Phosphatidylinositol 3-Kinase Is Required for Insulin-stimulated Tyrosine Phosphorylation of Shc in 3T3-L1 Adipocytes*

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The interactions between the phosphatidylinositol 3-kinase (PI 3-kinase) and Ras/MAPK kinase pathways have been the subject of considerable interest. In the current studies, we find that epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) lead to rapid phosphorylation of Shc (maximum at 1–2 min), whereas insulin-mediated Shc phosphorylation is relatively delayed (maximum at 5–10 min), suggesting that an intermediary step may be necessary for insulin stimulation of Shc phosphorylation. The Src homology-2 (SH2) domain of Shc is necessary for PDGF- and EGF-mediated Shc phosphorylation, whereas the phosphotyrosine binding (PTB) domain is critical for the actions of insulin. Because the Shc PTB domain can interact with phospholipids, we postulated that PI 3-kinase might be a necessary intermediary step facilitating insulin-stimulated phosphorylation of Shc. In support of this, we found that the PI 3-kinase inhibitors, wortmannin and LY294002, blocked insulin-stimulated but not EGF- or PDGF-stimulated Shc phosphorylation. Furthermore, overexpression of a dominant negative PI 3-kinase construct (p85N-SH2) blocked insulin, but not EGF- or PDGF-induced Shc phosphorylation. All three growth factors cause localization of Shc to the plasma membrane, but only the effect of insulin was inhibited by wortmannin, supporting the view that PI 3-kinase-generated phospholipids mediate insulin-stimulated Shc phosphorylation. Consistent with this, expression of a constitutively active PI 3-kinase (p110EAA) increased membrane localization of Shc, and this was completely blocked by wortmannin. A mutant Shc with a disrupted PTB domain (Shc S154D) did not localize to the membrane in p110EAA-expressing cells or after insulin stimulation and was not phosphorylated by insulin. In summary, 1) PI 3-kinase is a necessary early step in insulin-stimulated Shc phosphorylation, whereas the effects of EGF and PDGF on Shc phosphorylation are independent of PI 3-kinase. 2) PI 3-kinase-stimulated generation of membrane phospholipids can localize Shc to the plasma membrane through the Shc PTB domain facilitating phosphorylation by the insulin receptor.

Growth factor signaling initiates a variety of biologic responses, many of which are mediated through the PI 3-kinase and the Ras/MAP kinase pathway. PI 3-kinase is a dual protein and lipid kinase composed of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110). PI 3-kinase phosphorylates phosphoinositides at the 3' position of the inositol ring to generate phosphorylated lipid products and can also phosphorylate proteins on serine/threonine residues (1, 2). PI 3-kinase plays a central role in a diverse range of cellular responses, including cell growth, differentiation, protein synthesis, glucose uptake, lipogenesis, and membrane trafficking (3).

The Ras/MAP kinase pathway is another key component in the transduction of mitogenic signals. Activation of the insulin receptor or other growth factor receptors results in the tyrosine phosphorylation of Shc, which then interacts with the adapter protein Grb2, which is pre-associated with SOS, a guanine nucleotide exchange factor (4, 5). SOS stimulates formation of active GTP-bound Ras, which then initiates a sequence of phosphorylation events, activating a cascade of protein serine/threonine kinases. Ras activates Raf-1 kinase leading to phosphorylation and activation of mitogen-activated/extracellular signal-regulated kinase, which in turn phosphorylates and activates MAP kinase (6). Thus, in this pathway, Ras functions as a molecular switch converting tyrosine kinase signals into a serine/threonine kinase cascade (7).

Several investigators have demonstrated an interaction between the PI3-kinase and Ras/MAPK kinase pathways; however, the results have been somewhat conflicting. PI 3-kinase has been shown to stimulate Ras by some groups (8, 9) but to be a target of Ras by others (10, 11). Furthermore, inhibition of PI 3-kinase with wortmannin or dominant negative PI 3-kinase can block MAP kinase activation in some, but not, all cells (12–16).

To date, most of the attention has been focused on potential direct interactions between PI 3-kinase and Ras, but in this study, we have concentrated on an upstream activator of Ras, and we have explored potential interactions between PI 3-kinase signaling and Shc activation. These studies have shown that PI 3-kinase activity is necessary for insulin-stimulated tyrosine phosphorylation of Shc, whereas other growth factors, such as PDGF and EGF, can efficiently signal to Shc in the absence of the PI 3-kinase requirement. As such, these experiments demonstrate a novel mechanism of cross-talk between...
the PI 3-kinase and Ras/MAP kinase signaling pathways and demonstrate the specificity of this mechanism for the insulin action cascade.

EXPERIMENTAL PROCEDURES

Materials—Porcine insulin was kindly provided by Lilly. Phospho-specific Akt and anti-Akt antibodies were from New England Biolabs (Beverly, MA). Polyclonal anti-Shc, anti-Grb2, anti-PP2A, anti-PDGFR receptor, anti-EGRF receptor, and anti-phosphotyrosine (4G10) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-FLAG, anti-insulin receptor antibodies, the horseradish peroxidase-linked anti-rabbit, -mouse, and -goat antibodies, and protein A/G-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Shc antibody was from Transduction Laboratories (Lexington, KY). Wortmannin and LY294002 were from Calbiochem. Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum were obtained from Invitrogen. XAR-5 film was obtained from Eastman Kodak Co. All other reagents and chemicals were purchased from Sigma.

Cell Culture—3T3-L1 cells were cultured and differentiated as described previously (9). Prior to the experiment, the adipocytes were trypsinized and reseeded in the appropriate culture dishes. Rat 1 fibroblasts overexpressing human insulin receptors (HIRCB) were maintained as described previously (17). NIH/3T3 fibroblasts were grown in DMEM with 10% calf serum. The Ad-EIA-transformed human embryonic kidney cell line 293 cells were cultured as described previously (9).

Preparation of Recombinant Adenovirus and Cell Treatment—The adenovirus encoding the p110-CAAX and the N-SH2 domain of the p85α subunit of PI 3-kinase (p55N-SH2) were prepared as described previously (9, 16). 3T3-L1 adipocytes were infected with adenoviruses at the indicated multiplicity of infection (m.o.i.) for 6 h. Transduced cells were incubated for 60 h at 37 °C under 10% CO2 in DMEM high glucose medium with 2% heat-inactivated serum, followed by starvation for 18 h.

Preparation of Whole Cell Lysates and Immunoprecipitation—Starved cells were stimulated with ligands at 37 °C and lysed in solubilizing buffer. Whole cell lysates and antibody immunoprecipitates were resolved by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P; Bedford, MA). Membranes were blocked and probed with specified antibodies. Blots were subsequently incubated with horseradish peroxidase-linked secondary antibody followed by chemiluminescence detection, according to the manufacturer’s instructions (Pierce).

Subcellular Fractionation—Starved cells were stimulated with 100 ng/ml insulin, 50 ng/ml PDGF, or 10 ng/ml EGF for 5 min. Cells were scraped into ice-cold HES buffer (225 mM sucrose, 20 mM HEPES, pH 7.5, 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1 mM sodium vanadate, 50 mM sodium fluoride, 50 units of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4 °C. The cell lysates were centrifuged to remove insoluble materials. For immunoprecipitation, cell lysates were incubated with primary antibody for 6 h at 4 °C and protein A/G-agarose for an additional 2 h.

Immunoblotting—Whole cell lysates and antibody immunoprecipitates were resolved by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P; Bedford, MA). Membranes were blocked and probed with specified antibodies. Blots were subsequently incubated with horseradish peroxidase-linked second antibody followed by chemiluminescence detection, according to the manufacturer’s instructions (Pierce).

Transfection Study—The FLAG-tagged Shc expression vector, pRK5 Shc was a generous gift from Dr. Edward Y. Skolnik (Skirball Institute, New York). A mutant Shc cDNA (serine 154 to proline, S154P) was generated by PCR with a mutagenic oligonucleotide and subcloned into pRK5 as described previously (19). Transient transfection into HIRCB cells and NIH/3T3 cells was performed with SuperFECT (Qiagen, Valencia, CA) in accordance with the manufacturer’s instructions. After transfection, cells were allowed to grow for 24 h followed by serum starvation for additional 24 h, before conducting the experiment as described previously (16, 19).

RESULTS

Time Course of Insulin-, EGF-, and PDGF-stimulated Shc Tyrosine Phosphorylation—Growth factor stimulation leads to tyrosine phosphorylation of Shc, with downstream activation of the Ras/MAP kinase pathway (6, 20–22). We conducted time course experiments of Shc phosphorylation after stimulation by insulin, EGF, or PDGF. As shown in Fig. 1, although all three ligands cause phosphorylation of Shc, the time courses are decidedly different. Thus, the effects of EGF and PDGF are maximal by 1–2 min and begin to decline thereafter, while phosphorylation of Shc after insulin treatment is slower, reaching a maximal effect by 10 min. These results raise the possibility that an intermediate step exists between the insulin receptor and Shc phosphorylation, whereas the effects of the EGF- and PDGF-receptors on Shc phosphorylation are more direct. To assess this possibility, we examined the effects of PI 3-kinase inhibition on insulin-, EGF-, and PDGF-stimulated Shc phosphorylation.

Effect of PI 3-Kinase Inhibition on Shc Tyrosine Phosphorylation and Its Association with Grb2 in Response to Insulin, EGF, and PDGF—As shown in Fig. 2, the PI 3-kinase inhibitors wortmannin and LY294002 inhibit insulin-stimulated Shc phosphorylation as well as insulin-stimulated association of Shc with Grb2, whereas the effects of EGF- and PDGF are unchanged. Clearly, these results are consistent with a role for PI 3-kinase in insulin stimulation of Shc.

To explore further the role of PI 3-kinase activation in growth factor-stimulated Shc phosphorylation, we utilized an adenoviral vector containing the p55N-SH2 domain (16). When expressed in cells, the p55N-SH2 domain behaves as a dominant negative inhibitor of PI 3-kinase activity. Cells expressing this dominant negative PI 3-kinase construct were then stimulated with insulin, EGF, or PDGF followed by measurements of Shc phosphorylation and its association with Grb2. As shown in Fig. 3, expression of p55N-SH2 inhibits insulin-stimulated Shc phosphorylation, as well as Shc-Grb2 association, but did not affect EGF- or PDGF-stimulated Shc phosphorylation.
not influence the effects of EGF or PDGF. The level of expression of p85N-SH2 was the same in all conditions (data not shown), and as a control, the effectiveness of this dominant negative construct is demonstrated in Fig. 3B, which shows decreased insulin-stimulated Akt phosphorylation in p85N-SH2 domain-expressing cells.

We have shown recently that Shc proteins associate with protein phosphatase 2A (PP2A) in the basal state and that after growth factor-mediated Shc phosphorylation, Shc dissociates from PP2A (23). Fig. 4 demonstrates this effect showing that Shc is associated with PP2A in untreated cells and that insulin and EGF lead to Shc phosphorylation and dissociation of Shc from PP2A. In the presence of wortmannin, the effect of insulin is inhibited, whereas the actions of EGF on Shc phosphorylation are unimpaired. Because the dissociation of Shc from PP2A is dependent on Shc tyrosine phosphorylation (23), these experiments further support the importance of PI 3-kinase in the process of insulin-stimulated Shc phosphorylation.

p110CAAX Inhibits Insulin but Not PDGF- or EGF-stimulated Tyrosine Phosphorylation of Shc and Its Association with Grb2—Although insulin, EGF, and PDGF receptors all phosphorylate Shc, the mechanisms differ. The Shc PTB domain is responsible for binding to phosphorylated insulin and insulin-like growth factor-1 receptors (24–26); the Shc SH2 domain mediates binding to the PDGF receptor (27), and the EGF receptor requires both (25, 28). In addition, the Shc PTB domain can associate with phospholipids phosphorylated in the 3' position (i.e. phosphatidylinositol 3,4-biphosphate (PIP_3) and phosphatidylinositol 3,4,5-triphosphate (PIP_5)) (29, 30).

These findings raise the possibility that PI 3-kinase stimulation of membrane phospholipid content serves to localize Shc to the plasma membrane through the Shc PTB domain, facilitating phosphorylation by the insulin receptor. In contrast, because the Shc SH2 domain binds directly to the EGF and PDGF receptors, such an intermediate step is not necessary for signaling by these growth factors. To explore this further, we expressed constitutively active PI 3-kinase (p110CAAX) in cells using adenovirus gene transfer and then measured activation of Shc. Insulin-stimulated Shc phosphorylation and its association with Grb2 were inhibited by 44 and 40%, respectively, by p110CAAX expression (Fig. 5). PDGF and EGF-stimulated Shc phosphorylation and its association with Grb2 were not inhibited by p110CAAX. Shc protein expression was not altered by
p110<sup>CAAX</sup> expression (Fig. 5A, lower panel), and p110<sup>CAAX</sup> did not affect tyrosine autophosphorylation of the insulin receptor (data not shown). Taken together, p110<sup>CAAX</sup> inhibits insulin but not PDGF- or EGF-stimulated Shc phosphorylation, and this inhibition is distal to the insulin receptor.

Membrane Localization of Shc—The above results suggest that the lipid products generated by insulin or membrane-targeted p110<sup>CAAX</sup> expression may localize Shc to the membrane. To examine this idea, we determined the effect of insulin, EGF, PDGF, and p110<sup>CAAX</sup> on membrane localization of Shc. In control cells, a small amount of Shc was localized to the plasma membrane compartment; this increased markedly after insulin stimulation and was completely blocked by wortmannin (Fig. 6). Consistent with our hypothesis, basal plasma membrane localization of Shc was increased substantially in p110<sup>CAAX</sup>-expressing cells, and this was also blocked by wortmannin. These results indicate that the membrane phospholipids generated by PI 3-kinase recruit Shc to the plasma membrane. On the other hand, EGF- or PDGF-induced Shc membrane localization was not blocked by wortmannin. These findings support our hypothesis that the Shc SH2 domain binds directly to the EGF and PDGF receptors (causing membrane localization), and an intermediate step is not necessary for signaling by these growth factors.

The Shc PTB Domain Is Required for Membrane Localization and for Insulin but Not for EGF- or PDGF-stimulated Shc Phosphorylation—To assess more specifically the role of the PTB domain on plasma membrane localization of Shc, we constructed FLAG-tagged wild-type Shc (Shc WT) and a mutant Shc containing a serine to proline substitution at residue 154 in the PTB domain (Shc S154P), which prevents phosphotyrosine binding (19). These constructs were transiently expressed in uninfected and p110<sup>CAAX</sup>-infected HIRcB cells, followed by insulin stimulation. The plasma membrane and cytosolic fractions were then prepared and analyzed by Western blotting using anti-FLAG antibody. As shown in Fig. 7A, membrane localization of Shc WT was stimulated by insulin treatment and by p110<sup>CAAX</sup> expression. However, Shc S154P failed to localize to the plasma membrane in the absence or presence of insulin treatment or upon p110<sup>CAAX</sup> expression, indicating the importance of a functional Shc PTB domain for these interactions.

We next determined whether Shc S154P was tyrosine-phosphorylated in response to ligand stimulation. As expected, Shc WT was phosphorylated and associated with Grb2 after insulin, EGF, and PDGF stimulation (Fig. 7B, top two panels). On the other hand, Shc S154P was not phosphorylated after insulin stimulation but was phosphorylated and associated with Grb2 in response to EGF and PDGF (Fig. 7B, top two panels). Furthermore, Shc S154P did not associate with the insulin receptor after insulin stimulation but did
associate with the EGF and PDGF receptors (Fig. 7B, bottom panel). The receptors for insulin, EGF, and PDGF were phosphorylated normally after ligand stimulation (Fig. 7B, 4th panel), and the expression levels of both Shc WT and Shc S154P were comparable (Fig. 7B, 3rd panel). These results show that the PTB domain of Shc is required for phosphorylation, Grb2 association, and plasma membrane localization in response to stimulation by insulin but not by EGF or PDGF. Because the Shc PTB domain binds to PI 3-kinase-generated lipid products, which are abundant in p110CAAX cells, it seems reasonable to propose that in p110CAAX-expressing cells, Shc is targeted to membranes through its PTB domain, preventing association of Shc with the activated insulin receptor. However, because the Shc SH2 domain primarily mediates interactions with the PDGF and EGF receptors, the association between these receptors and Shc is not impaired in p110CAAX-expressing cells.

**DISCUSSION**

Growth factors, such as EGF, PDGF, and insulin bind to their cognate receptor tyrosine kinases (RTKs) leading to rapid tyrosine phosphorylation of Shc with subsequent activation of the Ras/MAP kinase pathway (6, 20, 21). Because Shc contains an SH2 and a PTB domain, current thinking is that following ligand-directed tyrosine phosphorylation of RTKs, these receptors directly bind to Shc leading to tyrosine phosphorylation (24-28, 31). The PI 3-kinase and Ras/MAP kinase pathways are clearly interconnected, but there is considerable debate and controversy as to sites and mechanisms of convergence (8, 10, 11). In the current studies, we provide evidence for a novel interaction pathway between PI 3-kinase and the Shc/Ras/MAP kinase cascade, with respect to insulin signaling. We find that PI 3-kinase stimulation is a necessary step mediating Shc phosphorylation by the insulin receptor. Our data indicate that PI 3-kinase stimulation leads to generation of plasma membrane lipid products that mediate localization of Shc to the plasma membrane through the Shc PTB domain. This PI3-kinase-dependent step is necessary for insulin receptor phosphorylation of Shc but not for interactions with the EGF or PDGF receptors.

RTK activation causes tyrosine phosphorylation of Shc, but the structural basis for Shc tyrosine phosphorylation is different for different growth factors (24-28). Shc can bind to phosphotyrosine residues through either the Shc PTB or SH2 domains. With respect to the insulin receptor, it is well established that the PTB domain is necessary for interaction and tyrosine phosphorylation (24, 26). For example, a mutant Shc with a disrupted PTB domain failed to bind to insulin receptors in the two-hybrid system and was not phosphorylated in vitro (24). Conversely, mutant insulin receptors with a disabled PTB domain-binding motif (NPXY domain) cannot bind to or phosphorylate Shc (26). In contrast to the insulin receptor, Shc associates with PDGF receptors via its SH2 domain (27), whereas both the PTB and the SH2 domain of Shc can bind to EGF receptors (25, 28). Membrane localization of Shc is also necessary for growth factor-stimulated phosphorylation (30), and the PTB domain of Shc binds to phospholipids in vitro (29, 30), potentially mediating membrane localization.

We found that EGF and PDGF treatment leads to very rapid phosphorylation of Shc, consistent with direct association of the Shc SH2 domain with these RTKs, which then phosphorylate Shc on tyrosine residues (27, 28). In contrast, the time course of insulin-induced Shc phosphorylation is relatively delayed compared with EGF and PDGF, raising the possibility of an intermediary step. This step can be explained by the idea that insulin leads to activation of PI 3-kinase with generation of plasma membrane phospholipids (3, 32), the Shc PTB can bind to or phosphorylate Shc (26). In contrast to the insulin receptor, X domain) cannot bind with the structural basis for Shc tyrosine phosphorylation is different for different growth factors (24-28). Shc can bind to phosphotyrosine residues through either the Shc PTB or SH2 domains. With respect to the insulin receptor, it is well established that the PTB domain is necessary for interaction and tyrosine phosphorylation (24, 26). For example, a mutant Shc with a disrupted PTB domain failed to bind to insulin receptors in the two-hybrid system and was not phosphorylated in vitro (24). Conversely, mutant insulin receptors with a disabled PTB domain-binding motif (NPXY domain) cannot bind to or phosphorylate Shc (26). In contrast to the insulin receptor, Shc associates with PDGF receptors via its SH2 domain (27), whereas both the PTB and the SH2 domain of Shc can bind to EGF receptors (25, 28). Membrane localization of Shc is also necessary for growth factor-stimulated phosphorylation (30), and the PTB domain of Shc binds to phospholipids in vitro (29, 30), potentially mediating membrane localization.

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membrane. Interestingly, insulin-stimulated Shc phosphorylation to undetectable levels, we cannot definitively rule out the possibility that there is a small component of PI-3 kinase-independent insulin signaling to Shc phosphorylation.

p110<sup>C</sup><sub>CAAX</sub> is a constitutively active, membrane-targeted form of the p110 catalytic subunit of PI-3 kinase (9), and our results show that adenoviral mediated expression of this protein results in membrane localization of Shc, consistent with the idea of the p110 catalytic subunit of PI-3 kinase (9), and our results show that this mutation ablated p110<sub>C</sub> activity.

The S154P Shc contains a disrupted PTB domain (19), and we speculated that the intact SH2 domain of S154P Shc is sufficient to localize Shc to cellular membrane fractions, including the plasma membrane, interfering with Shc-insulin receptor association, and inhibiting insulin mediated Shc phosphorylation. In this way, the excess PI-3 kinase-generated membrane phospholipids effectively compete with the insulin receptor for binding to the Shc PTB domain.

The S154P Shc contains a disrupted PTB domain (19), and this mutation ablated p110<sup>C</sup><sub>CAAX</sub> and insulin-stimulated membrane localization, consistent with the idea that the PTB domain is necessary for this process (30). We also found that insulin failed to phosphorylate this S154P Shc, whereas EGF and PDGF did. Because the Shc SH2 domain can interact with PDGF and EGF receptors (25, 27, 28), these results are consistent with the view that the intact SH2 domain of S154P Shc is sufficient to allow phosphorylation by the EGF and PDGF receptors.

In summary, these data demonstrate a novel insulin-specific mechanism whereby PI-3 kinase stimulation interacts with the Ras/MAP kinase pathway. Thus, PI-3 kinase stimulation is necessary for insulin-induced Shc phosphorylation which then facilitates downstream signaling to Ras. In contrast, the effects of PDGF and EGF on Shc phosphorylation are independent of PI-3 kinase. Thus, PI-3 kinase stimulation is necessary for insulin-induced Shc phosphorylation which then facilitates downstream signaling to Ras. In contrast, the effects of PDGF and EGF on Shc phosphorylation are independent of PI-3 kinase.

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