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Insulin-induced Activation of Phosphatidyl Inositol 3-Kinase

DEMONSTRATION THAT THE p85 SUBUNIT BINDS DIRECTLY TO THE COOH TERMINUS OF THE INSULIN RECEPTOR IN INTACT CELLS*

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Insulin activates the insulin receptor tyrosine kinase to phosphorylate signaling molecules such as insulin receptor substrate-1 (IRS-1). Phosphorylated IRS-1 binds to SH2 domains in the p85 regulatory subunit of phosphatidyl inositol (PI) 3-kinase, thereby stimulating the catalytic activity of PI 3-kinase. For most growth factor receptor tyrosine kinases (including receptors for epidermal growth factor and platelet-derived growth factor), the p85 regulatory subunit of PI 3-kinase binds directly to phosphorylated YXXM motifs contained in the cytoplasmic domain of the receptor itself. Previous studies in cell-free systems have shown that the phosphorylated YHTM sequence (amino acid residues 1322–1325) in the COOH terminus of the insulin receptor has the ability to bind to the p85 subunit of PI 3-kinase, thereby activating the enzyme. In this investigation, we demonstrate the occurrence of the same direct binding interaction in intact cells. Subsequent to insulin-stimulated phosphorylation of the insulin receptor, a complex is formed that contains the insulin receptor and PI 3-kinase. This complex can be immunoprecipitated by antibodies directed against either the insulin receptor or the p85 subunit of PI 3-kinase. The Δ43 mutant insulin receptor that lacks 43 amino acids at the COOH terminus does not bind p85. In addition, the Δ43 truncation impairs the ability of the receptor to mediate the activation of PI 3-kinase. Thus, by binding directly to p85, the phosphorylated YHTM motif in the COOH terminus of the insulin receptor contributes partially to mediating the effect of insulin to activate PI 3-kinase.

The tyrosine kinase activity of the insulin receptor plays a necessary role in mediating insulin action (1, 2). Like other receptor tyrosine kinases, ligand binding leads to autophosphorylation of the receptor (3, 4). Autophosphorylation of the insulin receptor is functionally significant in that it activates the receptor tyrosine kinase to phosphorylate other intracellular proteins such as insulin receptor substrate-1 (IRS-1)[1] (5, 6). IRS-1 serves as an intermediate docking protein that contains multiple phosphotyrosine residues, providing binding sites for multiple SH2 domain-containing proteins (7). However, unlike most other growth factor receptor tyrosine kinases, phosphotyrosine residues in the insulin receptor are generally not thought to serve as major binding sites for SH2 domain-containing proteins in intact cells. Nevertheless, one of the phosphorylation sites in the COOH-terminal domain of the insulin receptor is located in a YHTM sequence (amino acid residues 1322–1325) that conforms to the YXXM motif which defines binding sites for the p85 subunit of PI 3-kinase (8, 9). Furthermore, *in vitro* studies in cell-free systems have demonstrated that p85 can bind directly to phosphorylated insulin receptors (10, 11). In the present work, we conducted immunoprecipitation studies demonstrating that p85 binds directly to phosphorylated insulin receptors and that this interaction requires the COOH-terminal 43 amino acids of the insulin receptor. These data support the hypothesis that p85 binds directly to the phosphotyrosine residue in the YHTM sequence located in the COOH-terminal of the β-subunit of the insulin receptor. Furthermore, deletion of the COOH-terminal 43 amino acids partially impairs the ability of the receptor to mediate the action of insulin to activate PI 3-kinase. Nevertheless, these data do not necessarily contradict the hypothesis that IRS-1 is required for optimal activation of PI 3-kinase. For example, the presence of two SH2 domains in p85 may permit the molecule to bridge between the phosphorylated YHTM sequence in the insulin receptor and a phosphorylated YXXM motif in IRS-1.

MATERIALS AND METHODS

Expression of Insulin Receptors by Transfection of cDNA in Cultured Cells—NIH-3T3 cells were stably transfected with expression vector for human insulin receptor cDNA (12) or a truncated receptor lacking the 43 amino acid residues from the COOH terminus of the β-subunit as described previously (13). Expression of insulin receptors was assayed by measuring ^{125}I-insulin binding (14) and/or immunoblotting (15). Based upon Scatchard analysis of insulin binding data, we estimate that there are approximately 400,000 wild type human insulin receptors/cell or approximately 300,000 Δ43 truncated receptors/cell expressed on the cell surface of the transfected cells.

Immunoprecipitation—Confluent cells in 15-cm Petri dishes, grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were incubated in the presence or the absence of 10^{-7} insulin for 3 min at 37 °C. The cells were quickly washed once with ice-cold phosphate-buffered saline followed by two washes with washing buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM Na_3VO_4). Thereafter, the cells were solubilized in 1 ml of washing buffer containing Nonidet P-40 (1%), glycerol (10%), phenylmethylsulfonyl fluoride (2 mM). After normalization for protein concentration, about one-fifth of the cell lysate was immunoprecipitated using either a monoclonal antiphosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY) at a concentration of 2.5 μg/ml, or a polyclonal antibody directed against the p85 regulatory subunit of PI 3-kinase (Upstate Biotechnology Inc.) at a dilution of 1:250, or B-10, an anti-insulin receptor antibody directed against the α-subunit at a dilution of 1:100 or a mixture of antibodies directed against IRS-1 or a non-immune rabbit serum (1:100 dilution). To achieve efficient immunoprecipitation of IRS-1, we used a mixture of two different polyclonal antibodies, at a final dilution of 1:300 (Upstate Biotechnology Inc.) and a monclonal antibody, at a final concentration of 250 ng/ml (Transduction Laboratory). The immune complexes were precipitated with protein A-agarose

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[1] The abbreviations used are: IRS-1, insulin receptor substrate-1; PI, phosphatidyl inositol.

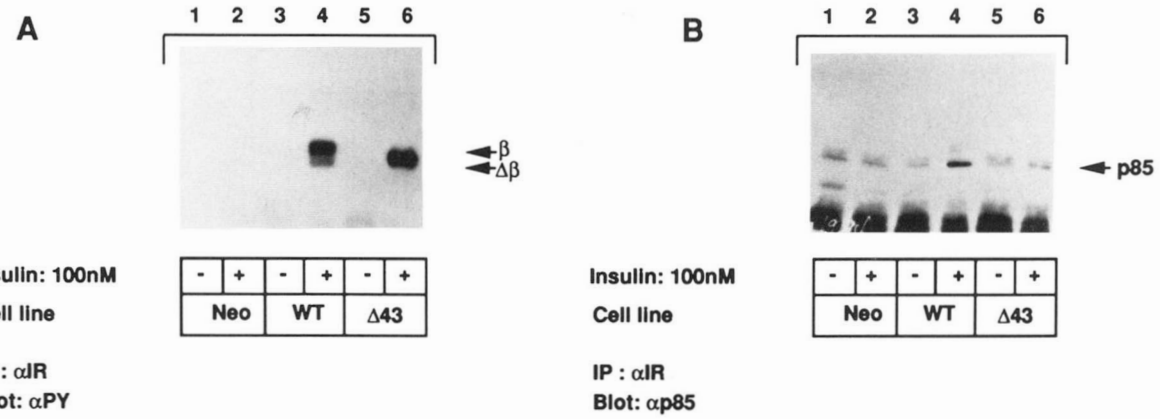

FIG. 1. **PI 3-kinase binds to the COOH terminus of the insulin receptor in intact cells.** Confluent monolayers (15-cm plates) of stably transfected NIH-3T3 cells expressing the neomycin resistance gene (*lanes 1* and *2*), the full-length insulin receptor (*lanes 3* and *4*), or the Δ43-truncated insulin receptor (*lanes 5* and *6*) were incubated in the absence (*lanes 1, 3,* and *5*) or in the presence (*lanes 2, 4,* and *6*) of insulin (10^{-7} M) for 3 min at 37 °C. The cells were lysed in 1 ml of lysis buffer as described under "Materials and Methods." After solubilization, 200 μl were immunoprecipitated using anti-insulin receptor antibody. The immunoprecipitates were analyzed by SDS-polyacrylamide (6.5%) gel electrophoresis and transferred to nitrocellulose sheets by electroblotting. Blots were probed with either antiphosphotyrosine antibody (*panel A*) or anti-p85 antibody (*panel B*). In *panel B*, there is a light band in the region of p85 in *lanes 1–3, 5,* and *6*. This light band, which was not detected in all experiments, may represent nonspecific immunoprecipitation of a small quantity of p85 by anti-insulin receptor antiserum B-10.

(Life Technologies, Inc.) in which nonspecific sites were saturated by washing with a buffer containing Tris-HCl (10 mM, pH 7.5) and albumin (10 mg/ml). The immunoprecipitates were washed once with phosphate-buffered saline containing Nonidet P-40, (0.1%, 1% for PI 3-kinase assay) and vanadate (100 mM), twice with a buffer containing Tris-HCl (100 mM, pH 7.5), $LiCl_2$ (500 mM), vanadate (100 mM), and once with a buffer containing Tris-HCl (10 mM, pH 7.5), NaCl (100 mM), EDTA (1 mM), and vanadate (100 μM).

Phosphatidylinositol 3-Kinase Activity—After the washings described above, the pellet was resuspended in 40 μl of a buffer containing Tris-HCl (10 mM, pH 7.5), NaCl (100 mM), EDTA (1 mM). To each tube was added 10 μl of $MnCl_2$ (100 mM) and 20 μg of phosphatidylinositol (Sigma). The phosphorylation reaction was started by the addition of 10 μl of ATP (440 μM) containing 30 μCi of [γ-^{32}P]ATP. After 10 min, the reaction was stopped by the addition of 20 μl of HCl (8 N) and 160 μl of $CHCl_3$/methanol (1:1). The organic phase was extracted and applied to a silica gel thin layer chromatography plate (Merck). Thin layer chromatography plates were developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (60:47:11.3:2), dried, and visualized by autoradiography. The radioactivity was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) (16, 17).

Immunoblotting—After immunoprecipitation, the complexes were boiled in 40 μl of Laemmli sample buffer containing dithiothreitol (80 mM) for 3 min. The samples were analyzed by SDS-polyacrylamide (6.5%) gel electrophoresis. Proteins were transferred from the gel to nitrocellulose sheets by electroblotting at 90 V for 1 h at 4 °C in a solution containing Tris (25 mM), glycine (192 mM), and methanol (20%). The immunoblots were probed either with a monoclonal antiphosphotyrosine antibody at a concentration of 250 ng/ml or a monoclonal antibody directed against the p85 subunit of PI 3-kinase at a concentration of 1 μg/ml (Upstate Biotechnology Inc.), or a rabbit antibody (rAb-53) directed against a peptide corresponding to the amino acids 1142–57 of the β-subunit of the human insulin receptor (1:2000), and proteins were detected by Enhanced Chemiluminescence using horseradish peroxidase-labeled anti-mouse γ-globulin (Amersham Corp.). Thereafter, where indicated, the blots were stripped off as described elsewhere (15) and reprobed as described above.

RESULTS

Insulin Receptor Coimmunoprecipitates with PI 3-Kinase—The p85 regulatory subunit of PI 3-kinase binds to phosphotyrosine residues in the context of YMXM or YXXM sequences (9, 18). Binding of p85 to phosphotyrosine residues leads to activation of PI 3-kinase (9, 19). Because the YHTM sequence (amino acid residues 1322–1325) in the COOH terminus of the insulin receptor represents a YXXM motif, we inquired whether PI 3-kinase binds to phosphorylated insulin receptor in intact cells. NIH-3T3 cells transfected with human insulin receptor cDNA were incubated in the presence or absence of 100 nM insulin (Fig. 1). Receptors were immunoprecipitated with anti-receptor antibody, and immunoblots were probed with either antiphosphotyrosine antibody (Fig. 1, *panel A*) or antibody to the p85 subunit of PI 3-kinase (Fig. 1, *panel B*). As expected, insulin increased the content of phosphotyrosine in the wild type insulin receptor (Fig. 1*A*, *lanes 3* and *4*). In addition, insulin increased the amount of p85 regulatory subunit of PI 3-kinase coimmunoprecipitated with the insulin receptor (Fig. 1*B*, *lanes 3* and *4*). Comparable studies were carried out in cells expressing the Δ43 truncated mutant form of the insulin receptor that lacks the 43 amino acids at the COOH terminus of the human insulin receptor (11, 20). The Δ43 mutant lacks Tyr^{1322} and the YXXM motif (20–22). Consistent with previous observations (20, 21), insulin increased the content of phosphotyrosine in the Δ43 mutant insulin receptor (Fig. 1*A*, *lanes 5* and *6*). Although, both the 3T3-WT and the 3T3-Δ43 cells expressed a similar amount of insulin receptor (Fig. 1*B*, *lanes 4* and *6*; Fig. 3*C*, *lanes 1* and *2*), the p85 subunit of PI 3-kinase was not coimmunoprecipitated with the Δ43 mutant insulin receptor (Fig. 1*B*, *lanes 5* and *6*). These observations are consistent with the conclusion that the p85 subunit of PI 3-kinase binds to the phosphotyrosine at position 1322 in the phosphorylated insulin receptor.

Similar results were obtained when the same cell extracts were immunoprecipitated with antibody directed against p85 (Fig. 2). In cells expressing full-length human insulin receptors, exposure of cells to insulin increased the quantity of insulin receptors coimmunoprecipitated by anti-p85 antibody (Fig. 2*A*, *lanes 3* and *4*). In contrast, phosphorylated Δ43 mutant insulin receptors were not coimmunoprecipitated by anti-p85 antibody (Fig. 2*A*, *lanes 5* and *6*). Immunoblots probed with anti-p85 antibody demonstrated that p85 was expressed in comparable levels in the transfected cells expressing either full-length insulin receptors (Fig. 2*B*, *lanes 3* and *4*) or Δ43 mutant insulin receptors (Fig. 2*B*, *lanes 5* and *6*).

Coimmunoprecipitation of PI 3-Kinase with IRS-1—It has been clearly shown that insulin stimulates phosphorylation of IRS-1, thereby stimulating binding of PI 3-kinase to phosphotyrosine residues in IRS-1 (19, 23–25). To investigate binding interactions among the insulin receptor, IRS-1, and PI 3-kinase, we carried out studies in which cell extracts were immunoprecipitated with antibody to IRS-1 (Fig. 3, *A* and *B*). Exposure of cells to 100 nM insulin led to comparable increases in the

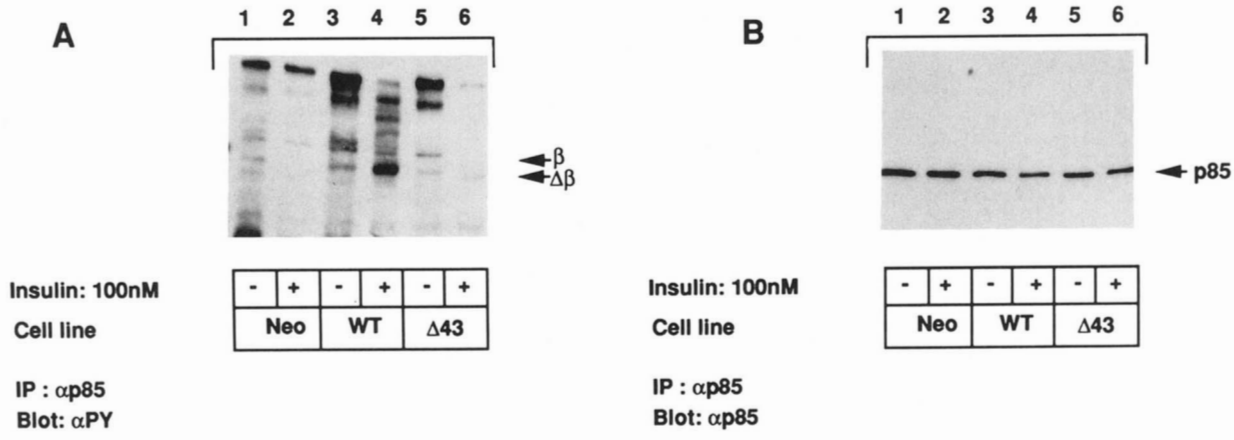

FIG. 2. **Insulin Receptor binds to the p85 subunit of PI 3-kinase in intact cells.** Cell extracts from the same experiment shown in Fig. 1 were used in this experiment. The experiment was identical to that described in the legend to Fig. 1 with the exception that cell extracts were immunoprecipitated using anti-p85 antibody instead of anti-insulin receptor antibody.

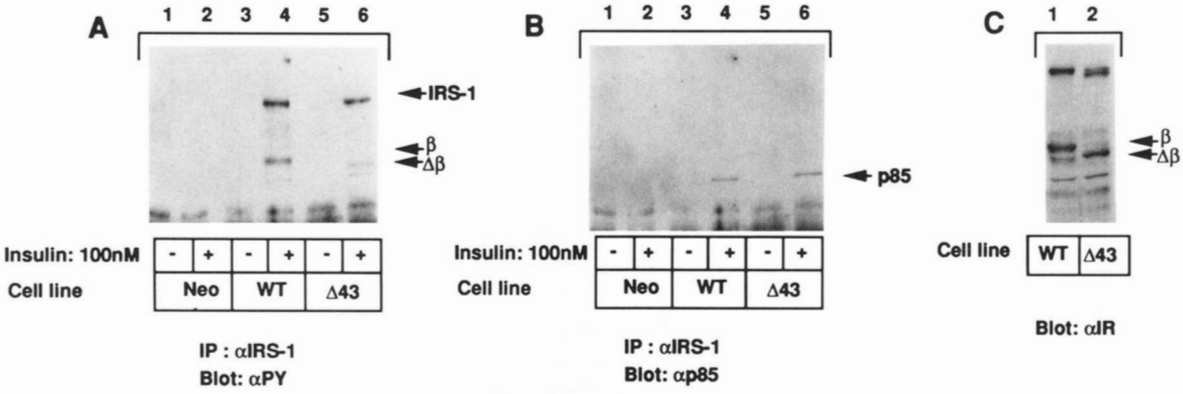

FIG. 3. **PI 3-kinase coimmunoprecipitates with IRS-1 in intact cells.** Cell extracts from the same experiment shown in Figs. 1 and 2 were used in this experiment. The experiment (*panels A* and *B*) was identical to that described in the legend to Fig. 1 with the exception that cell extracts were immunoprecipitated using anti-IRS-1 antibody. In addition, expression of insulin receptors was quantitated in *panel C*. Extracts (20 μl) from cells expressing either the full-length insulin receptor (*panel C, lane 1*) or the Δ43 truncated insulin receptor (*panel C, lane 2*) were analyzed by SDS-polyacrylamide gel electrophoresis, and the sheets were probed using rAb-53 (an antibody directed against the amino acid sequence 1142–1157).

phosphotyrosine content of both full-length insulin receptors (Fig. 3*A*, *lanes 3* and *4*) and Δ43 mutant insulin receptors (Fig. 3*A*, *lanes 5* and *6*). In addition, both forms of the insulin receptor (*i.e.* full-length and Δ43 mutant) were capable of phosphorylating IRS-1 (Fig. 3*A*, *lanes 3–6*). Furthermore, 100 nM insulin led to comparable increases in the content of p85 that bound to IRS-1 in cells expressing either full-length insulin receptors (Fig. 3*B*, *lanes 3* and *4*) or Δ43 mutant receptors (Fig. 3*B*, *lanes 5* and *6*). Thus, deletion of 43 amino acids from the COOH terminus of the insulin receptor inhibited the ability of PI 3-kinase to bind directly to the insulin receptor (Figs. 1 and 2). However, the COOH terminus of the insulin receptor was not required for insulin to stimulate binding of PI 3-kinase to the IRS-1 molecule.

Quantitative Comparisons of Immunoprecipitations with Various Antibodies—We carried out sequential immunoprecipitation studies to estimate the efficiencies of immunoprecipitation with various antibodies (Fig. 4). When two sequential immunoprecipitation steps were carried out with either anti-p85 antibody (Fig. 4, *lane 7*) or anti-IRS-1 antibody (Fig. 4, *lane 8*), we did not detect the cognate antigen in the pellet of the second immunoprecipitation. Thus, we conclude that, under our conditions, these two antibodies have immunoprecipitated ≈100% of the antigen (*i.e.* either p85 or IRS-1, respectively). In contrast, when samples were subjected to two sequential immunoprecipitations with anti-insulin receptor antibody B-10, approximately 30% of the insulin receptors were recovered in the pellet of the second immunoprecipitate (Fig. 4, *lane 9* and data not shown). Thus, under our experimental conditions, antibody B-10 immunoprecipitated ≈60–70% of the insulin receptors contained in the cell extract.

In the same experiment, we compared the quantity of p85 immunoprecipitated by anti-p85 and anti-insulin receptor antibodies (Fig. 4, *lanes 1–4*). Under our experimental conditions, both antibodies immunoprecipitated similar quantities of p85. However, it remains possible that IRS-1 is present in the complex that contains p85 and the insulin receptor. For example, it is possible that one SH2 domain of p85 binds IRS-1 while the second SH2 domain binds the insulin receptor.

Δ43 Truncation Impairs the Ability of Insulin to Activate PI 3-Kinase in Intact Cells—Insulin stimulates phosphorylation of YXXM motifs in the COOH terminus of the receptor as well as in IRS-1. As shown above, the p85 subunit of PI 3-kinase binds to both the receptor and IRS-1. If the association of PI 3-kinase with IRS-1 were the only mechanism involved in mediating the increase in PI 3-kinase activity, then deleting the YXXM motif from the COOH terminus of the receptor would not be predicted to alter the ability of insulin to activate PI 3-kinase. Thus, we designed an experiment to test this prediction (Fig. 5). Cells were incubated for 3 min in the presence or absence of 100 nM insulin. Although insulin had no detectable effect upon PI 3-kinase in cells transfected with only the neomycin resistance gene,

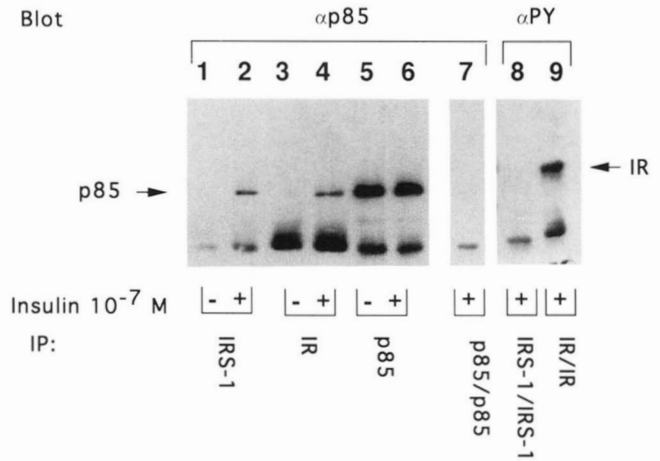

FIG. 4. **Quantitative comparison of immunoprecipitations with various antibodies.** Extracts of cells expressing wild type human insulin receptor were immunoprecipitated with one of the following antibodies: anti-IRS-1 (*lanes 1* and *2*), anti-human insulin receptor (B-10; *lanes 3* and *4*), or anti-p85 (*lanes 5* and *6*). In several cases, the supernatant of the immunoprecipitation was subjected to a second immunoprecipitation with the same antibody (*lanes 7–9*). Finally, the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with either anti-p85 (*lanes 1–7*) or antiphosphotyrosine antibodies (*lanes 8* and *9*). These experiments were carried out as described under "Materials and Methods" with two exceptions. First, we used a polyclonal anti-IRS-1 antibody directed against the COOH terminus of rat IRS-1 (1:100 dilution). Second, we used Ultra-Link Immobilized Protein A Plus supplied by Pierce rather than protein A-agarose supplied by Life Technologies, Inc.

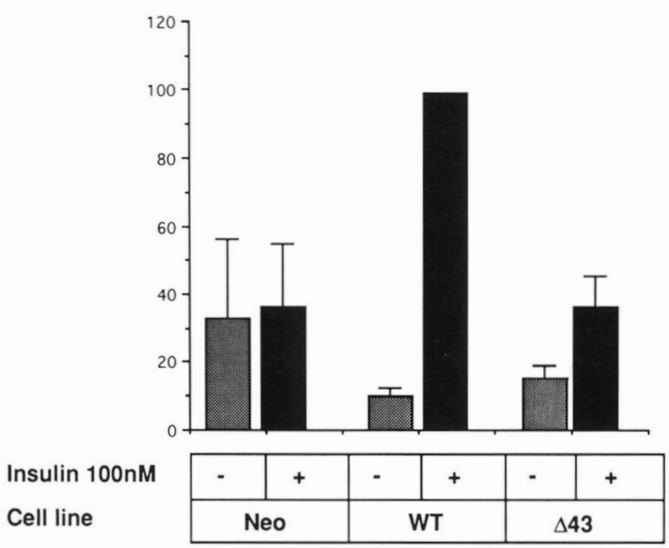

FIG. 5. **Δ43 truncation impairs the ability of insulin to activate PI 3-kinase in intact cells.** Antiphosphotyrosine immunoprecipitates from quiescent or insulin-treated cells expressing either the neomycin resistance gene alone, the wild type (*WT*) insulin receptor, or the Δ43 truncated insulin receptor were assayed for PI 3-kinase activity. Radioactivity was quantitated using a PhosphorImager. Data represent the mean ± S.E. and are representative of three separate experiments. Results are expressed as a percentage of the PI 3-kinase activity measured in insulin-stimulated cells expressing the wild type receptor.

transfection with full-length human insulin receptors allowed for insulin to stimulate PI 3-kinase activity 10-fold. In contrast, the effect of insulin upon PI 3-kinase activity was much smaller (≈2.2-fold) in cells expressing the Δ43 truncated receptor.

DISCUSSION

The p85 regulatory subunit of PI 3-kinase contains two SH2 domains that bind to phosphorylated YM*X*M and Y*X*X*M* motifs in several growth factor receptors, non-receptor tyrosine kinases, and other docking proteins such as IRS-1 (9, 19, 28). Indeed, it has been clearly demonstrated that PI 3-kinase binds to IRS-1 through one or more of its nine Y*X*X*M*/YM*X*M motifs (7, 28). Nevertheless, the presence of a phosphorylated YHTM motif in the COOH-terminal domain of the insulin receptor raises the possibility that this may also provide a binding site for SH2 domains of the p85 subunit of PI 3-kinase. In this study, we confirm the existence of signaling complexes containing insulin receptors, IRS-1, and p85 subunit of PI 3-kinase in intact cells (23, 26, 27). By using a coimmunoprecipitation technique, we characterize the nature of the binding interactions that stabilize these signaling complexes in intact cells. We demonstrate that deletion of the COOH-terminal YHTM sequence markedly decreased the coimmunoprecipitation of p85 by antibodies directed against the insulin receptor as well as the coimmunoprecipitation of the insulin receptor by antibodies directed against p85. These data strongly suggest that the COOH-terminal YHTM sequence is required for optimal binding of p85 to the insulin receptor in intact cells. Furthermore, deletion of the COOH-terminal 43 amino acids partially impairs the ability of the insulin receptor to mediate insulin action to activate PI 3-kinase. This suggests that binding of p85 to the COOH-terminal YHTM sequence has physiological significance.

Possible Binding Interactions to Form a Ternary Complex—It is well established that p85 can bind directly to phosphorylated IRS-1 (6, 24). Furthermore, deletion of the COOH-terminal 43 amino acids (including the YHTM sequence) of the insulin receptor does not inhibit the ability to coimmunoprecipitate IRS-1 with insulin receptor antibody (13) (also Fig. 3 of this report). Thus, it seems likely that IRS-1 can bind directly to both the insulin receptor and p85. Nevertheless, when the COOH-terminal YHTM sequence of the insulin receptor was deleted, this markedly inhibited the formation of complexes containing p85 plus the insulin receptor. Thus, there appears not to be a large quantity of ternary complex in which p85 and the insulin receptor bind simultaneously to IRS-1. Of course, it is possible that there may be a small quantity of ternary complex formed by binding of p85 to the IRS-1 component of a binary complex between the Δ43-truncated insulin receptor plus phosphorylated IRS-1. However, because only a relatively small percentage of total cellular IRS-1 is coimmunoprecipitated by anti-receptor antibody, most of the p85 bound directly to IRS-1 will be bound to IRS-1 molecules that are not coimmunoprecipitated by anti-receptor antibodies.

It is possible to propose an alternative structure for a ternary complex in which p85 serves as the nucleus on which to build the ternary complex. In this hypothetical structure, one SH2 domain of p85 binds to a Y*X*X*M* motif in IRS-1 while the other SH2 domain binds to the COOH-terminal YHTM sequence in the insulin receptor. This hypothesis leads to the prediction that deletion of the COOH terminus of the insulin receptor would prevent the formation of the ternary complex by abolishing the direct binding interaction between p85 and the insulin receptor. This model closely resembles the proposed model for binding of p85 to the PDGF receptor in which two phosphorylated Y*X*X*M* motifs are required for optimal binding of p85 (9), one phosphotyrosine-binding site for each of the two SH2 domains in p85.

Relative Roles of Insulin Receptor and IRS-1 in Activating PI 3-Kinase—While *in vitro* studies have suggested that p85 may bind directly to phosphotyrosine residues near the COOH terminus of the insulin receptor (10), this has not been demonstrated previously in intact cells. In fact, unlike our present observations, previous investigations did not detect any effect

of the Δ43 truncation upon the ability of the insulin receptor to mediate insulin's action to increase PI 3-kinase activity in intact cells (11, 20, 22). Nevertheless, in our experimental system, the Δ43 truncation partially impaired the ability to activate PI 3-kinase despite the fact that the truncation did not impair phosphorylation of IRS-1. These observations are consistent with the hypothesis that the direct binding interaction between p85 and the insulin receptor contributes importantly to the ability of insulin to activate PI 3-kinase. It is not clear how to explain apparent discrepancies between our data and previously reported observations. However, variations in the levels of expression of PI 3-kinase, IRS-1, and insulin receptors may modulate the relative contributions of the two mechanisms of PI 3-kinase activation, *i.e.* the direct mechanism mediated by binding of p85 to insulin receptors *versus* the indirect mechanism mediated by binding of p85 to IRS-1. In order to fully assess the physiological significance of these binding interactions, it will be necessary to carry out similar studies in physiologically relevant target tissues such as skeletal muscle, liver, and adipose tissue. Nevertheless, the present studies demonstrate that it is possible for insulin receptors to bind directly to p85 in intact cells and suggest that this binding interaction may contribute to the mechanism whereby insulin activates phosphatidyl inositol 3-kinase in transfected cells grown in tissues culture cells. It is now possible to express the Δ43 truncated receptor in transgenic mice; this would allow for experiments to address the physiological significance of direct binding interactions between the insulin receptor and p85 *in vivo*.

Conclusions—Over the past several years, considerable progress has been made in elucidating the signaling pathways downstream from tyrosine phosphorylation. It is becoming increasingly clear that these pathways are complex and contain many branches. From the beginning, it was obvious that the branches often diverged from a common point. One tyrosine kinase may phosphorylate many proteins; for example, in addition to autophosphorylation, the insulin receptor phosphorylates IRS-1 (5, 7), shc (29, 30), ecto-ATPase (31), etc. Similarly, a single phosphoprotein contains phosphotyrosine residues that bind multiple SH2 domain containing proteins; for example, phosphorylated IRS-1 binds PI 3-kinase (32), growth factor receptor binding protein-2 (GRB-2) (29), SH2-containing phosphotyrosine phosphatase two-dimensional (33), and nck (34). More recently, it has become clear that there are also converging branches in the pathways. For example, there are two pathways from the insulin receptor that lead to activation of GRB-2/m-SOS; one pathway involves phosphorylation of IRS-1 while the other involves phosphorylation of Shc (29). Similarly, in the present study, we demonstrate that there are two pathways whereby the insulin receptor can contribute to the activation of PI 3-kinase. In addition to the previously recognized indirect pathway that requires phosphorylation of IRS-1, our data suggest that the phosphorylated YXXM motif in the COOH terminus of the insulin receptor binds directly to the p85 regulatory subunit of PI 3-kinase and that this binding contributes to the activation of PI 3-kinase. This direct interaction between a phosphorylated receptor and PI 3-kinase closely resembles the major pathway whereby most growth factor receptor tyrosine kinases directly activate PI 3-kinase. It seems likely that this type of redundancy in the pathways may provide additional opportunities for regulation of crucial enzyme activities.

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REFERENCES

1. Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A., and Rosen, O. M. (1987) *J. Biol. Chem.* **262,** 1842–1847
2. Cama, A., Quon, M. J., Sierra, M. L., and Taylor, S. I. (1992) *J. Biol. Chem.* **267,** 8383–8389
3. Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M., and Kahn, C. R. (1982) *Nature* **298,** 667–669
4. Rosen, O. M. (1987) *Science* **237,** 1452–1458
5. White, M. F., Maron, R., and Kahn, C. R. (1985) *Nature* **318,** 183–186
6. Sun, X. J., Miralpeix, M., Myers, M. G., Glasheen, E. M., Backer, J. M., Kahn, C. R., and White, M. F. (1992) *J. Biol. Chem.* **267,** 22662–22672
7. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) *Nature* **352,** 73–77
8. Shoelson, S. E., Chatterjee, S., Chaudhuri, M., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2027–2031
9. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) *Cell* **64,** 281–302
10. Van Horn, D. J., Myers, M. G., and Backer, M. B. (1994) *J. Biol. Chem.* **269,** 29–32
11. Yonezawa, k., Yokono, K., Shii, K., Ogawa, W., Ando, A., Hara, K., Baba, S., Kavuragi, Y., Yamamoto-Honda, R., Momomura, K., Kadowaki, T., and Kasuga, M. (1992) *J. Biol. Chem.* **267,** 440–446
12. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y. C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., and Ramachandran, J. (1985) *Nature* **313,** 756–761
13. Levy-Toledano, R., Caro, L. H. P., Accili, D., and Taylor, S. I. (1994) *EMBO J.* **13,** 835–842
14. Kadowaki, H., Kadowaki, T., Cama, A., Marcus Samuels, B., Rovira, A., Bevins, C. L., and Taylor, S. I. (1990) *J. Biol. Chem.* **265,** 21285–21296
15. Levy-Toledano, R., Caro, L. H. P., Hindman, N., and Taylor, S. I. (1993) *Endocrinology* **133,** 1803–1808
16. Endemann, G., Yonezawa, K., and Roth, R. A. (1990) *J. Biol. Chem.* **265,** 396–400
17. Kato, H., Faria, T. N., Stannard, B., Roberts, C. T. J., and LeRoith, D. (1993) *J. Biol. Chem.* **268,** 2655–2661
18. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell* **72,** 767–778
19. Backer, J. M., Myers, M. J., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., and White, M. F. (1992) *EMBO J,* **11,** 3469–3479
20. Myers, M. G. J., Backer, J. M., Siddle, K., and White, M. F. (1991) *J. Biol. Chem.* **266,** 10616–10623
21. McClain, D. A., Maegawa, H., Levy, J., Huecksteadt, T., Dull, T. J., Lee, J., Ullrich, A., and Olefsky, J. M. (1988) *J. Biol. Chem.* **263,** 8904–8911
22. Maegawa, H., McClain, D. A., Freidenberg, G., Olefsky, J. M., Napier, M., Lipari, T., Dull, T. J., Lee, J., and Ullrich, A. (1988) *J. Biol. Chem.* **263,** 8912–8917
23. Backer, J. M., Myers, M. J., Sun, X. J., Chin, D. J., Shoelson, S. E., Miralpeix, M., and White, M. F. (1993) *J. Biol. Chem.* **268,** 8204–8212
24. Hadari, Y. R., Tzahar, E., Nadive, O., Rothemberg, P., Roberts, C. T., LeRoith, D., Yarden, Y., and Zick, Y. (1992) *J. Biol. Chem.* **267,** 17483–17486
25. Kelly, K. L., and Ruderman, N. B. (1993) *J. Biol. Chem.* **268,** 4391–4398
26. Zhang, W., Johnson, J. D., and Rutter, W. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11317–11321
27. Ruderman, N. B., Kapeller, R., White, M. F., and Cantley, L. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1411–1415
28. Shoelson, S. E., Chatterjee, S., Chaudhuri, M., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2027–2031
29. Skolnik, E. Y., Lee, C. H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. J., Jr., Backer, J. M., Ullrich, A., White, M. F., and Schlessinger, J. (1993) *EMBO J.* **12,** 1929–1936
30. Pronk, G. J., McGlade, J., Pelicci, G., Pawson, T., and Bos, J. L. (1993) *J. Biol. Chem.* **268,** 5748–5753
31. Perrotti, N., Accili, D., Marcus, S. B., Rees Jones, R. W., and Taylor, S. I. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3137–3140
32. Myers, M. J., Backer, J. M., Sun, X. J., Shoelson, S., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10350–10354
33. Kuhne, M. R., Pawson, T., Lienhard, G. E., and Feng, G.-S. (1993) *J. Biol. Chem.* **268,** 11479–11481
34. Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, M. G. J., White, M. F., Schlessinger, J., and Skolnik, E. Y. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11713–11717