The Shp-2 Tyrosine Phosphatase Has Opposite Effects in Mediating the Activation of Extracellular Signal-regulated and c-Jun NH₂-terminal Mitogen-activated Protein Kinases*

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Shp-2 is a widely expressed cytoplasmic tyrosine phosphatase with two SH2 domains. A targeted mutant allele of the Shp-2 gene with a deletion of 65 amino acids in the NH₂-terminal SH2 domain was created that leads to embryonic lethality at mid-gestation in homozygous mutant mice. To define the Shp-2 function in cell signaling, we have established mutant fibroblast cell lines, and have examined the effect of the Shp-2 mutation on extracellular signal-regulated kinase (ERK) and c-Jun NH₂-terminal kinase (JNK) mitogen-activated protein (MAP) kinase pathways. Insulin-like growth factor (IGF)-I-induced ERK activation was completely abolished, while ERK activity upon platelet-derived growth factor and epidermal growth factor stimulation was significantly reduced and shortened in mutant cells. Stimulation of ERK by phorbol 12-myristate 13-acetate was not affected in mutant cells, but the phorbole 12-myristate 13-acetate-induced ERK activity decayed much faster compared with that in wild-type cells. In contrast, JNK activation upon heat shock was significantly enhanced in Shp-2 mutant cells. Based on these results, we conclude that Shp-2 plays differential positive regulatory roles in various mitogenic signaling pathways leading to ERK activation, and that Shp-2 is a negative effector in JNK activation by cellular stress. This is the first evidence that a tyrosine phosphatase has opposite effects in mediating the activation of ERK and JNK MAP kinases.

In mammalian cells, the mitogen-activated protein (MAP)1 kinases comprise a family of three distinct groups: extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAP kinase (p38) (reviewed in Refs. 1 and 2). These MAP kinases are implicated in the regulation of fundamental cellular processes and are activated by distinct signaling pathways that are not fully understood.

Shp-2, a protein tyrosine phosphatase with two SH2 domains, appears to participate in signaling events proximal to receptor protein tyrosine kinases (3, 4). By a gene-targeting approach, we created a mutant allele of the murine Shp-2 gene with a deletion of exon 3, encoding for amino acids 46–110 in the NH₂-terminal SH2 domain (5, 6). A mutant protein with an internal deletion of 65 amino acids was expressed in heterozygous and homozygous mutants, although at a much lower level than the wild-type Shp-2. Homozygous mutant animals die at mid-gestation with severe defects in mesodermal patterning and body organization (5). Since homozygous mutant mice die at the same time as Shp-2 null mutants, the mutant Shp-2 molecule might be physiologically inert without the intact SH2 domains (5–7).

Previous experiments suggested Shp-2 might be a positive regulator in mitogenic signal transduction upstream of ERK. Expression of a catalytically inactive mutant of Shp-2 attenuated ERK activation by insulin, insulin-like growth factor-I (IGF-I), and fibroblast growth factor (8–10). Since Shp-2 might act downstream of a variety of receptor protein tyrosine kinases, an important issue to be resolved is whether Shp-2 plays the same role in different mitogenic signaling pathways leading to ERK activation. Given the potential physiological significance of prolonged ERK activation in cell growth and differentiation (2), a detailed examination of the kinetics of ERK activity is crucial to define a specific role of Shp-2 in each pathway. Another interesting question is whether Shp-2 also has a role in JNK activation under stress.

To address these questions, we have investigated Shp-2 functions in mediating the activation of ERK and JNK, using wild-type, heterozygous, and homozygous Shp-2 mutant embryonic fibroblast cell lines. Our results suggest for the first time that Shp-2 might have opposite effects in the control of ERK and JNK pathways. This SH2-containing protein tyrosine phosphatase appears to play different positive roles in various mitogenic signaling pathways leading to ERK activation, while it acts as a negative effector in JNK activation by heat shock.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents—**Mouse embryonic fibroblast cells were isolated by trypsinization of littermate embryos dissected at day 10.5 of gestation from crosses between heterozygous Shp-2 mutant animals. Primary cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, and immortalized by infection of a recombinant retrovirus expressing SV40 large T antigen (11). Genotyping of these cell lines was established by Southern blot and immunoblot analysis, as described previously (5, 6). Human recombinant platelet-derived growth factor (PDGF)-AA and mouse epidermal growth factor (EGF) were purchased from Collaborative Biomedical
RESULTS

Establishment of Wild-type and Mutant Cell Lines—To define the biological function of Shp-2 in cell signaling, we established wild-type, heterozygous (+/−), and homozygous (−/−) Shp-2 mutant origins were established as described in the text. Equal amounts of cell lysates from each cell line (40 μg) were separated on 8% SDS-polyacrylamide gel and immunoblotted by a polyclonal antibody raised against amino acid residues 576–593 in the COOH-terminal tail of Shp-2 (Santa Cruz Biotechnology, Inc.).

Products. Recombinant human IGF-I and phorbol 12-myristate 13-acetate (PMA) were from Sigma. Anti-tyrosine phosphorylated ERK (anti-FY-ERK) and anti-JNK1 antibodies came from New England Biolabs, Inc., and Santa Cruz Biotechnology, Inc., respectively.

Immunoprecipitation and Immunoblotting—Control or factor-stimulated cell lysates were made with cell lysis buffer (12), and protein concentration was quantitated using a Bio-Rad protein assay kit. For immunoprecipitation, cell lysates were incubated with specific antibodies in the presence of protein G/A-Sepharose beads. Samples of immunoprecipitates or cell lysates were resolved on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was then blotted with appropriate antibodies, and signals were detected by enhanced chemiluminescence (ECL kit, Amersham Corp.).

ERK Kinase Assay—Rabbit polyclonal antibody against ERK1 was raised by injection of a purified glutathione S-transferase (GST)-ERK1 fusion protein containing the full-length ERK1 (13). The antibody reacts with both ERK1 and ERK2 in immunoblots, and preferentially binds to ERK1 in immunoprecipitation. For kinase assay, ERK kinase was precipitated by the antibody and protein A-Sepharose 4B beads. The beads were washed twice with HNTG buffer (14), and once with the kinase buffer (20 mM Hepes, pH 7.5, 10 mM MgCl2, 2 mM mercaptoethanol, 1 mM Na3VO4). The assay was performed by mixing the beads with 1 mg/ml myelin basic protein (MBP), 80 μM ATP, 1.5 μCi of [γ-32P]ATP in the kinase buffer and incubated 10 min at 30 °C. After centrifugation, supernatants were spotted on P81 Whatman paper, and the papers were washed five times in 180 mM phosphoric acid and once in 100% ethanol, air-dried, and then counted.

JNK Kinase Assay—JNK kinase activity was measured following heat shock or UV irradiation. Cells were heat-shocked by incubation at 42 °C for 1 h and then recovered at 37 °C for the indicated time periods. For UV irradiation, cells were exposed to different doses of UV light followed by incubation for 1 h at 37 °C. Cells were lysed with the cell lysis buffer and the JNK activity was measured as described previously (15, 16), using GST-c-Jun (amino acids 1–223) as a substrate (a gift of Dr. Melanie H. Cobb). In brief, JNK was extracted from cell lysates by GST-c-Jun immobilized on glutathione-Sepharose beads, washed in washing buffer (phosphate-buffered saline, pH 7.4, 1% Nonidet P-40, 2 mM Na3VO4) and kinase buffer (16). The beads were incubated with 50 μM ATP, 5 μCi of [γ-32P]ATP in the kinase buffer for 20 min at 30 °C. Reactions were terminated by adding 1 × SDS loading buffer and boiling. After electrophoresis, gels were stained by Coomassie Blue, the GST-c-Jun band was cut out, and the incorporation of 32P was measured in a scintillation counter.

decreased was compared as with wild-type and heterozygous mutant cells (Fig. 3). This result suggests that Shp-2 is indeed a positive signal transducer in mitogenic stimulation of cell growth, consistent with previous observations that microinjection of Shp-2 SH2 domains or expression of a catalytically inactive mutant of Shp-2-suppressed mitogenic signaling (8, 17).

To explore the molecular mechanism for the reduced cell growth rate associated with the Shp-2 mutation, we examined growth factor-induced ERK activation in wild-type and mutant cells. Before performing the enzyme activity assay, we first determined the protein levels of ERK kinases and growth factor receptors. The expression of ERK1 and ERK2 was similar in wild-type (+/+ and Shp-2 mutant (−/−) cells and was not changed during the growth factor treatment (Fig. 3). Protein levels of EGF receptor, IGF-I receptor, and PDGF receptor (PDGFR-α) were not altered, while PDGFR-β was down-regulated in Shp-2 mutant cells. Therefore, subsequent experiments were performed with PDGFR-AA that activates PDGFR-α only.

Attenuated ERK Activation by EGF—To compare the cellular response to mitogenic stimulation, wild-type and mutant cells were treated with EGF for different time periods. Cell lysates were made and ERK1 activity was measured by immunoprecipitating the protein with its specific antibody and measuring phosphorylation of MBP. As shown in Fig. 4, EGF stimulated a transient ERK activation in wild-type cells, reaching maximum levels after treatment for 5 and 10

FIG. 1. Isolation of wild-type and Shp-2 mutant fibroblast cell lines. Fibroblast cell lines of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) Shp-2 mutant origins were established as described in the text. Equal amounts of cell lysates from each cell line (40 μg) were separated on 8% SDS-polyacrylamide gel and immunoblotted by a polyclonal antibody raised against amino acid residues 576–593 in the COOH-terminal tail of Shp-2 (Santa Cruz Biotechnology, Inc.).

FIG. 2. The growth rate for Shp-2+/+, Shp-2+/−, and Shp-2−/− embryonic fibroblast cells. Wild-type and mutant cells were seeded in 6-well cell culture plates at the density of 1.2 × 105 cells/well, and incubated in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Cell number was counted upon trypsinization at day 1, 2, 4, and 6 after plating. Results shown were averaged from duplicated plates. We have repeated this experiment at least three times and have obtained reproducible results.

FIG. 3. Immunoblot analysis of ERK kinases. Equal amounts of cell lysates (40 μg) of wild-type (+/+ and Shp-2−/−) cell lines (40 μg of wild-type (+/+ and Shp-2−/−) origins were resolved on SDS-polyacrylamide gel and immunoblotted by anti-ERK antibody. As shown, this antibody recognizes both ERK1 and ERK2 in immunoblotting. Lanes 1, control; 2–4, EGF stimulation for 10, 40, and 90 min; 5–7, IGF-I treatment for 10, 40, and 90 min; 8–10, PDGF treatment for 10, 40, and 90 min. Protein levels of ERK1 and ERK2 were similar in wild-type and mutant cells and were not changed during growth factor treatment.
Although EGF induction of ERK activity was initiated in Shp-2 mutant cells, the maximal level at 10 min was lowered and the activated ERK decayed much faster in comparison to that in wild-type cells.

To corroborate this altered cellular response to EGF in Shp-2 mutant cells, we further examined a number of other cell lines of 1/1, 1/2, and 2/2 origins, each of which was generated from a separate embryo. As shown in Fig. 5, very similar results of EGF-stimulated ERK activity were obtained for wild-type (+/+) and homozygous (2/2) cell lines, indicating that the Shp-2 mutation suppresses mitogenic stimulation by EGF. Although EGF-induced ERK activation was slightly decreased in heterozygotes (+/-), these cells exhibited a more prolonged ERK activation, as compared with that in Shp-2 2/2 cells (Fig. 5). This result might be due to a decreased level of the wild-type Shp-2 protein expressed in heterozygous cells rather than a dominant negative effect of the mutant Shp-2 protein.

The ERK kinases are activated upon phosphorylation of a Thr and a Tyr residue in the Thr-Glu-Tyr motif. Specific antibody against phosphorylated ERK (anti-PY-ERK) detects the...
activated form of ERK kinases by immunoblotting. Using this antibody (New England Biolabs, Inc.), we performed immunoblot analysis to examine the EGF stimulation of ERK activity. As shown in Fig. 6, similar results were obtained for the kinetics of ERK phosphorylation in response to EGF in wild-type and mutant cells, as compared with the data shown in Fig. 4. However, a faster loss of EGF-induced ERK phosphorylation was observed in Shp-2−/− cells. We have also conducted ERK kinase gel shift assays which measure ERK phosphorylation at the Thr and Tyr residues, and have obtained similar results for the EGF-induced ERK activity in wild-type and mutant cells (data not shown). Therefore, consistent results were achieved using three different ERK kinase assays. For the quantitative purpose, we feel that the kinase activity assay measuring phosphorylation of MBP is the method of choice.

Reduced ERK Activation by PDGF-AA—Results described above demonstrate that the Shp-2 mutation attenuates the EGF-stimulated ERK activity. By measuring the ERK activity on MBP as a substrate, we further compared the cellular response to PDGF-AA. Treatment of wild-type cells with PDGF-AA induced a similar profile of ERK activation as EGF, reaching a peak level at 10 min (Fig. 4). As described for the EGF induction, we have also found that ERK activation by PDGF-AA was severely reduced in mutant cells. The enzyme activity reached a peak level after PDGF treatment for 5 min, decreased by 10 min, and returned to a basal level at 20 min.

Block in IGF-I Stimulation of ERK Activity—Like PDGF and EGF, IGF-I induced a reversible ERK activation in wild-type cells, although the maximal stimulation fold was lower, approximately 6-fold instead of 15–20-fold for PDGF and EGF, when assessed using MBP as a substrate in the in vitro assay for ERK activity (Fig. 4). Notably, activation of ERK by IGF-I was completely blocked in Shp-2 mutant cells. This result indicates a requirement of Shp-2 in mediating IGF-I activation of ERK. We have also found that stem cell factor-induced ERK activation was abolished in homozygous Shp-2 mutant embryonic stem cell lines (6).

Altered ERK Activation by PMA—A more persistent ERK activity was induced by PMA in wild-type cells, as compared with growth factors, such as EGF, IGF-I, and PDGF-AA (Fig. 4). Although ERK activation also peaked at 5 and 10 min after PMA treatment, a relatively high level of activity, about 73% of the maximum, was detected at 90 min of stimulation. The initial phase of ERK activation by PMA seems not to be affected in Shp-2 mutant cells. However, PMA failed to sustain ERK activation at a high level in mutant cells. Although it was comparable between wild-type and mutant cells at 2 and 5 min, the ERK activity dropped quickly in mutant cells, to about 60% of maximum at 10 min and 36% at 90 min.

Enhanced JNK Activation by Heat Shock—Cells were heat-shocked at 42 °C for 1 h and allowed to recover at 37 °C for 0, 15, and 45 min. Cell lysates were prepared, and the JNK activity was assayed by GST-c-Jun as a substrate. As shown in Fig. 7, a 3–6-fold increase of JNK activity in wild-type (+/+), heterozygous (+/−), and homozygous (−/−) Shp-2 mutant cells.

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DISCUSSION

In this study, we report three interesting observations. (a) Shp-2 plays differential positive roles in mediating various growth factor-stimulated ERK activation. This SH2-containing protein tyrosine phosphatase is essential for IGF-I induction of ERK activity and is required for a maximal and sustained ERK activation by EGF and PDGF. (b) Although it is not required for PMA induction of ERK, Shp-2 contributes to a persistent activation of ERK by PMA. (c) More importantly, Shp-2 acts as a negative effector in JNK activation by heat shock.

These results suggest that Shp-2 is primarily a positive regulator in mitogenic signaling pathways leading to ERK activation. Although it is not clear how Shp-2 acts to promote mitogenic signals, the contribution of Shp-2 to each pathway is apparently variable, as mutation in the Shp-2 gene either blocked or reduced growth factor stimulation of ERK. This might be one molecular mechanism to define the specificity and redundancy in intracellular signal transduction. Since a sustained ERK activation might be required in some physiological processes (2), a decreased or abortive ERK activation may be enough to disrupt a coordinated cellular response to external stimulation.

PMA is a potent activator of ERK activity in a Ras-independent manner, presumably by activating protein kinase C (18). Noguchi et al. (9) reported that Shp-2 was not involved in mediating PMA stimulation of ERK, by detecting the kinase activity upon PMA treatment for 10 min. By performing a careful time course, we found that Shp-2 is indeed not required for the initial phase in PMA induction, since maximal ERK activation was achieved in mutant cells. However, the kinetics of a sustained ERK activation by PMA was altered in Shp-2 mutant cells, with a much faster decay of elevated ERK activity. These results suggest that Shp-2 might be required for sustained ERK activity, as well as its role in growth factor stimulation of ERK. Although growth factors and PMA can stimulate ERK via separate routes, a common mechanism for sustenance of ERK activity might be shared, which involves Shp-2 action.

The most interesting part of our results is that Shp-2 has opposite roles in mediating ERK and JNK MAP kinase pathways. In contrast to a positive effect in mitogenic stimulation of ERK, this phosphatase appears to be a negative regulator in mediating JNK activation to cellular stress, since JNK activity following heat shock was greatly enhanced in Shp-2 mutant cells. These results would raise an intriguing possibility that Shp-2 functions on a switching point that oppositely modulates ERK and JNK pathways. It is also interesting to note that JNK activation under UV irradiation was not changed in Shp-2 mutant cells. Thus, we would argue that the JNK kinase is activated by heat shock and UV irradiation via different pathways and that Shp-2 participates only in the cellular events mediating the heat shock induction of JNK activity. This is consistent with a previous observation that heat shock and UV irradiation induce JNK activity through distinct different mechanisms (19).

Consistent with a deficiency in mitogenic stimulation of ERK, a decreased growth rate for Shp-2 mutant cells was observed, although mutant cells eventually reached the same saturation density as wild-type cells. In addition, Shp-2 mutant cells exhibited changes in cell spreading, movement and cytoskeletal organization. Further experiments are in progress to determine the exact point(s) of Shp-2 in the Rasraf-MEK-ERK cascade. Recent identification of the putative Shp-2 (Csw) substrates, daughter of sevenless (DOS), and the SIRP family of transmembrane proteins including SHPS-1, also sheds light on the mechanism of Shp-2 function in cell signaling (20–23).

Conflicting data exist in the literature about whether JNK activation promotes cell death or protects cells from apoptosis (2). It will be of great interests to determine the role of Shp-2 in programmed cell death induced under different conditions.

In summary, our results on the opposite activities of Shp-2 in promoting ERK and suppressing JNK activation provide a fresh view on the dynamic regulation or cross-talk of these two MAP kinase pathways.

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