et al. (29), and we have previously reported that this receptor fusion construct binds insulin with biphasic binding curves (31), and the high affinity component of this receptor has affinity that is similar to what is found for the holo-receptor. The high affinity of the holo-receptor has been ascribed to the presence of two binding epitopes on the insulin molecule which are required to bridge the two receptor α-subunits to attain picomolar affinity binding (36, 37). In contrast to the biphasic binding curves obtained with the intact IRwt receptor, all deletion constructs yielded one-site binding curves with nanomolar affinity (Fig. 2 and Table I), similar to what is found for the intact IR ectodomain secreted from BHK cells. The loss of high affinity binding in the IR ectodomain has been suggested to be due to loss of contact between the two α-subunits so that insulin cannot bridge the two α-subunits (36, 37), and certainly in several of our constructs the α-α contact is lost resulting in monomeric α-subunit fragments.

In contrast to previous attempts to express free IR α-subunits (25), we have achieved good expression levels allowing characterization of deleted α-subunit fragments without purification. Two groups have previously expressed deletion mutants of the insulin holo-receptor. Kodawaki et al. (26) described an insulin receptor deleted of amino acids 486–569, and Sung et al. (27) expressed a receptor deleted of amino acids 485–599. Kodawaki et al. (26) found a 3-fold decrease in insulin affinity when deleting amino acids 486–569; furthermore, the Scatchard plots were nearly linear indicating that the deletion caused loss of high affinity binding also in the holo-receptor. Sung et al. (27) reported unchanged ligand binding with the deleted IR but a blunted tyrosine autophosphorylation response and concluded that the deleted region was a regulatory domain for insulin regulation of β-subunit functions. The two reports on deleted holoreceptors demonstrated that domains of the IR α-subunit can be deleted without compromising insulin binding, and we pursued this concept by deleting up to 246 amino acids and succeeded in identifying an insulin-binding receptor fragment of only 70 kDa (Fig. 6).

The receptor region investigated here (amino acids 469–685) has been suggested to contain cysteines that are responsible for
In the region deleted it has been suggested that residues 485–599 contain a regulatory domain for insulin regulation of β-subunit functions (27); this was based on observations of a blunted tyrosine autophosphorylation response in a holoreceptor deleted of residues 485–599. We only expressed soluble receptors so we cannot argue for regulatory functions, but deleting a region including one of the cysteines (Cys-524) involved in subunit interaction and in close proximity to other important cysteines could likely modify the conformational changes needed for tyrosine kinase activation.

The deleted region has also been implied in some cases of insulin resistance because it contains a major immunogenic region (amino acids 450–601) that includes an epitope recognized by anti-receptor antibodies (40). Zhang and Roth (40) found that several monoclonal antibodies and anti-receptor autoantibodies from all 15 patients with type B insulin resistance recognized epitopes within amino acids 450–601.

Another approach that has given valuable information on IR function is the naturally occurring IR mutants observed in patients (3, 4). Of the approximately 30 mutations described in the insulin receptor ectodomain, most are found in the first 412 residues. There are three mutations in the extracellular part of the β-subunit, and one in the proteolytic cleavage site (α-β junction), but only one mutation is recognized within residues 469–703, and this is a premature stop codon. This again indicates that this region is not of major importance for receptor function.

The best characterized ligand receptor interaction is that of growth hormone binding to its receptor. For this receptor system a naturally occurring receptor fragment has been identified in serum (41); this receptor fragment comprises the 246 amino acids ectodomain of the GH receptor. Upon binding of GH the receptor fragment dimerizes like the full-length receptor, and also the binding affinity of the full-length receptor is intact. By using a recombinant version of this receptor fragment (residues 1–238) expressed in _Escherichia coli_ the crystal structure of GH in complex with GH receptor fragment was solved (42). In the insulin receptor system the ectodomain is much larger (almost 1900 amino acids, 350–400 kDa); it is dimeric also in the absence of ligand, and no naturally occurring ligand binding fragment has been identified. Attempts to obtain smaller IR fragments that bind insulin have not been met with much success; only the full-length ectodomain has been expressed and purified in substantial quantities (16, 33). Given the size and complexity of the ectodomain protein, the identification of a minimal ligand-receptor complex is expected to facilitate generation of crystals suitable for detailed structural analysis by x-ray crystallography. In the present study we tried to minimize the ligand binding domain of the insulin receptor by deleting regions of the IR α-subunit. We succeeded in obtaining a 70-kDa monomeric receptor fragment that binds insulin with an affinity similar to what is found for the soluble ectodomain of the insulin receptor.

**Acknowledgments**—We thank Durita Simonsen, Ulla M. Jørgensen, Jytte Topp-Radzikowska, and Lene Drube for excellent technical assistance and Dr. Joseph Bass (University of Chicago) for providing IR-IgG cDNA.

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