Comparison of the Functional Insulin Binding Epitopes of the A and B Isoforms of the Insulin Receptor*

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Jonathan Whittaker‡, Heidi Sørensen‡, Vibeke L. Gadgebøll‡, and Jane Hinrichsen
From the Receptor Biology Laboratory, Hagedorn Research Institute, 2820 Gentofte, Denmark

The human insulin receptor is expressed as two isoforms that are generated by alternate splicing of its mRNA; the B isoform has 12 additional amino acids (718–729) encoded by exon 11 of the gene. The isoforms have been reported to have different ligand binding properties. To further characterize their insulin binding properties, we have performed structure-directed alanine-scanning mutagenesis of a major insulin binding site of the receptor, formed from the receptor L1 domain (amino acids 1–470) and amino acids 705–715 at the C terminus of the α subunit. Alanine mutants of each isoform were transiently expressed as recombinant secreted extracellular domain in 293 cells, and their insulin binding properties were evaluated by competitive binding assays. Mutation of Arg96 and Phe96 of each isoform resulted in receptors that were not secreted. The Kd's of unmutated receptors were almost identical for both isoforms. Several new mutations compromising insulin binding were identified. In L1, mutation of Leu111 decreased affinity 20- to 40-fold and mutations of Val110, Glu119, and Lys121 3 to 10-fold for each isoform. A number of mutations produced differential effects on the two isoforms. Mutation of Asn10 in the L1 domain and Phe714 at the C terminus of the α subunit inactivated the A isoform but only reduced the affinity of the B isoform 40- to 60-fold. At the C terminus of the α subunit, mutations of Asp707, Val711, and Val715 produced 7- to 16-fold reductions in affinity of the A isoform but were without effect on the B isoform. In contrast, alanine mutations of Tyr706 and Asn711 inactivated the B isoform but only reduced the affinities of the A isoform 11- and 6-fold, respectively. In conclusion, alanine-scanning mutagenesis of the insulin receptor A and B isoforms has identified several new side chains contributing to insulin binding and indicates that the energetic contributions of certain side chains differ in each isoform, suggesting that different molecular mechanisms are used to obtain the same affinity.

The human insulin receptor is expressed as two isoforms that are generated by alternate splicing of its mRNA (1–3). The two mature receptor proteins differ by the presence or absence of 12 amino acids at the C terminus of the extracellular α subunit; this insertion is encoded by the 36-nucleotide exon 11 of the receptor gene (4). The two isoforms have been reported to have different tissue distributions (3, 5) and to exhibit different functional properties (6–8).

The B isoform with the 12-amino acid insertion is the predominant form expressed in the classic insulin target tissues responsible for glucose homeostasis, i.e. fat, muscle, and liver, whereas the A isoform without the insertion predominates in non-classic target tissues, e.g. the pancreatic β cell and neural tissue (4, 5). The B isoform has been reported to signal more efficiently in response to insulin binding despite having a 2-fold lower affinity for insulin (6–9). A switch in isoform expression from A to B in hepatoma cells mediated by dexamethasone treatment leads to decreased insulin sensitivity (8). In addition, the aberrant regulation of alternate splicing observed in myotonic dystrophy leads to overexpression of the A isoform of the receptor in insulin target tissues and results in insulin resistance (10). Divergent isoform-dependent signaling mechanisms have been demonstrated in the pancreatic β cell, with differential activation of different phosphatidylinositol3 kinase and protein isoforms in response to insulin (11).

The ligand binding properties of the two isoforms also differ significantly. The A isoform has a 2-fold higher affinity for insulin than the B isoform. In addition, the kinetics of insulin binding to the two isoforms differ (6, 7). The A isoform has a higher affinity for insulin-like growth factor (IGF)-1 and -II than the B isoform (7, 12). Indeed, it has been proposed that it might be a physiological receptor mediating the growth-promoting effects of IGF-II (13). Taken together, these findings suggest that the structure of the ligand binding sites and the ligand receptor interface may differ.

Recently, a high resolution crystal structure of an N-terminal fragment of the homologous insulin-like growth factor I receptor (amino acids 1–460) has been reported (14). The molecule is composed of an extended bilobed structure composed of the two globular L domains flanking the cysteine-rich domain with dimensions of 40 × 48 × 105 Å. The N-terminal globular domain contacts the cysteine-rich domain along its length. In contrast, there is minimal contact between the C-terminal domain and the cysteine-rich domain. In the crystal structure, L1 and L2 occupy very different positions relative to the cysteine-rich domain. However, this could be an artifact of crystal packing in this fragment, and the position of L2 may be very different in the native molecule. It is possible that it is rotated into a position similar to that of L1 in relation to the cysteine-rich domain. However, irrespective of this, a cavity of ~30 Å diameter occupies the
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center of the molecule and possibly represents a binding pocket.

Each L domain resembles a loaf of bread with dimensions 24 x 32 x 37 A and is formed from a single right-handed beta helix capped at the ends by short alpha helices and disulfide bonds. The base of the loaf is formed from a six-stranded beta-sheet five residues in length. Both sides are formed from beta-sheets three amino acids in length, and the top is composed of irregular loops connecting the short beta-strands. As predicted from sequence comparisons, the cysteine-rich domain is composed of repetitive modules resembling parts of laminin and the tumor necrosis factor receptor (15). These form a rod-like structure connecting the two globular L domains from which a large mobile loop projects into the putative binding pocket.

Although this fragment and the corresponding part from the insulin receptor are devoid of ligand binding activity (14, 16), mutagenic studies indicate they contain amino acids whose side chains are involved in ligand contacts (17, 18). Affinity-labeling studies and alanine-scanning mutagenesis have also demonstrated that a C-terminal peptide of the alpha subunit, amino acids 705-715, is involved in insulin binding (19, 20). Further, fusion of this C-terminal fragment to the N-terminal 470 amino acids results in a recombinant protein that binds insulin with an affinity similar to that of the full-length secreted recombinant extracellular domain (21, 22), suggesting that these elements are sufficient to form an intact ligand binding site. Recently Kristensen et al. (24) have reported that an insulin binding site can be reconstituted from a fragment of the N terminus of the receptor encompassing amino acids 1-255 and the C-terminal peptide of the alpha subunit (amino acids 1-255), indicating that these elements are its minimal determinants.

In the present study, to further evaluate the molecular mechanisms responsible for the reported differences in insulin binding properties, we have compared their functional epitopes. We have used structure-directed alanine-scanning mutagenesis of the L1 domain and alanine-scanning mutagenesis of amino acids 705-715 for this purpose. The results of these studies reveal that previously unidentified amino acids in L1 are determinants of ligand binding of both isoforms. Mutation of Asp<sup>707</sup> and Val<sup>713</sup> result in compromises in affinity that are restricted to the A isoform. Mutations of amino acids Asn<sup>69</sup>, Phe<sup>84</sup>, Tyr<sup>709</sup>, Leu<sup>709</sup>, and Phe<sup>714</sup> have effects on receptor affinity that are isofrom-specific.

EXPERIMENTAL PROCEDURES

General Procedures—All molecular biological procedures, including agarose gel electrophoresis, restriction enzyme digestion, ligation, bacterial transformation, and DNA sequencing were performed by standard methods (25). All oligonucleotides were purchased from DNA Technology (Aarhus, Denmark). Restriction and modifying enzymes were from New England Biolabs (Beverly, MA). Recombinant human insulin and HPLC-purified moniosiodiated [<sup>125</sup>I]-Tyra<sup>14</sup>Insulin were from Novo Nordisk A/S. Protease inhibitors were from Roche Molecular Biochemicals. Medium and serum for tissue culture were from Invitrogen. PEAK Rapid cells (293 cells constitutively expressing SV40 large T antigen) by Drs. C. Kristensen and J. Brandt, Novo Nordisk A/S, Bagsværd, Denmark, and a cDNA encoding the A isoform of the insulin receptor was provided by Dr. Donald Steiner (University of Chicago).

Oligonucleotide-directed Mutagenesis—Oligonucleotide-directed mutagenesis was performed by a modification of the method of Kunkel (29, 30) using uracil containing single-stranded DNA prepared from phage rescued from Escherichia coli C1236 transformed with a cDNA encoding the full-length insulin receptor cloned into the phagemid pTZ18S as template. Restriction sites were deleted or introduced with the specific mutation to facilitate screening of the mutants. Successful mutagenesis was confirmed by DNA sequencing.

Expression of Mutant Receptor cDNAs—Recombinant mutant secreted insulin receptor cDNAs were reconstructed in the plasmid pcDNA3-zeo<sup>+</sup> for expression. DNA for transfection was prepared from 10 ml of overnight cultures by a boiling dectrytrimethyl-amonium bromide (CTAB) method (31) followed by purification using QiAwhell strips. The mutant receptor cDNAs were expressed transiently in Peak Rapid cells (293 cells constitutively expressing SV40 large T antigen) by transfection using FuGene 6 (Roche Molecular Biochemicals) according to the manufacturer’s directions. Conditioned medium was harvested 4 days post-transfection and, if necessary, concentrated prior to assay using Centricon 30 centrifugal concentrators (Millipore, Bedford, MA).

Receptor Binding Assays—Soluble insulin receptor binding assays were performed using a modification of the microtiter plate antibody capture assay that we have described previously (17, 18). Microtiter plates (Nunc Maxisorb, Roskilde, Denmark) were incubated overnight at 4°C with anti-insulin receptor antibody (18-44 IGG) (100 ng/well of 50 mu;g/ml solution in phosphate-buffered saline). Washing, blocking, and receptor binding were as previously described. Competitive binding assays with labeled and unlabelled insulin were carried out as previously, except that the incubation was for 16 h at 25°C. Binding data were analyzed by computer fitting to a one-site model to obtain the K<sub>d</sub> of the expressed protein.

Insulin Receptor Enzyme-linked Immunosorbent Assay—Microtiter plates were incubated overnight at 4°C with anti-insulin receptor antibody 18-44 IGG (26). Plates were then washed and blocked as for the receptor binding assay and incubated with varying dilutions of conditioned medium from transfected cells to immobilize the receptor. They were then incubated with biotinylated anti-FLAG M2 antibody (5 mu;g/ml) for 60 min at room temperature. After washing, plates were incubated with a 1:16,000 dilution of streptavidin horseradish peroxidase conjugate for a further 60 min at room temperature and then assayed spectrophotometrically.

RESULTS

Alanine Mutagenesis—We have constructed cDNAs encoding alanine mutants of the ligand-accessible amino acid side chains in the regions of the A and B isoforms of the human insulin receptor, the L1 domain (14), amino acids 1–150, and amino acids 705–715 at the C terminus of the alpha subunit (1, 2) that have been shown to be the minimal essential structural components of one of its major ligand binding sites (24). These were expressed transiently as recombinant secreted receptors with FLAG epitope tags in 293 Peak Rapid cells.

To evaluate receptor expression, receptor in conditioned medium was quantitated by enzyme-linked immunosorbent assay in a sandwich assay in which receptor was immobilized from conditioned medium of transfected cells by the anti-receptor monoclonal antibody 18–44 that is directed toward a linear epitope in the N terminus of the beta subunit of the receptor (26) and then detected with biotinylated anti-FLAG antibody and streptavidin-horseradish peroxidase. We found that, with the exception of the R86A and F96A mutants of both isoforms of the insulin receptor that were not secreted, all mutants were present in conditioned medium in approximately the same concentrations as those of control wild type receptor A and B isoforms (data not shown); we failed to detect R86A and F96A mutant receptors in conditioned medium even after 100-fold concentration of the medium. We have previously shown that the secretion of recombinant insulin receptor mutants, including the F96A mutant, is exquisitely sensitive to the integrity of the structure of the receptor protein (17). Thus it is probable that alanine mutations in these positions disrupt the structure of the receptor. This is supported by the report that a proline mutation of Arg<sup>60</sup> of the insulin receptor was the causative mutation in a patient with extreme insulin resistance (32, 33).
In that patient, processing of the full-length receptor was impaired with inefficient insertion in the plasma membrane. Equilibrium binding studies were performed on conditioned media from transfected cells to characterize insulin binding to both isoforms of wild type and mutant receptors. As previously described, insulin binding to both isoforms of the recombinant secreted receptor displayed simple kinetics and could be best fitted to a single-site model (data not shown). Computer analysis of binding data indicated a single population of binding sites with a $K_d$ of $2.1 \times 10^{-9}$ M for the A isoform and $1.8 \times 10^{-9}$ M for the B isoform (mean + S.E., $n = 8$). It should be noted that these values are higher than we have previously reported and probably reflect changes in assay conditions and computerized analysis of binding data (17, 20). Because studies utilizing alanine-scanning mutagenesis have demonstrated that meaningful changes in affinity, produced by a single alanine substitution, range from 2- to 100- (34), in the experiments described below we regarded any mutant with a greater than 2-fold increase in $K_d$, i.e. $K_d$ greater than $4.2 \times 10^{-9}$ M for the A isoform and $3.6 \times 10^{-9}$ M for the B isoform, as exhibiting a significant disruption of insulin-receptor interactions.

The effects of the individual alanine mutants on the $K_d$ for insulin of the A and B isoforms of the receptor are summarized in Fig. 1. In the L1 domain of the B isoform of the receptor, mutations of Asp72, Leu86, Leu97, Phe98, Phe99, Asn100, Tyr101, Glu102, and Glu122 produced a 2- to 10-fold increase in $K_d$ and mutations of Asn15, Gln34, Leu78, and Lys121 a 10- to 65-fold increase (Fig. IA). In addition, alanine mutations of Arg14 and Phe33 resulted in proteins with an affinity for insulin that was too low to be accurately determined (Fig. IA). In the L1 domain of the A isoform, with the exception of mutants of Asn15 and Leu78, similar effects were observed for all alanine mutations. Alanine mutation of Asn15 resulted in a receptor with unmeasurable affinity for insulin, and mutation of Leu78 had an effect that was half that in the B isoform (Fig. IA). In the cysteine-rich domain, alanine mutations of His217, Phe248, Gln249, and Asp253 failed to produce any significant compromise in the affinity of either the A or B isoform for insulin (data not shown). At the C terminus of the alpha subunit of the B isoform of the receptor, alanine mutations of Phe270, Glu271, Tyr272, His273, and Leu271 resulted in receptors with insulin binding that was too low to be accurately determined, and alanine mutations of Leu270 and Phe271 produced increases in $K_d$ for insulin of 40- and 80-fold, respectively (Fig. 1B). In the A isoform, alanine mutations of Phe270, Glu271, and His272 had the same profound effects on insulin binding as they did in the B isoform. Mutations of Asp277, Val278, and Val279 that were without effect on the B isoform produced increases of 7- to 16-fold in the $K_d$ for insulin of the A isoform. Also, mutation of Leu270 that resulted in an 80-fold increase in the $K_d$ of the B isoform produced an A isoform receptor devoid of insulin binding activity (Fig. 1B). In contrast, mutation of Asn271, which had a similar effect on the B isoform receptor, only produced a 45-fold increase in the $K_d$ of the A isoform (Fig. 1B).

**DISCUSSION**

In the present study we have used alanine-scanning mutagenesis to compare the functional epitopes of the A and B isoforms of the insulin receptor. In contrast to previously reported studies of insulin (6, 7), we have found that there is no significant difference between the affinities of the two isoforms for insulin. This is probably because of the different forms of the receptor that were studied. In previous studies, binding experiments were performed on native receptors in intact cells or solubilized native receptors, whereas in the present study they were performed on soluble recombinant receptors. These two types of preparation differ significantly in their binding properties (35, 36); the secreted receptors exhibit lower affinity for insulin and no negative cooperativity. These differences have been attributed to differences in the molecular mechanisms underlying binding; thus it is not surprising that the presence or absence of the alternately spliced exon should impact the binding properties of the two forms of the receptor differently. However, point mutations appear to have the same relative effects on both forms of the receptor (17).

The present study has identified a number of new mutations in the L1 domain (14) that compromise the affinity of both receptor isoforms for insulin. Two of these, alanine mutations of Leu27 and Glu97, were examined in our previous alanine-scanning study of the N-terminal binding domain of the B isoform of the receptor (17). In that study we reported that secretion of these mutant receptors was impaired and their affinity for insulin was too low for accurate determination. However, receptor secretion was not completely abolished as...
observed for the alanine mutants of Arg86 and Phe96 discussed above. It is likely that low receptor affinity combined with low transfection efficiency was the reason for our inability to determine the affinity of these mutants; the expression system used in the present study gives significantly higher yields of receptor (data not shown). Alanine mutations of Leu87, Glu120, and Lys121 were not examined in our previous study of the B isoform of the receptor and suggest that this region is the major contributor to the free energy of ligand binding for both, although certain mutants have differential effects on the A and the B isoforms. Alanine Mutations of Phe705, Glu706, and His710 inactivate both receptor isoforms. Alanine mutations of Tyr708 and Asn711 selectively have a greater effect on the affinity of the B isoform than the A, whereas the reverse is seen with alanine mutations of Leu709 and Phe714. Also, alanine mutations of Asp707, Val713, and Val715 moderately compromise the affinity of both receptor isoforms and suggest that this region is the major contributor to the free energy of ligand binding for both isoforms of the receptor and that both isoforms have almost identical affinities for insulin; alanine mutations of B and A isoforms appeared to make a greater contribution to the B isoform affinity for insulin; alanine mutations of B and A isoforms produced 40- and 20-fold reductions in affinity, respectively.

In the cysteine-rich domain, we analyzed the insulin binding properties of alanine mutants of four candidate residues, His247, Phe248, Gln249, and Asp250. These were selected because reconstitution studies had shown that amino acids 1-250 and 704-719 are the minimal determinants of this ligand binding site (24), and homology modeling based on the IGF-I receptor coordinates (14) showed these to be the only residues in the cysteine-rich domain between amino acids 1 to 250 that are accessible to ligand. Despite the fact that these residues correspond to residues in the IGF-I receptor cysteine-rich domain that have been implicated in IGF-I binding (18), their mutation had no significant effect on the affinity of either isoform of the insulin receptor for insulin. Thus it does not appear that insulin interactions with the cysteine-rich domain of either isoform of the insulin receptor make an energetically significant contribution to the insulin receptor interaction. This is in contrast to predictions made from a model of insulin receptor structure constructed from cryo-scanning tunnelling electron microscopic studies of the quaternary structure of the insulin receptor complex (38). In this study, the side chains of His247, Gln249, and Asp250 were reported to be contact sites for the side chains of the insulin residues, Glu21 and Arg22. Alanine mutations of these residues have been reported to increase the affinity of insulin for the receptor (39). Thus it would be predicted that alanine mutations of their putative contact sites in the receptor would increase the affinity of the receptor for insulin.

In the C-terminal domain of the α subunit, alanine mutations cause similar deleterious effects on ligand binding of both isoforms of the receptor and suggest that this region is the major contributor to the free energy of ligand binding for both, although certain mutants have differential effects on the A and the B isoforms. Alanine Mutations of Phe705, Glu706, and His710 inactivate both receptor isoforms. Alanine mutations of Tyr708 and Asn711 selectively have a greater effect on the affinity of the B isoform than the A, whereas the reverse is seen with alanine mutations of Leu709 and Phe714. Also, alanine mutations of Asp707, Val713, and Val715 moderately compromise the affinity of the A isoform for insulin but are without effect on the B.

The finding that alanine mutations in the insulin receptor ligand binding site have differential effects on the ligand affinity on the A and B isoforms of the receptor indicates that the structures of their binding sites must be different. This would be consistent with the observation that the kinetics of association and dissociation differ for the two receptor isoforms (6). Further support for this concept is provided by the finding that IGF-I and -II that bind to receptor sites that overlap those of the insulin binding site have very different affinities for the two receptor isoforms; their affinity is at least 10-fold greater for the A isoform than for the B isoform (7).

When these findings are taken together with the observation that both isoforms have almost identical affinities for insulin, it would appear that insulin-receptor interface exhibits significant plasticity, implying that either or both of the partners in the interaction can undergo significant conformational changes to accommodate the structural changes entrained by insertion of the additional 12 amino acids just adjacent to one of the subdomains of the binding site. Such a notion is supported by the relative independence of insulin affinity of the exact posi-

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**Fig. 2. Structure of the functional epitopes of the L1 and cysteine-rich domains.** The Co backbone of the L1 and cysteine-rich domains of a model of the insulin receptor constructed from the IGF-I receptor coordinates (14) by homology modeling (37) is shown as a ribbon representation. The amino acids mutated are shown in space-filling representation. Color coding is according to the results for the B isoform of the insulin receptor. Alanine mutations of amino acids colored green produced a 2- to 10-fold reduction in affinity, those colored yellow a 10- to 100-fold reduction, and those colored red >100-fold. Alanine mutations of residues colored white resulted in receptors that were not secreted in detectable amounts. This figure was prepared with the Swiss PDB Viewer (37).
tioning of the C-terminal peptide observed in recombinant receptors or in reconstitution experiments (21–23). Plasticity of protein-protein interfaces is not an uncommon phenomenon in nature (for review see Ref. 40). However, it is rare to observe the preservation of affinity that is observed in this situation. This presumably has arisen as a consequence of the co-evolution of insulin and its receptor.

In summary, we have demonstrated by comparative alanine-scanning mutagenesis that the A and B isoforms of the receptor have functional epitopes that differ qualitatively and quantitatively. Elucidation of the underlying bases for these differences at the atomic level will require the elucidation of the atomic structure of both isoforms of the receptor in complex with insulin.

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