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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 α subunit of the insulin receptor (IR) leads to autophosphorylation of the β subunit. The reaction proceeds as intramolecular transphosphorylation between αβ half-receptors of the heterotetrameric receptor dimer (αβpβ). Since IRS-1 is mobile in the plane of the plasma membrane, it is also possible that transphosphorylation may occur between adjacent holoreceptors (αβpβ) by an intermolecular reaction. To address this question, we cotransfected NIH-3T3 cells with two IR cDNA constructs: a truncated but functional IR lacking the C-terminal 43 amino acids (Δ43) and a full-length Leu23 mutant receptor that is expressed on the cell surface but does not bind insulin. A clonal cell line was selected from cells cotransfected with a 1:5 ratio of Δ43 cDNA/Leu23 cDNA. The two homodimers (Leu23 and Δ43) were expressed without detectable formation of hybrid receptors. By using specific antibodies, we demonstrate that in cells coexpressing both homodimers, the Leu23 mutant receptor was phosphorylated in vivo by the Δ43 IR in an insulin-dependent manner. However, when the Leu23 mutant receptor was expressed alone, no phosphorylation was detected. In addition, we demonstrate the association of the phosphorylated Leu23 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 Leu23 mutant receptor, that the phosphorylation of the Leu23 mutant receptor occurs by an intramolecular transphosphorylation mechanism, and, finally, that the Leu23 mutant receptor, once phosphorylated, can associate with insulin receptor substrate-1 and phosphatidylinositol 3-kinase.

Several lines of evidence suggest that the binding of insulin to one half of the α subunit dimer within a tetrameric holoreceptor (αβpβ) can result in intramolecular transphosphorylation of the opposite β subunit (3–7). We have recently presented evidence that strongly supports this concept by expressing hybrid insulin receptors in which one αβ half contains a mutation from a patient with extreme insulin resistance that is expressed on the cell surface but does not bind insulin (Leu23) and is not phosphorylated (8, 9). The other αβ half-receptor (αβpβ) has a wild-type (WT) α subunit and a truncated β 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 β 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 intramolecular 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 Leu23 mutant receptor and the truncated Δ43 IR. Using a clonal cell line that expresses homodimeric receptors (ααpββ or αβpαβ), we demonstrate that the Δ43 insulin receptor phosphorylates the Leu23 mutant receptor in response to insulin. Additionally we demonstrate that, once the Leu23 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 Leu23 mutant 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 β subunit of the insulin receptor (Δ43). WT and truncated cDNAs were ligated into pBluescript (Stratagene, Inc.) and a mixture of insulin receptor expression vector (20 μg) and an expression vector containing the neomycin phosphotransferase gene (pRSV-Neo; 0.5 μg) (15, 16). Cells were either transfected 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 (8). Stable transfected cells were selected with G418 (500 μg/ml; Life Technologies, Inc.). After selection, insulin receptor expression by stable transfecteds was assayed by measuring 125I-insulin binding and, in the case of cotransfected cells or cells expressing the Leu23 receptor alone, clones expressing both the full-length and the Δ43 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 α 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 β subunit of the human insulin receptor at a dilution of 1:50 (19). This peptide has been deleted from the Δ43 mutant insulin receptor; therefore, this antibody does not react with the Δ43 mutant. After electrophoretic transfer, 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 γ-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 MnCl₂ (100 mM), and 20 mg of phosphatidylinositol (Sigma). The phospholipid reaction was started by addition of 10 ml of ATP (440 µM) containing 30 mg of γ-phosphatidyl[γ-32P]ATP. After 10 min, the reaction was stopped by the addition of 20 ml of HCl (8 N) and 160 ml of CHCl₃/methanol (1:1). The organic phase was extracted and applied to a silica gel thin layer chromatography plate (Merck). Chromatography plates were developed in CHCl₃/CH₂OH/H₂O/NH₄OH (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 Δ43 IR, which has a deletion of 43 amino acids at the C terminus of the p subunit (14, 24, 25), or the Leu₃₂₃ mutant receptor or cotransfected with the truncated Δ43 IR and the Leu₃₂₃ mutant IR. In order to identify each specific transfected, 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 Leu₃₂₃ mutant IR cDNAs express an α subunit of an apparent molecular mass of 135,000 Da and a β subunit of 95,000 Da, whereas cells transfected with the Δ43 IR construct express a β 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 α subunit, two bands corresponding to the truncated β subunit (Mᵦ = 90,000) and the full-length β subunit (Mᵦ = 95,000) (Fig. 1). However, when immunoprecipitation was carried out with rAb50, only the 95-kDa band was detected, demonstrating that the Δ43 IR and the Leu₃₂₃ 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 lines, insulin binding to intact cells was measured. Specific insulin binding was measured after an overnight incubation of cells with 125I-insulin at 4°C. As expected, in cells expressing the Leu₃₂₃ mutant receptor, no significant binding was detected. By contrast, in cells expressing the WT IR, the truncated Δ43 IR, and in cells co-expressing the Leu₃₂₃ mutant and the Δ43 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 β subunit in all clonal cell lines, except those expressing only the Leu₃₂₃ mutant receptor (Fig. 3A). In cells expressing the Δ43 IR, insulin stimulated the phosphorylation of a band of 90 kDa, corresponding to the truncated Δ43 IR subunit and, as expected, in cells expressing the WT IR, a band of 95 kDa corresponding to the full-length β subunit was phosphorylated in response to insulin. In cells expressing both the Leu₃₂₃ mutant and the Δ43 homodimeric receptors, two bands were phosphorylated in response to insulin: the full-length β subunit of the Leu₃₂₃ mutant (Mᵦ = 95,000) and the truncated β subunit of the Δ43 IR (Mᵦ = 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 β subunit; this phosphorylation is insulin-dependent (Fig. 3B). In cells co-expressing the Leu₃₂₃ mutant IR and the truncated Δ43 IR, B10 immunoprecipitated two bands with apparent molecular masses of 95,000 and 90,000 Da, corresponding to the β subunits of the Leu₃₂₃ mutant receptor and to the Δ43 IR, respectively. When immunoprecipitation was carried out with rAb50, which does not interact with the Δ43 IR, only one band was immunoprecipitated, with an apparent molecular mass of 95,000 Da, corresponding to the β subunit of the Leu₃₂₃ mutant receptor. When the Leu₃₂₃ IR was expressed alone, no significant phosphoryl-
atation of the β subunit was detected in response to insulin. Therefore, the presence of a phosphorylated full-length β subunit in cells coexpressing both the Leu323 mutant IR and the Δ43 IR is the result of intermolecular transphosphorylation of the Leu323 homodimer by the Δ43 homodimer insulin receptors.

Association of IRS-1 with the Leu233 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 Leu323 mutant and Δ43 IRs (Fig. 4). In cells singly transfected with the Leu323 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 β subunit or the truncated β subunit co-immunoprecipitated with IRS-1 (Fig. 4). It is noteworthy that in cells expressing both the Leu323 mutant IR and the Δ43 IR, IRS-1 coimmunoprecipitated with two phosphorylated bands corresponding to the full-length β subunit of the Leu323 mutant receptor and the truncated β subunit of the Δ43 receptor.

Activation of PI 3-Kinase in Cells Coexpressing the Leu323 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 Leu323 mutant IR and in cells co-expressing both Leu323 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 Leu323 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

**Fig. 2. Determination of specific insulin binding to intact cells.** Confluent cells were incubated with 20,000 cpm of 125I-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), Leu323 mutant receptor (Leu323), or in cell surface of cells coexpressing Leu323 and Δ43 insulin receptors (Leu323/Δ43). Binding is expressed as specific binding over total activity (B/T, %). The result is the mean of four separate experiments ± S.E.

**Fig. 3. Insulin stimulates tyrosine phosphorylation of Leu323 and Δ43 homodimers in cotransfected cells.** NIH-3T3 cells expressing either wild-type receptor (WT), truncated Δ43 receptor (Δ43), Leu323 mutant receptor (Leu323), or coexpressing Leu323 and Δ43 insulin receptors (Leu323/Δ43). Immunoprecipitates were analyzed on SDS-PAGE (6.5%), followed by electrophoroblotting. The blot was probed with an anti-phosphotyrosine antibody, and bands were detected by ECL.

Both in vitro (26) and in intact cells,2 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 Leu323 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 αβ heterotetramer. The αβ dimer is required for high affinity binding, inasmuch as the α 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 (αβ) and a very low affinity mutated receptor half (αmutα2ββ). Furthermore, we have shown that, while the Leu323 holoreceptor neither binds insulin nor phosphorylates its β subunit, the αmutα2ββ hybrid receptor binds and phosphorylates in an insulin-de-
Insulin (10⁻⁸ M) | Cell Line | Neo | Δ43 | Leu323 | Δ43 | Leu323/Δ43 | WT
---|---|---|---|---|---|---|---
- | + | + | - | - | + | + | +
- | - | + | + | + | - | + | +
- | + | + | + | - | - | - | -
- | - | - | - | - | - | - | -

**FIG. 4.** Association of phosphorylated IRS-1 with Leu323 mutant insulin receptors in cells coexpressing Leu323 and Δ43 insulin receptors. NIH-3T3 cells expressing either the neomycin resistance gene alone (Neo), wild-type receptor (WT), Leu323 mutant (Leu323), truncated Δ43 receptor (Δ43), or coexpressing Leu323 and Δ43 insulin receptors (Leu323/Δ43) were incubated in the presence or absence of 10⁻⁸ M insulin for 1 min at 37 °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.

In a dependent fashion. This strongly supports the work of others that suggests that β subunit transphosphorylation occurs.

In the present experiments, cells were co-transfected with cDNA encoding the Leu323 mutant and the Δ43 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 Δ43 IR cDNA encoding the Leu323 mutant and the Δ43 IRSs. In addition, we have previously expressed in NIH-3T3 cells the Ledz3 mutant insulin receptor and demonstrated that insulin binds to the α subunit of the Δ43 half-receptor, and that the β subunit of the Leu323 mutant half-receptor is phosphorylated by an intramolecular mechanism. In the present study, we co-expressed both the Leu323 and the Δ43 IRSs without detectable hybrids and this was obtained when the ratio of Δ43 cDNA/Leu323 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 β subunit of the Leu323 mutant homodimer and the truncated β of the Δ43 IR. When rAb50 was used to immunoprecipitate IRSs, only one band, corresponding to the β subunit of the Leu323 mutant homodimer, was detected (Fig. 3, A and B). This confirms the absence of hybrid receptors and indicates that the Leu323 mutant IR is phosphorylated by the Δ43 IR. When the Leu323 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 Δ43 IRSs, 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 Δ43 construct. In cells co-expressing the Leu323 and Δ43 IRSs, both β and β3 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 Leu323 and the Δ43 IRSs. As expected in cells expressing only the Leu323 mutant IR, insulin only slightly stimulates PI 3-kinase (Fig. 5, upper and lower panels). Interestingly, in cells expressing only the Δ43 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 findings and consistent with the fact that the Δ43 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 Leu323 mutant and the Δ43 IRSs, the PI 3-kinase activity is similar to that of cells expressing the WT and higher than that of cells expressing only the Δ43 IR. One possible explanation is that the Leu323 IR, once phosphorylated by the Δ43 IRSs, 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 (Ile1153). This phosphorylated receptor, however, was not active toward downstream effects. These experiments are similar in that the β subunits of both the Ile1153 and Leu323 mutant receptors are phosphorylated. The Ile1153 mutant, however, cannot serve as a kinase because of the nature of its mutation, but the Leu323 β 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 Val382 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 Leu293 IR can then associate with IRS-1 and with PI 3-kinase. Thus, the transphosphorylated IRS-1 and the PI 3-kinase. This represents a novel form of gene therapy, at least the Ledz3 mutation is inherited in a recessive fashion. Contrary to mutations in the tyrosine kinase domain, the mutant insulin receptor can be transphosphorylated by a coexpressed normal insulin-binding IR. The transphosphorylated Ledz3 IR can then associate with IRS-1 and with PI 3-kinase. Thus, its downstream effects may be rescued by coexpressing of a normal mutant insulin receptor can be transphosphorylated by a coexpressed insulin-binding IR. The transphosphorylated Ledz3 IR can then associate with IRS-1 and with PI 3-kinase. Thus, the transphosphorylated IRS-1 and the PI 3-kinase. 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 Leu293 mutation is inherited in a recessive fashion.

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