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Structural Basis by Which a Recessive Mutation in the α-Subunit of the Insulin Receptor Affects Insulin Binding

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Recently, a mutation substituting Leu for Ser in the α-subunit of the human insulin receptor has been identified in an insulin-resistant patient. The Leu mutant leads to a severe impairment in insulin binding without significantly altering the processing or cell surface expression of the receptor. In order to study how αβ half-receptors interact to form the insulin-binding site, we cotransfected NIH-3T3 cells with two insulin receptor cDNA constructs: a truncated insulin receptor lacking the C-terminal 43 amino acids (Δ43) and the full-length Leu mutant receptor. A clonal cell line from cotransfected cells expresses a hybrid receptor consisting of a Leu half-receptor and a Δ43 half-receptor. We demonstrate that the Leu-Δ43 hybrid receptor binds insulin with high affinity. Furthermore, by cross-linking 125I-insulin to immobilized hybrid receptors, we show that only the αΔ half of the hybrid receptor binds insulin. Since the isolated half-insulin receptor has low affinity for insulin, this suggests that the addition of even a non-binding α-subunit can result in high affinity binding to the holoreceptor (αmutΔβ). Both β and β α-subunits of the Leu-Δ43 hybrid receptor are phosphorylated in vivo and in vitro in an insulin-dependent manner, suggesting an intramolecular transphosphorylation mechanism and that the presence of the Leu mutant receptor that lacks an intrinsic high affinity binding site does not prevent the associated β-subunit from functioning either as a tyrosine kinase or as a phosphate acceptor in the hybrid insulin receptor molecule (αmutβ). Furthermore, we show that the hybrid receptor can phosphorylate insulin receptor substrate-1 (IRS-1) in response to insulin and can be coimmunoprecipitated together with IRS-1 by anti-IRS-1 antibody.

The insulin receptor (IR) is a heterotetrameric glycoprotein composed of two identical αβ disulfide-linked half-receptors that form the αβ holoreceptor complex (1, 2). Insulin binding to the α-subunit results in autophosphorylation of the β-subunit on tyrosine residues. Formation of high affinity binding sites requires the cooperation of two α-subunits in an αβ oligomeric receptor molecule (3–6). The binding characteristics of insulin to its receptor, however, are complex as reflected by a curvilinear Scatchard plot. This has been explained by negative cooperativity. Insulin binding to the purified αβ holoreceptor exhibits negative cooperativity (7). Studies using insulin analogues as probes suggest that one molecule of insulin binds with high affinity to the αβ holoreceptor (4–6). However, when binding studies were carried out using purified αβ half-receptor, only the low affinity binding site was detected (3, 8). In all previous studies, however, the potential high affinity binding site of the unoccupied α-subunit half remained intact; and therefore, it is unclear as to whether this site plays a role in binding to the opposite α-subunit or only provides for a steric interaction between the two α-subunits in creating the high affinity binding site.

Recently, a mutation in the human insulin receptor in which Ser in the α-subunit was mutated to Leu has been identified in a patient with a genetic form of insulin resistance. This mutation severely impairs insulin binding without significantly altering IR processing or cell surface expression. In the present study, we have expressed a hybrid receptor consisting of the binding deficient Leu mutant half-receptor (αmutβ) with a high affinity binding truncated half-receptor (αΔβ) to form a holo αmutΔβ hybrid receptor. In doing so we have constructed an in vitro model for heterozygosity for the Leu mutation as found in the patient's mother. Inasmuch as the mother appears not to be insulin resistant, we conclude that the Leu mutation causes insulin resistance in a recessive fashion.

We demonstrate that the hybrid αmutΔβ insulin receptor binds insulin with high affinity. Furthermore, by cross-linking 125I-insulin to the immobilized hybrid receptors we show that only the wild type α-subunit can be cross-linked to insulin. This suggests that the addition of even a very low affinity α-subunit can result in high affinity binding to the holoreceptor (αmutβ). Furthermore, we show that both β-subunits (i.e. β and β) were phosphorylated in the hybrid receptors, probably by an intramolecular transphosphorylation mechanism. Finally, despite the presence of a non-binding αmut-subunit in this hybrid receptor, insulin binding leads to phosphorylation of IRS-1. Thus, the hybrid receptor has near normal function with respect to both insulin binding and tyrosine kinase activity. Thus, the studies presented in this report provide insights into the molecular mechanisms that explain the recessive effects of the Leu mutation.

MATERIALS AND METHODS

Expression of Insulin Receptors in Transfected NIH-3T3 Cells—To construct a vector for transfection of the human insulin receptor cDNA containing the Leu mutant receptor, genomic DNA from the patient was amplified by polymerase chain reaction. The upstream primer was

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‡ The abbreviations used are: IR, insulin receptor; IRS-1, insulin receptor substrate-1; WT, wild type; DTI, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
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a 43-mer which consisted of the final 20 base pairs of the 3' end of exon 3 followed the initial 23 base pairs of the 5' end of exon 4. The downstream primer was a 48-mer which consisted of the terminal 23 base pairs at the 3' end of exon 4 preceded by the initial 25 base pairs of the 5' end of exon 5. These primers were chosen in order to use that EcoRI and SalI restriction sites located in exon 3 and 5, respectively. The amplified fragment was then digested with EcoRI and SalI and ligated into pGEM4Z (Promega, Madison, WI) containing the wild type hIR cDNA which had been similarly digested. Competent Escherichia coli (DH5α cells, Life Technologies, Inc.) were transformed using the ligation mix and, since the presence of the mutation created a new BsrXI restriction site, plasmids containing the exon 4 mutation (Leu323) were identified by their ability to be digested with this enzyme. The hIR cDNA was excised from the construct using the restriction enzyme Sall and was ligated into a bovine papilloma virus expression vector (pBPV) which had been cut at its single XhoI site (pBPV-Leu323).

Using site-directed mutagenesis, we introduced a premature chain termination at codon 1301 of the insulin receptor cDNA (9), leading to a C-terminal deletion of 43 amino acid of the β-subunit of the insulin receptor (Δ43). WT and truncated cDNA were ligated into pBPV (Phar- macia LKB Biotechnology Inc.) as described elsewhere (10).

NIH-3T3 cells were transfected using lipofectin (Life Technologies, Inc.) and a mixture of insulin receptor expression vector (20 μg) plus expression vector containing the neomycin-resistance gene, pSvNeo, into the NIH-3T3 cells (11). A mixture of the expression vector for a single type of insulin receptor or cotransfected with a mixture of expression vectors for two types of insulin receptor (9). Stable transfecants were selected by cultivation in the presence of G418 (600 μg/ml) (Life Technologies, Inc.). After selection, insulin receptor expression by stable transfecants was assayed by measuring [3H]-insulin binding and, in the case of cotransfected cells, clones expressing both the full-length and the Δ43 insulin receptors were selected by Western blot analysis.

Biotinylation and Immunoprecipitation of Cell Surface Insulin Receptors—Confluent monolayers of transfected NIH-3T3 cells in Petri dishes (10-cm diameter) were biotinylated as described elsewhere (9). After cell solubilization, insulin receptors were immunoprecipitated using anti-receptor antibody B-10 directed against the α-subunit (13, 14) at a dilution of 1:50 or with a rabbit antibody (rAb50) directed against a peptide corresponding to amino acids 1321–1336 of the β-subunit of the human insulin receptor at a dilution of 1:50 (15). This peptide has been deleted from the Δ43 mutant insulin receptor, and, therefore, this antibody does not react with the Δ43 mutant. After electroblotting, nitrocellulose sheets were probed with horseradish peroxidase-linked streptavidin (Amersham Corp.) at a dilution of 1:500 in order to detect biotinylated insulin receptors, and enhanced chemiluminescence detection was performed (ECL Western blotting detection reagents, Amersham Corp.).

Insulin Binding to Immobilized Insulin Receptors—Transfected cells were solubilized in 500 μl of buffer (150 mM NaCl, 50 mM HEPES, pH 7.4) containing Triton X-100 (1%), phenylmethylsulfonyl fluoride (2 mM), and aprotinin (15 trypsin inhibitor units/ml), and then insulin receptor was immobilized on streptavidin (0.5 unit/ml). Receptors were immunoprecipitated with a rabbit antibody (rAb50) or rAb53 (rAb53 recognizes the β-subunit, and therefore immunoprecipitates both the Δ43 and the full-length β-subunit) at a dilution of 1:100. Immune complexes were precipitated with protein A-agarose (Life Technologies, Inc.), and the pellets were washed three times at 4°C in 1 ml of buffer (150 mM NaCl, 50 mM HEPES at pH 7.4) containing Triton X-100 (1%). After the last wash the immune precipitates were incubated in binding buffer (120 mM NaCl, 1 mM EDTA, 15 mM sodium acetate, 2.5 mM KCl, 1.2 mM MgSO4, 10 mM glucose, 50 mM HEPES) containing bovine serum albumin (10 μg/ml) and 100 μM insulin (0.1 ng/ml) as tracer at 4°C for 16–18 h. Tracer binding was inhibited by increasing concentrations of unlabeled insulin. The reaction was stopped by centrifugation at maximum speed in a microfuge for 5 min at 4°C; the pellets were washed three times with binding buffer and then counted in a gamma counter.

Insulin Cross-linking to Insulin Receptor—Transfected NIH-3T3 cells were solubilized in 500 μl of buffer (150 mM NaCl, 50 mM HEPES, pH 7.4) containing Triton X-100 (1%), phenylmethylsulfonyl fluoride (2 μM), aprotinin (15 trypsin inhibitor units/ml), and antipain (0.1 μg/ml, aprotinin and antipain was omitted for quantification of disuccinimidyl suberate (10 μM sodium bicarbonate) was added to give a final concentration of 0.1 μM. After incubation on ice for 20 min, the reaction was quenched by addition of four volumes of Tris (10 mM), EDTA (1 mM) in phosphate-buffered saline (16). The complex was pelleted by centrifugation. The pellets were washed and reprecipitated in 75 mM Tris at pH 8.5 containing 1.25 mM DTT for 30 min at room temperature as described previously (17). The reaction was quenched by adding a 5-fold excess of ice-cold HEPES buffer (30 mM HEPES, pH 7.6, 0.02% sodium azide). The pellets were again washed and were resuspended in Lammli buffer with or without DTT, electroblotted with 10% SDS-PAGE, and transferred to nitrocellulose sheets for 2 h at room temperature as described previously (18). The blots were probed with horseradish peroxidase-linked streptavidin (Amersham Corp.) at a dilution of 1:500 in order to detect biotinylated insulin receptors, and enhanced chemiluminescence detection was performed (ECL Western Blotting detection reagents, Amersham Corp.).

Phosphorylation of Insulin Receptors and IRS-1 in Intact Cells—Phosphorylation of insulin receptors in intact cells was conducted as described elsewhere (9). Insulin receptors were immunoprecipitated with antibody B-10 or rAb50. Endogenous IRS-1 was immunoprecipitated with a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY) and horseradish peroxidase-linked anti-mouse γ-globulin (Amersham Corp.). Filters were washed and ECL was performed as described previously (9).

In Vitro Phosphorylation—Insulin receptors from transfected cells were immunoprecipitated with rAb50 or rAb53 and immobilized on protein A-agarose as we described previously (Materials and Methods). Immobilized receptors were incubated in the presence or in absence of 10−6 M insulin at room temperature for 30 min. Thereafter, phosphorylation was initiated by the addition of 5 mM ATP, 1 mM CTP, and 3 mM manganese acetate. The reaction was stopped by the addition of Laemmli sample buffer containing DTT (80 mM) and boiling for 5 min. The samples were analyzed by SDS-PAGE (6.5%) gel electrophoresis, and proteins were transferred to nitrocellulose. The membranes were incubated with antiphosphotyrosine antibody (as described above), and ECL detection was performed.

RESULTS

Expression of Wild Type, Mutant, and Leu323Δ43 Hybrid Insulin Receptors—Cells were first singly transfected with the wild type insulin receptor, the truncated Δ43 receptor which has a deletion of 43 amino acids at C-terminal of the β-subunit (18, 19) or the Leu323 mutant receptor. After biotinylation of cell

![Image](https://via.placeholder.com/150)
surface insulin receptors and immunoprecipitation with either B10 or rAb50, blotting with horseradish peroxidase-streptavidin showed that cells expressing WT or Leu232 mutant insulin receptors express a β-subunit of an apparent molecular mass of 95 kDa, whereas the truncated β-subunit of the Δ43 IR has an apparent molecular mass of 90 kDa (Fig. 1). Similar experiments were performed on cells cotransfected with Δ43 and Leu232 receptors (Fig. 1). Following immunoprecipitation with antibody B10 and with rAb50, a clonal cell line was identified which expressed hybrid receptors as indicated by the presence of two bands: one of 90 kDa corresponding to the truncated β-subunit of the Δ43 receptor, and one of 95 kDa corresponding to the β-subunit of the Leu232 mutant receptor. Since rAb50 does not recognize the Δ43 mutant, the presence of these two bands indicates the presence of the Leu232-Δ43 hybrid receptor (Fig. 1, right panel). In addition to the Leu232-Δ43 hybrid receptor, it is likely that this clone may also express IR homodimers (Δ43-Δ43 or Leu232-Δ235).

**Insulin Binding to the Leu232-Δ43 Hybrid Insulin Receptor**

Δ235-Insulin binding to intact cells and to immunoprecipitated insulin receptors was measured as described under "Materials and Methods." Insulin binding to intact cells was performed overnight at 4°C in the presence or absence of increasing concentrations of unlabeled insulin. The B/T (specific binding over total radioactivity added) ratio was expressed as function of added insulin in different cell lines as reported in A. Using the same batch of cells, cells were lysed in detergent, and insulin receptors were immunoprecipitated with either rAb50 (directed against the amino acids 1321-1336) (panel B) or rAb55 (raised against the C-terminal of the β-subunit) (panel C) overnight at 4°C and immobilized on protein A-agarose. Insulin binding assay was conducted with the immune complex immobilized on protein A-agarose-rAb50 or rAb53-insulin receptors in the presence or absence of increasing concentrations of unlabeled insulin. The maximum binding measured in the absence of unlabeled insulin on intact cells or immunoprecipitated receptors from different clonal cell lines was summarized in panel D where the maximum binding is expressed as percent of Bo/Te specific binding in the absence of unlabeled insulin over total radioactivity added. The affinity of the hormone to bind to Leu232-Δ43 hybrid receptors was finally compared to WT IR on immunoprecipitates (panel E). These data are the mean of two experiments in which each point was measured in triplicate for binding data on intact cells and in duplicate for data concerning immobilized receptors.

The presence of Δ43 homodimers in cells expressing hybrid receptors could account for a part of insulin binding seen in these cells. To distinguish between insulin binding to the Δ43 homodimers and to the Leu232-Δ43 hybrid receptors, immunoprecipitation using either rAb53 or rAb50 was performed. rAb53, which is directed against the insulin receptor β-subunit and recognizes Δ43 homodimers and rAb50 which was raised against the C-terminal portion of the insulin receptor β-subunit and does not immunoprecipitate the truncated β-subunit of Δ43 receptor. After solubilization of cells and incubation of detergent extracts with antibody, insulin receptors were immobilized on protein A-agarose. The immunoprecipitation efficiencies of rAb53 and rAb50 were similar, as both antibody immunoprecipitates showed identical specific binding on lysates from cells expressing WT insulin receptor (Fig. 2D). Similarly rAb53 immunoprecipitates from cells expressing the Δ43 holoreceptors and the Leu232-Δ43 hybrid IR showed similar specific binding (Fig. 2D). In cells expressing Leu232 mutant receptor, as expected, no significant binding was detected using either antibodies (less than 10% of the binding to WT receptors). 125I-Insulin binding to rAb50 immunoprecipitates showed that lysates from cells expressing Leu232 mutant receptors has no detectable binding. Lysate of cells expressing the Δ43 IR has insulin binding activity that can be immunoprecipitated by rAb53 but not by rAb50. In contrast, rAb50 and rAb53 immunoprecipitates from cells expressing Leu232-Δ43 hybrid receptors exhibited specific insulin binding. The affinity of the Leu232-Δ43 hybrid receptor for insulin was estimated by measuring 125I-insulin binding on WT and on hybrid receptors immobilized by rAb50 antibody and protein A-agarose. The
No bands were detected in the Leu²³³ insulin immunoprecipitates (Fig. 3A) which is in good agreement with the absence of insulin binding to the immunoprecipitated Leu²³³ holoreceptor (Fig. 3B). rAb53 immunoprecipitates from cells expressing the Δ43 IR or the Leu²³³-Δ43 hybrid IR showed a band corresponding to the α-subunit of IR which was specifically labeled with ¹²⁵I-insulin; this labeling was completely inhibited in the presence of an excess of unlabeled insulin. The presence of labeled α-subunit in rAb53 immunoprecipitates is well correlated with binding of insulin to the immunoprecipitated holoreceptors (the Δ43 or the Leu²³³-Δ43 IR) (Fig. 3B). Furthermore, the amount of labeled α-subunit was similar in cells expressing the Δ43 and in cells cotransfected with both Leu²³³ and Δ43 IR as shown by the phosphorimager analysis (Fig. 3B). In rAb50 immunoprecipitates from cells expressing the Δ43 or the Leu²³³-Δ43 IR, no bands were detected. Whereas, when insulin binding was measured on the hybrid holoreceptor immunoprecipitated with rAb50, a specific binding of 31% (expressed as B/T) was detected (Fig. 3B). Thus, the absence of labeled α-subunit after rAb50 immunoprecipitation indicates that in the Leu²³³-Δ43 hybrid IR, insulin binds to the α-subunit contributed by the Δ43 half-receptor. In both lanes, a band of higher molecular weight (>200) corresponding to cross-linked oligomers of receptor subunits (e.g., ααmut) were also detected.

Transphosphorylation of Leu²³³-Δ43 Hybrid IR—Insulin markedly increased insulin receptor phosphorylation in all transfected clonal cell lines except in cells singly transfected with the Leu²³³ mutant IR (Fig. 4). In cells expressing wild type and Δ43 receptors, we observed phosphorylation of both the 95 and 90 kDa bands. When rAb50 was applied to lysates of cells expressing the Leu²³³-Δ43 hybrid IR, two bands were phosphorylated in an insulin-dependent manner. This corresponds to the β-subunit (95 kDa) of the Leu²³³ mutant receptor and to the β-subunit (90 kDa) of the Δ43 receptor that, together, constitute the hybrid IR. As expected antibody B10 precipitated the same two bands (95 and 90 kDa) in cells expressing the Leu²³³-Δ43 hybrid IR. When the precipitation was carried out with antibody rAb50, no band was detected in cells expressing only the Δ43 receptors. However, in lysates from cells expressing WT receptor, rAb50 antibody precipitated a band of 95 kDa which is phosphorylated. In each case phosphorylation is insulin dose-dependent.

Since the insulin receptor is mobile in the plane of the plasma membrane. Phosphorylation of the Leu²³³ half of the hybrid IR could occur by an intramolecular mechanism or by an intermolecular event involving adjacent mobile receptors. To investigate this further, receptors from transfected cells were immunoprecipitated with either rAb50 or rAb53, immobilized on protein A-agarose, and insulin-stimulated phosphorylation measured in vitro. In immunoprecipitates from cells expressing WT receptors, insulin increased the phosphorylation of the β-subunit, whereas in those from cells expressing Leu²³³ mutant, no phosphorylated bands were detected (Fig. 5). Immunoprecipitates from cells expressing hybrid receptors, both rAb53- and rAb50-immobilized receptors showed two bands (95 and 90 kDa) which were phosphorylated in an insulin-dependent manner. This finding suggests that the phosphorylation of Leu²³³ can be mediated by an intramolecular mechanism involving the tyrosine kinase of the Δ43 half-receptor. This does not exclude further intermolecular events that might occur in vivo.

Phosphorylation and Association of Leu²³³ Mutant Insulin Receptors and Leu²³³-Δ43 Hybrid Insulin Receptors with IRS-1—To investigate the phosphorylation of IRS-1, transfected cells were stimulated with insulin and solubilized in detergent. IRS-1 was immunoprecipitated with an anti-IRS-1 antibody (20, 21). Immunoprecipitates were subjected to SDS-PAGE and
Recessive Mutation Affects Insulin Binding

**DISCUSSION**

The insulin receptor has two identical α-subunits, each with a potential binding site for insulin. However, studies using insulin analogues cross-linked to the holo-receptor have suggested that only one molecule of insulin binds to the receptor with high affinity and that this is sufficient to activate a reaction cascade starting with transphosphorylation and leading to a biological response (6, 22). Nevertheless, when insulin binding was measured on receptor halves (αβ), only low affinity binding was seen (3, 8). Therefore, it is clear that two α-subunits must interact in order to create a high affinity binding site for insulin. The availability of the Leu323 mutant receptor that does not bind insulin with high affinity provides a novel opportunity to investigate the subunit interactions that are required for high affinity insulin binding.

**Interactions between Two α-Subunits to Form High Affinity Binding Site for Insulin**—Based upon analogy to the binding of growth hormone to its receptor, it is possible to propose a molecular model to explain how the two α-subunits of the insulin receptor cooperate to form a high affinity binding site. The growth hormone molecule possesses two distinct binding domains (23). This enables growth hormone to bind simultaneously to two receptors. Similarly, it is possible that the insu-
lin molecule binds simultaneously to two α-subunits in the αβ2 insulin receptor oligomer (Fig. 7). This model implies that the α-subunit possesses two potential binding sites, denoted H (high affinity) and L (low affinity) (e.g., WT IR), the mutated α-subunit has lost the H site (e.g., the mutant homodimer), and the hybrid receptor (heterodimer) possesses one normal α-subunit (H and L sites) and one mutated α-subunit (L site).

Recessive Inheritance of Insulin Resistance Due to the Leu323 Mutation—The data presented in this paper are entirely consistent with this model. Whereas both the H and L sites are normal in the wild type α-subunit, we assume that one site (e.g., the H site) is defective in the Leu323 mutant α-subunit. Despite the presence of two L sites in the homotypic (αmutβ)2, Leu323 mutant receptor, the absence of a functional H site abolishes the normal high affinity binding of the heterotetrameric receptor. However, high affinity binding is reconstituted in the hybrid αmutββ receptor because the Leu323 mutant α-subunit contributes an L site and the wild type α-subunit contributes an H site (Fig. 7). It is noteworthy that the Leu323 mutation was identified in a patient with extreme insulin resistance due to the virtual absence of insulin binding to the patient’s cells. While the patient’s mother is heterozygous for the Leu323 mutation, her cells have normal insulin binding, and she appears to have a normal insulin response. The ability of the hybrid Leu323-mutant/wild type receptor to bind insulin with apparently normal affinity may provide a molecular explanation for the observation that the Leu323 mutation causes insulin resistance in a recessive fashion.

Formation of Hybrid Receptors—In previous studies, we have investigated the formation of hybrid receptors in NIH-3T3 cells cotransfected with two expression vectors containing insulin receptor cDNA. We did not detect formation of hybrids in cells co-expressing Δ43-truncated insulin receptors plus either wild type insulin receptors or kinase-deficient Ile153 mutant receptors (9). In contrast, we did detect the formation of hybrids between Δ43-truncated insulin receptors and receptors with the Val323 mutation located in the α-subunit (9). Similarly, in the present work, we detected hybrid formation between Δ43-truncated insulin receptors and Leu323 mutant receptors, another mutation located in the α-subunit. It is also noteworthy that, as with the Leu323 mutation, insulin resistance due to the Val323 mutation is inherited in a recessive fashion (24). Unfortunately, reagents are not available to determine whether hybrid receptors are formed in vivo in patients who are heterozygous for mutations in the insulin receptor gene. Nevertheless, it has been possible to demonstrate formation of αmutββΔβmutβΔIGF hybrids between insulin receptors and IGF-1 receptors in normal tissues (e.g., human placenta) (25–27). These hybrid insulin/IGF-1 receptors retain the ability to bind both insulin and IGF-1. However, while the hybrid receptors bind IGF-1 with the same affinity as the IGF-1 receptor, the affinity of insulin binding to hybrid receptors is 10-fold lower than the affinity of insulin binding to the insulin receptor (25). These observations suggest that, unlike insulin receptors (including the Leu323 mutant receptor), IGF-1 receptors do not possess an L site for insulin (Fig. 7).

Insulin-stimulated Phosphorylation of Receptors and Substrate IRS-1—In order to study the effect of the non-binding α-subunit on the tyrosine kinase activity and the autophosphorylation of both β-subunits of the hybrid receptor, we investigated the effect of insulin to stimulate phosphorylation of hybrid receptors in intact cells and cell free extracts. We demonstrated that both βα and ββ-subunits were phosphorylated in intact cells in an insulin-dependent manner. This phosphorylation could have occurred either via an intramolecular or an intermolecular mechanism. Cells expressing the hybrid receptor also express the Δ43 homodimer. By using specific antibodies, we were able to measure the autophosphorylation of immobilized hybrid receptor in immunoprecipitates. We demonstrated that both β-subunits were phosphorylated by an intramolecular transphosphorylation, but this does not exclude that an intermolecular transphosphorylation may also operate in intact cells. The intramolecular transphosphorylation of the Leu323-Δ43 hybrid IR is distinguished from previous studies that reported the asymmetrical phosphorylation of the IR because in these studies both α-subunits were able to bind insulin. In our study, the hybrid receptors are asymmetrical, consisting of a normal α-subunit together with the Leu323 mutant receptor.

To investigate further downstream events after the phosphorylation of the heterodimer Leu323-Δ43, we studied the phosphorylation of IRS-1. The Leu323-Δ43 insulin hybrid receptor phosphorylated IRS-1 in an insulin-dependent manner and anti-IRS-1 antibody immunoprecipitated, two bands of 90 and 95 kDa corresponding to the βΔ and ββ-subunits of the hybrid receptor. In cells expressing the Leu323 holoreceptor, a weak but significant phosphorylation of IRS-1 was detected. This cannot be attributed to the endogenous receptors since in immunoprecipitates from Neo cells no band corresponding to IRS-1 was detected. Thus, despite the lack of insulin binding, the Leu323 mutant is associated with IRS-1 and can phosphorylate this protein in an insulin-dependent manner, possibly through the formation of hybrid receptor between the mutant and the endogenous IR.

In summary, the expression of Leu323-Δ43 insulin hybrid receptor demonstrates that an α-subunit with a major defect in its ability to bind insulin can nevertheless combine with a wild type receptor to reconstituted high affinity insulin binding and normal phosphorylation of both β-subunits of the IR. Previously, molecular mechanisms of dominant mutations that have
have been reported in the β-subunit have been extensively studied (9, 28). However, this is the first demonstration that a mutation of the IR that causes insulin resistance in a recessive fashion can function relatively normally when expressed together with functional IR under conditions where the receptor assembles into oligomers so as to form hybrid receptors. Therefore, this in vitro cell model may reflect the situation in vivo in heterozygous carriers of the Leu222 mutant receptor.

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