Mutation of the High Cysteine Region of the Human Insulin Receptor \(\alpha\)-Subunit Increases Insulin Receptor Binding Affinity and Transmembrane Signaling*

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Our previous studies indicated that amino acid residues 240–250 in the cysteine-rich region of the human insulin receptor \(\alpha\)-subunit constitute a site in which insulin binds (Yip, C. C., Hsu, H., Patel, R. G., Hawley, D. M., Maddux, B. A., and Goldfine, I. D. (1988) Biochem. Biophys. Res. Commun. 157, 321–329). We have now constructed a human insulin receptor mutant in which 3 residues in this sequence were altered (Thr-Cys-Pro-Pro-Pro-Tyr-Tyr-His-Phe-Gln-Asp to Thr-Cys-Pro-Arg-Arg-Tyr-Tyr-Asp-Phe-Gln-Asp) and have expressed this mutant in rat hepatoma (HTC) cells. When compared with cells transfected with normal insulin receptors, cells transfected with mutant receptors had an increase in insulin-binding affinity and a decrease in the dissociation of bound \(^{125}\)I-insulin. Studies using solubilized receptors also demonstrated that mutant receptors had a higher binding affinity than normal receptors. In contrast, cells transfected with either mutant or normal receptors bound monoclonal antibodies against the receptor \(\alpha\)-subunit with equal affinity. When receptor tyrosine kinase activity and \(\alpha\)-aminoisobutyric acid uptake were measured, cells transfected with mutant insulin receptors were more sensitive to insulin than cells transfected with normal receptors. These findings lend further support therefore to the hypothesis that amino acid sequence 240–250 of the human insulin receptor \(\alpha\)-subunit constitutes one site that interacts with insulin, and they indicate that mutations in this site can influence insulin receptor binding and transmembrane signaling.

Insulin regulates the metabolic and mitogenic functions of many cells. Insulin initially interacts with its specific receptor located in the plasma membranes (1–4). The insulin receptor is a disulfide-linked tetrmeric glycoprotein composed of two extracellular 130-kDa \(\alpha\)-subunits containing the insulin-binding site(s) and two transmembrane 95-kDa \(\beta\)-subunits containing tyrosine kinase activity in their intracellular domains (1–4). The \(\alpha\) - and the \(\beta\)-subunits are synthesized as a precursor protein with a minimal molecular mass of 155 kDa as deduced from cDNA analysis (5, 6). When insulin binds to the \(\alpha\)-subunit, tyrosine kinase activity in the \(\beta\)-subunit is activated, and insulin action ensues (1–4).

We have reported recently the localization of a potential insulin-binding site on the receptor \(\alpha\)-subunit (7). We postulated that this site resides within a receptor fragment spanning residues 205–318. This sequence 240–250 in this fragment was of particular interest since its direct binding to agareshape insulin beads was inhibited by solubilized affinity-purified insulin receptors (7). This sequence therefore may play a role in the binding of insulin to its receptor.

Accordingly, we have now constructed a human insulin receptor mutant in which Pro\(^{245}\) Pro\(^{244}\), and His\(^{246}\) are changed to Arg\(^{245}\), Arg\(^{244}\), and Asp\(^{247}\), respectively. If the sequence 240–251 is important for insulin binding, then the introduction of charged amino acids in this region should greatly affect the binding of insulin by the mutant receptor. Herein, we report that when transfected into rat HTC cells, this mutant insulin receptor has higher affinity for insulin and is more effective in transmembrane signaling than the normal human insulin receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—HEPES, Triton X-100, polyethylene glycol 8000, sodium orthovanadate, ATP, poly(Glu-Tyr) 4:1, aprotinin, and phenylmethylsulfonyl fluoride were from Sigma. Bovine serum albumin fraction V was from Reheis, Chicago, IL; porcine insulin was from Eli Lilly, Indianapolis, IN; \(\alpha\)-[3H]-aminoisobutyric acid (\(\alpha\)-[3H]-AIB) was obtained from Du Pont-New England Nuclear. Oligonucleotides for site-directed mutagenesis were from the Biomolecular Resource Center, University of California, San Francisco. Monoclonal antibody (mAb) 20 was prepared and radiolabeled as described previously (8).

**Construction of Vectors**—A full length expression vector for the human insulin receptor was synthesized as described previously (9). A 5.5-kilobase insert of this vector containing the entire coding sequence of the receptor was cloned into M13mp19 (10). Oligonucleotide-directed mutagenesis was carried out on this construct (11), with the oligonucleotide CTTGAAATCGTCGATACCAGCGCGGCGG-CAGGT, containing 3-base pair changes from the sequence determined by Ullrich et al. (5). A suitable mutant clone was selected and its sequence confirmed by dideoxysequencing in M13 using a separate upstream primer (11). The final construct encoded the amino acid sequence Thr-Cys-Pro-Arg-Arg-Tyr-Tyr-Asp-Phe-Gln-Asp spanning residues 240–250. The mutated receptor fragment was reinserted into its original position in the expression vector, under the control of a Rous sarcoma virus promoter, and the SV40 early gene splicing and termination elements.

* The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AIB, \(\alpha\)-aminoisobutyric acid; mAb, monoclonal antibody; IR, insulin receptor(s).

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Transfection and Selection of Clones—HTC rat hepatoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum as described previously (12). Stable transfected cell lines were obtained using the calcium phosphate/glycerol shock method and co-selection for the neomycin resistance gene in pSV2neo as described previously (13). Resistant colonies were subcloned, and stable lines were tested for insulin binding (12). Those cell lines exhibiting the highest binding of 125I-insulin were subcloned. The presence of human receptors in these transfected cells were demonstrated further by the binding of 125I-labeled monoclonal antibodies specific for the human insulin receptor as described previously (8). Radioimmunoassay (14) of the insulin receptor indicated that HTC cells transfected with normal human receptors (IR) and HTC cells transfected with mutant receptors (IR mutant) contained 83,000 and 120,000 sites/cell, respectively.

Insulin Binding to Intact Cells—HTC-IR cells and HTC-IR mutant cells were grown to confluence on 1-cm² plastic multiwell plates. Cells were then incubated for 3 h at 15°C in bicarbonate-free Dulbecco’s modified Eagle’s H-16 medium supplemented with 15 mM HEPES (pH 7.4) and 10% fetal bovine serum with 6 pM 125I-insulin and various concentrations of unlabeled insulin. After incubation, cells were washed with 150 mM NaCl, 10 mM Tris-HCl (pH 7.6), and specific binding of insulin was determined as described previously (12). Data obtained were analyzed by Scatchard plots (15).

Dissociation of Bound Insulin—The rate of dissociation of the insulin receptor complex was determined as follows. Confluent cell cultures were incubated in bicarbonate-free HEPES-buffered Dulbecco’s modified Eagle’s H-16 medium containing 15 mM HEPES (pH 7.6) and 10% fetal bovine serum and 0.7 nM 125I-insulin at 15°C. The cells were then washed with medium at 4°C to remove unbound hormone. Fresh medium was added to each well, and the bound insulin was allowed to dissociate at 15°C. At various times, the medium was removed, and the cell-associated radioactivity was determined as described previously (12).

Insulin Binding to Solubilized Receptors—Insulin receptors from transfected cells were solubilized in 1% Triton X-100 containing 50 mM HEPES (pH 7.6), 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, and 0.1 mg/ml aprotinin. Cell debris was removed by centrifugation at 100,000 × g for 1 h, and the receptors were partially purified and concentrated by wheat germ agglutinin chromatography as described previously (16). Binding of 125I-insulin to solubilized receptors was determined by incubating 2 nM of insulin receptor (determined by radioimmunoassay) in 220 µl of 0.1% Triton X-100 with 8 µM labeled insulin and various concentrations of unlabeled insulin for 20 h at 4°C. Next, 180 µl of 21% polyethylene glycol 8000 was added, and the mixture was allowed to stand for 10 min at 4°C. The receptor-hormone complexes were separated from unbound hormone by centrifugation for 15 min at 1,500 × g. In all assays, nonspecific binding was less than 5% of total binding.

Activation of Insulin Receptor Kinase—Mutant and normal insulin receptors from transfected cells were partially purified by wheat germ agglutinin chromatography as described (16). Receptor protein was quantitated by radioimmunoassay, and the concentration of receptor was adjusted to 50 µg/ml. Insulin-dependent tyrosine kinase activity was determined by preincubating 2 nM of receptor with various concentrations of insulin for 16 h at 4°C in buffer containing 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 0.25% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride. Next, 15 µl of 2 mM MnCl₂, 10 mM MgCl₂ and 10 µM [γ-32P]ATP were added for 30 min. The synthetic substrate poly(Glu-Tyr) was then added at a final concentration of 2.0 mg/ml, and the incubation was continued for 30 min at 20°C. Finally, aliquots were spotted on 4-cm² discs of Whatman 3MM paper, and the dried discs were washed in four changes of 10% trichloroacetic acid containing 10 mM sodium phosphate. Radioactivity was determined by scintillation counting. The background controls were determined from duplicate reactions lacking poly(Glu-Tyr).

Insulin Binding—We first studied the binding of labeled insulin to cells expressing the mutant receptor (IR mutant) and the normal receptor (IR) (Fig. 1A). In cells with the mutant receptor, the IC₅₀ was 2.6 ± 0.7 nM (mean ± S.E., n = 3), and in the normal receptor it was 7.3 ± 0.6 nM. Scatchard analysis (Fig. 1B) demonstrated that cells transfected with the mutant receptor bound insulin with a higher affinity than cells transfected with the normal receptor (Kᵰ of 3.4 ± 0.3 nM versus 6.5 ± 0.6 nM, mean ± S.E., n = 3). Similar results were obtained when insulin binding was carried out at either 4°C for 20 h or at 22°C for 2 h, suggesting that the difference in binding affinity was independent of the incubation temperature.

The higher affinity of binding observed with the mutant receptor was due to a slower rate of dissociation of the bound hormone (Fig. 2). The half-life of dissociation of 125I-insulin from the mutant receptor was increased when compared with the normal receptor (60 ± 2 min versus 27 ± 1 min; mean ± S.E., n = 3).

FIG. 1. Binding of 125I-insulin to intact transfected HTC cells. A, competition inhibition curves of the binding of 125I-insulin to HTC cells transfected with normal human (IR) and mutant (IR mutant) receptors. B, Scatchard plots of the same data. Specific 125I-insulin binding was determined as described under “Experimental Procedures.” HTC cells were incubated with labeled and unlabeled insulin at 15°C for 3 h. A representative of three separate experiments is shown.

FIG. 2. Dissociation of bound 125I-insulin from HTC cells transfected with either normal (IR) or mutant (IR mutant) receptors. Cells were incubated with 22 pM 125I-insulin at 15°C for 3 h. The cells were then washed to remove unbound insulin, and dissociation was allowed to dissociate in medium without insulin. After the indicated times, cell-associated radioactivity was determined. Results are expressed as percent of original insulin bound at time 0. A representative of three separate experiments is shown.
The binding affinity of the mutant receptor was accompanied by enhanced transmembrane signaling. When solubilized cells transfected with the mutant receptor (Fig. 5). An also increased (Fig. 3) when compared with normal receptors affinity of insulin binding to solubilized mutant receptors was was an intrinsic property of the receptor structure since the sensitivity than the normal receptor (EDb0 of 0.75 ± 0.01 nM versus 0.95 ± 0.06 nM, mean ± S.E., n = 3). This increased sensitivity of the mutant receptor kinase to insulin was paralleled by an increased insulin sensitivity of AIB uptake by cells transfected with the mutant receptor (Fig. 5). An EDb0 of 0.1 ± 0.01 nM (mean ± S.E., n = 3) was obtained in cells transfected with the mutant receptor, compared with an EDb0 of 0.9 ± 0.04 nM (mean ± S.E., n = 3) obtained in cells transfected with the normal receptor.

**Binding of Antireceptor Monoclonal Antibodies**—In order to investigate whether the increase in the binding affinity observed in the mutant receptor was caused by a major alteration in the structure of the receptor α-subunit, we examined the ability of an α-subunit mAb 20 (8) to bind to both mutant and normal receptors. There was no difference between normal and the mutant receptors in the binding of labeled antibody (Fig. 6). Similar results were obtained when we used another monoclonal antibody, mAb 10 (8) (data not shown).

**DISCUSSION**

In our previous study, we bound a photolabeled insulin analogue to the human insulin receptor and obtained a photolabeled 23-kDa insulin receptor fragment by exhaustive digestion of the human receptor α-subunit with the endoproteinase Glu-C (7). We postulated that this fragment was derived from residues 205–316 and contained an insulin-binding site (7). We then focused on the receptor sequence 240–251 as a possible specific site of insulin binding. In preliminary experiments (29), we find that an antiserum

**FIG. 3.** Scatchard plot of 125I-insulin binding to solubilized insulin receptors prepared from HTC cells transfected with either normal (IR) or mutant (IR mutant) insulin receptors. Solubilized receptors were prepared as described under "Experimental Procedures" and incubated with 8 pM 125I-insulin for 20 h at 4°C.

**FIG. 4.** Tyrosine kinase activity of the insulin receptor toward the synthetic substrate poly(Glu-Tyr) in HTC cells transfected with normal (IR) and mutant (IR mutant) receptors. Solubilized receptors were prepared as described under "Experimental Procedures" and preincubated with insulin for 16 h at 4 °C; 10 μM [32P]ATP was then added for 30 min at 20 °C. Next, poly(Glu-Tyr) (2.5 mg/ml final concentration) was added for 30 min at 20 °C. The reaction was stopped by spotting duplicate aliquots on Whatman 3MM papers and washing the papers in trichloroacetic acid. Basal activity was 5–10% of maximal stimulation. Under basal conditions, 0.55 ± 0.06 and 0.93 ± 0.11 pmol of ATP (mean ± S.E., n = 3) were incorporated into poly(Glu-Tyr) by the mutant and normal receptors, respectively. Insulin-stimulated incorporation was 17.5 ± 1.0 and 23.3 ± 1.5 pmol of ATP in the mutant and normal receptors, respectively. A representative of three separate experiments is shown.

The increase in binding affinity observed with intact cells was an intrinsic property of the receptor structure since the affinity of insulin binding to solubilized mutant receptors was also increased (Fig. 3) when compared with normal receptors (Kd of 0.33 ± 0.09 nM versus 0.95 ± 0.06 nM, mean ± S.E., n = 3).

**Receptor Kinase Activity and AIB Uptake**—The increase in the binding affinity of the mutant receptor was accompanied by enhanced transmembrane signaling. When solubilized receptors were examined for their ability to phosphorylate the exogenous tyrosine kinase substrate poly(Glu-Tyr) in response to insulin (Fig. 4), the mutant receptor was more sensitive than the normal receptor (ED50 of 0.75 ± 0.1 nM versus 8.3 ± 0.9 nM, mean ± S.E., n = 3). This increased sensitivity of the mutant receptor kinase to insulin was paralleled by an increased insulin sensitivity of AIB uptake by cells transfected with the mutant receptor (Fig. 5). An ED50 of 0.1 ± 0.01 nM (mean ± S.E., n = 3) was obtained in cells transfected with the mutant receptor, compared with an ED50 of 0.9 ± 0.04 nM (mean ± S.E., n = 3) obtained in cells transfected with the normal receptor.

**FIG. 5.** Stimulation of [3H]AIB uptake. HTC-IR and HTC-IR mutant cells were preincubated for 24 h at 37 °C in serum-free medium (9). Cells were then washed and incubated for 2 h in the absence and presence of insulin. Next, 1.0 μCi/ml [3H]AIB was added, and uptake was measured. The basal uptake was 0.81 ± 0.1 and 0.54 ± 0.05 pmol of AIB (mean ± S.E., n = 3) in the HTC normal and mutant cells, respectively. Following insulin stimulation, AIB uptake was 1.7 ± 0.1 pmol in normal and 2.8 ± 0.025 pmol in mutant cells. A representative of three experiments is shown.

**FIG. 6.** Competition inhibition plot of the binding of mAb 20 (MA 20) to HTC cells transfected with normal (IR) and mutant (IR mutant) receptors. Cells were incubated for 3 h at 15 °C, using labeled mAb 20 and various concentrations of unlabeled mAb 20.
produced against a synthetic peptide containing the sequence of residues 243–252 (Pro-Pro-Tyr-Tyr-His-Phe-Gln-Asp-Trp-Arg) can immunoprecipitate the aforementioned photo-labeled 23-kDa fragment, thus confirming its origin in the high cysteine region of the insulin receptor α-subunit.

In the present study, we have examined the properties of a human insulin receptor that was mutated in this sequence. Experiments were carried out on HTC cells transfected with either the normal or mutant insulin receptors. Untransfected HTC cells have low numbers of insulin receptors and are relatively insensitive to insulin (12). However, after transfection with normal human insulin receptors, both sensitivity and responsiveness to insulin are markedly increased (9). The results of our present experiments indicated that the mutant receptor exhibited a 2–3-fold increase in insulin-binding affinity, which could be accounted for by a decrease in the rate of ligand dissociation. Moreover, the increased receptor binding affinity was associated with an enhancement of transmembrane signaling. Both insulin activation of receptor kinase activity and insulin stimulation of AIB uptake in cells transfected with mutant receptors occurred at 5–10-fold lower insulin concentrations than in cells transfected with normal receptors. Thus, the increase in binding affinity was associated with an even greater increase in cellular sensitivity to insulin. These results from our present study on the mutant receptor in which this sequence has been altered by site-specific mutagenesis lend further support, therefore, to our original hypothesis that sequence 240–250 may constitute a site at which insulin binds.

Studies on the structure and function of the hormone insulin have led to the hypothesis that the receptor-binding domain of insulin includes 1) the hydrophobic region involved in dimerization and 2) other exposed polar residues outside this region (18). This hydrophobic region involved in dimerization includes residues B24–B26 (Phe-Phe-Tyr) of the carboxyl end of the B chain of insulin. The receptor sequence 240–250 (Thr-Cys-Pro-Pro-Tyr-His-Phe-Gln-Asp) could provide, therefore, the required hydrophobic interaction with this hydrophobic region of the insulin B chain. However, such a hydrophobic interaction is not sufficient to ensure optimal receptor binding and transmembrane signaling. In naturally occurring insulins of low binding affinity and biological potency such as guinea pig insulin (19) and Atlantic hagfish insulin (20) the hydrophobic region of the B chain is conserved. Also, the low biological activity of these insulins is apparently not the result of gross structural changes since the three-dimensional structures of these insulins (as revealed by high resolution x-ray crystallography and by other spectroscopic methods) are nearly identical to those of other fully active insulins. Also in spite of the conservation of its B chain hydrophobic region, guinea pig insulin does not dimerize (19). Thus, the structural properties required for the dimerization of insulin are not necessarily the same as those required for its interaction with the receptor.

Other factors, therefore, such as charge and hydrophobicity of other residues are required for optimal interaction of insulin with its receptor. In two studies, the biological potency of a truncated and amidated analogue of insulin, des-(B26-B30)-[PhεNεNH₂]insulin, was found to be comparable to that of normal insulin (21, 22). Also, the replacement of PhεNεNH₂ with either tyrosine or histidine in this truncated insulin analogue resulted in a 2–3-fold increase in biological potency. Results from another study on the effect of pH on insulin binding (23) and another on the inhibition of receptor insulin-binding activity by diethyl pyrocarbonate (24) also indicated the importance of charge interactions between insulin and its receptor.

Insulin analogues with enhanced potencies have been reported. Recent studies have shown that an insulin analogue in which HisB⁹⁰ was replaced by aspartic acid was superactive with increased affinity of binding and increased biological activity (25, 26). In contrast, replacement of HisB⁹⁰ with asparagine resulted in reduced biological activity (27) (it is relevant to note that HisB⁹⁰ is replaced by asparagine in guinea pig insulin). One of the possible explanations for the super-activity of the AspB¹º insulin analogue is that this negatively charged AspB¹º residue interacts directly with a positively charged site on the receptor. More recently, it was reported (28) that the analogue, des-(B26-B30)⋅[AspB¹º, Tyr⁴⁵¹, NH₂] insulin was 11–13-fold more potent than intact insulin with respect to receptor binding affinity and stimulation of lipogenesis. These studies suggested, therefore, that residues B10 and B25 could be components of two distinct receptor-binding domains.

In our present study, we have observed a 2–3-fold increase in the affinity of insulin binding by the mutant receptor in which the sequence Thr-Cys-Pro-Pro-Pro-Tyr-Arg-Asp-Gln-Asp has been mutated to Thr-Cys-Pro-Arg-Asp-Tyr-Tyr-Asp-Gln-Asp, resulting in a net gain of a positive charge. The introduction of additional positive charges could enhance the charge interaction of the mutant receptor with normal insulin, for instance through the B10 residue and thus increase its binding affinity for insulin. It is possible that the mutations could have produced structural changes favorable to insulin binding. However, these structural changes, if present, were small and not detected by monoclonal antireceptor antibodies.

In conclusion, in our present study we have demonstrated that alterations of the residues in the sequence 240–250 by mutagenesis of the human insulin receptor both increased the affinity of insulin binding and enhanced insulin receptor transmembrane signaling. Although we cannot be certain that the sequence 240–250 of the receptor α-subunit interacts directly with insulin, our observations are consistent with the hypothesis that residues in this sequence play an important functional role in the interaction of the receptor with insulin. However, since the binding of insulin to its receptor most likely involves a conformational fit, it remains possible that there may be other site(s) for ligand interaction on the receptor α-subunit.

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