SHP2, an SH2-containing Protein-tyrosine Phosphatase, Positively Regulates Receptor Tyrosine Kinase Signaling by Dephosphorylating and Inactivating the Inhibitor Sprouty*

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Shp2 is a widely expressed protein-tyrosine phosphatase (Shp2) function as a positive effector in receptor tyrosine kinase (RTK) signaling immediately proximal to activated receptors. However, neither its physiological substrate(s) nor its mechanism of action in RTK signaling has been defined. In this study, we demonstrate that Sprouty (Spry) is a possible target of Shp2. Spry acts as a conserved inhibitor of RTK signaling, and tyrosine phosphorylation of Spry is indispensable for its inhibitory activity. Shp2 was able to dephosphorylate fibroblast growth factor receptor-induced phosphotyrosines on Spry both in vivo and in vitro. Shp2-mediated dephosphorylation of Spry resulted in dissociation of Spry from Grb2. Furthermore, Shp2 could reverse the inhibitory effect of Spry on FGF-induced neurite outgrowth and MAP kinase activation. These findings suggest that Shp2 acts as a positive regulator in RTK signaling by dephosphorylating and inactivating Spry.

Receptor tyrosine kinase signaling regulates a wide variety of biological processes in response to extracellular signals, including cellular growth, differentiation and metabolism (1–4). For example, fibroblast growth factor (FGF)1 stimulates the receptor tyrosine kinase activity of the FGF receptor (FGFR), leading to tyrosine phosphorylation of the docking protein FRS2, consequent recruitment of multiple Grb2-Sos complexes, and activation of the Ras-mitogen-activated protein (MAP) kinase signaling pathway (5–7). Precise control of MAP kinase activation is critical for such cellular responses, and several molecules have been identified that serve to regulate the activation of MAP kinase either positively or negatively.

Shp2 is a widely expressed protein-tyrosine phosphatase (PTPase) that seems to play a positive role in the activation of MAP kinase in response to growth factors (8–14). In response to growth factor stimulation, Shp2 becomes tyrosine-phosphorylated and binds to Grb2, where it seems to act as an adaptor protein to recruit the Grb2-Sos complex to the plasma membrane. This, in turn, leads to Ras activation (6, 9, 15–17). However, several studies have shown that mutation of Shp2 in the constitutively active and catalytically inactive mutants of Shp2 (E76A and C459S), Glu-76 and Cys-459 were replaced by Ala and Ser, respectively. These mutants were constructed by PCR-based mutagenesis.

EXPERIMENTAL PROCEDURES

Construction of the Constitutively Active and Catalytically Inactive Mutants of Shp2—The constitutively active and catalytically inactive mutants of Shp2 (E76A and C459S), Glu-76 and Cys-459 were replaced by Ala and Ser, respectively. These mutants were constructed by PCR-based mutagenesis.
Cell Cultures and Transfection—C2C12 cells were cultured in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum. PC12 cells were cultured on poly-L-lysine-coated plates in Dulbecco's modified Eagle's medium supplemented with 0.35% glucose, 10% fetal calf serum, and 5% heat-inactivated horse serum. These cells were split on 35-mm or 60-mm dishes at 2 × 10^5 or 5 × 10^5 cells per dish, respectively. After 19 h, cells were transfected using LipofectAMINE Plus reagent (Invitrogen) or FuGENE 6 reagent (Roche Applied Science) according to the manufacturer's protocol, respectively.

Antibodies, Immunoprecipitation, and Immunoblotting—Antibodies were purchased from the following sources: anti-phospho-ERK from New England Biolabs, anti-Grb2 and anti-Myc from Santa Cruz Biotechnology, anti-phospho-Tyr from Upstate Biotechnology, and anti-HA from Babco. Cells were lysed in a buffer consisting of 20 mM Tris, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM Na₃VO₄, 0.5% Nonidet P-40, 2 mM dithiothreitol, 1 mM Na₄P₂O₇, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 10% glycerol. Cell lysates (20 μl per lane) were subjected to immunoprecipitation and immunoblotting with the indicated antibodies.

Bacterial Expression and Purification of Recombinant Shp2—For bacterial expression, the open reading frame of Shp2 (WT, E76A, or C459S, respectively) was amplified by PCR and subcloned into pGEX6P3 (Amersham Biosciences). Escherichia coli cells transformed with pGEX-Shp2 WT, E76A, or C459S were grown overnight to saturation in 10 ml of LB medium containing 50 μg/ml ampicillin. The cells were grown in 3 liters of LB medium containing 50 μg/ml ampicillin at 37 °C to reach A₆₀₀ = 0.6. One hour after the temperature shift to 25 °C, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and cells were cultured for 12 h. Purification of glutathione S-transferase-Shp2 WT, E76A, or C459S was performed by the method described previously (29).

Luciferase Assay—Cells were treated for 15–24 h with or without 25 ng/ml bFGF, and the luciferase activity in cell lysates was measured by using a luciferase assay system (Promega) in a Berthold Lumat LB 9507 luminometer. Relative luciferase activities were normalized by co-expressed β-galactosidase activities.

Neurite Outgrowth—PC12 cells possessing one or more neurites of a length of more than 1.5-fold the diameter of the cell body were scored as positive. More than 200 cells were scored in each point and the averages from three independent experiments are shown.

RESULTS

Shp2 Promotes Tyrosine Dephosphorylation of Spry in Vivo—To test the hypothesis that Spry is a possible target of Shp2, we first determined the effect of Shp2 on tyrosine phosphorylation of mouse Spry2 (mSpry2). As Shp2 is normally inactive because of auto-inhibition by its N-terminal SH2 domain (30), we used a constitutively active mutant of Shp2 (Shp2 E76A), in which Glu-76 was replaced by Ala. C2C12 cells were co-transfected with FGFR, Myc-mSpry2, and increasing amounts of Shp2 WT or Shp2 E76A. After 24 h, cell lysates were subjected to immunoprecipitation with anti-Myc antibody followed by immunoblotting with anti-pTyr antibody to detect tyrosine phosphorylation levels on mSpry2 protein. As reported previously (27), mSpry2 was tyrosine phosphorylated in an FGFR-dependent manner (Fig. 1, upper panel). The experiments were repeated three times with or without 5 mM vanadate. The reaction mixtures were then immunoblotted with anti-pTyr antibody to detect tyrosine-phosphorylated mSpry2 (WT, E76A, or C459S) for the indicated times with or without 5 mM vanadate. The reaction mixtures were then immunoblotted with anti-pTyr antibody to detect tyrosine-phosphorylated mSpry2 (upper panel) or with anti-Myc antibody to confirm equivalent levels of mSpry2 expression (lower panel). The experiment was repeated three times with similar results.

![Fig. 1. Shp2 dephosphorylates Spry in vivo. C2C12 cells were co-transfected with Myc-mSpry2 (A) or Myc-xSpry1 (B), FGFR, and increasing amounts of HA-Shp2 WT or HA-Shp2 E76A. After 24 h, cell lysates were subjected to immunoprecipitation with anti-Myc antibody and then immunoblotted with anti-pTyr antibody to detect tyrosine-phosphorylated mSpry2 (upper panels), with anti-Myc antibody to confirm equivalent levels of mSpry2 expression (lower panels), or with anti-HA antibody to detect Shp2 (middle panels). The experiment was repeated three times with similar results.](http://www.jbc.org/)

Shp2 E76A also decreased the levels of tyrosine phosphorylation on mSpry2 drastically (Fig. 1, lanes 5 and 6). Next, we tested the effect of Shp2 on Xenopus Spry1 (xSpry1). Similarly to mSpry2, expression of FGFR induced tyrosine phosphorylation on xSpry1 (Fig. 1B, lane 2). Co-expression of Shp2 WT resulted in reduced FGFR-induced tyrosine phosphorylation of xSpry1 in a dose-dependent manner (lanes 3 and 4). Furthermore, co-expression of Shp2 E76A decreased the levels of tyrosine phosphorylation on xSpry1 efficiently (lanes 5 and 6). Taken together, these results suggested that Shp2 promotes tyrosine dephosphorylation of Spry in vivo.

![Fig. 2. Shp2 dephosphorylates Spry in vitro. C2C12 cells were co-transfected with Myc-xSpry1 and FGFR. Myc-xSpry1 was immunoprecipitated with anti-Myc antibody and incubated at 37 °C with 0.1 μg of glutathione S-transferase-Shp2 (WT, E76A, or C459S) for the indicated times with or without 5 mM vanadate. The reaction mixtures were then immunoblotted with anti-pTyr antibody to detect tyrosine-phosphorylated xSpry1 (upper panel) or with anti-Myc antibody to confirm equivalent levels of xSpry1 expression (lower panel). The experiment was repeated three times with similar results.](http://www.jbc.org/)

![Fig. 3. Shp2 induces dissociation of Spry from Grb2. C2C12 cells were co-transfected with Myc-mSpry2 (A) or Myc-xSpry1 (B), FGFR, and increasing amounts of Shp2 WT or Shp2 E76A. After 24 h, cell lysates were subjected to immunoprecipitation with anti-Grb2 antibody and then immunoblotted with anti-Myc antibody (upper panels) or anti-Grb2 antibody (middle panels). To confirm that equivalent levels of mSpry2 (A) or xSpry1 (B) were expressed, aliquots of total lysates were immunoblotted with anti-Myc antibody (lower panels). The experiments were repeated three times with similar results.](http://www.jbc.org/)
Control of Sprouty by Shp2

Fig. 4. Shp2 suppresses the inhibitory activity of Spry on FGF signaling. PC12 cells were co-transfected with Gal4-Elk1, the Gal4-E1b-luciferase reporter and the indicated plasmids, and treated with FGF (25 ng/ml). After 24 h, cells were harvested and assayed for luciferase activity. The relative luciferase activities (means ± S.D.; n = 3) are presented.

Fig. 5. Shp2 and Spry together determine the duration of MAP kinase activation. A, C2C12 cells were co-transfected with the indicated combinations of plasmids and stimulated with FGF (100 ng/ml) for the indicated times. Cell lysates were immobilized with anti-phospho-ERK-specific antibody to monitor MAP kinase activation (p-ERK) or with anti-ERK antibody to confirm equivalent levels of ERK expression (ERK). Cell lysates were also subjected to immunoprecipitation with anti-Myc antibody and then immunoblotted with anti-pTyr antibody to detect tyrosine-phosphorylated xSpry1 (pY-xSpry1). The experiment was repeated three times with similar results. B, the relative MAP kinase activity.

Fig. 6. Effects of Shp2 and Spry on FGF-induced neurite outgrowth. PC12 cells were co-transfected with pEGFP-C1 together with an empty expression vector pCS or the indicated combinations of xSpry1, Shp2 E76A, and Shp2 C459S. After 24 h, the cells were treated with 12 or 25 ng/ml FGF. At 60 h after FGF treatment, the transfected cells were identified by green fluorescent protein fluorescence. The percentage of cells with neurites is shown.

FGF resulted in induction of the reporter (Fig. 4). However, expression of xSpry1 or mSpry2 inhibited induction by FGF. Spry-mediated inhibition of FGF signaling was partially relieved by co-expression of Shp2 E76A but not by Shp2 WT. Taken together, these results suggest that Shp2 acts as a positive regulator in the FGF signaling pathway by dephosphorylating and inactivating the inhibitor Spry.

Shp2 and Spry Function Together to Control the Duration of MAP Kinase Activation—Because Spry controls the duration of MAP kinase activation in a tyrosine phosphorylation-dependent manner (27), we examined the effect of Shp2 on the kinetics of MAP kinase activation in response to FGF stimulation. In C2C12 cells, activation of MAP kinase was detected at 5 min and was maximal at 10 min after FGF stimulation. After this, the activation of MAP kinase decreased gradually (Fig. 5, A and B). When xSpry1 was expressed in cells, tyrosine phosphorylation of xSpry1 peaked at 15 min after FGF treatment, and the activity of MAP kinase consequently decreased at 15–30 min after treatment (Fig. 5, A and B). Co-expression of Shp2 E76A
caused a drastic decrease in FGF-induced tyrosine phosphorylation levels in xSpry1. As a result, activation of MAP kinase persisted for as long as that seen in the absence of xSpry1 expression (Fig. 5, A and B). Expression of catalytically inactive Shp2 C459S failed to block the inhibitory activity of xSpry1 on MAP kinase activation, and indeed was seen to potentiate it slightly (Fig. 5, A and B). Thus, Shp2 is able to counteract the inhibitory effect of xSpry1 on MAP kinase activation.

In PC12 cells, FGF treatment induces neurite outgrowth by means of the activation of MAP kinase (6). Therefore, we examined the effects of Shp2 and xSpry1 on neurite outgrowth in response to FGF stimulation (Fig. 6). As observed previously, expression of xSpry1 blocked neurite outgrowth induced by FGF stimulation in PC12 cells. Co-expression of Shp2 E76A, but not of Shp2 C459S, reversed the inhibitory effect of xSpry1 on neurite outgrowth in response to FGF stimulation. These results are consistent with those obtained above with respect to the effects of Shp2 and xSpry1 on FGF-mediated MAP kinase activation.

**DISCUSSION**

The present studies suggest a mechanism underlying the requirement of Shp2 PTPase activity in MAP kinase activation. Recently, it was reported that the recruitment of Shp2 PTPase to the membrane is required for its stimulation of MAP kinase activation (26). In response to growth factor stimulation, Spry is recruited to the membrane is required for its stimulation of MAP kinase activation by dephosphorylating Spry at the plasma membrane and inactivating the inhibitory activity of Spry. These findings also suggest that the co-operation between Shp2 and Spry controls the duration of MAP kinase activation, which is crucial to determining cell fate.

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