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# Rescue and Activation of a Binding-deficient Insulin Receptor

EVIDENCE FOR INTERMOLECULAR TRANSPHOSPHORYLATION\*

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Binding of insulin to the  $\alpha$  subunit of the insulin receptor (IR) leads to autophosphorylation of the  $\beta$  subunit. The reaction proceeds as intramolecular transphosphorylation between  $\alpha\beta$  half-receptors of the heterotetrameric receptor dimer ( $\alpha_2\beta_2$ ). Since IRs are mobile in the plane of the plasma membrane, it is also possible that transphosphorylation may occur between adjacent holoreceptors ( $\alpha_2\beta_2$ ) by an intermolecular reaction. To address this question, we cotransfected NIH-3T3 cells with two IR cDNA constructs: a truncated but functionally normal IR lacking the C-terminal 43 amino acids ( $\Delta 43$ ) and a full-length Leu<sup>323</sup> mutant receptor that is expressed on the cell surface but that does not bind insulin. A clonal cell line was selected from cells cotransfected with a 1/5 ratio of  $\Delta 43$  cDNA/Leu<sup>323</sup> cDNA. The two homodimers (Leu<sup>323</sup> and  $\Delta 43$ ) were expressed without detectable formation of hybrid receptors. By using specific antibodies, we demonstrate that in cells coexpressing both homodimers, the Leu<sup>323</sup> mutant receptor was phosphorylated *in vivo* by the  $\Delta 43$  IR in an insulin-dependent manner. However, when the Leu<sup>323</sup> mutant receptor was expressed alone, no phosphorylation was detected. In addition, we demonstrate the association of the phosphorylated Leu<sup>323</sup> mutant receptor with insulin receptor substrate-1 and with phosphatidylinositol 3-kinase. These findings indicate that insulin binding is not required for phosphorylation of the Leu<sup>323</sup> mutant receptor, that the phosphorylation of the Leu<sup>323</sup> mutant receptor occurs by an intermolecular transphosphorylation mechanism, and, finally, that the Leu<sup>323</sup> mutant receptor, once phosphorylated, can associate with insulin receptor substrate-1 and phosphatidylinositol 3-kinase.

The insulin receptor (IR)<sup>1</sup> is a transmembrane protein consisting of two identical disulfide-linked half-receptors. Each half ( $\alpha\beta$ ) consists of an extracellular  $\alpha$  subunit that binds insulin and a transmembrane  $\beta$  subunit that contains a tyrosine kinase domain (1, 2). Insulin binding to the  $\alpha$  subunit results in autophosphorylation of the  $\beta$  subunit, activation of receptor tyrosine kinase activity, and tyrosine phosphorylation of intracellular targets of insulin action.

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<sup>1</sup> The abbreviations used are: IR, insulin receptor; IRS-1, insulin receptor substrate-1; WT, wild-type; PI 3-kinase, phosphatidylinositol 3-kinase; rAb, rabbit antibody; PAGE, polyacrylamide gel electrophoresis.

Several lines of evidence suggest that the binding of insulin to one half of the  $\alpha$  subunit dimer within a tetrameric holoreceptor ( $\alpha\beta\alpha\beta$ ) can result in intramolecular transphosphorylation of the opposite  $\beta$  subunit (3–7). We have recently presented evidence that strongly supports this concept by expressing hybrid insulin receptors in which one  $\alpha\beta$  half contains a mutation from a patient with extreme insulin resistance that is expressed on the cell surface but does not bind insulin (Leu<sup>323</sup>) and is not phosphorylated (8, 9). The other  $\alpha\beta$  half-receptor ( $\alpha\beta_{\Delta}$ ) has a wild-type (WT)  $\alpha$  subunit and a truncated  $\beta$  subunit lacking 43 amino acids in the C terminus; this truncation does not impair IR tyrosine kinase activity. The fact that this hybrid IR binds insulin with high affinity and phosphorylates the  $\beta$  subunit of the mutant half-receptor demonstrates this intramolecular transphosphorylation event.

The insulin receptor, like the epidermal growth factor receptor (10–12), is mobile in the plane of the plasma membrane. The epidermal growth factor receptor, a single chain monomer, appears to be phosphorylated and activated by an intermolecular reaction involving receptor aggregation (13). While phosphorylation of insulin receptors does not require interaction among individual receptor heterotetramers, IR interactions could augment the receptor signal.

In the present study, we have co-transfected NIH-3T3 cells with constructs encoding for the Leu<sup>323</sup> mutant receptor and the truncated  $\Delta 43$  IR. Using a clonal cell line that expresses homodimeric receptors ( $\alpha_{mut}\beta_{mut}$ ) and ( $\alpha_{\Delta}\beta_{\Delta}$ ), we demonstrate that the  $\Delta 43$  insulin receptor phosphorylates the Leu<sup>323</sup> mutant receptor in response to insulin. Additionally we demonstrate that, once the Leu<sup>323</sup> mutant receptor is phosphorylated, it behaves similarly to a WT receptor in terms of association with IRS-1 and activation of PI 3-kinase.

## EXPERIMENTAL PROCEDURES

**Expression of Insulin Receptors in Transfected NIH-3T3 Cells**—The human IR cDNA carrying the Leu<sup>323</sup> mutation was constructed as described previously (8, 9).

Using site-directed mutagenesis, we introduced a premature chain termination codon at codon 1301 of the insulin receptor cDNA (14), leading to a C-terminal deletion of 43 amino acids of the  $\beta$  subunit of the insulin receptor ( $\Delta 43$ ). WT and truncated cDNAs were ligated into pBPV (Pharmacia Biotech Inc.) as described elsewhere (15).

NIH-3T3 cells were transfected using Lipofectin (Life Technologies, Inc.) and a mixture of insulin receptor expression vector (20  $\mu$ g) and an expression vector containing the neomycin phosphotransferase gene (pRSV-Neo; 0.5  $\mu$ g) (15, 16). Cells were either transfected with an expression vector for a single type of insulin receptor or co-transfected with a mixture of expression vectors for two types of insulin receptor (8). Stable transfectants were selected with 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 or cells expressing the Leu<sup>323</sup> receptor alone, clones expressing both the full-length and the  $\Delta 43$  insulin receptors and cells expressing only the full-length insulin receptor, receptor expres-

sion was assayed 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 (8, 14). After cell solubilization, insulin receptors were immunoprecipitated using anti-receptor antibody B-10 directed against the  $\alpha$  subunit (17, 18) at a dilution of 1:50 or with a rabbit antibody (rAb50) 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 (19). This peptide has been deleted from the  $\Delta 43$  mutant insulin receptor; therefore, this antibody does not react with the  $\Delta 43$  mutant. After electroblotting, nitrocellulose sheets were probed with horseradish peroxidase-linked streptavidin (Amersham Corp.) at a dilution of 1:500. Biotinylated insulin receptors were detected by ECL (Amersham Corp.).

**Insulin Binding to Intact Cells**—Insulin binding to intact cells was performed at 4 °C overnight in the presence of labeled insulin, and specific binding was determined in the presence of an excess of unlabeled insulin as described previously (20).

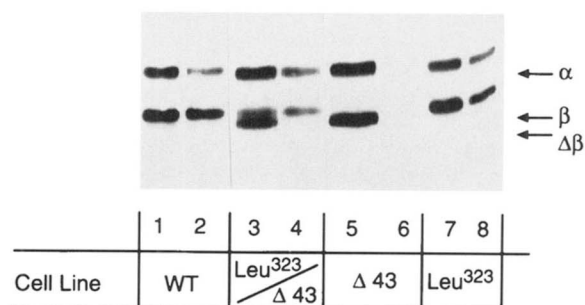
**Phosphorylation of Insulin Receptors and IRS-1 in Intact Cells**—Phosphorylation of insulin receptors in intact cells was conducted as described elsewhere (21). Insulin receptors were immunoprecipitated either with B10 or with rAb50. Endogenous IRS-1 was immunoprecipitated with 1:100 dilution of a rabbit antibody directed against the rat IRS-1 protein (rAb-IRS-1) (Upstate Biotechnology, Inc., Lake Placid, NY). Following SDS-PAGE and electrotransfer, proteins containing phosphotyrosine were detected by sequential incubation with a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.) and horseradish peroxidase-linked anti-mouse  $\gamma$ -globulin (Amersham Corp.). Filters were washed and ECL was performed as described previously (8).

**Association of Insulin Receptor with PI 3-Kinase**—PI 3-kinase activity associated with the insulin receptor expressed in NIH-3T3 cells was determined in immunoprecipitated proteins as described previously (22, 23). Briefly, after cell solubilization and immunoprecipitation with anti-phosphotyrosine antibody, pellets were washed and resuspended in 40 ml of a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA. To each tube was added 10 ml of  $MnCl_2$  (100 mM) and 20 mg of phosphatidylinositol (Sigma). The phosphorylation reaction was started by addition of 10 ml of ATP (440 mM) containing 30 mCi of [ $\gamma$ - $^{32}P$ ]ATP. After 10 min, the reaction was stopped by the addition of 20 ml of HCl (8 N) and 160 ml of  $CHCl_3$ /methanol (1:1). The organic phase was extracted and applied to a silica gel thin layer chromatography plate (Merck). Chromatography plates were developed in  $CHCl_3/CH_3OH/H_2O/NH_4OH$  (60:47:11.3:2) and visualized by autoradiography for 2 h. The radioactivity was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

## RESULTS

**Expression and Coexpression of Wild-type and Mutant Insulin Receptors**—Cells were either singly transfected with expression plasmids encoding the WT IR, the truncated  $\Delta 43$  IR, which has a deletion of 43 amino acids at the C terminus of the  $\beta$  subunit (14, 24, 25), or the  $Leu^{323}$  mutant receptor or cotransfected with the truncated  $\Delta 43$  IR and the  $Leu^{323}$  mutant IR. In order to identify each specific transfectant, cell-surface insulin receptors were biotinylated. Cells were then solubilized and immunoprecipitated with either B10 (Fig. 1) or rAb50 (Fig. 1). Cells transfected with the WT IR or the  $Leu^{323}$  mutant IR cDNAs express an  $\alpha$  subunit of an apparent molecular mass of 135,000 Da and a  $\beta$  subunit of 95,000 Da, whereas cells transfected with the  $\Delta 43$  IR construct express a  $\beta$  subunit of an apparent molecular mass of 90,000 Da, consistent with its truncation (Fig. 1). In co-transfected cells, immunoprecipitation with B10 showed, in addition to the  $\alpha$  subunit, two bands corresponding to the truncated  $\beta$  subunit ( $M_r = 90,000$ ) and the full-length  $\beta$  subunit ( $M_r = 95,000$ ) (Fig. 1). However, when immunoprecipitation was carried out with rAb50, only the 95-kDa band was detected, demonstrating that the  $\Delta 43$  IR and the  $Leu^{323}$  mutant IRs are expressed on the surface of this clonal cell line as homodimers, and the formation of hybrid receptors does not occur.

**Insulin Binding to the Wild-type and Mutant Insulin Receptors**—Following the isolation of these four clonal cell

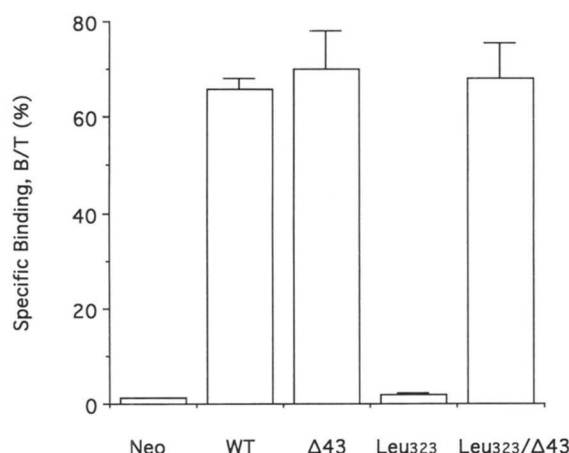


**FIG. 1. Detection of biotinylated cell-surface insulin receptors by streptavidin blotting.** Proteins on the surface of stably transfected NIH-3T3 cells were biotinylated. Clonal cell lines expressed either one or two forms of insulin receptor as follows: cells expressing wild-type insulin receptor (WT), truncated  $\Delta 43$  insulin receptor ( $\Delta 43$ ),  $Leu^{323}$  mutant receptor ( $Leu^{323}$ ), or cells coexpressing  $Leu^{323}$  mutant and  $\Delta 43$  insulin receptors ( $Leu^{323}/\Delta 43$ ). After cell biotinylation, insulin receptors were immunoprecipitated using either antibody B10 directed against the  $\alpha$  subunit (lanes 1, 3, 5, and 7) or a rabbit antibody (rAb50) directed against a peptide corresponding to amino acids 1321–1336 of the  $\beta$ -subunit (lanes 2, 4, 6, and 8). 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  $\alpha$  subunit of both full-length and truncated receptors has the same mobility, the  $\beta$  subunit of the truncated  $\Delta 43$  insulin receptor migrated faster than the  $\beta$  subunit of the full-length receptor.

lines, insulin binding to intact cells was measured. Specific insulin binding was measured after an overnight incubation of cells with  $^{125}I$ -insulin at 4 °C. As expected, in cells expressing the  $Leu^{323}$  mutant receptor, no significant binding was detected. By contrast, in cells expressing the WT IR, the truncated  $\Delta 43$  IR, and in cells co-expressing the  $Leu^{323}$  mutant and the  $\Delta 43$  IR, similar levels of specific insulin binding were measured (Fig. 2).

**Intermolecular Transphosphorylation of Insulin Receptor**—Cells were incubated in the presence or absence of insulin for 1 min and lysed. Insulin receptors were immunoprecipitated with B10. Following SDS-PAGE and electrotransfer, phosphorylated receptors were detected by an anti-phosphotyrosine antibody. Insulin markedly increased the phosphorylation of the IR  $\beta$  subunit in all clonal cell lines, except those expressing only the  $Leu^{323}$  mutant receptor (Fig. 3A). In cells expressing the  $\Delta 43$  IR, insulin stimulated the phosphorylation of a band of 90 kDa, corresponding to the truncated  $\Delta 43$   $\beta$  subunit and, as expected, in cells expressing the WT IR, a band of 95 kDa corresponding to the full-length  $\beta$  subunit was phosphorylated in response to insulin. In cells expressing both the  $Leu^{323}$  mutant and the  $\Delta 43$  homodimeric receptors, two bands were phosphorylated in response to insulin: the full-length  $\beta$  subunit of the  $Leu^{323}$  mutant ( $M_r = 95,000$ ) and the truncated  $\beta$  subunit of the  $\Delta 43$  IR ( $M_r = 90,000$ ) (Fig. 3).

In separate experiments, after stimulation with insulin and cell solubilization, receptors were immunoprecipitated either with B10 or rAb50. Following SDS-PAGE and electrotransfer, phosphorylated receptors were blotted with an anti-phosphotyrosine antibody. In cells expressing WT IRs, both antibodies immunoprecipitated a band of an apparent molecular mass of 95,000 Da, corresponding to the  $\beta$  subunit; this phosphorylation is insulin-dependent (Fig. 3B). In cells co-expressing the  $Leu^{323}$  mutant IR and the truncated  $\Delta 43$  IR, B10 immunoprecipitated two bands with apparent molecular masses of 95,000 and 90,000 Da, corresponding to the  $\beta$  subunits of the  $Leu^{323}$  mutant receptor and to the  $\Delta 43$  IR, respectively. When immunoprecipitation was carried out with rAb50, which does not interact with the  $\Delta 43$  IR, only one band was immunoprecipitated, with an apparent molecular mass of 95,000 Da, corresponding to the  $\beta$  subunit of the  $Leu^{323}$  mutant receptor. When the  $Leu^{323}$  IR was expressed alone, no significant phosphoryl-



**FIG. 2. Determination of specific insulin binding to intact cells.** Confluent cells were incubated with 20,000 cpm of  $^{125}\text{I}$ -insulin in the presence or absence of an excess of unlabeled insulin ( $10^{-7}$  M). After incubation of cells at 4 °C overnight, specific insulin binding was determined in cells expressing neomycin resistance alone (Neo), wild-type insulin receptor (WT), truncated Δ43 insulin receptor (Δ43), Leu<sup>323</sup> mutant receptor (Leu<sup>323</sup>), or in cell surface of cells coexpressing Leu<sup>323</sup> and Δ43 insulin receptors (Leu<sup>323</sup>/Δ43). Binding is expressed as specific binding over total activity (B/T, %). The result is the mean of four separate experiments  $\pm$  S.E.

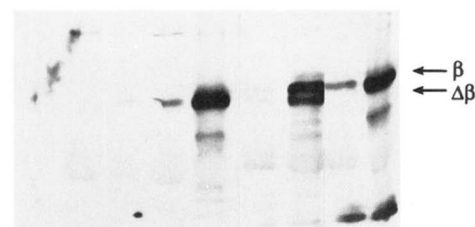
ation of the  $\beta$  subunit was detected in response to insulin. Therefore, the presence of a phosphorylated full-length  $\beta$  subunit in cells coexpressing both the Leu<sup>323</sup> mutant IR and the Δ43 IR is the result of intermolecular transphosphorylation of the Leu<sup>323</sup> homodimer by the Δ43 homodimer insulin receptors.

**Association of IRS-1 with the Leu<sup>323</sup> Mutant IR in Cotransfected Cells**—In order to assess the phosphorylation of IRS-1 and its association with the IR, intact cells were incubated in the presence or absence of insulin for 1 min. Following cell solubilization, cell lysates were immunoprecipitated with anti-IRS-1 antibody. Phosphorylated IRS-1 and phosphotyrosine-containing proteins potentially associated with IRS-1 were detected with an anti-phosphotyrosine antibody. Insulin increased markedly the phosphorylation of IRS-1 in cells expressing WT or Δ43 IRs, and in cells coexpressing the Leu<sup>323</sup> mutant and Δ43 IRs (Fig. 4). In cells singly transfected with the Leu<sup>323</sup> mutant IR, phosphorylation of IRS-1 is only slightly increased, similar to that observed in Neo cells (Fig. 4). In cells expressing the WT or the Δ43 IR, both the phosphorylated full-length  $\beta$  subunit or the truncated  $\beta$  subunit co-immunoprecipitated with IRS-1 (Fig. 4). It is noteworthy that in cells expressing both the Leu<sup>323</sup> mutant IR and the Δ43 IR, IRS-1 coimmunoprecipitated with two phosphorylated bands corresponding to the full-length  $\beta$  subunit of the Leu<sup>323</sup> mutant receptor and the truncated  $\beta$  subunit of the Δ43 receptor.

**Activation of PI 3-Kinase in Cells Coexpressing the Leu<sup>323</sup> Mutant IR and the Truncated Δ43 IR**—To investigate downstream events following IR phosphorylation, PI 3-kinase activity was measured after insulin stimulation of cells expressing the WT IR, the Δ43 IR, or the Leu<sup>323</sup> mutant IR and in cells co-expressing both Leu<sup>323</sup> mutant IR and Δ43 IR. As expected, insulin markedly stimulated PI 3-kinase activity in cells expressing the WT IR and had only a slight effect, just above that seen in the Neo cells, in cells singly transfected with the Leu<sup>323</sup> mutant IR (Fig. 5, upper and lower panels). In cells expressing the Δ43 IR, insulin stimulated PI 3-kinase activity, but not as efficiently as in cells expressing the WT IR (Fig. 5, upper and lower panels). This is in good agreement with previous studies

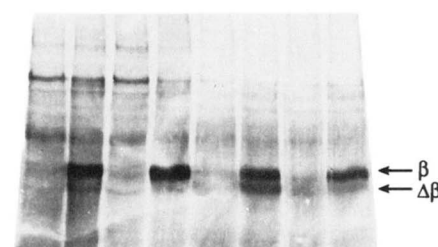
**A**

IP: B10  
Blot:  $\alpha$ PY



Insulin ( $10^{-8}$ M)	-	+	-	+	-	+	-	+
Cell Line	Leu <sup>323</sup>		Δ43		Leu <sup>323</sup> /Δ43		WT	

**B**



Insulin (10 <sup>-8</sup> M)	-	+	-	+	-	+	-	+
IP	B10		rAb50		B10		rAb50	
Cell Line	WT				Leu <sup>323</sup> Δ 43			

**FIG. 3. Insulin stimulates tyrosine phosphorylation of Leu<sup>323</sup> and Δ43 homodimers in cotransfected 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 coexpressing Leu<sup>323</sup> and Δ43 insulin receptors were incubated in the presence or absence of  $10^{-8}$  M insulin at 37 °C for 1 min. Cells were solubilized, and insulin receptors were immunoprecipitated with either B10 (panels A and B) or rAb50 (panel B) for cells expressing WT insulin receptors or coexpressing Leu<sup>323</sup> and Δ43 homodimers (Leu<sup>323</sup>/Δ43). Immunoprecipitates were analyzed on SDS-PAGE (6.5%), followed by electrophoretic transfer to a nitrocellulose membrane. The blot was probed with an anti-phosphotyrosine antibody, and bands were detected by ECL.

both *in vitro* (26) and in intact cells,<sup>2</sup> showing that deletion of 43 amino acids in the Δ43 IR removes a potential site of direct interaction of the IR and the p85 regulatory subunit of PI 3-kinase. In cells co-expressing the Leu<sup>323</sup> mutant IR and the Δ43 IR, the activation of PI 3-kinase was similar to that obtained in cells expressing the WT IR (Fig. 5, upper and lower panels).

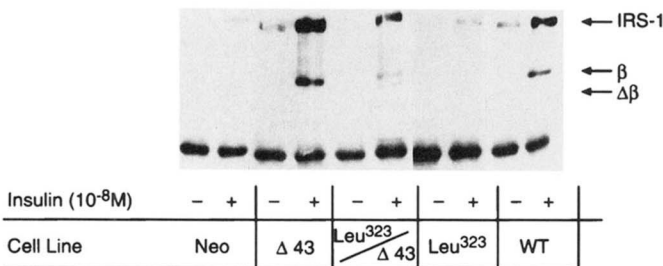
## DISCUSSION

The insulin receptor gene encodes a single-chain polypeptide that is processed and inserted into the plasma membrane of the cell as an  $\alpha_2\beta_2$  heterotetramer. The  $\alpha_2$  dimer is required for high affinity binding, inasmuch as the  $\alpha$  subunit monomer binds insulin with low affinity (27–30). We have recently demonstrated, however, that high affinity binding can be reconstituted in a hybrid between a wild-type receptor half ( $\alpha\beta$ ) and a very low affinity mutated receptor half ( $\alpha_{\text{Leu-323}}\beta$ ). Furthermore, we have shown that, while the Leu<sup>323</sup> holoreceptor neither binds insulin nor phosphorylates its  $\beta$  subunit, the  $\alpha_{\text{mut}}\beta\alpha\beta$  hybrid receptor binds and phosphorylates in an insulin-de-

<sup>2</sup> R. Levy-Toledano, M. Taouis, D. H. Blaettler, P. Gorden, and S. I. Taylor, submitted for publication.



IP:  $\alpha$ IRS-1  
Blot:  $\alpha$ PY



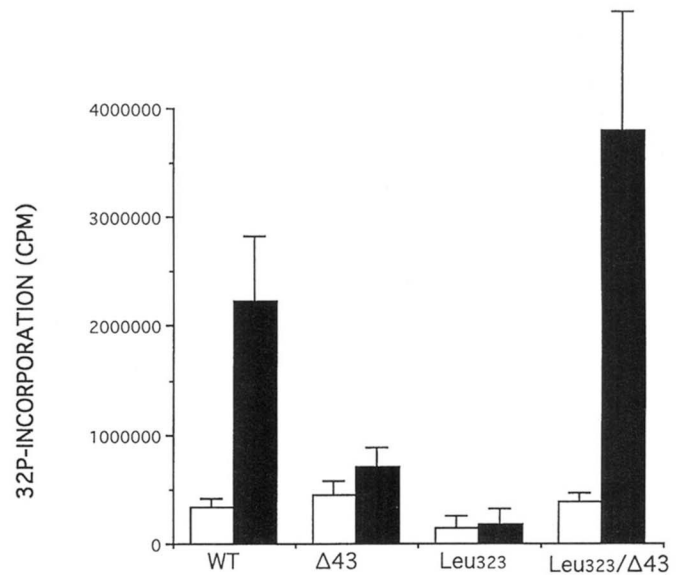
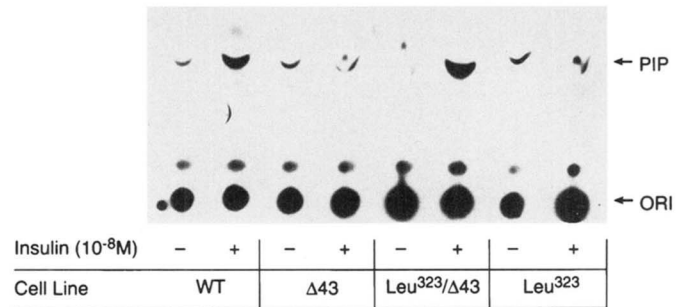
**FIG. 4. Association of phosphorylated IRS-1 with  $Leu^{323}$  mutant insulin receptors in cells coexpressing  $Leu^{323}$  and  $\Delta 43$  insulin receptors.** NIH-3T3 cells expressing either the neomycin resistance gene alone (Neo), wild-type receptor (WT),  $Leu^{323}$  mutant ( $Leu^{323}$ ), truncated  $\Delta 43$  receptor ( $\Delta 43$ ), or coexpressing  $Leu^{323}$  and  $\Delta 43$  insulin receptors ( $Leu^{323}/\Delta 43$ ) were incubated in the presence or absence of  $10^{-8}$  M insulin for 1 min at  $37^\circ\text{C}$ . IRS-1 was immunoprecipitated using rAb-IRS-1 as described under "Materials and Methods." The blot was probed with an anti-phosphotyrosine antibody, and bands were detected by ECL.

pendent fashion. This strongly supports the work of others that suggests that  $\beta$  subunit transphosphorylation occurs.

In the present experiments, cells were co-transfected with cDNA encoding the  $Leu^{323}$  mutant and the  $\Delta 43$  IRs. In addition, control cells were singly transfected with one construct. By combining biotinylation and differential immunoprecipitation using a specific antibody that does not react with the  $\Delta 43$  IR homodimer, we were able to select a clonal cell line that expressed both  $Leu^{323}$  and  $\Delta 43$  homodimers ( $\alpha\alpha\beta\beta_\Delta$  and  $\alpha_{mut}\alpha_{mut}\beta\beta$ ) but no detectable heterodimer ( $\alpha\alpha_{mut}\beta\beta_\Delta$ ) (8).

We have previously expressed in NIH-3T3 cells the  $Leu^{323}$ - $\Delta 43$  hybrid insulin receptor and demonstrated that insulin binds to the  $\alpha$  subunit of the  $\Delta 43$  half-receptor, and that the  $\beta$  subunit of the  $Leu^{323}$  mutant half-receptor is phosphorylated by an intramolecular mechanism. In the present study, we co-expressed both the  $Leu^{323}$  and the  $\Delta 43$  IRs without detectable hybrids and this was obtained when the ratio of  $\Delta 43$  cDNA/ $Leu^{323}$  cDNA is 1/5. However, if the ratio is inverted, the formation of hybrid receptors is possible as we have shown previously (8). The explanation of this phenomenon is not understood. We demonstrate, in cells co-expressing both homodimers, that insulin stimulates the phosphorylation of the full-length  $\beta$  subunit of the  $Leu^{323}$  mutant homodimer and the truncated  $\beta$  of the  $\Delta 43$  IR. When rAb50 was used to immunoprecipitate IRs, only one band, corresponding to the  $\beta$  subunit of the  $Leu^{323}$  mutant homodimer, was detected (Fig. 3, A and B). This confirms the absence of hybrid receptors and indicates that the  $Leu^{323}$  mutant IR is phosphorylated by the  $\Delta 43$  IR. When the  $Leu^{323}$  IR was singly expressed, no phosphorylation of the mutant was detected (Fig. 3A).

In order to investigate post-receptor events in cells coexpressing both the mutant and the  $\Delta 43$  IRs, we measured the phosphorylation of IRS-1 and insulin-induced PI 3-kinase. Phosphorylation of IRS-1 was insulin-dependent in all clonal cell lines, except in cells singly transfected with the  $Leu^{323}$  construct. In cells co-expressing the  $Leu^{323}$  and  $\Delta 43$  IRs, both  $\beta$  and  $\beta_\Delta$  coimmunoprecipitate with IRS-1. We also measured insulin-induced PI 3-kinase. Insulin markedly stimulates PI 3-kinase activity in cells expressing the WT IR and in cells co-expressing the  $Leu^{323}$  and the  $\Delta 43$  IRs. As expected in cells expressing only the  $Leu^{323}$  mutant IR, insulin only slightly stimulates PI 3-kinase (Fig. 5, upper and lower panels). Interestingly, in cells expressing only the  $\Delta 43$  IR, insulin stimulates PI 3-kinase activity but not as efficiently as in cells expressing the WT IR. This is in good agreement with previous *in vitro*



**FIG. 5. Activation of PI 3-kinase in cells coexpressing  $Leu^{323}$  mutant and truncated  $\Delta 43$  insulin receptors.** NIH-3T3 cells expressing wild-type insulin receptor (WT), truncated  $\Delta 43$  insulin receptor ( $\Delta 43$ ),  $Leu^{323}$  mutant receptor ( $Leu^{323}$ ), or coexpressing  $Leu^{323}$  and  $\Delta 43$  insulin receptors ( $Leu^{323}/\Delta 43$ ) were incubated in the presence (+) or absence (-) of  $10^{-8}$  M insulin and lysed. Lysates were immunoprecipitated with an anti-phosphotyrosine antibody. PI 3-kinase activity associated with each of the immunoprecipitates was assessed as described under "Materials and Methods." The upper panel shows a typical autoradiogram of the thin-layer chromatogram of PI 3-kinase activity associated with the autophosphorylated insulin receptor, where ORI represents the origin and PIP the migration of labeled phosphatidylinositol phosphate. The lower panel shows the mean result of four separate experiments, where  $^{32}\text{P}$  incorporation into phosphatidylinositol phosphate was measured by PhosphorImager analysis  $\pm$  S.E.  $\square$ , - insulin;  $\blacksquare$ , + insulin.

findings and consistent with the fact that the  $\Delta 43$  IR has lost a specific domain that reacts with the SH2 domain of PI 3-kinase (26). It is noteworthy that in cells expressing both the  $Leu^{323}$  mutant and the  $\Delta 43$  IRs, the PI 3-kinase activity is similar to that of cells expressing the WT and higher than that of cells expressing only the  $\Delta 43$  IR. One possible explanation is that the  $Leu^{323}$  IR, once phosphorylated by the  $\Delta 43$  IRs, behaves like a WT IR and fully activates the PI-3 kinase either directly or via IRS-1 phosphorylation.

The present work has both similarities and differences from a previous demonstration of intermolecular phosphorylation (14). In the previous study the receptor phosphorylates a kinase mutant receptor (Ile<sup>1153</sup>). This phosphorylated receptor, however, was not active toward downstream effects. These experiments are similar in that the  $\beta$  subunits of both the Ile<sup>1153</sup> and  $Leu^{323}$  mutant receptors are phosphorylated. The Ile<sup>1153</sup> mutant, however, cannot serve as a kinase because of the nature of its mutation, but the  $Leu^{323}$   $\beta$  subunit is normal as a kinase toward other substrates once it is activated. It is in this special context that genetic rescue is possible. It has also been

shown *in vitro* that the Val<sup>382</sup> mutant IR can be phosphorylated by the WT IR; however, this mutant is expressed weakly on the cell surface (31).

In summary, we have demonstrated that a binding-deficient mutant insulin receptor can be transphosphorylated by a coexpressed insulin-binding IR. The transphosphorylated Leu<sup>323</sup> IR can then associate with IRS-1 and with PI 3-kinase. Thus, contrary to mutations in the tyrosine kinase domain, the Leu<sup>323</sup> mutation has no dominant-negative effect. Thus, its downstream effects may be rescued by co-expressing of a normal receptor. This represents a novel form of gene therapy, at least to cultured cells. Furthermore, it provides a molecular explanation for the observation that insulin resistance due to the Leu<sup>323</sup> mutation is inherited in a recessive fashion.

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