Shp2 Is Required for Protein Kinase C-dependent Phosphorylation of Serine 307 in Insulin Receptor Substrate-1

Karsten Müssig, Harald Staiger, Hendrik Fiedler, Klaus Moeschel, Alexander Beck, Monika Kellerer, and Hans-Ulrich Häring

From the Division of Endocrinology, Metabolism, and Pathobiochemistry, Department of Internal Medicine, University Hospital of Tübingen, Tübingen 72076, Germany

The function of insulin receptor substrate-1 (IRS-1), a key molecule of insulin signaling, is modulated by phosphorylation at multiple serine/threonine residues. Phorbol ester stimulation of cells induces phosphorylation of two inhibitory serine residues in IRS-1, i.e. Ser-307 and Ser-318, suggesting that both sites may be targets of protein kinase C (PKC) isoforms. However, in an in vitro system using a broad spectrum of PKC isoforms (α, β1, β2, δ, ε, η, χ, μ), we detected only Ser-318, but not Ser-307 phosphorylation, suggesting that phorbol ester-induced phosphorylation of this site in intact cells requires additional signaling elements and serine kinases that link PKC activation to Ser-307 phosphorylation. As we have observed recently that the tyrosine phosphatase Shp2, a negative regulator of insulin signaling, is a substrate of PKC, we studied the role of Shp2 in this context. We found that phorbol ester-induced Ser-307 phosphorylation is reduced markedly in Shp2-deficient mouse embryonic fibroblasts (Shp2−/−) whereas Ser-318 phosphorylation is unaltered. The Ser-307 phosphorylation was rescued by transfection of mouse embryonic fibroblasts with wild-type Shp2 or with a phosphatase-inactive Shp2 mutant, respectively. In this cell model, tumor necrosis factor-α-induced Ser-307 phosphorylation as well depended on the presence of Shp2. Furthermore, Shp2-dependent phorbol ester effects on Ser-307 were blocked by wortmannin, rapamycin, and the c-Jun NH2-terminal kinase (JNK) inhibitor SP600125. This suggests an involvement of the phosphatidylinositol 3-kinase/mammalian target of rapamycin cascade and of JNK in this signaling pathway resulting in IRS-1 Ser-307 phosphorylation. Because the activation of these kinases does not depend on Shp2, it is concluded that the function of Shp2 is to direct these activated kinases to IRS-1.

Protein kinases C (PKCs), a family of 12 structurally and functionally related serine/threonine kinases, have been shown to play a critical role in insulin resistance and type 2 diabetes (for review, see Ref. 1). PKC activity is increased in insulin-sensitive tissues (muscle, liver) of diabetic patients (2, 3). The PKC isoforms β1, β2 (4), α, ε, η, ι (5), δ, and θ (6) are reported as potential candidates for negative regulation of insulin signaling. Activation of PKCs by hyperglycemia and phorbol ester inhibits insulin receptor tyrosine kinase activity (7, 8) and attenuates insulin-induced tyrosine phosphorylation of downstream mediators of the insulin signaling pathway, such as insulin receptor substrate-1 (IRS-1) (9). Upon activation of PKC, IRS-1 is phosphorylated on serine and threonine residues that have been shown to contribute to the development of insulin resistance (10, 11).

Among several potential serine phosphorylation sites of IRS-1, serine 307 (rat IRS-1 Ser-307 corresponds to Ser-312 of human IRS-1) is a key regulatory site because phosphorylation of Ser-307 inhibits insulin-mediated activation of the phosphatidylinositol 3 (PI 3)-kinase and mitogen-activated protein kinase (MAPK) pathways by disturbing the interaction of IRS-1 with the insulin receptor (12). c-Jun NH2-terminal kinase (JNK) (13) and inhibitor of B kinase-(IKK) (14) have been described to phosphorylate IRS-1 at Ser-307. Recently, it was reported that phorbol ester-stimulated PKC activation promotes phosphorylation of IRS-1 at Ser-307 (15, 16). However, we and others have recently shown using in vitro phosphorylation assays that rat IRS-1 Ser-318 (corresponding to Ser-323 of human IRS-1), but not Ser-307, serves as a major PKC phosphorylation site (17, 18). Thus, PKC-dependent Ser-307 phosphorylation in intact cells is probably not direct and requires further downstream effectors.

One candidate mediator is the cytoplasmic protein-tyrosine phosphatase Shp2, which was shown previously (19, 20) to be upstream of potential IRS-1 serine/threonine kinases, such as the MAPK family members extravascular signal-regulated kinases 1 and 2 (ERK1/2) (21, 22). Furthermore, Shp2 mRNA, protein, and activity were shown to be increased in liver and skeletal muscle of animal models of insulin resistance and diabetes (23, 24). In 3T3-L1 fibroblasts, overexpression of wild-type Shp2 impairs insulin-stimulated tyrosine phosphorylation of IRS-1 as well as PI 3-kinase activation, resulting in decreased glycogen synthesis (25). Structurally, Shp2 is characterized by a catalytic subunit at the carboxyl terminus and two Src homology 2 (SH2) domains at the amino terminus (26). With its SH2 domains, Shp2 binds tyrosine-phosphorylated proteins including components of the insulin signaling pathway, such as the insulin receptor (27), IRS-1 (28), and IRS-2 (29). Therefore, Shp2 may modulate insulin-dependent pathways through direct interaction with the insulin receptor (30) and the insulin receptor substrates (31). Furthermore, the insulin-like actions of vanadate (32) have been shown to be partly mediated by inhibition of Shp2 (33). Treatment with vanadate reverses the effects of hyperglycemia on insulin receptor and IRS-1 phosphorylation status by blocking Shp2 association with IRS-1 (34).

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Based on the ability of Shp2 to regulate insulin signaling negatively and our recent finding that Shp2 is a substrate of classical and novel PKC isoforms (35), we investigated in this study whether Shp2 is a mediator of PKC-induced phosphorylation of Ser-307 in IRS-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents and fetal calf serum were from Cambrex Bioscience (Verviers, Belgium). 12-O-Tetradecanoylphorbol-13-acetate (TPA), aprotinin, phenylmethylsulfonyl fluoride, Na3VO4, Triton X-100, dimethyl sulfoxide, dithiothreitol, anisomycin, bisindolylmaleimide I, wortmannin, as well as secondary anti-mouse, anti-rabbit, and anti-goat antibodies were purchased from Sigma. Reagents for SDS-PAGE and Western blotting were from Rockland (Karlruhe, Germany) and Bio-Rad. Geneticin was from Invitrogen, enhanced chemiluminescence reagent and nitrocellulose were from Amersham Biosciences, and gel blotting papers were from Schleicher & Schuell. Recombinant human insulin was from Lilly (Indianapolis). Recombinant human tumor necrosis factor-α (TNF-α) was from TEBU (Offenbach, Germany). SP600125 was from Apotex (San Diego), PD98059 was from Alexis Biochemical (Carlsbad, CA), and farnesylthiosalicylic acid was from Biomol (Plymouth Meeting, PA). SB20358, 15-deoxyprostaglandin I2 (15dPGI2), FTI-277, rapamycin, and a JNK Activity Immunoassay Kit were from Calbiochem. d-myo-Phosphatidylinositol 3,4-bisphosphate (PtdIns3,4P2) was from Echelon Biosciences (Salt Lake City, UT). Thin layer chromatography plates (Silica Gel 60) were from Merck. X-AR 5 film was from Kodak.

The rabbit polyclonal IRS-1 phosphoserine 307-specific antibody was a gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). The mouse monoclonal IRS-1 antibody and the rabbit polyclonal IRS-1 phosphoserine 318-specific antibody were produced in our laboratory. The rabbit polyclonal Shp2 (NH2 terminus) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal Akt phosphothreonine 389-specific antibody, the rabbit polyclonal c-Jun phosphoserine 73-specific antibody, the rabbit polyclonal c-Jun phosphothreonine 389-specific antibody, and the rabbit polyclonal p70S6 kinase antibody were purchased from Cell Signaling Technology (Beverly, MA). Protein G-Sepharose CL-4B and [γ-32P]ATP were purchased from Amersham Biosciences. All HPLC-grade solvents were obtained from Merck.

**Purification and In Vitro Phosphorylation of GST-IRS-1N2 with PKC Isoforms**—The glutathione S-transferase (GST) fusion protein with the amino-terminal fragment of rat IRS-1 (IRS-1N2; amino acids 265–522; size 53.8 kDa), was generated as described previously (18). In brief, the fragment was ligated to GST and cloned into the pGEX-2T vector (Amersham Biosciences). The DNA encoding this region of IRS-1 was synthesized by PCR using rat IRS-1 cDNA as template and pairs of oligonucleotide primers that contain appropriate restriction sites bordering these fragments (36). For mutation of the codon for amino acid position 318 serine to alanine, base substitutions were made by oligonucleotide-mediated mutagenesis. The mutagenesis upstream primer

**Stimulation and Cell Lysis**—MEFs were stimulated with 100 nM TPA for 10 to 120 min, or 100 nM insulin, 5 nM TNF-α, 25 μM glucose, 25 μg/ml anisomycin, or 10 μM PDIns3,4,5P3 for 30 min at 37 °C. In some experiments, cells were preincubated with 20 μM INK inhibitor SP600125, 1 μM p38 kinase inhibitor SB203580, 20 μM MAPK/ERK kinase (MEK) 1 inhibitor PD98059, 500 nM PKC inhibitor bisindolylmaleimide I, 15 μM Ikk-B inhibitor 15dPGJ2, 10 and 100 nM PI 3-kinase inhibitor wortmannin, or 25 μM mammalian target of rapamycin (mTOR) inhibitor rapamycin each for 10 min at 37 °C, with 25 μM Ras inhibitor farnesylthiosalicylic acid or 1 μM Ras inhibitor FTI-277 each for 24 h at 37 °C. After washing with phosphate-buffered saline, the cells were lysed (500 μl/10 cm diameter dish) with ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10 μg/ml aprotinin, 100 mM NaF, 10 mM Na3VO4, 2 mM Na2VO3). Cell lysates were cleared by centrifugation at 12,000 × g for 10 min at 4 °C.
Western Blotting—After separation by SDS-PAGE (100 μg of total protein/lane), proteins were transferred to nitrocellulose membranes by semi-dry electroblotting. Membranes were incubated in gelatin buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton X-100, and 0.25% gelatin, pH 7.4) for 1 h at room temperature, probed with the appropriate primary antibody overnight at 4 °C, washed three times with gelatin-conjugated secondary antibody for 1.5 h at room temperature. Signals were detected by enhanced chemiluminescence.

PL 3-Kinase Assay—PL 3-kinase assays were performed as described by Morgan et al. (40), with some modifications. Briefly, PL 3-kinase was immunoprecipitated from MEF cell lysates. Immunoprecipitates were washed twice in phosphate-buffered saline, 1% Nonidet P-40, and 100 μmol/liter Na3VO4; twice in 500 mmol/liter LiCl, 100 mmol/liter Tris-HCl, pH 7.5, and 100 mmol/liter Na3VO4 and twice in 10 mmol/liter Tris-HCl, pH 7.5, 100 mmol/liter NaCl, 1 mmol/liter EDTA, and 100 mmol/liter Na3VO4. The beads were resuspended in 25 μl of 60 mmol/liter Tris-HCl, pH 7.5, 300 mmol/liter NaCl, 12 mmol/liter MgCl2, and 12.5 μl of phosphatidylinositols (1 mg/ml). The kinase reaction was started by adding 12.5 μl of 40 μmol/liter ATP containing 10 μCi [γ-32P]ATP, and samples were incubated for 20 min at room temperature. The reaction was stopped by the addition of 150 μl of a mix of 1 N HCl. Lipids were extracted once with 450 μl of chloroform:methanol (1:1, v/v), and the phospholipid-containing organic phase was resolved on thin layer chromatography plates and developed in chloroform:methanol:acetone:glacial acetic acid (40:13:15:12.7, v/v). Thin layer chromatography plates were dried and exposed on film.

JNK Activity Immunoassay—JNK activity immunoassay was performed according to the manufacturer’s protocol. In brief, JNK was immunoprecipitated from MEF cell lysates, immunoprecipitates were washed three times, the kinase reaction was started by adding c-Jun protein and an ATP mixture, and samples were incubated for 25 min at 30 °C. Proteins were separated by SDS-PAGE, and Western blotting was performed as described above using a c-Jun phospho-specific antibody.

Results

In Vitro Phosphorylation of IRS-1N2 by Phorbol Ester-inducible PKC Isoforms—Based on mass spectrometry, we and others have recently identified Ser-318 as a major in vitro phosphorylation site of PKC isoforms ζ and δ in the IRS-1N2 fragment (rat IRS-1 sequence comprising amino acids 265–522) (17, 37). However, we did not detect Ser-307 phosphorylation. Therefore, we tested whether other phorbol ester-inducible PKC isoforms, such as PKC-α, -β1, -β2, -ζ, -θ, and μ, are able to phosphorylate Ser-307 directly in the same experimental setting. Using these isoforms in recombinant form, the fragment IRS-1N2 was again phosphorylated at Ser-318, but not at Ser-307 (data not shown).

From these results, we conclude that IRS-1 Ser-307 is not a direct substrate for PKC and that phorbol ester-induced phosphorylation of this site in intact cells requires additional signaling elements.

Involvement of Shp2 in TPA-induced Phosphorylation of IRS-1 Ser-307—Because we have recently observed that the tyrosine phosphatase Shp2 is a substrate of PKC, we studied the role of Shp2 in this context. In mock-transfected MEF Shp2−/− cells, TPA only weakly stimulated Ser-318 phosphorylation of IRS-1. However, after reexpression of wild-type Shp2, phosphorylation of Ser-307 was enhanced markedly (Fig. 1A). By contrast, there was no difference in TPA-induced Ser-318 phosphorylation between mock-transfected MEF Shp2−/− cells and Shp2-reexpressing cells (Fig. 1B). These data suggest that Shp2 is specifically involved in TPA-induced phosphorylation of Ser-307. To confirm the involvement of PKC, TPA-treated MEF cells reexpressing wild-type Shp2 were preincubated with the PKC inhibitor bisindolylmaleimide I. This treatment caused markedly diminished TPA-induced Ser-307 phosphorylation (Fig. 2).

In MEF cells transfected with human wild-type Shp2, not only stimulation with the PKC activator TPA (100 nm), but also treatment with insulin (100 nm), TNF-α (5 nm), and high glucose (25 mM), resulted in increased IRS-1 Ser-307 phosphorylation. These phosphorylation events were diminished in MEF Shp2−/− cells (Fig. 3, A and B). However, only the TPA- and TNF-α-induced Ser-307 phosphorylation signals significantly depended on the presence of Shp2 (p < 0.05, Bonferroni’s post hoc test, n = 5, Fig. 3B). In conclusion, TPA- and TNF-α-induced phosphorylation of IRS-1 Ser-307 requires Shp2, whereas this protein-tyrosine phosphatase is not involved in PKC-stimulated Ser-318 phosphorylation.

Densitometry and Statistical Analysis—For quantification of signal intensity of phospho-specific immunoblots, the EasyWin32 Herolab Software was used. Densitometric data obtained from phospho-specific signals were normalized to the densitometric values obtained from the corresponding immunoblots detecting protein expression. At least five independent experiments were performed to obtain mean values ± S.E. A one-way analysis of variance with Bonferroni’s post hoc multiple comparison test was used to detect differences between the groups. p < 0.05 was considered to be statistically significant.
Role of Serine Residues 576 and 591 and the Phosphatase Activity of Shp2 in TPA-stimulated IRS-1 Ser-307 Phosphorylation—Because Shp2 is phosphorylated at serine residues 576 and 591 by PKC (35), we tested the involvement of these phosphorylation sites in TPA-stimulated phosphorylation of IRS-1 Ser-307. Furthermore, we tested the requirement of phosphatase activity in TPA-induced IRS-1 phosphorylation at Ser-307. To this end, we introduced mutant forms of Shp2, i.e. the double mutant S576A/S591A and the phosphatase-negative mutant C459A, into MEF Shp2-/- cells. In contrast to the mock-transfected MEF Shp2-/- cells, all Shp2 constructs (wild-type, double mutant S576A/S591A, and phosphatase-negative form) promoted significant TPA-induced Ser-307 phosphorylation. However, there was no significant difference in the degree of TPA-dependent Ser-307 phosphorylation between wild-type and mutant Shp2 forms (Fig. 4, A and B). This suggests that neither the PKC phosphorylation sites nor the phosphatase activity is necessary for Shp2 mediation of PKC-induced IRS-1 Ser-307 phosphorylation.

Role of JNK in Shp2-dependent IRS-1 Ser-307 Phosphorylation—JNK and IKK-β have been described as phosphorylating IRS-1 at Ser-307 (13, 14). Accordingly, incubation of MEF cells with the specific JNK activator anisomycin induced a marked IRS-1 Ser-307 phosphorylation both in wild-type Shp2-expressing cells and in mock-transfected cells (Fig. 5A). Preincubation with the JNK inhibitor SP600125 markedly decreased TPA-induced IRS-1 Ser-307 phosphorylation in MEF cells reexpressing wild-type Shp2, whereas incubation with the IKK-β inhibitor 15dPGJ2 did not alter this phosphorylation (Fig. 5B). This suggests a critical role of JNK, but not of IKK-β, in Shp2-regulated phosphorylation of Ser-307 in IRS-1. Testing the involvement of MAPK, such as ERK1/2 and p38, we found that preincubation with the p38 inhibitor SB203580 or with the MEK1 kinase inhibitor PD098059, respectively, left the phosphorylation of IRS-1 Ser-307 unaffected (Fig. 5B), suggesting that p38 and ERK1/2 are not involved in Shp2-mediated phosphorylation of Ser-307 in IRS-1.

Role of the PI 3-Kinase/mTOR Pathway in Shp2-dependent IRS-1 Phosphorylation at Ser-307—Because it is known that Ras and PI 3-kinase can activate JNK (41, 42), we tested their involvement in Shp2-dependent phosphorylation of Ser-307 in IRS-1. Preincubation of MEF cells reexpressing wild-type Shp2 with the farnesylation inhibitors farnessylthiosalicylic acid and FTI-277 had no influence on TPA-induced Ser-307 phosphorylation in IRS-1 (data not shown). By contrast, treatment with the PI-3 kinase inhibitor wortmannin dose-dependently decreased phorbol ester-stimulated IRS-1 phosphorylation at Ser-307 (Fig. 6A), suggesting a critical role of PI-3 kinase, but not of Ras, in Shp2-regulated IRS-1 Ser-307 phosphorylation. Treatment with the PI 3-kinase product PtdIns(3,4)P2 strongly increased IRS-1 Ser-307 phosphorylation both in MEF cells transfected with wild-type Shp2 or mock-
ponent of this signaling pathway. Furthermore, PtdIns(3,4)P2-induced phosphorylation, providing evidence that mTOR is indeed a com-
depicted in Fig. 7, rapamycin pretreatment abolished TPA-induced Ser-
involved in Shp2-dependent IRS-1 Ser-307 phosphorylation, TPA-
downstream target of PI 3-kinase and a potential IRS-1 kinase (43), is
Shp2 were treated with 100 nM TPA (30 min) with or without preincubation (10 min) with
20 μM JNK inhibitor SP600125, 1 μM p38 kinase inhibitor SB203580, 20 μM MEK1 inhibitor
PD98059, or 15 μM IKK-β inhibitor 15dPGJ2, respectively. Representative immunoblots are shown.

activity immunoassay showed a TPA-induced phosphorylation of c-Jun in wild-type Shp2-expressing cells as well as in MEF Shp2−/− cells (Fig. 8A). Incubation with TPA induced an Akt Thr-308 phosphorylation and p70S6 kinase (S6K) Thr-389 phosphorylation both in wild-type Shp2-expressing cells and in MEF Shp2−/− cells (Fig. 8B). After stimulation with TNF-α, there was also an Akt Thr-308 phosphorylation and S6K Thr-389 phosphorylation as well as a phosphorylation of c-Jun in the JNK activity immunoassay both in wild-type Shp2-expressing cells and in MEF Shp2−/− cells (data not shown). The PI 3-kinase assay did not show a difference in PI 3-kinase activity after stimulation with TPA and TNF-α between wild-type Shp2-expressing cells and mock-transfected cells (data not shown). These results suggest that activation of JNK, PI-3 kinase, and mTOR after TPA and TNF-α treatment was independent of the presence of Shp2, whereas directing these kinases to the end point IRS-1 Ser-307 phosphorylation requires the presence of Shp2.

**DISCUSSION**

Previous studies have identified serine residues 307 and 318 in IRS-1 as targets of several protein kinases, among these also members of the PKC family. In the present study we show that the PKC activators TPA and TNF-α induce phosphorylation of both serine residues. However, only the phosphorylation of Ser-307 depended on the presence of pro-

![FIGURE 5. JNK mediates TPA-stimulated IRS-1 Ser-307 phosphorylation. A, MEF cells reexpressing human wild-type (wt) Shp2 and MEF Shp2−/− cells were treated with 25 μg/ml anisomycin (30 min). B, MEF cells reexpressing wild-type Shp2 and mock-transfectants were treated with 10 μM PtdIns(3,4)P2 (30 min). Representative immunoblots are shown.](image)

![FIGURE 6. TPA-induced phosphorylation of IRS-1 Ser-307 is regulated by PI 3-kinase. A, MEF cells transfected with wild-type (wt) Shp2 were treated with 100 nM TPA (30 min) with or without preincubation (10 min) with 10 or 100 nM PI 3-kinase inhibitor wortmannin. B, MEF cells reexpressing wild-type Shp2 and mock-transfectants were treated with 10 μM PtdIns(3,4)P2 (30 min). Representative immunoblots are shown.](image)

![FIGURE 7. mTOR mediates Shp2-dependent IRS-1 Ser-307 phosphorylation. MEF cells transfected with human wild-type (wt) Shp2 were treated with 100 nM TPA or 10 μM PtdIns(3,4)P2 (30 min), respectively, with or without preincubation (10 min) with 25 nM mTOR inhibitor rapamycin. A representative immunoblot is shown.](image)
tein-tyrosine phosphatase Shp2. High glucose and insulin, also known to activate PKC, stimulated Ser-307 phosphorylation as well but did not reveal clear Shp2 dependence. The reason for this discrepancy is unknown but might reflect the existence of alternative Shp2-independent PKC signaling pathways resulting in IRS-1 Ser-307 phosphorylation. This is further evidenced by the finding that, even in the complete absence of Shp2, residual Ser-307 phosphorylation signals are seen with all external stimuli tested (Fig. 3A).

Neither the phosphatase activity of Shp2 nor its serine residues 576 and 591, which have been described previously to be phosphorylated by PKC (35) are required for phorbol ester-induced Ser-307 phosphorylation. The observation that Shp2 can modulate signaling independently of its phosphatase activity is in agreement with previous studies demonstrating that Shp2 functions as an adaptor or scaffolding molecule that brings into close proximity upstream regulators, such as growth factor receptors, and downstream effectors, such as Ras (44, 45), Src (46), and PI 3-kinase (47, 48). In accordance with this, Shp2 has previously been shown to have a phosphatase-independent adaptor function in insulin receptor/IRS-1 interaction as well allowing the formation of a multiprotein signaling complex (49). Furthermore, Noguchi et al. (20) demonstrated that upon insulin stimulation, a catalytically inactive mutant of Shp2 did not affect PI 3-kinase activation, but attenuated MAPK activation. By contrast, Ugi et al. (51) reported that expression of a mutant Shp2 that lacked the entire catalytic domain inhibited insulin-induced PI 3-kinase activity. The detected differences may be explained by the different tertiary structures of the Shp2 mutants. Although we as well as Noguchi et al. (20) expressed a catalytically inactive Shp2 that was point mutated at cysteine 459, a highly conserved cysteine residue in all tyrosine phosphatases which is crucial for catalytic activity (52), Ugi et al. (51) used a Shp2 that consisted only of the two SH2 domains and lacked the complete catalytic domain. Therefore, the interaction of this truncated Shp2 mutant with binding partners may be disturbed, or unspecific associations with nonphysiological binding partners may occur. We propose that Shp2 as a molecular linker brings together PKC and its downstream mediators of IRS-1 Ser-307 phosphorylation.

Consistent with recent studies (13, 16), we have found that PKC-induced Shp2-dependent IRS-1 Ser-307 phosphorylation depends on JNK, a well known downstream effector of TNF-α. Other kinases that have been described to phosphorylate IRS-1 such as IKK-β (14) and MAPK (53) do not seem to be involved in this phosphorylation event in our cell system. The exact mechanism of JNK activation is still unclear. Several potential upstream activators of JNK, such as the small GTP-binding protein Ras (41) and PI 3-kinase (42), have been described. However, our data do not indicate a critical role of Ras in Shp2-dependent IRS-1 Ser-307 phosphorylation. By contrast, we found that PI 3-kinase and mTOR are involved in Ser-307 phosphorylation, consistent with previous inhibitor studies showing that inhibition of PI-3 kinase or mTOR decreased insulin-induced Ser-307 phosphorylation in IRS-1 (43, 50, 53). TPA and TNF-α treatment induced a phosphorylation of AKT and p70S6K, known substrates of PI 3-kinase and mTOR respectively, in all external stimuli tested (Fig. 3B).

Although we as well as others have previously shown to have a phosphatase-independent adaptor function in insulin receptor/IRS-1 interaction as well allowing the formation of a multiprotein signaling complex (49), the exact mechanism of JNK activation is still unclear. Several potential upstream activators of JNK, such as the small GTP-binding protein Ras (41) and PI 3-kinase (42), have been described. However, our data do not indicate a critical role of Ras in Shp2-dependent IRS-1 Ser-307 phosphorylation. By contrast, we found that PI 3-kinase and mTOR are involved in Ser-307 phosphorylation, consistent with previous inhibitor studies showing that inhibition of PI-3 kinase or mTOR decreased insulin-induced Ser-307 phosphorylation in IRS-1 (43, 50, 53).

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