CHARACTERIZATION OF THE FUNCTIONAL INSULIN BINDING EPITOPES OF
THE FULL LENGTH INSULIN RECEPTOR

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Running Title: Insulin Receptor Binding Site

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SUMMARY

Mutational analyses of the secreted recombinant insulin receptor extracellular domain have identified a ligand binding site comprised of residues located in the L1 domain (amino acids 1-470) and at the C-terminus of the α subunit (amino acids 705-715). In order to evaluate the physiological significance of this ligand binding site, we have transiently expressed cDNAs encoding full length receptors with alanine mutations of the residues forming the functional epitopes of this binding site and determined their insulin binding properties. Insulin bound to wild type receptors with complex kinetics which were fitted to a two component sequential model; the Kd of the high affinity component was 0.03nM and that of the low affinity component was 0.4nM. Mutations of Arg14, Phe64, Phe705, Glu706, Tyr708, Asn711 and Val715 inactivated the receptor. Alanine mutation of Asn15 resulted in a 20-fold decrease in affinity while mutations of Asp12, Gln34, Leu37, Leu87, Phe89, Tyr91, Lys121, Leu709 and Phe714 all resulted in 4 to 10-fold decreases. When the effects of the mutations are compared to those of the same mutations of secreted recombinant receptor, significant differences are observed for Asn15, Leu37, Asp707, Leu709, Tyr708, Asn711, Phe714 and Val715 suggesting that different molecular basis of the interaction of each form of the receptor with insulin differs. We also examined the effects of alanine mutations of Asn15, Gln34, Phe89 and Leu709 on insulin induced receptor auto-phosphorylation. They had no effect on the maximal response to insulin but produced an increase in the EC50 commensurate with their effect on the affinity of the receptor for insulin.

INTRODUCTION

The initiating event in the insulin signaling cascade is the binding of insulin to a specific plasma membrane receptor. This interaction has been extensively studied and has been found to be extremely complex (for review see ref.(1)). Scatchard plots (2) of equilibrium binding data are concave and curvilinear, suggesting heterogeneity of ligand binding sites, negatively cooperative site-site interactions or a combination of both (1). These properties and high affinity interactions with insulin are dependent on the dimeric structure of the receptor; insulin binds non-cooperatively to a single population of binding sites in the monomeric receptor (3-5). The stoichiometry of binding to the native receptor appears to be one insulin molecule to one receptor dimer (6). The secreted recombinant extracellular domain of the receptor exhibits similar properties to those of the receptor monomer and has a stoichiometry of two insulin molecules to one receptor dimer (6;7).

A number of hypothetical models of insulin-receptor interactions have been proposed to explain these findings (7-9). The model that best explains the experimental findings is that of De Meyts (1). This proposes that insulin has two topographically distinct receptor binding sites and that the receptor has two topographically distinct insulin binding sites per monomer. In this model insulin binds asymmetrically to the insulin dimer with each of its binding sites contacting its cognate receptor site on a separate monomer. While the detailed structural basis of this model has yet to be elucidated, the structure-function relationships of the insulin molecule and the receptor have been extensively studied and provide support for the essentials of the model.
Insulin is a 6Kd globular protein with disulfide linked A and B chains (for review see ref.(10)). The consensus of published studies is that it has a receptor binding site composed of Gly A1, Ile A2, Val A3, Gln A5, Tyr A19, Val B12, Tyr B16, Gly B23, Phe B24, Phe B25 and Tyr B26 (1;10). However hagfish insulin, in which these residues and the tertiary structure of the molecule are conserved (11), displays anomalous receptor binding behavior (11;12), suggesting that other residues outside this region might also be involved in receptor interactions. This is supported by the finding that two recombinant insulin analogues with substitutions in residues located on the opposite side of the molecule, Leu A13 to Ser and Leu B17 to Gln, exhibited similar receptor binding behavior to hagfish insulin (7;8).

The structure and structure-function relationships of the insulin receptor are less well documented. It is a dimeric trans-membrane protein: see ref. (1) for review. Each monomer consists of disulfide linked α and β subunits. The α subunits are wholly extracellular and contain the ligand binding site(s) and the β subunit has an extracellular component, a single transmembrane helix and an intracellular component with tyrosine kinase catalytic activity. The structure of the N-terminus of the homologous IGF-I receptor has been determined (13); amino acids 1-460 in IGFR, corresponding to amino acids 1-470 of IR. It consists of two globular β helical domains (L1 and L2) flanking a cysteine rich domain (CRD). Comparative homology modeling suggests the remainder of the extracellular domain to be composed of three type 3 fibronectin repeats FnIII0, FnIII1 and FnIII2 (14-16). FnIII1 is atypical because it contains a 120 amino acid insert domain of indeterminate structure(15). Affinity labeling studies, studies with chimeric receptors and the epitopes of anti-receptor antibodies which inhibit insulin binding strongly suggest the each receptor monomer contains two topologically discrete ligand binding sites (17-22).

We have characterized a binding site of the secreted recombinant insulin receptor, using alanine scanning mutagenesis (17;18;23-25). This is composed of two functional epitopes, one located on the base of the L1 domain and the other at the C-terminus of the α subunit. As discussed above, the secreted recombinant receptor exhibits anomalous binding properties, suggesting monovalent insulin binding (7). In order to evaluate the function of this binding site in the holo-receptor, i.e. where there is bivalent binding, we have expressed alanine mutants of the residues that comprise this functional epitope as full length proteins. We found that all mutations with the exception of two residues which were minor contributors to the epitope of the secreted receptor impaired binding. All hot spot residues were conserved but an additional residue was present in the holo-receptor hot spot. In addition a number of residues which we had previously shown to vary in their contributions to the free energy of binding of the two isoforms of the secreted receptor also differed in their contribution to the free energy of binding of the holo-receptor. In three selected mutants, the effects of the mutations on the sensitivity of insulin regulated receptor auto-phosphorylation were commensurate with their effects on affinity for insulin.

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(26). Restriction and modifying enzymes were from New England Biolabs. Recombinant human insulin was from NovoNordisk A/S. HPLC-purified monoiodinated [125I-TyrA14] insulin was from Amersham. Protease inhibitors were from Roche Molecular Biochemicals. PEAK Rapid cells were purchased from Edge Biosystems (Gaithersburg, MD). Medium and serum for tissue culture were from Cellgro. The mammalian expression vector pcDNA3.Zeo+ was from InVitrogen. Coding sequences for a triple repeat of the AU5 epitope tag (TDFYLK) was introduced between the BamHI and XbaI sites of the pcDNA3.Zeo+ polylinker by cassette mutagenesis. The cDNA for the insulin receptor was as previously described (27). It was modified for sub-cloning into the epitope tag expression vector by elimination of the existing Bam HI site by a silent mutation and introduction of an in frame Bam HI site encoding a Gly-Ser linker at the 3’ end of the coding sequence by site-directed mutagenesis.
Monoclonal anti-AU5 IgG was purchased from Covance.

Construction of receptor mutants- Alanine mutations of the cDNA encoding the recombinant secreted receptor extra-cellular domain have been described previously (23-25). These were used to generate full length epitope-tagged mutants of the full length receptor by standard sub-cloning techniques.

Expression of Receptor cDNAs- Plasmid DNA for transfection was prepared as previously described (28). The receptor cDNAs were expressed transiently in PEAK rapid cells using Transit 293 (Mirus) according to the manufacturers’ directions. Cells were harvested by lysis in 0.15M NaCl, 0.1M Tris pH 8 containing 1% Triton X-100 and protease inhibitor cocktail 72h post-transfection and an enriched glycoprotein fraction was obtained by wheat germ agglutinin affinity chromatography (29). These were stored at -80°C until assay.

Receptor Binding Assays- Insulin competitive receptor binding assays were performed by a modification of the microtiter plate antibody capture assay that we have described previously (24). Microtiter strip plates (Nunc Maxisorb) were incubated overnight at 4°C with AU5 IgG (100 µl/well of 40mg/ml in PBS). Washing, blocking, receptor binding and competitive binding assays with labeled and unlabeled peptides were performed as described. Binding data were analyzed by a 2 site sequential model (8;30).

Receptor Autophosphorylation- Insulin stimulated receptor tyrosine autophosphorylation was evaluated with a modification of a time resolved fluorescence assay of insulin-like growth factor receptor auto-phosphorylation (31;32). Concentrations of mutant and wild type receptors in enriched glycoprotein fractions from transfected cell lysates were determined by receptor ELISA (24). Equal concentrations of mutant and wild type receptors were incubated with varying concentrations of insulin in 0.1ml 0.15M NaCl, MgCl2 10mM, HEPES 25mM pH 7.8 , Triton X-100 0.05% (v/v) for 2h at 25°C. Autophosphorylation was initiated by addition of Na2VO4 and MgCl2 to final concentrations of 2mM and 10mM respectively. The reaction was stopped after 20 minutes by the addition of EDTA and EGTA to final concentrations of 25mM. The reaction mixture was then transferred to Delfia micro-titer assay plates (Perkin-Elmer) coated with anti-AU5 IgG and incubated overnight at 4°C to immobilize insulin receptor. After washing with PBS Tween-20 0.02%, receptors were incubated with Europium labeled PY20 (Perkin-Elmer) according to the manufacturers directions. After washing, phosphotyrosine containing receptor was quantitated by time resolved fluorescence using a Victor 3 (Perkin Elmer) plate reader. EC50s (concentrations of insulin producing half maximal response) were obtained from the dose response data by non linear least squares analysis using Prism (GraphPad Software).

RESULTS

Insulin Binding- We have previously characterized the functional epitopes of the secreted recombinant insulin receptor extracellular domain by alanine scanning mutagenesis (23-25). In order to determine significance of these epitopes for insulin binding in a physiological context, we sub-cloned the alanine mutations used to characterize them into the full length B isoform receptor cDNA tagged at its 3’ end with the coding sequence for a triple repeat of the AU-5 epitope tag. The mutated cDNAs were transiently transfected in PEAK 293 cells. We then confirmed expression of the transfected cDNAs by determining tracer [125I-Tyr A14]insulin I binding to detergent lysates of the transfected cells immobilized in microtiter plates coated with anti-AU5 IgG; no insulin binding was observed with lysates from mock transfected cells (data not shown). Detectable insulin binding was observed for all mutant cDNAs except those with alanine mutations of Arg14, Phe64, Phe705, Glu706, Tyr708, His710, Asn711 and Val715 (data not shown). Expression of these receptor mutants was subsequently confirmed and shown to be comparable of that of wild type receptor by Western blotting with anti-AU5 antibody (data not shown). We have previously shown that these mutants are also expressed normally as the recombinant secreted extracellular domain of the receptor(23-25). In order to characterize the equilibrium binding properties of the mutant receptors, detergent lysates of transfected cells were depleted of receptor pre-cursor by wheat germ agglutinin affinity chromatography (29). Insulin equilibrium
binding assays were then performed on receptor isolated from the resulting glycoprotein fractions and dissociation constants and IC50s were determined by computerized curve fitting using a two site sequential model as described under “Methods”. For wild type insulin receptor, Kd1, the dissociation constant of the high affinity component of binding, was 0.03 ± 0.001 nM; Kd2, the dissociation constant of the low affinity component of binding, was 0.4 ± 0.01 nM and the IC50 was 0.09 ± 0.01 nM (mean ± SEM, n=8; Table 1). These binding properties differ significantly from those of the secreted recombinant insulin receptor determined under similar experimental conditions. With this form of the receptor, insulin binds to a single population of binding sites with a lower affinity, Kd = 2.1nM,(24). This is consistent with the findings reported in other studies (7;17).

The dissociation constants and IC50s of alanine mutants of residues in the L1 domain and in the C-terminus of the α–subunit are summarized in Tables 1A and 1B respectively. In the L1 domain, alanine mutations of Arg14 and Phe64 appear to produce receptors that are devoid of insulin binding activity; no significant tracer binding was detected for these mutants even after 20-fold concentration by wheat germ agglutinin chromatography (29) despite comparable expression to wild type receptor in transiently transfected cells. Alanine mutation of Asn15 resulted in a greater than 20-fold decrease in affinity for insulin. Alanine mutations of Asp12, Gln34, Leu36, Leu37, Leu87, Phe89, Asn90, Tyr91 and Lys121 all resulted in 4 to 10-fold decreases. Mutations of Glu97 and Glu120 were without significant effect.

In the C-terminus of the α subunit, alanine mutations of Phe705, Glu706, Tyr708, His710, Asn711 and Val715 had similar detrimental effects on insulin binding to those described for alanine mutants of Arg14 and Phe64 in the L1 domain. Alanine mutations of Leu709 and Phe714 both produced a 4 to 5-fold decrease in affinity for insulin. Alanine mutations of Asp707, Val712 and Val713 had no significant impact.

For those mutations where we have been able to provide a quantitative analysis of effects on insulin affinity, there is impairment of both high (Kd1) and low affinity (Kd2) components of binding. Further the magnitude of impairment of both components is similar.

**Insulin Receptor Autophosphorylation**—To evaluate the role of this functional epitope in insulin receptor signaling we evaluated insulin regulated receptor autophosphorylation of alanine mutants of Asn15, Gln34 and Phe69; as discussed above these mutations result in a 4 to 24-fold reduction in affinity for insulin. Wheat germ agglutinin enriched glycoprotein fractions of transfected cell lysates were incubated with varying concentrations of insulin and then autophosphorylated. Tyrosine phosphorylation was quantitated by isolating receptor in microtiter plates coated with ant-AU5 antibody and quantitating phosphor-tyrosine content of the immobilized receptors with europium labeled anti-PY20 antibody. For all receptors studied, the dose response curve for auto-phosphorylation was bell-shaped (Fig. 1). Insulin produced a maximal 4.4 to 6.5 stimulation of auto-phosphorylation and there were no significant differences between receptors (data not shown). The mutant receptors produced right shifts in the dose response curve (Figure 1) and the EC50s for receptor auto-phosphorylation corresponded reasonably well with the IC50s for insulin binding (Table 2).

**DISCUSSION**

In order to compare the relative impact of the alanine mutations on the affinities of the full length receptor and the secreted recombinant receptor extra-cellular domain, the ratios of the IC50 of the mutant to the wild type IC50 for each mutation were compared for each type of receptor (Fig 1). Alanine mutations Arg14, Phe64, Phe705, Glu706, Tyr708, His710 and Asn711 which appear to inactivate the insulin binding function of the secreted recombinant receptor also do so for the full length receptor. However due to technical limitations of the assays it is impossible to assess whether this is due to quantitatively similar disruption of insulin affinity. Rather surprisingly alanine mutation of Val715, which has no impact on the affinity of the secreted receptor inactivates the full length receptor. Quantitatively similar changes in affinity in both forms of the receptor (4 to 10-fold) result from alanine mutations of Gln34, Leu36, Leu57, Asn90, and Tyr91 in the L1 domain. Mutations of Asp12, Leu77, Glu97, and Glu120 in the
L1 domain had a 2 to 3-fold greater effect on the affinity of the secreted extra-cellular domain for insulin than on the full length receptor. In contrast alanine mutation of Phe\textsuperscript{89} had a slightly larger effect on the affinity of the full length receptor (10-fold vs 6-fold). In the C-terminus of the α subunit, alanine mutations of Leu\textsuperscript{709} and Phe\textsuperscript{714} had significantly greater effects on the affinity of secreted receptor than the full length receptor (80-fold and 40-fold vs 5-fold and 6-fold respectively). Alanine mutations of Asp\textsuperscript{707}, Val\textsuperscript{712} and Val\textsuperscript{713} had no significant effect on affinity of either form of the receptor.

Thus, consistent with previous findings, we found that the affinity of the native insulin receptor for insulin is higher than that of the secreted recombinant receptor and its binding kinetics are more complex (7;17). While the functional epitopes of the equivalent ligand binding site of both forms of receptor are similar, certain amino acids appear to make significantly different contributions the free energy of binding of each form of the receptor. It is worth considering these findings in the context of current models of insulin/receptor interactions. Both De Meyts and Schaffer have proposed similar bivalent mechanisms of insulin binding to the receptor (7;8). Both propose that there are two distinct receptor binding sites on the insulin molecule and that there are two distinct insulin binding sites on each α subunit(1;7). Insulin first binds to one site on one α subunit and then cross-links the two heterodimers by binding to the non-equivalent site on the second subunit, generating the high affinity component of the receptor interaction. Binding of a second insulin molecule in this manner disrupts the cross-linking of the first and accelerates its dissociation (negative cooperativity), giving rise to the complex binding behavior exhibited by insulin interactions with the full length receptor. In contrast, in the recombinant secreted receptor and the isolated heterodimer, it is postulated that insulin only binds to the site with higher affinity and hence this interaction displays a lower affinity than that of the native receptor and simple binding kinetics. From the data presented by Shaffer (7), it is apparent that the affinity of this site is very much greater than that of the other. Thus it would be expected mutations producing changes in the affinity of this binding site for insulin, in the recombinant secreted receptor would also produce comparable changes in affinity of the holo-receptor. In general, this appears to be the case. Further this model would also suggest that alanine mutations compromising the higher affinity site, i.e. the binding site of the secreted recombinant receptor would have similar effects on the high and low affinity components of insulin binding in the full length receptor as is generally observed in the present study.

The sequential binding mechanism may also provide an explanation for the differences in the functional epitopes observed for the two forms of the receptor. Insulin has been observed to have varying conformations in different crystallographic and NMR solution structures (33;34). Thus it is possible that the initial binding event in the full length receptor may lead to a change in its conformation from that in solution thus producing the differences in effects of the alanine mutations from those observed with the secreted receptor. Further previous studies from this laboratory comparing the functional epitopes of the A and B isoforms of the receptor have provided evidence for plasticity in the conformation of the ligand binding site of the receptor (24). It is perhaps significant that the mutations which produce the most divergent effects in the full length and secreted receptor involve those residues that are most divergent in the functional epitopes of the A and B isoforms of the secreted receptor (24).

We also compared the effects of mutations of Asn\textsuperscript{15}, Gln\textsuperscript{34} and Phe\textsuperscript{89} on insulin induced receptor auto-phosphorylation. The dose- response curve for auto-phosphorylation was bell-shaped for all receptors. Such bell-shaped curves are indicative of bivalent binding mechanisms involving ligand cross-linking of binding sites (1). There were no significant differences in the maximal response to insulin, indicating that the coupling between ligand binding and the conformational changes resulting in activation of the receptor tyrosine kinase catalytic activity is intact in these mutants. This further confirms the structural integrity of the mutants. However these mutations did result in shifts of the concentration dependence of insulin regulated receptor auto-phosphorylation, which correlated reasonably well with the changes in affinity for insulin. This is consistent with the results of studies of the biological activities of insulin analogues which bind to this site, in which
it was found that changes in potency correlated with changes in affinity for the receptor (35).

In summary we have demonstrated that alanine mutations of amino acids in the functional epitope of the secreted recombinant receptor largely have similar effects on the affinity of the full length receptor for insulin. Further, we have shown that selected mutations cause changes in the insulin sensitivity of receptor proportional to their effects on affinity for insulin.

FOOTNOTES
This work was supported in part by grants from the National Institutes of Health (5 R01 DK065890) and the Juvenile Diabetes Research Foundation International (1-2000-198) to J.W.

Abbreviations
The abbreviations used are: [125I-Tyr A14] insulin, insulin radio-iodinated at Tyrosine14 of the A polypeptide chain of insulin.

REFERENCES


FIGURE LEGENDS

Figure 1: **Insulin regulated auto-phosphorylation of mutant receptors.** 293 PEAK cells were transiently transfected with insulin receptor cDNAs. Seventy two hours post-transfection, cells were harvested by detergent lysis and enriched glycoprotein fractions of cell lysates were prepared by wheat germ agglutinin chromatography(29). Insulin receptors were autophosphorylated in the presence of varying concentrations of insulin as described under “Experimental Procedures”. Results are expressed as % maximal stimulation and represent the mean S.E.M. of three independent experiments. The mutants are designated by the amino acid being mutated in single-letter code, followed by the number indicating its position in the insulin receptor sequence, followed by alanine.

Figure 2: **Comparison of the functional epitopes of the wild type and secreted recombinant insulin receptors.** Data for the the wild type insulin receptor (IR) from Table 1 were expressed as the ratio of the IC50 of the alanine mutant receptor to IC50 of the wild type receptor (IC50 MUT/ IC50 WT). Data for the secreted receptor (sIR) were taken from ref.(24). Results represent the mean of 4 independent experiments for each receptor. Amino acids mutated to alanine are designated by the single letter code. Those mutants with an affinity for insulin that was too low to be determined accurately were arbitrarily assigned a value for IC50MUT/ IC50 WT of 100.
**Table 1**

**Insulin binding to mutant insulin receptors.** 293PEAK cells were transiently transfected with cDNAs encoding alanine mutations of the B isoform of the insulin receptor. Equilibrium binding studies with insulin and [125I-Tyr A14]insulin were performed with receptors isolated from detergent lysates of transfected cells. Data were analyzed by curve fitting to a two site sequential model as described under “Methods” (8;30). Results are mean ± S.E.M of 8 independent determinations for wild type and 4 for mutants. The mutants are designated by the amino acid being mutated in single letter code, followed by the number indicating its position in the insulin receptor sequence, followed by alanine. IC50 is the insulin concentration that results in 50% inhibition of [125I-Tyr A14]insulin binding to the receptor. ND indicates that specific tracer [125I-Tyr A14]insulin binding to the mutant receptor was undetectable even after 20-fold concentration of the cell lysate.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Dissociation Constants</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kd1 (M x 10^-9)</td>
<td>Kd2 (M x 10^-9)</td>
</tr>
<tr>
<td><strong>A. L1 Domain</strong></td>
<td></td>
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<tr>
<td>WT</td>
<td>0.03 ± 0.001</td>
<td>0.4 ± 0.01</td>
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<tr>
<td>D12A</td>
<td>0.19 ± 0.017</td>
<td>3.6 ± 0.54</td>
</tr>
<tr>
<td>R14A</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>N15A</td>
<td>0.93 ± 0.13</td>
<td>11.8 ± 1.16</td>
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<tr>
<td>Q34A</td>
<td>0.24 ± 0.06</td>
<td>4.0 ± 0.41</td>
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<tr>
<td>L36A</td>
<td>0.15 ± 0.03</td>
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<td>L37A</td>
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<td>L87A</td>
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<td>N90A</td>
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<td>Y91A</td>
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<td>E120A</td>
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<td><strong>B. C-terminus of α-subunit</strong></td>
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<td>F705A</td>
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<td>E706A</td>
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<td>D707A</td>
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<td>H710A</td>
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<tr>
<td>N711A</td>
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<tr>
<td>V712A</td>
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<td>F714A</td>
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<td>V715A</td>
<td>ND</td>
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Table 2

Comparison of EC50s for receptor autophosphorylation and IC50s for insulin of mutant receptors. 
EC50s for insulin regulated receptor auto-phosphorylation were obtained by non-linear least squares 
analysis of the data in Figure 1. Data for relative IC50s are from Table 1.

<table>
<thead>
<tr>
<th>RECEPTOR</th>
<th>EC50 (nM)</th>
<th>EC50 relative to WT</th>
<th>IC50 relative to WT</th>
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<tr>
<td>WT</td>
<td>0.7 ± 0.2</td>
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<td>100</td>
</tr>
<tr>
<td>N15A</td>
<td>14.7 ± 0.3</td>
<td>20.1</td>
<td>20.4</td>
</tr>
<tr>
<td>Q34A</td>
<td>2.0 ± 0.2</td>
<td>2.8</td>
<td>6.5</td>
</tr>
<tr>
<td>F89A</td>
<td>3.7 ± 0.4</td>
<td>5.4</td>
<td>9.6</td>
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Figure 2: IC50MUT/IC50WT for different mutants in L1 and CTα domains.


- IR and sIR are represented by different line styles.
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J. Biol. Chem. published online March 30, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M411320200

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