Complementation Analysis Demonstrates That Insulin Cross-links Both α Subunits in a Truncated Insulin Receptor Dimer*

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The insulin receptor is a homodimer composed of two αβ half receptors. Scanning mutagenesis studies have identified key residues important for insulin binding in the L1 domain (amino acids 1–150) and C-terminal region (amino acids 704–719) of the α subunit. However, it has not been shown whether insulin interacts with these two sites within the same α chain or whether it cross-links a site from each α subunit in the dimer to achieve high affinity binding. Here we have tested the contralateral binding mechanism by analyzing truncated insulin receptor dimers (midi-hIRs) that contain complementary mutations in each α subunit. Midi-hIRs containing Ala14, Ala64, or Gly714 mutations were fused with Myc or FLAG epitopes at the C terminus and were expressed separately by transient transfection. Immunoblots showed that R14A+FLAG, F64A+FLAG, and F714G+Myc mutant midi-hIRs were expressed in the medium but insulin binding activity was not detected. However, after co-transfection with R14A+FLAG/F714G+Myc or F64A+FLAG/F714G+Myc, hybrid dimers were obtained with a marked increase in insulin binding activity. Competitive displacement assays revealed that the hybrid mutant receptors bound insulin with the same affinity as wild type and also displayed curvilinear binding activity. These results demonstrate that a single insulin molecule can contact both α subunits in the insulin receptor dimer during high affinity binding and this property may be an important feature for receptor signaling.

The metabolic actions of insulin are initiated by its binding to the insulin receptor, a transmembrane protein whose structure has been studied extensively and is now well characterized. The insulin receptor (IR)2 is a homodimer composed of two identical αβ half receptors. A single disulfide bond covalently links the αβ subunits in each half receptor, and the half receptors are also linked by multiple disulfide bonds between the α subunits.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotide primers were prepared using an Applied Biosystems DNA Synthesizer. PCR amplification and ligation of DNA fragments, preparation of plasmid DNA, and DNA sequence analyses were performed using standard techniques. Cultured 293H cells were purchased from Invitrogen. 125I(A14)-insulin was obtained from Amersham Biosciences. All chemicals were molecular biology or reagent grade.

Construction and Expression of midi-hIR—The midi-hIR cDNA corresponds to mIR.Fn0/Ex10 as described by Brandt et al. (6) and was constructed by PCR amplification and ligation of DSS, disuccinimidyl suberate; DTT, dithiothreitol; WT, wild type; aa, amino acid.
DNA fragments using a cloned hIR cDNA available in our laboratory as the template. The resultant WT+FLAG and WT+Myc cDNAs encoded hIR signal peptide and residues 1–601 to residues 650–719 together with an epitope tag sequence FLAG (DYKDDDDK) or Myc (EQKLISEEDL) at the C terminus and were inserted into mammalian expression vector pcDNA 3.1 (Invitrogen). DNA sequencing confirmed the plasmids contained the canonical hIR sequence (9) except for coding mutations H144Y and K224R due to allelic variations present in our cloned hIR cDNA (sequences available upon request). Mutant receptors R14A+FLAG (CGG mutated to GCG at residue 14), F64A+FLAG (TTC mutated to GCC at residue 64), and F714G+Myc (TTC mutated to GGC at residue 714) were generated by in vitro mutagenesis of the WT midi-hIR plasmid using the QuikChange site-directed mutagenesis kit from Stratagene. The entire cDNA insert in each mutant midi-hIR was verified by DNA sequencing.

The mid-hIR constructs were transiently expressed by transfection into 293H cells using Lipofectamine 2000 reagent (Invitrogen). Medium was collected 72 and 144 h post-transfection, concentrated 7-fold by ultrafiltration (Amicon Ultra 15; Millipore), adjusted to 50 mM Hepes, pH 7.5, 0.02% NaN₃, and stored at 4 °C until used.

**Insulin Binding Assay**—Binding of $^{125}\text{I}(A14)$-insulin by the midi receptors was assayed by the polyethylene glycol precipitation method (10). Samples containing midi-hIR were incubated in 200 μl of binding buffer (100 mM Hepes, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.25% (w/v) bovine serum albumin, 0.025% (w/v) Triton X-100) containing 30,000 cpm (~10 pm) $^{125}\text{I}(A14)$-insulin overnight at 4 °C. 500 μl of 0.2% γ-globulin and 500 μl of 30% (v/v) polyethylene glycol M₄000 were added to co-precipitate the insulin-receptor complex, the sample was microfuged for 10 min, the supernatant was removed by aspiration, and the pellet was counted in a γ counter. Specific binding was determined by subtracting background radioactivity obtained with only binding buffer. For competitive displacement assays, unlabeled human insulin was added to the samples in the concentrations indicated.

**Chemical Cross-linking and Immunoprecipitation**—Disuccinimidyl suberate (DSS) was used to covalently cross-link $^{125}\text{I}(A14)$-insulin to the midi-hIR. After an overnight incubation in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 mM DSS (freshly dissolved in binding buffer at 4 °C, samples containing the insulin-receptor complex were treated with 10 M

**SDS-PAGE and Immunoblotting**—Samples were dissolved in Laemmli loading buffer in the presence (reducing) or absence (non-reducing) of 2% β-mercaptoethanol, heated at 90 °C for 3 min, and electrophoresed on 6% acrylamide slab gels. For autoradiography, gels were fixed for 20 min in 10% acetic acid/25% isopropanol (v/v), dried onto Whatman 3MM paper, and exposed to x-ray film (Kodak Biomax MS) according to the manufacturer’s instructions. Mobility of a 220-kDa marker protein is shown. B, $^{125}\text{I}(A14)$-insulin binding activity. Media from 293H cells transiently transfected with WT, R14A+FLAG, F64A+FLAG, F714G+Myc, or co-transfected with R14A+FLAG/F714G+Myc, F64A+FLAG/F714G+Myc, or R14A+FLAG/F64A+FLAG were assayed for $^{125}\text{I}(A14)$-insulin binding as described under “Experimental Procedures.” Results show average values for percent bound of $^{125}\text{I}(A14)$-insulin obtained with duplicate samples of 5 μl of WT and 10 μl of mutant midi-hIR medium with the range values too small to be shown. NB, no binding detected.

**FIGURE 1. Expression and insulin binding activity of WT and mutant midi-hIRs. A, immunoblot of WT, R14A+FLAG, F64A+FLAG, and F714G+Myc. 3 μl of medium from 293H cells transiently transfected with the indicated midi-hIRs were separated on a non-reducing 6% SDS-PAGE, transferred onto Hybond-N membrane (Amersham Biosciences), and probed with anti-hIR N20. The immunoblot was visualized using an ECL kit (Amersham Biosciences) according to the manufacturer’s instructions. Mobility of a 220-kDa marker protein is shown. B, $^{125}\text{I}(A14)$-insulin binding activity. Media from 293H cells transiently transfected with WT, R14A+FLAG, F64A+FLAG, F714G+Myc, or co-transfected with R14A+FLAG/F714G+Myc, F64A+FLAG/F714G+Myc, or R14A+FLAG/F64A+FLAG were assayed for $^{125}\text{I}(A14)$-insulin binding as described under “Experimental Procedures.” Results show average values for percent bound of $^{125}\text{I}(A14)$-insulin obtained with duplicate samples of 5 μl of WT and 10 μl of mutant midi-hIR medium with the range values too small to be shown. NB, no binding detected.**
The expressed midi-hIR sequence corresponded to the mIR. Fno/Ex10 construct as described by Brandt et al. (6) and contained the hIR signal peptide plus aa 1–601 fused to aa 650–719 of the h9251 subunit. In addition, a Myc (EQKLISEEDL) or FLAG (DYKDDDDK) epitope tag sequence was fused to the C terminus to uniquely label each receptor. After transfection with WT/h11001 FLAG or WT/h11001 Myc into 293H cells, immunoblot analysis showed that a predominately 260-kDa truncated IR dimer was secreted into the medium, which was reduced by mercaptoethanol to the 130-kDa monomeric form. The medium also contained high affinity insulin binding activity with a calculated IC\text{50} 0.27 nM (see Fig. 5), consistent with results presented by Brandt et al. (6).

Mutant midi-hIRs R14A/FLAG, F64A/FLAG, and F714G/Myc were prepared by in vitro mutagenesis and analyzed for expression after transient transfection. ImmunobLOTS revealed that each mutant receptor was expressed as a 260-kDa dimer in similar amounts to WT, but no insulin binding was detected (Fig. 1). These results show that alanine mutations at Arg14, Phe64, and glycine mutation at Phe714 that markedly...
reduced insulin binding in the holo-IR (7, 8) also inactivated insulin binding in the midi-hIR. However, when 293H cells were co-transfected with two complementary constructs, R14A + FLAG and F714G + Myc, or F64A + FLAG and F714G + Myc, insulin binding activity was recovered in the medium. A control co-transfection performed with two non-complementary mutants, R14A + FLAG and F64A + FLAG, yielded no detectable insulin binding activity, and this provided evidence that the mini-hIRs must contain complementary mutations (Fig. 1). However, we always obtained 2–6-fold less insulin binding activity than would be expected from the amount of hybrid receptor formed by random dimerization of each mutant α subunit. The explanation for this discrepancy is not known, but one possibility is that mutant receptors may preferentially form homodimers due to subtle conformational changes introduced by the presence of different epitope tags at the C terminus or to the presence of the mutated residues. Thus, the amount of hybrid receptor present in the medium after co-transfection with two mutant midi-hIR may be less than expected based on the immunoblot result.

To demonstrate that the recovery of insulin binding activity was due to the formation of hybrid mutant receptors, several experiments were performed to characterize these receptors. First, when media from single mutant transfections were combined, no insulin binding activity was recovered, indicating that co-expression of the complementary mutant receptors in the same cell was necessary as would be the case in order to form hybrid receptors.

Truncated Hybrid Mutant Insulin Receptors

FIGURE 5. Competitive insulin binding assays for WT( ), R14A + FLAG/F714G + Myc( ), and F64A + FLAG/ F714G + Myc( ). Upper panel, media containing the indicated receptors were assayed for 125I(A14)-insulin binding in the presence of increasing concentrations of unlabeled insulin. Each data point represents average value obtained from duplicate samples. A Scatchard analysis of each competitive binding assay is shown in the lower panels. IC50 ± S.E. values calculated from three to five independent experiments were as follows. WT = 0.27 nM ± 0.38, R14A + FLAG/F714G + Myc = 0.13 nM ± 0.09, F64A + FLAG/F714G + Myc = 0.38 nM ± 0.37.

PAGE revealed that substantially all of the insulin-labeled receptors were immunoreactive with both antibodies, and this provides strong evidence that the active receptor is a covalent hybrid dimer (Fig. 3). However, if the 125I(A14)-insulin-labeled R14A + FLAG/F714G + Myc receptor was first reduced to monomeric form by incubation with 2 mM DTT and subsequently immunoprecipitated with anti-FLAG and anti-Myc, only the R14A + FLAG α subunit was detected (Fig. 4). This result demonstrates that DSS cross-links 125I(A14)-insulin specifically to the R14A + FLAG α subunit and therefore insulin was bound asymmetrically to the hybrid receptor. We have recently performed the same analysis with a hybrid midi-hIR in which the epitope tags were reversed, i.e. R14A + Myc/ F714G + FLAG, and in this case the cross-linked insulin was immunoprecipitated only with anti-Myc (data not shown). This result demonstrates that insulin cross-linking was not dependent on the epitope tag sequence.

Competitive displacement assays were performed to compare the binding affinity of the hybrid mutant receptors with that of WT. Fig. 5 shows representative competitive assays performed with WT, R14 + FLAG/F714A + Myc, and F64A + FLAG/ F714G + Myc. There were no significant differences in the calculated values for affinity binding constants with WT = 0.27 nM ± 0.38, R14A + FLAG/F714G + Myc = 0.13 nM ± 0.09, and F64A + FLAG/F714G + Myc = 0.38 nM ± 0.37. In addition, Fig. 5 shows that curvilinear Scatchard plots were also obtained for both WT and hybrid receptors. These results demonstrate
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that the hybrid mutant receptors bound insulin with complex kinetic properties similar to WT mini-hIR.

DISCUSSION

Utilizing alanine-scanning mutagenesis, Whittaker et al. (7, 8) have reported that mutations at Arg\(^{14}\), Phe\(^{64}\), and Phe\(^{714}\) in the holo-IR resulted in a marked loss of insulin binding activity. Here we have shown that secreted truncated IR dimer constructs (mini-hIRs) with an alanine or glycine mutation at Arg\(^{14}\), Phe\(^{64}\), or Phe\(^{714}\) were efficiently expressed and correctly folded but also exhibited no detectable insulin binding activity. However, by co-expressing two mini-hIRs with complementary mutations, R14A + FLAG/F714G + Myc or F64A + FLAG/F714G + Myc, a hybrid receptor was obtained that bound insulin with high affinity similar to wild type mini-hIR and yielded a curvilinear Scatchard plot.

Several control experiments were performed to ensure that the insulin binding activity was attributable to the formation of a hybrid receptor. Thus, we showed that treatment with 2 mM DTT reduced the α-α disulfide bond in the dimer to yield inactive mini-hIR monomer. Similarly, insulin binding activity was not recovered when two preformed mutant mini-hIRs were incubated in vitro, and the co-expression of two noncomplementary mutants, i.e. R14A + FLAG and F64A + FLAG, did not yield active hybrid receptor.

We have also shown that $^{125}$I(A14)-insulin is bound asymmetrically to the hybrid mini-hIRs containing R14A + FLAG/F714G + Myc or F64A + FLAG/F714G + Myc. After cross-linking with DSS and reduction, the labeled A-chain was selectively immunoprecipitated with anti-FLAG. Because DSS can cross-link with the primary amine at Gly\(^{A3}\), this implies that the insulin N-terminal A-chain binds to the C-terminal region of the α-subunit. This result is consistent with the recent finding reported by Wan et al. (14), who used photo-affinity-labeled insulin analogue to identify that Val\(^{A3}\) interacts with the C-terminal region of the IR α chain.

The recovery of high affinity insulin binding activity in the hybrid mutant mini-hIR suggests that insulin can bind contrateralaterally to the two α subunits in the holo-IR and this property may be important for IR signaling. It is now clear that the signaling mechanisms for a number of hormones, including growth hormone and the epidermal growth factors, involve ligand-mediated receptor dimerization (15–17). In the case of insulin, the conundrum has been that the IR already exists as a covalently linked dimer. Nonetheless, several models have been proposed in which a single insulin molecule is shown to bind both α subunits, and this binding induces a conformational change in the IR to activate the intrinsic tyrosine kinase (18–21). Our results suggest that by constructing a hybrid holo-IR with a complementary mutation in each α subunit using sequences analogous to those described here, the validity of a cross-linking mechanism can be tested by characterizing hybrid holo-IR insulin binding and tyrosine kinase activities.

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