Modulation of Insulin Receptor Substrate-1 Tyrosine Phosphorylation by an Akt/Phosphatidylinositol 3-Kinase Pathway*

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Serine/threonine phosphorylation of insulin receptor substrate 1 (IRS-1) has been implicated as a negative regulator of insulin signaling. Prior studies have indicated that this negative regulation by protein kinase C involves the mitogen-activated protein kinase and phosphorylation of serine 612 in IRS-1. In the present studies, the negative regulation by platelet-derived growth factor (PDGF) was compared with that induced by endothelin-1, an activator of protein kinase C. In contrast to endothelin-1, the inhibitory effects of PDGF did not require mitogen-activated protein kinase or the phosphorylation of serine 612. Instead, three other serines in the phosphorylation domain of IRS-1 (serines 632, 662, and 731) were required for the negative regulation by PDGF. In addition, the PDGF-activated serine/threonine kinase called Akt was found to inhibit insulin signaling. Moreover, this inhibition required the same IRS-1 serine residues as the inhibition by PDGF. Finally, the negative regulatory effects of PDGF and Akt were inhibited by rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), one of the downstream targets of Akt. These studies implicate the phosphatidylinositol 3-kinase/Akt kinase cascade as an additional negative regulatory pathway for the insulin signaling cascade.

After insulin binds to its receptor, it activates an intrinsic tyrosine kinase activity that mediates the tyrosine phosphorylation of a variety of endogenous substrates including insulin receptor substrates 1–4 (1). Binding of these tyrosine-phosphorylated substrates to the Src homology (SH)2 domain-2 of the regulatory subunit of the heterodimeric p85/p110 phosphatidylinositol (PI) 3-kinase leads to a 3–5-fold stimulation in its enzymatic activity and an increase in the PI 3,4-bisphosphate and 3,4,5-trisphosphate in the cell (2, 3). A variety of data from several approaches has demonstrated a role for the production of these lipid products in mediating many, if not all, of the subsequent actions of insulin including for example the stimulation of glucose uptake, activation of both the glycogen synthase and the 70-kDa S6 kinase (4). The effects of PI 3,4,5-trisphosphate may in part be mediated through the activation of the Ser/Thr kinases called Akt or one of the atypical protein kinase Cs (4, 5).

Because of the critical role of the activation of this lipid kinase in inducing subsequent biological responses, increasing interest has focused on the regulation of this process. Indeed, several mechanisms for regulating this process have been identified. First, long term treatment of cells with insulin or the glucocorticoid dexamethasone has been shown to induce the degradation of IRS-1 (6, 7). Second, increased serine phosphorylation of IRS-1 has been observed after treatment of cells with either activators of protein kinase C, Ser/Thr phosphatase inhibitors like okadaic acid, platelet-derived growth factor (PDGF), insulin, angiotensin II, or activation of cellular stress pathways by tumor necrosis factor and other cytokines (8–15). This increased serine phosphorylation of IRS-1 has been shown to inhibit the subsequent ability of this substrate to be tyrosine-phosphorylated by the insulin receptor and to bind and activate the PI 3-kinase (8–15). In addition, serine phosphorylation of IRS-1 has been shown to interfere with the ability of the IRS-1 to interact with the insulin receptor (16).

In the case of cells treated with an activator of protein kinase C, an increase in the serine phosphorylation of a particular serine, serine 612, in the IRS-1 molecule was found to play a prominent role in this inhibition (12). This residue appeared to be phosphorylated by one of the MAP kinases (also called extracellular signal-regulated kinases), and an increase in MAP kinase activity was shown to inhibit subsequent signaling by the insulin receptor kinase (17). However, as noted above, a number of other stimuli also appear to negatively regulate the ability of the insulin receptor to tyrosine-phosphorylate IRS-1 by serine phosphorylation. In particular, PDGF has recently been shown to stimulate the Ser/Thr phosphorylation of IRS-1 and to inhibit the ability of insulin to stimulate the subsequent tyrosine phosphorylation of IRS-1 and its association with PI 3-kinase (14, 15). Since PDGF can activate multiple signaling pathways including the MAP kinase cascade (18), the present studies were therefore designed to examine whether PDGF was affecting IRS-1 via the same pathway as was observed after PKC activation. In particular, we have tested whether the negative regulation of insulin signaling by PDGF was also mediated via MAP kinase and the phosphorylation of Ser-612 in the IRS-1 molecule. We have found that treatment of cells with PDGF inhibits the insulin signaling pathway via a distinct mechanism, possibly by activation of a serine kinase modulated by the Akt pathway, which appears to require the serine phosphorylation of distinct residues on the IRS-1 molecule. In contrast, we find that endothelin-1, an activator of PKC (19), inhibits the insulin signaling pathway via the MAP kinase pathway and the serine phosphorylation of Ser-612 in IRS-1. These results indicate that there are MAP kinase-dependent and -independent pathways that regulate the insulin-stimulated association of IRS-1 with PI 3-kinase.
IRS-1 Modulation by Akt

EXPERIMENTAL PROCEDURES

Plasmid Construction—A Myc-tagged wild type IRS-1 was obtained by PCR using pCLDN/IRS-1 as the template and the same primers described previously (12). The SUFI-digested PCR product was subcloned into the retroviral vector pWZL-neo (20). The single mutants of IRS-1 were generated by using either the Transformation-based mutagenesis kit from CLONTECH or a PCR-based strategy. In order to make the triple mutant of IRS-1 (S632A/S662A/S731A), two initial PCR reactions were performed using puc18/IRS-1/S632A and puc18/IRS-1/S731A as templates. One set of primers consisted of a 5'-internal S662A mutagenesis primer; the other set consisted of a 5'-internal S662A mutagenesis primer; the other set consisted of a 3'-flanking primer and a 3'-flanking primer. Both PCR products were combined in a final PCR reaction with only the flanking 5' and 3' primers. This amplified fragment was then subcloned into the pWZL-neo vector. The quadruple IRS-1 mutant (S612A/S632A/S662A/S731A) was made by introducing S612A single mutation into the IRS-1 triple mutant. The sequence of the constructs were confirmed by restriction mapping and DNA sequencing.

Generation of Stable Cell Lines Expressing the Wild Type and Mutant IRS-1—3T3-L1 preadipocytes were infected with the pWZL-expressing IRS-1 constructs as described previously (17). In brief, 70% confluent Phoenix packaging cells were transiently transfected using the calcium phosphate precipitation method as described (17). After the final media change, the cells were incubated for 3 days at 30 °C, 5% CO2, to generate the cells. The cells were then stimulated with 100 nM insulin for 5 min as indicated. Activated MAP kinase was analyzed by immunoblotting cell lysates with an anti-phospho-MAP kinase antibody. B, modulation of PI 3-kinase activation by PDGF. The cell lysates from experiments like those shown in panel A were adsorbed with antibodies to the Myc tag, and the precipitated Myc-tagged IRS-1 was assayed for the associated PI 3-kinase activity. Where indicated, rapamycin was added to a final concentration of 200 nM. Results shown are means ± S.E. from three experiments normalized to the amount of activity present in the precipitates from cells treated with only insulin (i.e. maximum activation). C, modulation of PI 3-kinase activation by endothelin-1. The cell lysates from experiments like those shown in panel A except using 1 μM endothelin-1 in place of PDGF were processed as described in the legend to panel B. D, effect of wortmannin on the PDGF and endothelin inhibition of the insulin-stimulated association of IRS-1 and PI 3-kinase. 3T3-L1 cells were pretreated with 1 μM wortmannin for 30 min as indicated, then 50 ng/ml PDGF or 1 μM endothelin were added and after an additional 20 min, 100 nM insulin was added. After 5 min, the cells were lysed, the IRS-1 was immunoprecipitated and the precipitates were immunoblotted with an antibody to the p85 subunit of PI 3-kinase (upper panel). The same blot was stripped and reprobed with an antibody to IRS-1 (lower panel). Results shown are representative of three experiments.

Cell Culture and Treatments—3T3-L1 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% calf serum, at 37 °C, 5% CO2. The cells were serum-starved overnight before performing each experiment. PDGF (the B/B isoform, from Boehringer Mannheim), endothelin-1 (Sigma), and PD98059 and rapamycin (Life Technologies, Inc.) were added as described in the figure legends.

Akt and PI 3-Kinase—The Akt kinase assay of the expressed HA-tagged MER-Akt was performed after immunoprecipitation with anti-HA antibodies and using the GSK-3 peptide (sequence GRPRTSS-FAEG) as substrate as described (21). The phosphorylated peptide was separated on a 40% urea gel, and the identified peptide band was cut out and counted. IRS-associated PI 3-kinase was assayed after immunoprecipitation of the cell lysates with control antibodies, anti-Myc antibodies (BabCo), anti-IRS-1 antibodies (1D6), or anti-IRS-2 antibodies. The precipitates were assayed for PI 3-kinase activity as described (12) or visualized by immunoblotting with an antibody to the p85 subunit of the PI 3-kinase (Transduction Laboratories).

Assessing IRS-1 Expression and MAP Kinase Activation—Confluent cells were lysed in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM NaPPi, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1% Nonidet P-40). The lysates were incubated with either anti-Myc antibodies or normal mouse IgG prebound to protein G-agarose (Pharmacia), washed twice each with lysis buffer and a low salt buffer (20 mM Tris, pH 7.4, 100 mM NaCl). The bound proteins were eluted by boiling in sample buffer, analyzed on a 10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The blots were developed with either anti-IRS-1 antibodies (Upstate Biotechnology Inc.) or anti-phosphotyrosine antibodies (Transduction Laboratories). Measurements of the formation of phospho-MAP kinase were performed by immunoblotting total cell lysates with antibodies specific to the activated phospho-MAP kinase (Promega).
RESULTS

PDGF and Endothelin-1 Modulate Insulin-stimulated IRS-1 Association with PI 3-Kinase via Distinct Mechanisms—As recently reported (14, 15), PDGF treatment of 3T3-L1 cells rapidly inhibited the subsequent ability of insulin to stimulate the association of an expressed Myc-tagged IRS-1 with PI 3-kinase by about 80% (Fig. 1B). To test whether this inhibition was mediated via MAP kinase, we utilized the MEK inhibitor PD98059 (22). This compound had no significant effect on the inhibitory response induced by PDGF (Fig. 1B). A control Western blot verified that in these cells PDGF was stimulating the formation of the activated, phosphorylated MAP kinase and this stimulation was completely inhibited by the MEK-inhibitor, PD98059 (Fig. 1A).

Since prior studies have indicated that endothelin-1, an activator of the PKC and the MAP kinase pathways (19), could also induce insulin resistance in rat adipocytes (23), we tested whether this hormone would inhibit the insulin-stimulated increase in IRS-1-associated PI 3-kinase. Endothelin-1 was found to inhibit by 50% the insulin-stimulated association of IRS-1 with PI 3-kinase. In agreement with the above studies, both PDGF and endothelin-1 (Fig. 1D) were found to stimulate their tyrosine phosphorylation (Fig. 2 and data not shown). The levels of expressed IRS-1 were comparable to those of the endogenous IRS-1, as demonstrated by the approximate 2-fold increase in immunoreactive IRS-1 observed in the blots of total lysates of infected cells in comparison to the control cells (Fig. 2). Of most interest was the finding that, although PDGF inhibited the insulin-stimulated tyrosine phosphorylation of the wild type expressed IRS-1, it did not inhibit the insulin-stimulated tyrosine phosphorylation of the quadruple IRS-1 mutant (S612A/S631A/S662A/S731A) (Fig. 2B). In addition, PDGF did not inhibit the subsequent insulin-stimulated association of the quadruple mutant of IRS-1 with PI 3-kinase (Fig. 3A). To verify that these transfected cells were still responsive to PDGF, the supernatants from the anti-IRS-1 precipitates were adsorbed with antibodies to IRS-2. The insulin-stimulated association of IRS-2 with the PI 3-kinase was inhibited by PDGF in the cells expressing both the wild type IRS-1 as well as the quadruple IRS-1 mutant (Fig. 3B), indicating that the cells expressing the mutant IRS-1 had not lost their PDGF responsiveness.

To further test this hypothesis, we compared the ability of the PI 3-kinase inhibitor, wortmannin, to block the PDGF and endothelin-1 effects. To accomplish this, we measured the ability of wortmannin to affect the ability of PDGF and endothelin to inhibit the insulin-induced association of IRS-1 with the PI 3-kinase. In agreement with the above studies, both PDGF and endothelin inhibited the insulin-stimulated association of IRS-1 with PI 3-kinase, with PDGF being more potent then endothelin-1 (Fig. 1D). Wortmannin blocked the effect of PDGF but had no significant effect on the inhibition observed with endothelin-1 (Fig. 1D). Controls verified that the amount of IRS-1 precipitated in each case was the same. These results support the hypothesis that the ability of PDGF to interfere with the insulin pathway utilizes the PI 3-kinase cascade whereas the endothelin-1 effect is via a distinct pathway.

Expression of Mutant IRS-1 Molecules in 3T3-L1 Cells and Their Regulation by PDGF and Endothelin-1—3T3-L1 cells were infected with retroviral vectors encoding Myc-tagged wild type IRS-1 molecules or various mutant IRS-1 molecules in which particular serine residues have been changed to alanine. In particular, mutant IRS-1 molecules were expressed in which either serine 612 (S612A), serines 632, 662, and 731 (S632A/S662A/S731A), or serines 612, 632, 662, and 731 (S612A/S631A/S662A/S731A) were changed to alanine. In each case, expression of the mutant molecules was confirmed by Western blotting with antibodies to the Myc epitope and insulin was found to stimulate their tyrosine phosphorylation (Fig. 2 and data not shown). The levels of expressed IRS-1 were comparable to those of the endogenous IRS-1, as demonstrated by the approximate 2-fold increase in immunoreactive IRS-1 observed in the blots of total lysates of infected cells in comparison to the control cells (Fig. 2). Of most interest was the finding that, although PDGF inhibited the insulin-stimulated tyrosine phosphorylation of the wild type expressed IRS-1, it did not inhibit the insulin-stimulated tyrosine phosphorylation of the quadruple IRS-1 mutant (S612A/S631A/S662A/S731A) (Fig. 2B). In addition, PDGF did not inhibit the subsequent insulin-stimulated association of the quadruple mutant of IRS-1 with PI 3-kinase (Fig. 3A). To verify that these transfected cells were still responsive to PDGF, the supernatants from the anti-IRS-1 precipitates were adsorbed with antibodies to IRS-2. The insulin-stimulated association of IRS-2 with the PI 3-kinase was inhibited by PDGF in the cells expressing both the wild type IRS-1 as well as the quadruple IRS-1 mutant (Fig. 3B), indicating that the cells expressing the mutant IRS-1 had not lost their PDGF responsiveness.

The quadruple mutation in the IRS-1 molecule could affect the activation of the PI 3-kinase independently of its association. To test this possibility, we also directly examined the insulin-stimulated association of PI 3-kinase with the wild type and quadruple mutant IRS-1 molecules. As observed for the PI 3-kinase enzymatic activity, the insulin-stimulated association of PI 3-kinase with the wild type IRS-1 was inhibited by PDGF whereas the insulin-stimulated association of PI 3-kinase with the quadruple mutant IRS-1 was not affected by PDGF (Fig. 3C).

To further characterize the role of particular serines in the ability of PDGF to regulate the insulin-stimulated association of PI 3-kinase with the mutant IRS-1 molecules, cells expressing the single, triple, or quadruple mutation were examined. PDGF treatment was found to inhibit the insulin-stimulated
association of the single mutant IRS-1 (S612A) with PI 3-kinase by about 80%, a level comparable to that observed with the wild type IRS-1 (Fig. 4). In contrast, the endothelin-1 inhibition of the insulin-stimulated association of IRS-1 with PI 3-kinase was essentially abolished with the same single mutation (data not shown). However, the mutation of three other serines (Ser-631, Ser-662, and Ser-731) dramatically reduced the inhibition with PDGF treatment (Fig. 4).

Regulation of the Insulin-stimulated Association of IRS-1 with PI 3-Kinase by Akt—Since PDGF can stimulate the PI 3-kinase/Akt pathway as well as the MAP kinase cascade (18), we tested whether Akt could also modulate the insulin-stimulated association of IRS-1 with PI 3-kinase. 3T3-L1 cells expressing a hydroxytamoxifen-regulatable form of Akt (called MER-Akt) (21) were stimulated with insulin either with or without a prior treatment of hydroxytamoxifen and lysed, and the Myc-tagged IRS-1 was immunoprecipitated and tested for its associated PI 3-kinase activity (Fig. 5). The inhibition in the MER-Akt-expressing cells was dependent upon the dose of hydroxytamoxifen used to stimulate the cells and paralleled the increase in Akt activity in these cells (Fig. 6).

To test whether the inhibition observed via Akt activation was mediated via the mTOR pathway, we utilized the mTOR inhibitor rapamycin. As was observed for PDGF inhibition, rapamycin significantly blocked the inhibitory response observed with Akt activation (Fig. 7A).

Since the PDGF inhibition of the insulin-stimulated association of PI 3-kinase and IRS-1 was dependent upon the presence of specific serine residues in the phosphorylation domain (Fig. 4), we tested whether a similar requirement was also present for the inhibition via Akt. To this end, the quadruple IRS-1 mutant (S612A/S632A/S662A/S731A) as well as the epitope-tagged wild type IRS-1 were expressed in the 3T3-L1 cells containing the MER-Akt. These cells were stimulated with insulin either with or without a prior activation of the MER-Akt and lysed, and the amount of IRS-1-associated PI 3-kinase was measured. The activation of the MER-Akt was
found to inhibit the insulin-stimulated association of PI 3-kinase with the wild type expressed IRS-1 but not with the expressed quadruple IRS-1 mutant (Fig. 7B).

DISCUSSION

Prior studies have demonstrated that increased Ser/Thr phosphorylation of IRS-1 can be stimulated by a number of factors including activators of protein kinase C, Ser/Thr phosphatase inhibitors like okadaic acid, PDGF, insulin, or activation of cellular stress pathways by tumor necrosis factor and other cytokines (8–17). This serine phosphorylation may be a contributing factor in the development of insulin resistance since an increase in serine phosphorylation interferes with the tyrosine phosphorylation of IRS-1 by the insulin receptor and its subsequent association with PI 3-kinase. Thus, it is important to identify the kinase cascades responsible for the serine phosphorylation of IRS-1.

Prior studies have identified several kinases that can phosphorylate IRS-1. These include casein kinase II (25), glycogen synthase kinase 3 (26), MAP kinase (17), and even a lipid kinase, the phosphatidylinositol 3-kinase (27, 28). The case of MAP kinase, a particular serine (serine 612) has been identified in the IRS-1 molecule as being responsible for causing the subsequent inhibition of insulin-stimulated association with PI 3-kinase (17). In this prior work, the MAP kinase was activated by phorbol esters, a potent but non-physiological activator of protein kinase C. In the present work, endothelin-1, a normal regulator of protein kinase C (19), was also found to inhibit the ability of insulin to stimulate the IRS-1 association with PI 3-kinase. This inhibition also appeared to be mediated via the activation of MAP kinase since the MEK inhibitor had essentially no effect on the PDGF-induced negative regulation of the insulin signaling cascade. Although PDGF did induce the activation of MAP kinase in the 3T3-L1 cells, this pathway did not appear to be required for the negative regulation of the insulin signaling cascade. In contrast to the results with endothelin-1, the MEK inhibitor had essentially no effect on the PDGF-induced negative regulation of the insulin signaling cascade. In the present studies, we have also examined the pathway that is involved in the PDGF-induced negative regulation of the insulin signaling cascade. Although PDGF did induce the activation of MAP kinase in the 3T3-L1 cells, this pathway did not appear to be required for the negative regulation of the insulin signaling cascade. In contrast to the results with endothelin-1, the MEK inhibitor had essentially no effect on the PDGF-induced negative regulation of the insulin signaling cascade.
induced inhibition in IRS-1-associated PI 3-kinase, although it completely inhibited the ability of PDGF to stimulate MAP kinase in these cells. This finding of a lack of inhibition by the MEK inhibitor of the PDGF response was also recently reported by Staub et al. (15).

These results indicate that PDGF is most likely regulating the insulin-induced increase in IRS-1-associated PI 3-kinase by a pathway independent of the MAP kinase cascade. Further evidence to support this hypothesis is provided by the finding that the IRS-1 mutant lacking Ser-612 is still negatively regulated by PDGF. However, a mutant IRS-1 lacking three other serines in the same domain (Ser-632, -662, and -731) was found to lose its ability to be negatively regulated by PDGF treatment of cells. These results are consistent with the hypothesis that the negative regulation occurs via the serine phosphorylation of IRS-1; however, the sites required for this effect appear to differ from that required for the negative regulation by MAP kinase.

In addition to activating the MAP kinase cascade, PDGF also stimulates the PI 3-kinase/Akt pathway. PI 3-kinase activity of this enzyme has been shown to be regulated by Akt, insulin, and PDGF (33, 35). In addition, the sites phosphorylated by this enzyme in one of its substrates, PHAS-I (for properties of heat and acid stability-1), are similar to the regulatory sites identified in IRS-1 in that both have a (Ser/Thr)-Pro motif (36). Finally, the finding that rapamycin at least partly inhibits the negative regulation by both PDGF and Akt is consistent with this hypothesis.

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