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# Structural Basis by Which a Recessive Mutation in the $\alpha$ -Subunit of the Insulin Receptor Affects Insulin Binding\*

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Recently, a mutation substituting Leu for Ser<sup>323</sup> in the  $\alpha$ -subunit of the human insulin receptor has been identified in an insulin-resistant patient. The Leu<sup>323</sup> mutation 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  $\alpha\beta$  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 ( $\Delta 43$ ) and the full-length Leu<sup>323</sup> mutant receptor. A clonal cell line from cotransfected cells expresses a hybrid receptor consisting of a Leu<sup>323</sup> half-receptor and a  $\Delta 43$  half-receptor. We demonstrate that the Leu<sup>323</sup>- $\Delta 43$  hybrid receptor binds insulin with high affinity. Furthermore, by cross-linking <sup>125</sup>I-insulin to immobilized hybrid receptors, we show that only the  $\alpha\beta_{\Delta}$  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  $\alpha$ -subunit can result in high affinity binding to the holoreceptor ( $\alpha\alpha_{mut}\beta_{\Delta}\beta$ ). Both  $\beta$  and  $\beta_{\Delta}$ -subunits of the Leu<sup>323</sup>- $\Delta 43$  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<sup>323</sup> mutant receptor that lacks an intrinsic high affinity binding site does not prevent the associated  $\beta$ -subunit from functioning either as a tyrosine kinase or as a phosphate acceptor in the hybrid insulin receptor molecule ( $\alpha\alpha_{mut}\beta_{\Delta}\beta$ ). 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)<sup>1</sup> is a heterotetrameric glycoprotein composed of two identical  $\alpha\beta$  disulfide-linked half-receptors that form the  $\alpha_2\beta_2$  holoreceptor complex (1, 2). Insulin binding to the  $\alpha$ -subunit results in autophosphorylation of the  $\beta$ -subunit on tyrosine residues. Formation of high affinity binding sites requires the cooperation of two  $\alpha$ -subunits in an  $\alpha_2\beta_2$  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  $\alpha_2\beta_2$  holoreceptor exhibits negative cooperativity (7). Studies using insulin analogues as probes suggest that one molecule of insulin binds with high affinity to the  $\alpha_2\beta_2$  holoreceptor (4–6). However, when binding studies were carried out using purified  $\alpha\beta$  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  $\alpha$ -subunit half remained intact; and therefore, it is unclear as to whether this site plays a role in binding to the opposite  $\alpha$ -subunit or only provides for a steric interaction between the two  $\alpha$ -subunits in creating the high affinity binding site.

Recently, a mutation in the human insulin receptor in which Ser<sup>323</sup> in the  $\alpha$ -subunit was mutated to Leu has been identified in a patient with a genetic form of the insulin resistance.<sup>2</sup> 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<sup>323</sup> mutant half-receptor ( $\alpha_{mut}\beta$ ) with a high affinity binding truncated half-receptor ( $\alpha\beta_{\Delta}$ ) to form a holo  $\alpha\alpha_{mut}\beta_{\Delta}\beta$  hybrid receptor. In doing so we have constructed an *in vitro* model for heterozygosity for the Leu<sup>323</sup> mutation as found in the patient's mother. Inasmuch as the mother appears not to be insulin resistant, we conclude that the Leu<sup>323</sup> mutation causes insulin resistance in a recessive fashion.

We demonstrate that the hybrid  $\alpha\alpha_{mut}\beta_{\Delta}\beta$  insulin receptor binds insulin with high affinity. Furthermore, by cross-linking <sup>125</sup>I-insulin to the immobilized hybrid receptors we show that only the wild type  $\alpha$ -subunit can be cross-linked to insulin. This suggests that the addition of even a very low affinity  $\alpha$ -subunit can result in high affinity binding to the holoreceptor ( $\alpha\alpha_{mut}\beta_{\Delta}\beta$ ). Furthermore, we show that both  $\beta$ -subunits (*i.e.*  $\beta$  and  $\beta_{\Delta}$ ) were phosphorylated in the hybrid receptors, probably by an intramolecular transphosphorylation mechanism. Finally, despite the presence of a non-binding  $\alpha_{mut}$ -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<sup>323</sup> 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<sup>323</sup> mutant receptor, genomic DNA from the patient was amplified by polymerase chain reaction. The upstream primer was

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<sup>1</sup> The abbreviations used are: IR, insulin receptor; IRS-1, insulin receptor substrate-1; WT, wild type; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

<sup>2</sup> P. Roach, Y. Zick, P. Formisano, D. Accili, S. I. Taylor, and P. Gorden, manuscript submitted for publication.

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 *EspI* restriction sites located in exon 3 and 5, respectively. The amplified fragment was then digested with *EcoRI* and *EspI* and ligated into pGEM4Z (Promega, Madison, WI) containing the wild type hIR cDNA which had been similarly digested. Competent *Escherichia coli* (DH5  $\alpha$  cells, Life Technologies, Inc.) were transformed using the ligation mix and, since the presence of the mutation created a new *BstXI* restriction site, plasmids containing the exon 4 mutation (*Leu*<sup>323</sup>) were identified by their ability to be digested with this enzyme. The hIR cDNA was excised from the construct using the restriction enzyme *SalI* and was ligated into a bovine papilloma virus expression vector (pBPV) which had been cut at its single *XhoI* site (pBPV-*Leu*<sup>323</sup>).

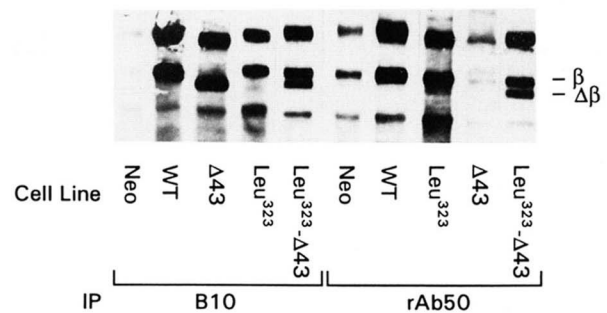
Using site-directed mutagenesis, we introduced a premature chain termination at codon 1301 of the insulin receptor cDNA (9), leading to a C-terminal deletion of 43 amino acid of the  $\beta$ -subunit of the insulin receptor ( $\Delta 43$ ). WT and truncated cDNA were ligated into pBPV (Pharmacia LKB 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  $\mu$ g) plus an expression vector containing the neomycin phosphotransferase (pRSV-Neo, 0.5  $\mu$ g) (10–12). Cells were transfected either with an 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 transfectants were selected by cultivation in the presence of G418 (600  $\mu$ g/ml, Life Technologies, Inc.). After selection, insulin receptor expression by stable transfectants was assayed by measuring <sup>125</sup>I-insulin binding and, in the case of cotransfected cells, clones expressing both the full-length and the  $\Delta 43$  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  $\alpha$ -subunit (13, 14) at a dilution of 1:50 or with a rabbit antibody (rAb-50) directed against a peptide corresponding to amino acids 1321–1336 of the  $\beta$ -subunit of the human insulin receptor at a dilution of 1:50 (15) (this peptide has been deleted from the  $\Delta 43$  mutant insulin receptor, and therefore, this antibody does not react with the  $\Delta 43$  mutant). After electrophoresis, 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  $\mu$ l of buffer (150 mM NaCl, 50 mM HEPES, pH 7.4) containing Triton X-100 (1%), phenylmethylsulfonyl fluoride (2 mM), aprotinin (15 trypsin inhibitor units/ml), antipain (0.1 mM  $\alpha$ -macroglobulin (0.5 unit/ml). Receptors were immunoprecipitated with a rabbit antibody (rAb50) or rAb53 (rAb53 recognizes the  $\beta$ -subunit, and therefore immunoprecipitates both the  $\Delta 43$  and the full-length  $\beta$ -subunits) 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 (0.1%). After the last wash the immunoprecipitates were incubated in binding buffer (120 mM NaCl, 1 mM EDTA, 15 mM sodium acetate, 2.5 mM KCl, 1.2 mM MgSO<sub>4</sub>, 10 mM glucose, 50 mM HEPES) containing bovine serum albumin (10 mg/ml) and <sup>125</sup>I-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 microcentrifuge for 5 min at 4 °C; the pellets were washed three times with binding buffer and then counted in a gamma counter.

**<sup>125</sup>I-Insulin Cross-linking to Insulin Receptor**—Transfected NIH-3T3 cells were solubilized in 500  $\mu$ l of buffer (150 mM NaCl, 50 mM HEPES, pH 7.4) containing Triton X-100 (1%), phenylmethylsulfonyl fluoride (2 mM), aprotinin (15 trypsin inhibitor units/ml), antipain (0.1 mM  $\alpha$ -macroglobulin (0.5 unit/ml) and then insulin receptors were immunoprecipitated with rAb53 or with rAb50 antibody at 4 °C overnight. The antibody-insulin receptor complex was added to protein A-agarose. To this final complex, we added <sup>125</sup>I-insulin, and binding was performed at 4 °C overnight in the absence or presence of an excess of unlabeled insulin. After centrifugation at maximum speed in microcentrifuge at 4 °C for 5 min, the bound complex was divided into two samples, the



**FIG. 1. Detection of biotinylated cell surface insulin receptors by streptavidin blotting.** Proteins on the surface of stably transfected NIH-3T3 cells were biotinylated. Clonal cell lines expressed either one or two forms of insulin receptor as follows: cells expressing neomycin resistance gene alone (*Neo*); wild type insulin receptor (*WT*); truncated  $\Delta 43$  insulin receptor ( $\Delta 43$ ); *Leu*<sup>323</sup> mutant insulin receptor (*Leu*<sup>323</sup>) or *Leu*<sup>323</sup> $\Delta 43$  insulin hybrid receptor (*Leu*<sup>323</sup> $\Delta 43$ ). Biotinylated cell surface insulin receptors were immunoprecipitated using either antibody B10 directed against the  $\alpha$ -subunit (left panel) or a rabbit antibody (rAb50) directed against a peptide corresponding to amino acids 1321–1336 of the  $\beta$ -subunit (right panel). The immunoprecipitates were analyzed by SDS-PAGE (6.5%) and transferred to nitrocellulose filter by electroblotting. The blot was probed with horseradish peroxidase-labeled streptavidin. While the  $\alpha$ -subunits of both full-length and truncated receptors have the same mobility (upper band), the  $\beta$ -subunit of the truncated  $\Delta 43$  insulin receptor migrated faster than the  $\beta$ -subunit of full-length receptor.

first sample was used to determine insulin binding to the immunoprecipitated holoreceptors, and the second sample was washed with binding buffer and bovine serum albumin had been omitted. A sufficient quantity of disuccinimidyl suberate (10 mM in dimethyl sulfoxide) was added to give a final concentration of 0.1 mM. After incubation on ice for 20 min, the reaction was quenched by addition of 4 volumes of Tris (10 mM), EDTA (1 mM) in phosphate-buffered saline (16). The complex was pelleted by centrifugation. The pellets were washed and were resuspended in 75 mM Tris at pH 8.5 containing 1.25 mM DTT for 30 min at room temperature as described previously (3). 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 Laemmli buffer with or without DTT, electrophoresed through either 3–10% gradient SDS-PAGE in non-reducing conditions (17) or to 6% SDS-PAGE in the presence of DTT (80 mM). The gel was autoradiographed after 2 days of exposure.

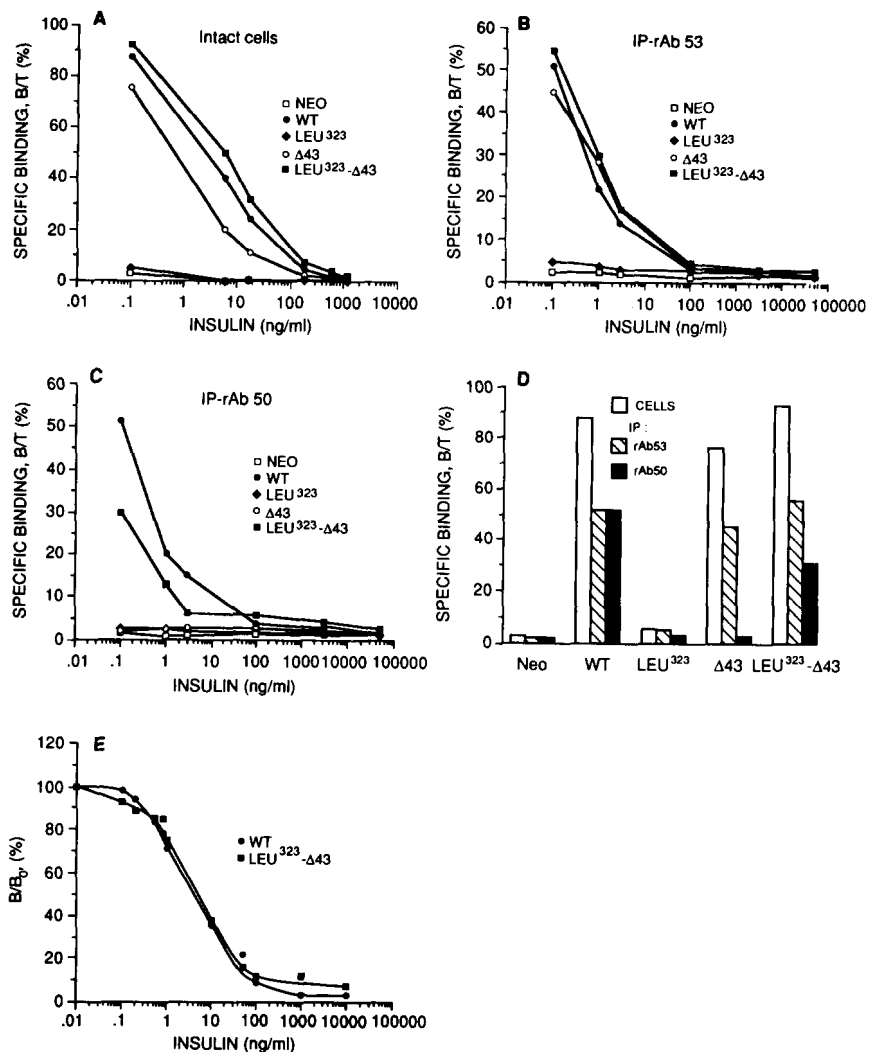
**Phosphorylation of Insulin Receptors and IRS-1 in Intact Cells**—Phosphorylation of insulin receptors in intact cells was conducted as described elsewhere (9). Insulin receptors were immunoprecipitated either with antibody B10 or rAb50. Endogenous IRS-1 was immunoprecipitated with 1:100 dilution of a rabbit antibody directed against the rat IRS-1 protein (rAb-IRS-1) (Upstate Biotechnology Inc., Lake Placid, NY). Proteins containing phosphotyrosine were detected by sequential incubation with a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY) and horseradish peroxidase-linked anti-mouse  $\gamma$ -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 under "Materials and Methods." Immobilized receptors were incubated in the presence or in absence of 10<sup>-8</sup> 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 *Leu*<sup>323</sup> $\Delta 43$  Hybrid Insulin Receptors**—Cells were first singly transfected with the wild type insulin receptor, the truncated  $\Delta 43$  receptor which has a deletion of 43 amino acids at C-terminal of the  $\beta$ -subunit (18, 19) or the *Leu*<sup>323</sup> mutant receptor. After biotinylation of cell

**FIG. 2.  $^{125}$ I-Insulin binding to intact cells and to immobilized insulin receptors.** Insulin binding to intact cells and to immobilized 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 rAb53 (raised against the C-terminal of the  $\beta$ -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 immobilized receptors from different clonal cell lines was summarized in *panel D* where the maximum binding is expressed as percent of B<sub>0</sub>/T (specific binding in the absence of unlabeled insulin over total radioactivity added). The affinity of the hormone to bind to Leu<sup>323</sup>- $\Delta$ 43 hybrid receptors was finally compared with WT IR on rAb50 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.

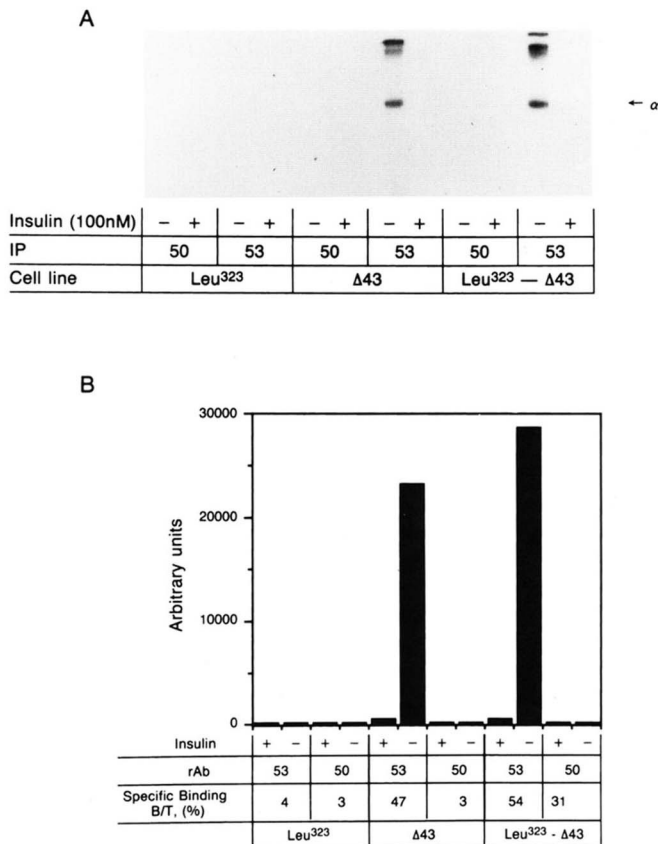


surface insulin receptors and immunoprecipitation with either B10 or rAb50, blotting with horseradish peroxidase-streptavidin showed that cells expressing WT or Leu<sup>323</sup> mutant insulin receptors express a  $\beta$ -subunit of an apparent molecular mass of 95 kDa, whereas the truncated  $\beta$ -subunit of the  $\Delta$ 43 IR has an apparent molecular mass of 90 kDa (Fig. 1). Similar experiments were performed on cells cotransfected with  $\Delta$ 43 and Leu<sup>323</sup> 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  $\beta$ -subunit of the  $\Delta$ 43 receptor, and one of 95 kDa corresponding to the  $\beta$ -subunit of the Leu<sup>323</sup> mutant receptor. Since rAb50 does not recognize the  $\Delta$ 43 mutant, the presence of these two bands indicates the presence of the Leu<sup>323</sup>- $\Delta$ 43 hybrid receptor (Fig. 1, *right panel*). In addition to the Leu<sup>323</sup>- $\Delta$ 43 hybrid receptor, it is likely that this clone may also express IR homodimers ( $\Delta$ 43- $\Delta$ 43 or Leu<sup>323</sup>-Leu<sup>323</sup>).

**Insulin Binding to the Leu<sup>323</sup>- $\Delta$ 43 Hybrid Insulin Receptor—** $^{125}$ I-Insulin binding to intact cells and to immunoprecipitated insulin receptors was measured. As expected insulin binding to cells expressing the Leu<sup>323</sup> mutant receptors was barely detectable, similar to that seen in NEO cells (Fig. 2, A and D). Cells expressing the  $\Delta$ 43 IR or the Leu<sup>323</sup>- $\Delta$ 43 hybrid IR showed specific insulin binding with high affinity comparable to that seen in cells expressing the WT IR (Fig. 2, A and D).

The presence of  $\Delta$ 43 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  $\Delta$ 43 homodimers and to the Leu<sup>323</sup>- $\Delta$ 43 hybrid receptors, immunoprecipitation using either rAb53 or rAb50 was performed. rAb53, which is directed against the insulin receptor  $\beta$ -subunit and recognizes  $\Delta$ 43 homodimers and rAb50 which was raised against the C-terminal portion of the insulin receptor  $\beta$ -subunit and does not immunoprecipitate the truncated  $\beta$ -subunit of  $\Delta$ 43 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  $\Delta$ 43 holoreceptors and the Leu<sup>323</sup>- $\Delta$ 43 hybrid IR showed similar specific binding (Fig. 2D). In cells expressing Leu<sup>323</sup> mutant receptor, as expected, no significant binding was detected using either antibodies (less than 10% of the binding to WT receptors).  $^{125}$ I-Insulin binding to rAb50 immunoprecipitates showed that lysates from cells expressing Leu<sup>323</sup> mutant receptors has no detectable binding. Lysate of cells expressing the  $\Delta$ 43 IR has insulin binding activity that can be immunoprecipitated by rAb53 but not by rAb50. In contrast, rAb50 and rAb53 immunoprecipitates from cells expressing Leu<sup>323</sup>- $\Delta$ 43 hybrid receptors exhibited specific insulin binding. The affinity of the Leu<sup>323</sup>- $\Delta$ 43 hybrid receptor for insulin was estimated by measuring  $^{125}$ I-insulin binding on WT and on hybrid receptors immobilized by rAb50 antibody and protein A-agarose. The



**FIG. 3. Cross-linking of  $^{125}\text{I}$ -insulin to IR.** NIH-3T3 cells expressing the Leu<sup>323</sup>, the Δ43 or the Leu<sup>323</sup>-Δ43 IR were solubilized and IR immunoprecipitated with either rAb50 or rAb53. The immunoprecipitates were divided into two samples, the first one used for cross-linking and the second used for determination of insulin binding to the immunoprecipitated holoreceptors. **A** and **B**, after cross-linking of  $^{125}\text{I}$ -insulin to the immunoprecipitated and immobilized IR, the complexes were subjected to mild reduction using DTT in order to obtain half-receptors. After, washing the complexes were subjected to a 6% SDS-PAGE under reducing conditions. Specific cross-linking of  $^{125}\text{I}$ -insulin to the  $\alpha$ -subunit is shown in the autoradiograph (**A**). The incorporated radioactivity into the  $\alpha$ -subunit was measured by using phosphorimager analysis and is expressed in arbitrary units (**B**). Insulin binding to the immunoprecipitated and immobilized receptors was measured using labeled insulin and was expressed as percent of specific binding (**B/T**, %).

shapes of the binding competition curves were similar, suggesting that the affinity for insulin was similar between WT and Leu<sup>323</sup>-Δ43 hybrid IRs. In both cases, a concentration of 5 ng/ml of insulin inhibited  $^{125}\text{I}$ -insulin by 50% (Fig. 2E). These results demonstrate that Leu<sup>323</sup>-Δ43 insulin hybrid receptor heterodimer binds insulin with high affinity.

**$^{125}\text{I}$ -Insulin Cross-linking to IR**—To determine directly the relative contributions to binding of the different  $\alpha\beta$  monomers of the Leu<sup>323</sup>-Δ43 hybrid receptor, transfected NIH-3T3 cells were solubilized and insulin receptors immunoprecipitated with rAb53 or rAb50 in the presence of protein A-agarose. The immunoprecipitates were divided into two parts: the first part served to determine insulin binding, and the second part was used for the cross-linking of labeled insulin to the IR. For cross-linking,  $^{125}\text{I}$ -insulin was incubated with the antibody-IR complex at 4 °C in the presence or absence of an excess of cold insulin and cross-linked to the IR in the presence of disuccinimidyl suberate. Receptors were then subjected to mild reduction in the presence of 1.25 mM DTT in order to disrupt the disulfide bonds that associate the  $\alpha\beta$  half-receptors while minimizing the reduction of other disulfide bonds. This was followed by extensive washing of the beads. Finally, we analyzed by 6% SDS-PAGE under reducing conditions.

No bands were detected in the Leu<sup>323</sup> immunoprecipitates (Fig. 3A) which is in good agreement with the absence of insulin binding to the immunoprecipitated Leu<sup>323</sup> holoreceptor (Fig. 3B). rAb53 immunoprecipitates from cells expressing the Δ43 IR or the Leu<sup>323</sup>-Δ43 hybrid IR showed a band corresponding to the  $\alpha$ -subunit of IR which was specifically labeled with  $^{125}\text{I}$ -insulin; this labeling was completely inhibited in the presence of an excess of unlabeled insulin. The presence of labeled  $\alpha$ -subunit in rAb53 immunoprecipitates is well correlated with binding of insulin to the immunoprecipitated holoreceptors (the Δ43 or the Leu<sup>323</sup>-Δ43 IR) (Fig. 3B). Furthermore, the amount of labeled  $\alpha$ -subunit was similar in cells expressing the Δ43 and in cells cotransfected with both Leu<sup>323</sup> and Δ43 IR as shown by the phosphorimager analysis (Fig. 3B). In rAb50 immunoprecipitates from cells expressing the Δ43 or the Leu<sup>323</sup>-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  $\alpha$ -subunit after rAb50 immunoprecipitation indicates that in the Leu<sup>323</sup>-Δ43 hybrid IR, insulin binds to the  $\alpha$ -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.  $\alpha\alpha_{\text{mut}}$ ) were also detected.

**Transphosphorylation of Leu<sup>323</sup>-Δ43 Hybrid IR**—Insulin markedly increased insulin receptor phosphorylation in all transfected clonal cell lines except in cells singly transfected with the Leu<sup>323</sup> 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 Leu<sup>323</sup>-Δ43 hybrid IR, two bands were phosphorylated in an insulin-dependent manner. This corresponds to the  $\beta$ -subunit (95 kDa) of the Leu<sup>323</sup> mutant receptor and to the  $\beta$ -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 Leu<sup>323</sup>-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 Leu<sup>323</sup> 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  $\beta$ -subunit, whereas in those from cells expressing Leu<sup>323</sup> mutant, no phosphorylated bands were detected (Fig. 5). In 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 Leu<sup>323</sup> 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 Leu<sup>323</sup> Mutant Insulin Receptors and Leu<sup>323</sup>-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



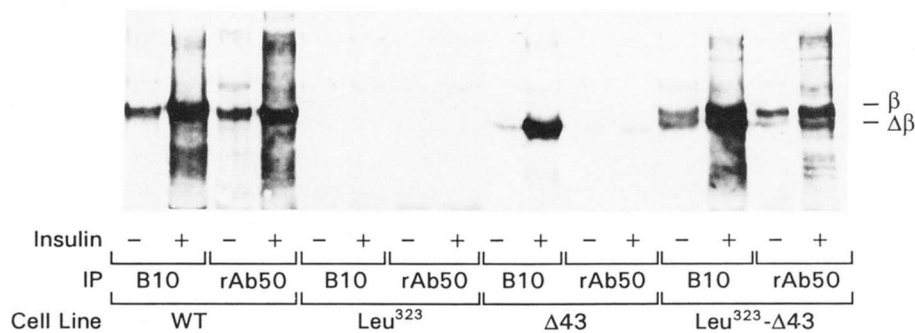


FIG. 4. Insulin stimulates tyrosine phosphorylation of Leu<sup>323</sup>-Δ43 insulin hybrid receptor in intact cells. NIH-3T3 cells expressing either wild type receptor (WT), truncated Δ43 receptor (Δ43), Leu<sup>323</sup> mutant receptor (Leu<sup>323</sup>) or Leu<sup>323</sup>-Δ43 insulin hybrid receptor were incubated for 1 min in the presence of 10<sup>-7</sup> M of insulin at 37 °C. Cells were solubilized, and insulin receptors were immunoprecipitated with either B10 or rAb50. The immunoprecipitates were analyzed on SDS-PAGE (6.5%) followed by electroblotting. The blot was probed by antiphosphotyrosine antibody and bands were detected by ECL.

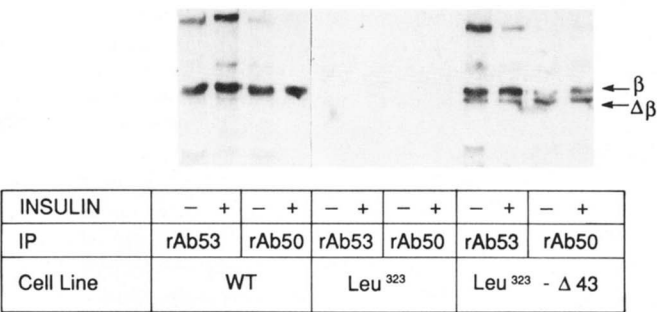


FIG. 5. Insulin stimulates the phosphorylation of both halves of Leu<sup>323</sup>-Δ43 insulin hybrid receptor *in vitro*. NIH-3T3-transfected cells were solubilized, and insulin receptors were immunoprecipitated by either rAb50 or rAb53 which are directed against the β-subunit. The immunoprecipitated receptors were immobilized on protein A-agarose as previously described and incubated for 30 min at room temperature in the presence or absence of 10<sup>-8</sup> M of insulin. Phosphorylation was initiated by addition of ATP, CTP, and manganese acetate. The reaction was stopped by addition of Lammeli sample buffer containing DTT. After SDS-PAGE and electroblotting, the blots were probed with antiphosphotyrosine antibody and ECL detection was performed. The locations of the β and Δβ are indicated.

transferred to nitrocellulose. Filters were then probed with antiphosphotyrosine antibody. In immunoprecipitates from cells expressing either wild type IR or Δ43 receptors, insulin increased the phosphotyrosine content of a 170 kDa band presumably corresponding to IRS-1 (Fig. 6, upper panel). Furthermore, the anti-IRS-1 antibody coimmunoprecipitated phosphotyrosine containing bands of 95 and 90 kDa (Fig. 6, upper panel), corresponding to the β-subunits of the wild type insulin receptor and Δ43 insulin receptor, respectively. In cells expressing the Leu<sup>323</sup> mutant receptor, insulin increased only slightly the phosphorylation of IRS-1. Furthermore, almost no signal was detected at 95 kDa in cells singly transfected with the Leu<sup>323</sup> mutant receptor (Fig. 6, upper and lower panels). In contrast, in cells expressing Leu<sup>323</sup>-Δ43 hybrid receptors, insulin markedly increased the phosphorylation of the 170 kDa band corresponding to IRS-1 and the β-subunits of the Δ43 and the Leu<sup>323</sup> half-receptors (90 and 95 kDa).

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 holoreceptor 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-sub-

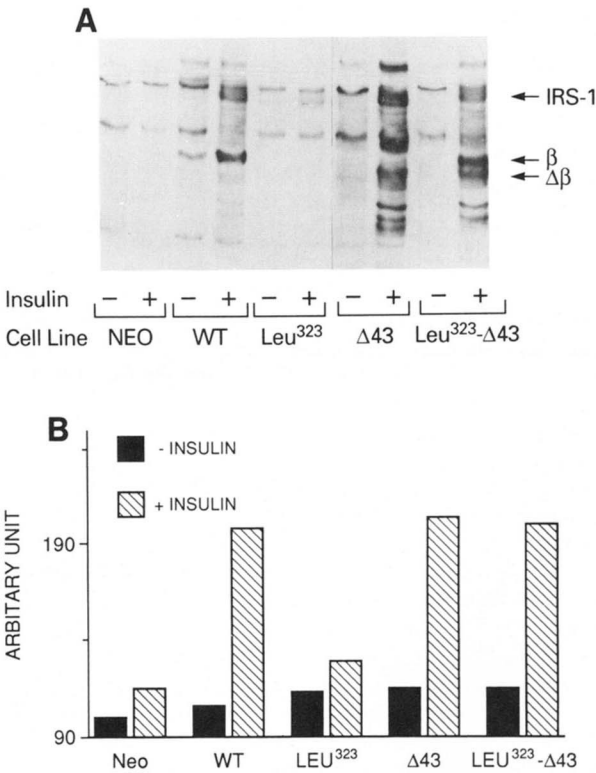


FIG. 6. Phosphorylation of IRS-1 in intact cells. Panel A, NIH-3T3 cells expressing either the neomycin resistance gene alone (alone), wild type receptor (WT), Leu<sup>323</sup> mutant (Leu<sup>323</sup>), truncated Δ43 receptor (Δ43) or Leu<sup>323</sup>-Δ43 insulin receptor hybrid were incubated in the presence of 10<sup>-8</sup> M of insulin during 1 min at 37 °C. IRS-1 was immunoprecipitated using rAb-IRS-1 as described under "Materials and Methods." The blot was probed with antiphosphotyrosine antibody. Panel B, IRS-1 phosphorylation of the blot shown in A was quantified by capturing a video image that was analyzed using Image 1.49 software.

units must interact in order to create a high affinity binding site for insulin. The availability of the Leu<sup>323</sup> 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-

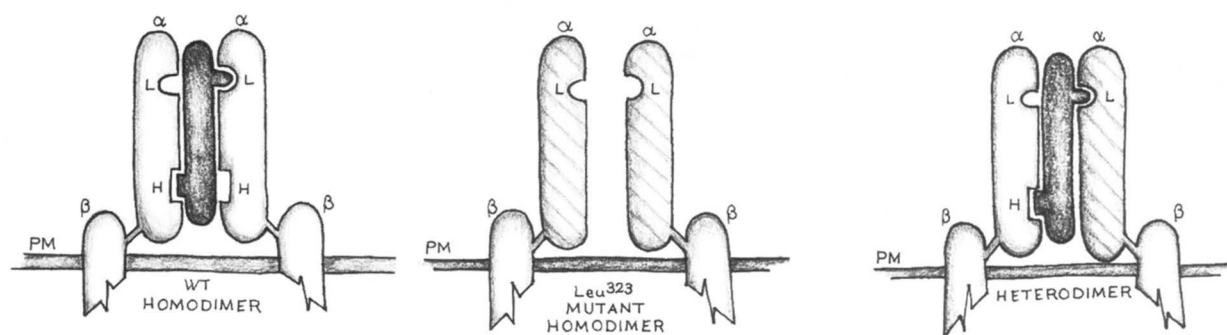


FIG. 7. Schematic presentation of WT,  $\text{Leu}^{323}$  mutant, and  $\text{Leu}^{323}$ - $\Delta 43$  hybrid IRs. The wild type  $\alpha$ -subunit possesses two potential binding sites, denoted H (high affinity) and L (low affinity) (e.g. WT IR), the mutated  $\alpha$ -subunit has lost the H site (e.g. the mutant homodimer), and the hybrid receptor (heterodimer) possesses one normal  $\alpha$ -subunit (H and L sites) and one mutated  $\alpha$ -subunit (L sites).

lin molecule binds simultaneously to two  $\alpha$ -subunits in the  $\alpha_2\beta_2$  insulin receptor oligomer (Fig. 7). This model implies that the  $\alpha$ -subunit possesses two potential binding sites, denoted H and L. According to this hypothesis, high affinity binding of insulin results from the sum of the binding interactions with the H site on one  $\alpha$ -subunit and the L site on the other  $\alpha$ -subunit. The lower affinity of the  $\alpha\beta$  half-receptor results from the fact that only one  $\alpha$ -subunit is available to interact with insulin. Confirmation of this type of model will require structural data.

**Recessive Inheritance of Insulin Resistance Due to the  $\text{Leu}^{323}$  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  $\alpha$ -subunit, we assume that one site (e.g. the H site) is defective in the  $\text{Leu}^{323}$  mutant  $\alpha$ -subunit. Despite the presence of two L sites in the homotypic ( $\alpha_{\text{mut}}$ ) $_2\beta_2$   $\text{Leu}^{323}$  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  $\alpha_{\text{mut}}\alpha_{\text{WT}}\beta_2$  receptor because the  $\text{Leu}^{323}$  mutant  $\alpha$ -subunit contributes an L site and the wild type  $\alpha$ -subunit contributes an H site (Fig. 7). It is noteworthy that the  $\text{Leu}^{323}$  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  $\text{Leu}^{323}$  mutation, her cells have normal insulin binding, and she appears to have a normal insulin response. The ability of the hybrid  $\text{Leu}^{323}$ -mutant/wild type receptor to bind insulin with apparently normal affinity may provide a molecular explanation for the observation that the  $\text{Leu}^{323}$  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  $\Delta 43$ -truncated insulin receptors plus either wild type insulin receptors or kinase-deficient  $\text{Ile}^{1153}$  mutant receptors (9). In contrast, we did detect the formation of hybrids between  $\Delta 43$ -truncated insulin receptors and receptors with the  $\text{Val}^{382}$  mutation located in the  $\alpha$ -subunit (9). Similarly, in the present work, we detected hybrid formation between  $\Delta 43$ -truncated insulin receptors and  $\text{Leu}^{323}$  mutant receptors, another mutation located in the  $\alpha$ -subunit. It is also noteworthy that, as with the  $\text{Leu}^{323}$  mutation, insulin resistance due to the  $\text{Val}^{382}$  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  $\alpha_{\text{ins}}\alpha_{\text{IGF}}\beta_{\text{ins}}\beta_{\text{IGF}}$  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  $\text{Leu}^{323}$  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  $\alpha$ -subunit on the tyrosine kinase activity and the autophosphorylation of both  $\beta$ -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  $\beta_A$  and  $\beta_B$ -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  $\Delta 43$  homodimer. By using specific antibodies, we were able to measure the autophosphorylation of immobilized hybrid receptor in immunoprecipitates. We demonstrated that both  $\beta$ -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  $\text{Leu}^{323}$ - $\Delta 43$  hybrid IR is distinguished from previous studies that reported the asymmetrical phosphorylation of the IR because in these studies both  $\alpha$ -subunits were able to bind insulin. In our study, the hybrid receptors are asymmetrical, consisting of a normal  $\alpha$ -subunit together with the  $\text{Leu}^{323}$  mutant receptor.

To investigate further downstream events after the phosphorylation of the heterodimer  $\text{Leu}^{323}$ - $\Delta 43$ , we studied the phosphorylation of IRS-1. The  $\text{Leu}^{323}$ - $\Delta 43$  insulin hybrid receptor phosphorylated IRS-1 in an insulin-dependent manner and anti-IRS-1 antibody coimmunoprecipitated, two bands of 90 and 95 kDa corresponding to the  $\beta_A$  and  $\beta_B$ -subunits of the hybrid receptor. In cells expressing the  $\text{Leu}^{323}$  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  $\text{Leu}^{323}$  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  $\text{Leu}^{323}$ - $\Delta 43$  insulin hybrid receptor demonstrates that an  $\alpha$ -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  $\beta$ -subunits of the IR. Previously, molecular mechanisms of dominant mutations that have

been reported in the  $\beta$ -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 a 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 Leu<sup>323</sup> mutant receptor.

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