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

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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, 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),2 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 (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 protein-tyrosine phosphatase Shp2, which was shown previously (19, 20) to be upstream of IRS-1, causes IRS-1 Ser-307 phosphorylation. Phosphorylation of IRS-1 Ser-307 on 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 can associate with the insulin receptor substrate-1; IRS-1H2, amino-terminal fragment of IRS-1; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; mTOR, mammalian target of rapamycin; PI 3-kinase, phosphatidylinositol 3-kinase; PtdIns(3,4)P2, d-myophosphatidylinositol 3,4-bisphosphate; 56K, p70 S6 kinase; SH2, Src homology-2 domain-containing protein-tyrosine phosphatase-2; TNF-α, tumor necrosis factor-α; TPA, 1,2-O-tetradecanoylphorbol-13-acetate.

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2 The abbreviations used are: PKC, protein kinase C; 15dPGJ2, 15-deoxy-prostaglandin J2; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; IKK-β, inhibitor of κB kinase-β; IRS-1, insulin receptor substrate-1; IRS-1H2, amino-terminal fragment of IRS-1; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; mTOR, mammalian target of rapamycin; PI 3-kinase, phosphatidylinositol 3-kinase; PtdIns(3,4)P2, d-myophosphatidylinositol 3,4-bisphosphate; 56K, p70 S6 kinase; SH2, Src homology 2; SH2 domain-containing protein-tyrosine phosphatase-2; TNF-α, tumor necrosis factor-α; TPA, 1,2-O-tetradecanoylphorbol-13-acetate.
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 Roth (Karlsruhe, 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 Apotech (San Diego), PD98059 was from Alexis Biochemical (Carlsbad, CA), and farnesylsaccharic 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 (PtdIns(3,4)P2) 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 p70S6 kinase phosphothreonine 308-specific antibody, the rabbit polyclonal Akt antibody, and the rabbit polyclonal c-Jun antibody, were produced in our laboratory. The mouse monoclonal IRS-1 antibody and the rabbit polyclonal IRS-1 phosphoserine 266-specific antibody were 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 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 used was 5’-GGTGGGAAAACGAGGTCCTTCAGGGTGCGTCC-3’ with the wild-type IRS-1 expression vector as template. Positive clones were verified by sequencing. The PCR products were isolated, digested with appropriate restriction enzymes, and subcloned into pGEX-2T vectors, which were used to transform Escherichia coli BL21. The fusion protein was expressed and purified by affinity chromatography. The in vitro phosphorylation assays were performed at 30 °C for 90 min with 1 nmol of the isolated GST-IRS-1N2 protein fragments (wild-type and S318A mutant), which were phosphorylated in vitro by recombinant PKC isoforms α, β1, β2, ε, θ, and μ (Calbiochem) in 55 mM HEPES, pH 7.5, 1.25 mM EGTA, 1 mM EDTA, 5 mM MgCl2, 1 mM dithiothreitol, 5 μg/ml l-α-phosphatidyl-l-serine, 1 μg/ml 1,2-dioctanoyl-sn-glycerol, 100 μM ATP, and 6 μCi of [γ-32P]ATP. The samples were analyzed by 7.5% SDS-PAGE and visualized by autoradiography.

In-gel Protein Digestion and Reversed Phase HPLC Peptide Separation—The GST-IRS-1N2 protein was excised and in-gel digested as described previously (37). Peptides eluted from the polyacrylamide matrix were separated on a 100 × 2.0 (inner diameter) mm C4/C18 reversed phase column (Grom, Herrenberg, Germany) using a 10A HPLC system (Shimadzu, Duisburg, Germany) equipped with a Uvicord 2215 UV-Detector (Amersham Biosciences). The gradient program used was 0–5 min 3% B, 5–10 min 15% B, and 10–80 min 60% B, where A was 0.02% trifluoroacetic acid in water and B was 0.023% trifluoroacetic acid, 80% acetonitrile in water at a flow rate of 250 μl/min. Elution was monitored at 214 nm. Collected fractions (1 cm cut) were analyzed in a Trilux 1450 MicroBeta Plus β-counter (PerkinElmer Life Sciences).

**Cell Culture**—Shp2 knock-out mouse embryonic fibroblasts (MEF Shp2−/−), kindly provided by Dr. Tony Pawson, Toronto, Canada, and the human cell line BOSC 23 were maintained in Dulbecco’s modified Eagle’s medium (1 g/liter glucose), supplemented with 10% fetal calf serum. Packaging cells GP+E86 were grown in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) containing 10% fetal calf serum.

**Generation of Recombinant Retroviruses**—The cDNAs for Shp2, the serine-to-alanine mutant Shp2 S576A/S591A, and the phosphatase-negative Shp2 (C459A), described previously (35), were excised from the cytomegalovirus early promoter–based expression plasmid pRK5 as EcoRI/Xhol fragments and ligated into EcoRI/Xhol-digested retroviral expression vector pLXSN (38). After analyzing the sequences of the plasmid constructs by sequencing, plasmid DNA was prepared using a Qiagen Plasmid Kit. To receive retroviral stocks by transient transfection (39), BOSC 23 cells were transfected with each of these constructs. To increase the titer of the recombinant retroviruses, 105 exponentially growing packaging cells GP+E86/6-well plate were incubated with supernatants of BOSC 23 cells containing the recombinant retroviruses for 12 h at 37 °C, 5% CO2, and in the presence of 8 μg/ml Polybrene. Virus-containing medium was then removed, and cells were grown for selection in medium containing 750 μg/ml Geneticin for 2–3 weeks.

**Retrovirus-mediated Gene Transfer**—105 MEF Shp2−/− cells/6-well plate were incubated with the supernatants of retrovirus-expressing GP+E86 cells in the presence of 8 μg/ml Polybrene for 24 h at 37 °C, 5% CO2. Virus-containing medium was then removed, and cells were selected in medium containing 750 μg/ml Geneticin for 2 weeks. Geneticin-resistant clones were transferred to 10-cm dishes and analyzed for the expression of the transduced gene.

**Stimulation and Cell Lysis**—MEF cells were stimulated with 100 nM TPA for 10 to 120 min, or 100 nM insulin, 5 nM TNF-α, 25 mM glucose, 25 μg/ml anisomycin, or 10 μM PtdIns(3,4)P2 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 IκB-β inhibitor 15dPGJ2, 10 and 100 nM PI 3-kinase inhibitor wortmannin, or 25 mM mammalian target of rapamycin (mTOR) inhibitor rapamycin each for 10 min at 37 °C, with 25 μM Ras inhibitor farnesylsaccharic 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% glycerol, 1% Triton X-100) containing a protease/phosphatase inhibitor mix (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 100 mM NaF, 10 mM Na3VO4, 2 mM Na2MoO4). 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 electrophoretic blotting. 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) at 1 h at room temperature, probed with the appropriate primary antibody overnight at 4°C, washed three times with gelatin buffer followed by incubation with peroxidase-conjugated secondary antibody for 1.5 h at room temperature. Signals were detected by enhanced chemiluminescence.

**PI 3-Kinase Assay**—PI 3-kinase assays were performed as described by Morgan et al. (40), with some modifications. Briefly, PI 3-kinase immunoprecipitated from MEF cell lysates. Immunoprecipitates were washed twice in phosphate-buffered saline, 1% Nonidet P-40, and 100 mM Tris, pH 7.5, and 1% Triton X-100, and 0.25% gelatin, pH 7.4) at 1 h at room temperature, probed with the appropriate primary antibody overnight at 4°C, washed three times with gelatin buffer followed by incubation with peroxidase-conjugated secondary antibody for 1.5 h at room temperature. Signals were detected by enhanced chemiluminescence.

**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 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-1 by Phorbol Ester-inducible PKC Isomers—Based on mass spectrometry, we and others have recently identified Ser-318 as a major site of in vitro phosphorylation site of PKC isoforms in many cell lines. However, we did not detect Ser-307 phosphorylation. Therefore, we tested whether other phorbol ester-inducible PKC isoforms, such as PKC-α, -β1, -β2, -ε, -γ, and -μ, are able to phosphorylate Ser-307 directly in the same experimental setting. Using these isoforms in recombinant form, the fragment IRS-1 (1-1100) 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—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-307 phosphorylation of IRS-1. However, after reexpression of wild-type Shp2, phosphorylation of Ser-307 was enhanced markedly (Fig. 1, A). 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.
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 farnesylthiosalicylic acid and FTI-277 had no influence on TPA-induced Ser-307 phosphorylation (data not shown). By contrast, pretreatment with the PI-3 kinase inhibitor wortmannin dose-dependently decreased phorbol ester-stimulated IRS-1 phosphorylation at Ser-307 (Fig. 6A), 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 farnesylthiosalicylic acid and FTI-277 had no influence on TPA-induced Ser-307 phosphorylation in IRS-1 (data not shown). By contrast, pretreatment 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-transfected with human wild-type Shp2 and mock-transfected cells were treated with 100 nM TPA, 100 nM insulin, 25 mM glucose, or 5 nM TNF-α (30 min), respectively. A shows a representative immunoblot. In B, the results of the densitometric analysis of five independent experiments are given (n = 5). Significant differences compared with vector control (p < 0.05, Bonferroni’s post hoc test) are marked with an asterisk.

FIGURE 3. TPA- and TNF-α-induced IRS-1 Ser-307 phosphorylation depends on Shp2. MEF cells transfected with human wild-type (wt) Shp2 and mock-transfected cells were treated with 100 nM TPA, 100 nM insulin, 25 mM glucose, or 5 nM TNF-α (30 min), respectively. A shows a representative immunoblot. In B, the results of the densitometric analysis of five independent experiments are given (n = 5). Significant differences compared with vector control (p < 0.05, Bonferroni’s post hoc test) are marked with an asterisk.

FIGURE 4. Serine residues 576 and 391 as well as the phosphatase activity of Shp2 are not required for TPA-induced phosphorylation of IRS-1 Ser-307. MEF cells transfected with human wild-type (wt) Shp2, the mutant Shp2-S576A/S591A, a phosphatase-negative Shp2 (PTPase−), or control vector were treated with 100 nM TPA (30 min). A shows a representative immunoblot. In B, the results of the densitometric analysis of 10 independent experiments are given (n = 10). Significant differences compared with vector control (p < 0.05, Bonferroni’s post hoc test) are marked with an asterisk.
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-
stimulated Shp2-reexpressing cells were pretreated with rapamycin. As
involved in Shp2-dependent IRS-1 Ser-307 phosphorylation, TPA-
downstream target of PI 3-kinase and a potential IRS-1 kinase (43), is
are shown.

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 Shp2
wt cells transfected with human wild-type Shp2 and mock-transfectants were
treated with 10 µM PtdIns(3,4)P2 (30 min). Representative immunoblots are shown.

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 and control cells were
treated with 100 nM TPA (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.

activity immunoassay showed a TPA-induced phosphorylation of c-Jun
in wild-type Shp2-expressing cells as well as in MEF Shp2 wt (Fig. 8A). Incubation with TPA induced an Akt Thr-308 phosphorylation and
p70S6 kinase (S6K) Thr-389 phosphorylation as well as a phosphorylation of c-Jun in the
MEF Shp2 wt 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 wt 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-

Shp2 in PKC-dependent IRS-1 Ser-307 Phosphorylation

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.
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. 3).

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 our cell system. The PI 3-kinase assay as well as the JNK activity immunoassay did also not show a discrepancy is indeed difficult to explain. On the other hand, as already discussed above, we detect weak TPA-induced phosphorylation of IRS-1 Ser-307 even in the absence of Shp2 (Figs. 1A and 3, A and B), and this suggests that alternative Shp2-independent pathways exist. It is conceivable that anisomycin and PtdIns(3,4)P2 are strong activators of the kinases by these pharmacological stimuli.

In conclusion, we have found that phorbol ester- and TNF-α-induced phosphorylation of Ser-307 in IRS-1 is not a direct PKC-mediated event but depends on the presence of Shp2. Future experiments will evaluate the relevance of these findings in vivo.

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