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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 (αβpβp). Since IRs are mobile in the plane of the plasma membrane, it is also possible that transphosphorylation may occur between adjacent homoreceptors (αβpβp) by an intermolecular reaction. To address this question, we cotransfected NIH-3T3 cells with two IR cDNA constructs: a truncated but functional αβpβp 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/Leu323 cDNA. The two homodimers (Leu323 and Δ43) were expressed without detectable formation of hybrid receptors. By using specific antibodies, we demonstrate that in cells coexpressing both homodimers, the Leu323 mutant receptor was phosphorylated in vivo by the Δ43 IR in an insulin-dependent manner. However, when the Leu323 mutant receptor was expressed alone, no phosphorylation was detected. In addition, we demonstrate the association of the phosphorylated Leu323 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 Leu323 mutant receptor, that the phosphorylation of the Leu323 mutant receptor occurs by an intramolecular transphosphorylation mechanism, and, finally, that the Leu323 mutant receptor, once phosphorylated, can associate with insulin receptor substrate-1 and phosphatidylinositol 3-kinase.

The insulin receptor (IR) is a transmembrane protein consisting of two identical disulfide-linked half-receptors. Each half (αβpβp) consists of an extracellular α subunit that binds insulin and a transmembrane β subunit that contains a tyrosine kinase domain (1, 2). Insulin binding to the α subunit results in autophosphorylation of the β subunit, activation of receptor tyrosine kinase activity, and tyrosine phosphorylation of intracellular targets of insulin action. Several lines of evidence suggest that the binding of insulin to one half of the α subunit dimer within a tetrameric holoreceptor (αβpβp) 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 αβpβp half contains a mutation from a patient with extreme insulin resistance that is expressed on the cell surface but does not bind insulin (Leu323) and is not phosphorylated (8, 9). The other αβpβp half-receptor (αβpβp) 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 Leu323 mutant receptor and the truncated Δ43 IR. Using a clonal cell line that expresses homodimeric receptors (αβpβp), we demonstrate that the Δ43 insulin receptor phosphorylates the Leu323 mutant receptor in response to insulin. Additionally we demonstrate that, once the Leu323 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 Leu323 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 β subunit of the insulin receptor (Δ43). WT and truncated cDNAs were ligated into pSV40 (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 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 co-transfected with a mixture of expression vectors for two types of insulin receptor (8). Stable transfecants were selected with G418 (600 μg/ml; Life Technologies, Inc.). After selection, insulin receptor expression by stable transfecants was assayed by measuring 125I-insulin binding and, in the case of cotransfected cells or cells expressing the Leu323 receptor alone, cloning expressing both the full-length and the Δ43 insulin receptors and cells expressing only the full-length insulin receptor, receptor expres-
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), or 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 electroblotting, nitrocellulose sheets were probed with horseradish peroxidase-labeled 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 phoshatidylinositol (Sigma). The phosphorylation reaction was started by addition of 10 ml of ATP (440 mM) containing 30 mM (γ32P)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/CH3OH/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 β 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 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 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 (M, = 90,000) and the full-length β subunit (M, = 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 β subunit and, as expected, in cells expressing the WT IR, a band of 96 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 (M, = 95,000) and the truncated β subunit of the Δ43 IR (M, = 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-
Therefore, the presence of a phosphorylated full-length insulin receptor mutant receptor (Leu232), or in cell surface of cells coexpressing Leu232 and Δ43 insulin receptors (Leu232/Δ43). Binding is expressed as specific binding over total activity (B/T, %). The result is the mean of four separate experiments ± S.E.

**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), Leu232 mutant receptor (Leu232), or in cell surface of cells coexpressing Leu232 and Δ43 insulin receptors (Leu232/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 Leu232 and Δ43 homodimers in cotransfected cells.** NIH-3T3 cells expressing either wild-type receptor (WT), truncated Δ43 receptor (Δ43), Leu232 mutant receptor (Leu232), or coexpressing Leu232 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 Leu232 and Δ43 homodimers (Leu232/Δ43). Immunoprecipitates were analyzed on SDS-PAGE (6.5%), followed by electrophoretic transfer. The blot was probed with anti-phosphotyrosine antibody, and bands were detected by ECL.

**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 heterotrimer. The αα 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 (αmutβ). Furthermore, we have shown that, while the Leu232 holoreceptor neither binds insulin nor phosphorylates its β subunit, the αmutβ hybrid receptor binds and phosphorylates in an insulin-de-
Blot: aPY

**Fig. 4.** Association of phosphorylated IRS-1 with Leu235 mutant insulin receptors in cells coexpressing Leu235 and Δ43 insulin receptors. NIH-3T3 cells expressing either the neomycin resistance gene alone (Neo), wild-type receptor (WT), Leu235 mutant (Leu235), truncated Δ43 receptor (Δ43), or coexpressing Leu235 and Δ43 insulin receptors (Leu235/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 the present experiments, cells were co-transfected with cDNA encoding the Leu235 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 A43 IR subunit of the mutant homodimer, we were able to select a clonal cell line that expressed both Leu235 and Δ43 homodimers (ααββ and ααααββ) but no detectable heterodimer (ααααααββ) (8).

We have previously expressed in NIH-3T3 cells the Leu235, Δ43 hybrid insulin receptor and demonstrated that insulin binds to the α subunit of the Δ43 half-receptor, and that the β subunit of the Leu235 mutant half-receptor is phosphorylated by an intramolecular mechanism. In the present study, we co-expressed both the Leu235 and the Δ43 IRs without detectable hybrids and this was obtained when the ratio of Δ43 cDNA/Leu235 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 Leu235 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 Leu235 mutant homodimer, was detected (Fig. 3, A and B). This confirms the absence of hybrid receptors and indicates that the Leu235 mutant IR is phosphorylated by the Δ43 IR. When the Δ43 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 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 Δ43 construct. In cells co-expressing the Leu235 and Δ43 IRs, both β and β2 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 Leu235 and the Δ43 IRs. As expected in cells expressing only the Leu235 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 Leu235 mutant and the Δ43 IRs, the 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 32P incorporation into phosphatidylinositol phosphate was measured by PhosphorImager analysis ± S.E. □, insulin; ■, + insulin.

**Fig. 5.** Activation of PI 3-kinase in cells coexpressing Leu235 mutant and truncated Δ43 insulin receptors. NIH-3T3 cells expressing wild-type insulin receptor (WT), truncated Δ43 insulin receptor (Δ43), Leu235 mutant receptor (Leu235), or coexpressing Leu235 and Δ43 insulin receptors (Leu235/Δ43) 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 assayed 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 32P incorporation into phosphatidylinositol phosphate was measured by PhosphorImager analysis ± S.E. □, insulin; ■, + insulin.
shown in vitro that the Val^{282} 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^{282} IR can then associate with IRS-1 and with PI 3-kinase. Thus, contrary to mutations in the tyrosine kinase domain, the Leu^{282} mutation has no dominant-negative effect. Thus, its downstream effects may be rescued by coexpressing of a normal mutant insulin receptor can be transphosphorylated by a coexpressed insulin receptor. This represents a novel form of gene therapy, at least in vitro in cultured cells. Furthermore, it provides a molecular explanation for the observation that insulin resistance due to the Val^{382} mutation is inherited in a recessive fashion. Thus, its downstream effects may be rescued by coexpressing a normal mutant insulin receptor can be transphosphorylated by a coexpressed insulin receptor.

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