Requirement for Protein-tyrosine Phosphatase SHP-2 in Insulin-induced Activation of c-Jun NH$_2$-terminal Kinase*

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Mitogen-activated protein kinases, including extracellular signal-regulated kinases and c-Jun NH$_2$-terminal kinases (JNKs), are activated by insulin. Although the mechanism by which the insulin receptor activates extracellular signal-regulated kinases is relatively well defined, the pathway that leads to JNK activation is poorly understood. Overexpression of a catalytically inactive mutant (SHP-2/C) of the protein-tyrosine phosphatase SHP-2 in Rat-1 fibroblasts that also express human insulin receptors has now revealed that activation of JNKs by insulin and epidermal growth factor, but not that by anisomycin or sorbitol, requires SHP-2. A dominant negative mutant (RasN17) of Ha-Ras blocked insulin-induced JNK activation, whereas a dominant negative mutant (RacN17) of Rac1 or a specific inhibitor (LY294002) of phosphoinositide 3-kinase did not, indicative of involvement of both Ras and Rac in JNK activation. Furthermore, expression of myristoylated SOS, which functions as a potent activator of Ras, induced JNK activation even when SHP-2 was inactivated. These results suggest that SHP-2 contributes to JNK activation in response to insulin by positively regulating the Ras signaling pathway at the same level as, or upstream from, SOS.

Activation of the insulin receptor (IR)† results in tyrosine phosphorylation of its cellular substrates, including members of the IRS family of proteins and SHC. These phosphorylated proteins then bind various Src homology (SH) 2 domain-containing signaling molecules, which in turn propagate the signals that underlie the pleiotropic biological effects of insulin (1, 2). One of the best characterized insulin signaling pathways is that which links the IR to extracellular signal-regulated kinases (ERKs), which constitute one class of the mitogen-activated protein kinases (MAPks). The GRB2-SOS complex binds to tyrosine-phosphorylated SHC or IRS1 and then activates Ras by catalyzing the conversion of the inactive, GDP-bound form to the active, GTP-bound form (3). Activated Ras subsequently triggers a protein kinase cascade that includes Raf1, MEK, and ERKs (4).

The c-Jun NH$_2$-terminal kinases (JNKs), also known as stress-activated protein kinases, and p38 constitute another class of MAPks and were originally shown to be activated by various environmental stresses and inflammatory cytokines such as tumor necrosis factor-a (5–8). Subsequent studies revealed that growth factors, such as epidermal growth factor (EGF) (9, 10), nerve growth factor (11), and platelet-derived growth factor (12), as well as ligands for G protein-coupled receptors (13, 14) activate JNKs. Insulin has also been shown to activate both JNKs and p38. It activates JNKs in cultured Rat-1 fibroblasts and Chinese hamster ovary cells that overexpress human IRs (15, 16) and activates JNKs and p38 in L6 myotubes and rat adipocytes (17, 18), and administration of insulin to mice induces rapid activation of JNKs and p38 as well as of ERKs in skeletal muscle, with activation of these kinases correlating with activation of glycogen synthase (19). These observations implicate JNKs and p38 as potential effectors of the biological activities of insulin. However, the mechanism by which insulin activates these enzymes remains poorly understood, although Ras and phosphoinositide (PI) 3-kinase have been suggested as mediators of JNK activation in response to insulin (15, 16).

SHP-2 is a nontransmembrane-type protein-tyrosine phosphatase that contains two SH2 domains (20). SHP-2 binds directly to growth factor receptors, including those for platelet-derived growth factor and EGF, in response to receptor stimulation with the corresponding ligand and undergoes tyrosine phosphorylation (21, 22). Furthermore, SHP-2 binds through its SH2 domains to tyrosine-phosphorylated docking proteins such as IRS1, IRS2, and GAB1 in response to insulin (23–25). We and others have previously shown that SHP-2 plays an essential role in insulin-, EGF-, or lysophosphatidic acid-induced activation of Ras and ERKs, DNA synthesis, or cell proliferation (26–30). Disruption of the Shp2 gene in mice results in failure of normal gastrulation and subsequent death in the early stages of embryogenesis, as well as in marked impairment of fibroblast growth factor-induced activation of ERKs (31). Thus, SHP-2 may play a crucial role in intracellular signaling elicited by various hormones and growth factors, probably by contributing to activation of the Ras-ERK pathway. However, the role of SHP-2 in activation of other types of
MAPKs has remained unclear.

In the present study, we sought to determine whether, and if so how, SHP-2 contributes to the insulin signaling pathway that leads to the activation of JNKs. With the use of a dominant negative mutant of the enzyme, we show that SHP-2 positively regulates this pathway by facilitating the activation of Ras. By transfecting cells with vectors encoding constitutively active forms of Ras and SOS, we also show that this effect of SHP-2 appears to occur at the same level as, or upstream from, SOS.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Reagents—Rat-1 fibroblasts that overexpress human IRs (Rat-1-IR cells) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Rat-1-IR cells that overexpress a catalytically inactive mutant of SHP-2 (Rat-1-SHP-2C/S cells) were generated as described previously (32). Polyclonal antibodies that react specifically with activated forms of p44 and p42 MAPKs (ERK1 and ERK2), activated forms of p54 and p46 JNKs, the activated form of Akt, or c-Jun phosphorylated at Ser63, as well as polyclonal antibodies to ERKs or JNKs that detect the enzymes in a manner independent of phosphorylation state, were obtained from New England Biolabs. Monoclonal antibodies (mAbs) to c-Jun or to Ha-Ras were obtained from Transduction Laboratories. Polyclonal antibodies to Ras, to p130Cas, and to Akt as well as horseradish peroxidase-conjugated mAb PY20 to phosphotyrosine were from Santa Cruz Biotechnology. A mAb to the hemagglutinin epitope (HA) was prepared from the culture supernatant of 12Ca5 hybridoma cells. Insulin, asinosycin, sorbitol, andLY294002 were obtained from Sigma, and EGF was from Calbiochem.

Expression Plasmids and Transfection—The cDNA3 vectors encoding HA-tagged JNK1 (HA-JNK), HA-tagged ERK2 (HA-ERK), and the Myc-tagged myristoylated Cdc25 domain of SOS (myr-SOS) (9) were obtained from Transduction Laboratories. Polyclonal antibodies to Ras, to p130Cas, and to Akt as well as horseradish peroxidase-conjugated mAb PY20 to phosphotyrosine were from Santa Cruz Biotechnology. A mAb to the hemagglutinin epitope (HA) was prepared from the culture supernatant of 12Ca5 hybridoma cells. Insulin, asinosycin, sorbitol, andLY294002 were obtained from Sigma, and EGF was from Calbiochem.

RESULTS

Effects of Overexpression of a Catalytically Inactive Mutant of SHP-2 on JNK Activation Induced by Insulin, EGF, or Cellar Stress—We have previously shown that a catalytically inactive mutant of SHP-2 (SHP-2C/S) inhibits insulin-induced activation of Ras and ERKs in a dominant negative manner in Chinese hamster ovary cells overexpressing human IRs (28). To examine the effect of this mutant on insulin-induced activation of JNKs, we used Rat-1 fibroblasts that overexpress IRs (Rat-1-IR cells) because insulin was shown to induce marked activation of JNKs in these cells (15). We monitored JNK activation with the use of an in vitro kinase assay in which a GST-c-Jun (1–79) fusion protein serves as substrate, as well as by immunoblot analysis with antibodies that recognize the enzymes only when they are activated by phosphorylation at Thr183 and Tyr185. Given that both assays gave essentially identical results, we mostly present data obtained by immunoblot analysis. Insulin-induced activation of p44 and p42 isoforms of MAPK (ERK1 and ERK2) was almost completely abolished in Rat-1-SHP-2C/S cells (Fig. 1A), verifying that SHP-2C/S acts as a dominant negative mutant in Rat-1-IR cells. Consistent with the results of a previous study (15), insulin-induced marked activation of p54 and p46 isoforms of endogenous JNK in Rat-1-IR cells (Fig. 1A), with the maximal effect apparent 15 min after stimulation (data not shown). In contrast, at all time points up to 20 min, the extent of insulin-induced JNK activation in Rat-1-SHP-2C/S cells was greatly reduced compared with that apparent in Rat-1-IR cells (Fig. 1, A and B; data not shown). The total amounts of JNKs or ERks in each lane were similar, as revealed by immunoblot analysis with antibodies to both enzymes to the corresponding enzymes.

We transiently transfect Rat-1-IR or Rat-1-SHP-2C/S cells with an expression vector encoding HA-tagged JNK1 (HA-JNK). After 48 h, cells were incubated in the absence or presence of insulin, lysed, and subjected to immunoprecipitation with antibodies to HA. Immunoblot analysis of the resulting precipitates with antibodies to activated JNK revealed that insulin induced marked activation of HA-JNK in Rat-1-IR cells but that this effect was greatly reduced in Rat-1-SHP-2C/S cells (Fig. 1C). The presence of similar amounts of immunoprecipitated JNK1 in each lane was confirmed by immunoblot analysis with antibodies to HA. Activation of endogenous JNKs by EGF was also markedly attenuated in Rat-1-SHP-2C/S cells compared with that apparent in Rat-1-IR cells (Fig. 1D). In
In contrast, sorbitol-induced osmotic shock activated JNKs in Rat-1-SHP-2C/S cells to a similar extent as that observed in Rat-1-IR cells (Fig. 2). Furthermore, the extent of JNK activation by anisomycin was greater in Rat-1-SHP-2C/S cells than in Rat-1-IR cells (Fig. 2). These results suggest that functional SHP-2 is required for JNK activation in response to insulin and EGF but not for that in response to cellular stress induced by anisomycin or sorbitol.

Effects of LY294002 and Dominant Negative Mutants of Ras and Rac on Insulin-induced Activation of JNK—To clarify the mechanism by which SHP-2 contributes to insulin-induced activation of JNKs, we first investigated which signaling pathway triggered by insulin (PI 3-kinase pathway, Ras pathway, or other pathway) mediates JNK activation. Treatment of Rat-1-IR cells with LY294002, a specific inhibitor of PI 3-kinase, almost completely blocked insulin-induced activation of Akt, a downstream effector of PI 3-kinase (Fig. 3A). In contrast, this compound did not substantially affect JNK activation in response to insulin (Fig. 3A). Coexpression of a dominant negative mutant of Rac1 (RacN17) also did not affect activation of HA-JNK induced by insulin (Fig. 3B). In contrast, coexpression of a dominant negative mutant of Ha-Ras (RasN17) markedly inhibited insulin-induced activation of HA-JNK (Fig. 3B). These results suggest that neither PI 3-kinase nor Rac is required for insulin activation of JNKs.

Analysis with antibodies specific for the activated forms of either p54 and p46 JNK (α-P-JNK) or p44 (ERK1) and p42 (ERK2) MAPK (α-P-MAPK). The same blots were also probed with antibodies to JNK (α-JNK) or to ERK (α-MAPK) that react with these enzymes in a manner independent of activation state, to confirm that equal amounts of each enzyme were present in each lane. B, cell lysates prepared as in A were incubated with 2 μg of GST-c-Jun(1-79) fusion protein bound to glutathione-Sepharose beads for 3 h at 4 °C, and the resultant precipitates were subjected to in vitro assay of kinase activity. JNK activity was detected by immunoblot analysis with antibodies that specifically recognize c-Jun phosphorylated at Ser63 (α-P-c-JUN) (upper panel) and was quantified by scanning densitometry with the NIH image program (lower panel). Data are expressed as fold activation by insulin. C, Rat-1-IR or Rat-1-SHP-2C/S cells were transiently transfected (or not) with 1 μg of pcDNA3 vector encoding HA-tagged JNK1 (HA-JNK) and subsequently incubated for 15 min in the presence of 100 nM insulin. Cell lysates were then subjected to immunoprecipitation (IP) with mAb 12CA5 to HA (αHA), and the resulting precipitates were subjected to immunoblot analysis with α-P-JNK. The same blot was also probed with αHA to confirm the presence of equal amounts of HA-JNK in each lane. D, Rat-1-IR or Rat-1-SHP-2C/S cells were incubated for 15 min in the absence or presence of EGF (100 ng/ml), after which cell lysates were subjected to immunoblot analysis with α-P-JNK for detection of activated forms of JNKs. The same blot was also probed with αJNK.
level as, or upstream from, Ras to mediate insulin-induced JNK activation. To confirm that SHP-2C/S inhibits insulin-induced activation of Ras in a specific manner, we examined the effects of this mutant on tyrosine phosphorylation and dephosphorylation events in response to insulin. Insulin induces the tyrosine dephosphorylation of pp125 focal adhesion kinase and p130Cas and thereby disrupts the Crk-p130Cas complex (35–37). Tyrosine dephosphorylation of the prominent band at ~130 kDa, the major components of which might be focal adhesion kinase and p130Cas, as well as tyrosine phosphorylation of cellular substrates such as IRS1, in response to insulin occurred to similar extents in both Rat-1-IR and Rat-1-SHP-2CS cells (Fig. 4B, left panel). Furthermore, insulin treatment equally induced the dissociation of the Crk-p130Cas complex in the two cell lines (Fig. 4B, right panel). These results indicate that inhibition of insulin-induced Ras activation by SHP-2C/S does not result from nonspecific sequestration of tyrosine-phosphorylated proteins.

**Effects of SHP-2C/S on JNK Activation by Oncogenically Activated Ras or Myristoylated SOS**—Previous studies have

whereas Ras appears to be essential for signaling between activated IRs and JNKs.

**Effect of SHP-2C/S on Insulin-induced Activation of Ras**—We next tested the possibility that the negative effect of SHP-2C/S on JNK activation results from inhibition of insulin-induced activation of Ras. Insulin markedly activated Ras in Rat-1-IR cells, as revealed by a precipitation assay with a GST fusion protein containing the Ras-binding domain of c-Raf1 (33) (Fig. 4A). In contrast, the extent of insulin-induced Ras activation was substantially reduced in Rat-1-SHP-2CS cells, indicating that functional SHP-2 is required for this effect of insulin (Fig. 4A). Given that activation of JNKs by insulin depends on Ras, these results suggest that SHP-2 may act at the same
Role of SHP-2 in Insulin-induced Activation of JNK

**DISCUSSION**

With the use of Rat-1 fibroblasts overexpressing IRs as a model system, we have shown that a catalytically inactive mutant of SHP-2 (SHP-2/C/S) blocked JNK activation induced by insulin or EGF. In contrast, SHP-2/C/S did not inhibit activation of JNK in response to cellular stresses such as anisomycin treatment or sorbitol-induced osmotic shock, consistent with the results of a previous study showing that SHP-2 is not required for stress-induced activation of JNK (40). Thus, it is likely that the requirement for SHP-2 in JNK activation is specific for signaling events elicited by ligands that act at receptor-type tyrosine kinases. However, SHP-2 was shown not to be required for EGF-induced activation of JNK in COS-7 cells (38). The reason for this apparent discrepancy with our data is unclear, but it might be attributable to the difference in cell lines studied.

The mechanism by which SHP-2 contributes to insulin-induced JNK activation is unknown. However, our results with Rat-1-IR cells suggest a role for Ras in this mechanism. A dominant negative mutant of Ras (RasN17) blocked JNK activation induced by insulin, whereas a constitutively active mutant of Ras (RasV12) markedly induced JNK activation in the absence of insulin stimulation. These results suggest that Ras activation is both necessary and sufficient for JNK activation in response to insulin in Rat-1-IR cells, consistent with the results of previous studies demonstrating the Ras-dependent activation of JNK in response to EGF (9–11), nerve growth factor (11), insulin (15), or interleukin-3 (31) in other cell types. Insulin-induced activation of Ras, in turn, requires functional SHP-2, as indicated by the observation that SHP-2/C/S specifically blocked this effect of insulin. Thus, SHP-2/C/S-induced inhibition of JNK activation by insulin likely results, at least in part, from the inhibitory effect of this mutant on Ras activation in response to insulin.

Previous studies have shown that PI 3-kinase participates in the JNK signaling pathway initiated by receptor-like tyrosine kinases, including the EGF receptor (42), the platelet-derived growth factor receptor (12), and the IR (16), or by the βγ subunits of heterotrimeric G proteins (43). In addition, Rac has been shown to mediate JNK activation induced by EGF (9, 10) or by the G protein-coupled muscarinic receptor m2 (44). Moreover, Crk, an adapter protein that contains both SH2 and SH3 domains, mediates EGF-induced JNK activation in a Rac-dependent manner (45). Insulin activates divergent signaling pathways, which include PI 3-kinase and Rac pathways in addition to the Ras pathway (2, 46). However, neither a dominant negative mutant of Rac nor a specific inhibitor of PI 3-kinase, LY294002, affected insulin activation of JNK in Rat-1-IR cells, indicating that neither PI 3-kinase nor Rac is required for the insulin signaling pathway that leads to JNK activation in this cell line.
Corkscrew, the Drosophila homolog of SHP-2, was shown to act either upstream (47) or downstream (48) of Ras1 in signaling by the Torso receptor tyrosine kinase. We have previously demonstrated that SHP-2 regulates an upstream element necessary for Ras activation in response to insulin in Chinese hamster ovary cells overexpressing human IRs (28). SHP-2 has also been shown to act immediately downstream from Ras to mediate ERK activation induced by insulin (49). Thus, the mode of action, in relation to Ras, of SHP-2 in growth factor signaling has remained unclear. We assumed that if SHP-2 acts downstream from Ras, then SHP-2C/S would block JNK activation by constitutive activation of Ras. However, SHP-2C/S did not inhibit the activity of RasV12 to activate JNK, suggesting that the signaling pathway from activated Ras to JNK does not require functional SHP-2 in Rat-1-IR cells. Furthermore, SHP-2C/S did not block JNK activation induced by myr-SOS, which functions as a potent activator of Ras, suggesting that SHP-2 may act at the same level as, or upstream from, SOS, thereby possibly promoting cell growth.

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