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A Role for the SHP-2 Tyrosine Phosphatase in Nerve Growth Factor-induced PC12 Cell Differentiation

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SHP-1 and SHP-2 are intracellular protein tyrosine phosphatases containing two adjacent src homology 2 domains that target these phosphatases to cell surface receptor signaling complexes and play a role in receptor signal transduction. In this report the PC12 cell system was used to investigate the potential roles of SHP-1 and SHP-2 in the induction of neuronal differentiation by nerve growth factor (NGF). By using neurite outgrowth as a marker for differentiation, the effects of transfected constructs of SHP-1 and SHP-2 were assessed. Overexpression of a catalytically inactive SHP-2, but not a catalytically inactive SHP-1, blocked NGF-stimulated neurite outgrowth. The mitogen-activated protein kinase (MAPK) signaling cascade is important for the morphological differentiation in PC12 cells, and both SHP-1 and SHP-2 have been implicated to act upstream of MAPK in other receptor signaling systems. A positive role for SHP-2 but not SHP-1 in the activation of MAPK by NGF was demonstrated by introduction of the SHP-2 phosphatase mutants along with hemagglutinin-tagged MAPK. Coexpression studies with the SHP-2 mutant along with mutant forms of MAPK kinase suggested that SHP-2 functions upstream of MAPK kinase and MAPK in NGF-induced neurite outgrowth.

INTRODUCTION

The protein tyrosine kinase activity of cell surface receptors such as the nerve growth factor (NGF) receptor plays a critical role in cellular response to growth factors and hormones. Activation of the receptor kinase upon stimulation results in the autophosphorylation of the receptor on tyrosine residues, providing binding sites for other proteins containing Src homology 2 domain (SH2) and protein tyrosine-binding motifs, generating multiprotein signaling complexes at the membrane (Pawson, 1994; van der Geer and Pawson, 1995). A large family of protein tyrosine phosphatases have been identified and have been implicated in modulating the effects of tyrosine kinase activation through alteration of phosphotyrosine-based protein–protein association or through regulation of the catalytic activity of signaling molecules.

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The SH2-containing family of intracellular protein tyrosine phosphatases have been shown to localize to receptor signaling complexes via their SH2 domains (e.g., Kazlauskas *et al.*, 1993; Tomic *et al.*, 1995; Vambutas *et al.*, 1995). SHP-2 (formerly PTP2C, PTP1D, SH-PTP2, and syp; Freeman *et al.*, 1992; Ahmad *et al.*, 1993; Feng *et al.*, 1993; Vogel *et al.*, 1993) is a member of the family that is expressed in virtually all tissues and cell types (reviewed in Neel, 1993). SHP-1 (formerly PTP1C, SH-PTP1, HCP, and SHP; Shen *et al.*, 1991; Matthews *et al.*, 1992; Plutzky *et al.*, 1992; Yi *et al.*, 1992) is a member that is expressed predominantly in hematopoietic cells yet is also expressed from a distinct promoter in other cell types (Banville *et al.*, 1995). SHP-1 and SHP-2 share extensive structural homology with two SH2 domains at the N terminus, followed by a tyrosine phosphatase catalytic domain and a C-terminal tail. The association of SHP-1 and SHP-2 with receptor complexes can induce tyrosine phosphorylation (Feng *et al.*, 1993; Lechleider *et al.*, 1993a; Vogel *et*

al., 1993; Bouchard *et al.*, 1994; Vambutas *et al.*, 1995; Kon-Kozlowski *et al.*, 1996; Krautwald *et al.*, 1996) and activation of the phosphatases (Lechleider *et al.*, 1993b; Vogel *et al.*, 1993; Bennett *et al.*, 1994; Vambutas *et al.*, 1995; Kon-Kozlowski *et al.*, 1996; Krautwald *et al.*, 1996).

Mutations in SHP-1 in the *motheaten* and *motheaten viable* mouse strains illustrate important functions for this phosphatase in multiple signaling pathways in embryonic development and in hematopoiesis (Bignon and Siminovitch, 1994). SHP-1 has been shown to function specifically to down-regulate signaling from cytokine receptors such as the erythropoietin receptor (Klingmuller *et al.*, 1995). Down-regulation of the cytokine-induced JAK-STAT signaling pathway is through a direct dephosphorylation of the JAK kinases (Jiao *et al.*, 1996). SHP-1 also attenuates signaling from the T-cell receptor through dephosphorylation of Zap70 (Plas *et al.*, 1996). In addition, SHP-1 has recently been shown to have a positive role in tyrosine kinase signaling from the colony-stimulating factor-1 receptor in macrophages (Krautwald *et al.*, 1996) and in epidermal growth factor (EGF) signaling in nonhematopoietic cells (Su *et al.*, 1996).

Corkscrew (*csw*), the *Drosophila* homologue of SHP-2, has been shown to play a critical role in the transduction of signals from the tyrosine kinase receptors, torso and *SEVENLESS*, which are required during *Drosophila* development in the induction of tail structures (Perkins *et al.*, 1992) and eye ommatidia (Allard *et al.*, 1996), respectively. In early *Xenopus* development, the process of mesoderm induction by fibroblast growth factor requires functional SHP-2 (Tang *et al.*, 1995). SHP-2 phosphatase activity has been shown to be required to transduce mitogenic signals from the tyrosine kinase growth factor receptors for platelet-derived growth factor (PDGF), EGF, insulin-like growth factor, and insulin in several mammalian tissue culture cell lines (Milarski and Saltiel, 1994; Noguchi *et al.*, 1994; Xiao *et al.*, 1994; Rivard *et al.*, 1995; Zhao *et al.*, 1995b; Bennett *et al.*, 1996; Roche *et al.*, 1996). This positive role of SHP-2 in mitogenesis appears not to generalize to all systems, however; SHP-2 activity was found to be dispensable (Bazenet *et al.*, 1996; Bennett *et al.*, 1996) or even to play a negative role (Reeves *et al.*, 1995) in mitogenic signaling in some cells. SHP-2 also functions as an inhibitor of growth factor–induced membrane ruffling in fibroblasts (Cossette *et al.*, 1996).

Despite of their high degree of structural similarity, SHP-1 and SHP-2 appear to have distinct functional roles in signaling mechanisms. SHP-1 could not complement a SHP-2 defect in the *Xenopus* system (Tang *et al.*, 1995). Conversely, although SHP-2 is also expressed in hematopoietic cell types, it apparently cannot complement for the loss of SHP-1 protein (Bignon and Siminovitch, 1994). The placement of SHP-1 and SHP-2 in known signaling pathways is still uncertain. In general, one or the other (or both) of these phosphatases appears to be required for activation of mitogen-activated protein kinase (MAPK), depending on the receptor (Milarski and Saltiel, 1994; Noguchi *et al.*, 1994; Xiao *et al.*, 1994; Sawada *et al.*, 1995; Tang *et al.*, 1995; Zhao *et al.*, 1995; Bennett *et al.*, 1996; Krautwald *et al.*, 1996; Su *et al.*, 1996). In some cases SHP-2 was required for activation of Ras (Noguchi *et al.*, 1994), but in other cases it appears to act downstream of or on an independent pathway from Ras (Sawada *et al.*, 1995). Through phosphorylation of the C-terminal tail, SHP-2 has been shown to act as an adapter molecule, mediating the association of Grb2 with the PDGF receptor (Li *et al.*, 1994). Yet in response to insulin, SHP-2 is not phosphorylated or bound to Grb2 but is associated with insulin receptor substrate-1 (IRS-1) and functions in the activation of MAPK cascade by insulin via an unknown mechanism that required SHP-2 phosphatase activity (Noguchi *et al.*, 1994). In the one case where it has been examined, SHP-1 appears to function downstream of Ras but upstream of MAPK kinase (MAPKK; Krautwald *et al.*, 1996). It appears that SHP-1 and SHP-2 have multiple roles in receptor signaling that are not all shared by all receptors. In most all cases where it has been examined, the catalytic activity of the phosphatases was required for signal transduction. Several substrates for SHP-2 have now been identified, including Src (Peng and Cartwright, 1995), DOS (Herbst *et al.*, 1996; Raabe *et al.*, 1996), and SHPS-1 (Fujioka *et al.*, 1996). Known SHP-1 substrates include the JAK2 (Jiao *et al.*, 1996), ZAP70 (Plas *et al.*, 1996) kinases, and the EGF receptor (Tenev *et al.*, 1997).

The PC12 cell line is one of few mammalian systems in which differentiation can be induced in vitro by activation of a tyrosine kinase growth factor receptor. PC12 cells will differentiate into sympathetic neurons in response to NGF (Greene and Tischler, 1976). Both SHP-1 and SHP-2 are endogenously expressed in PC12 cells (Vambutas *et al.*, 1995). SHP-1 has been directly implicated to function in NGF signaling because it is tyrosine phosphorylated and activated by NGF stimulation of PC12 cells (Vambutas *et al.*, 1995). The induction of neurite outgrowth PC12 cells has been shown to require the activities of Src, Ras (Kremer *et al.*, 1991), MAPKK (Cowley *et al.*, 1994), and MAPK (Fukuda *et al.*, 1995).

To investigate the possible roles the SHP-1 and the SHP-2 phosphatases play in a mammalian differentiation system, the activities of SHP-1 and SHP-2 were altered in PC12 cells, and the effects on neurite outgrowth in response to NGF were assessed. We determined that SHP-2 activity played a positive role in the induction of neurite outgrowth by NGF. In contrast, SHP-1 activity was not required for the differentiation. Cotransfection experiments with mutant forms of

MAPKK and with hemagglutinin (HA)-tagged MAPK revealed that SHP-2 functioned in the activation of MAPK in NGF receptor signaling in PC12 cells and that this may be a critical pathway that is blocked in the NGF-induced morphological differentiation.

MATERIALS AND METHODS

Cell Culture

PC12 cells were obtained from the laboratory of M. Bothwell (University of Washington). Cell stocks were maintained in DMEM with 10% horse serum and 5% fetal calf serum at 37°C and in an atmosphere of 5% CO_2.

DNA Constructs

The human SHP-1 (Shen *et al.*, 1991) and SHP-2 (Ahmad *et al.*, 1993) were subcloned into the Rc-cytomegalovirus (CMV) mammalian expression vector (Invitrogen, San Diego, CA) under the control of a CMV promoter. Point mutations converting the catalytic cysteine to a serine were generated in both phosphatase genes as described previously (Bouchard *et al.*, 1994; Zhao *et al.*, 1995b). This mutation has been shown to render the phosphatase catalytically inactive (Guan and Dixon, 1991). HA-tagged versions of the phosphatases were generated by subcloning both the wild-type and mutant forms of SHP-1 and SHP-2 into a modified pBluescript vector generated by Benjamin Tubb in the laboratory of Thomas Kirchhausen (Harvard Medical School). This subcloning resulted in the introduction of an HA-tag on the C termini of each of the four proteins. In the SHP-1 constructs, 9 amino acids were removed and replaced with the 13-amino acid HA tag. For the SHP-2 constructs, only three amino acids were eliminated in the replacement. The HA-tagged versions of the four proteins were then cloned into pcDNA3 for mammalian cell expression (Invitrogen; a later version of Rc-CMV).

The constitutively active MAPKK1a mutant was generated by changing serines 218 and 222, to glutamic acids (MAPKK SESE) as described previously (Seger *et al.*, 1994). The dominant negative MAPKK1a mutants were generated by changing serine 222 to alanine (MAPKK SA) or lysine 97 to alanine (MAPKK KA) as described (Seger *et al.*, 1994). The MAPKK1 mutants were expressed using either pcDNA1 or pcDNA3 (Invitrogen). HA-tagged ERK1 (Meloche *et al.*, 1992) was obtained from the laboratory of Dr. Neil Nathanson (University of Washington).

Transfections

PC12 cells were transfected using Lipofectamine (Life Technologies, Gaithersburg, MD) according to the specifications of the supplier. DNA constructs containing wild-type or mutant versions of the *SHP-2*, *SHP-1*, or *MAPKK1a* genes were introduced in a 5:1 ratio with a plasmid containing a CMV promoter-driven β-galactosidase (*β-gal*) gene (Chamberlain *et al.*, 1994), which served as a marker for transfection. Ten micrograms of the SHP constructs and 2 μg of CMV-β-gal were used per 10-cm dish of cells. In the cotransfection experiments with MAPKK mutants, 10 μg of each SHP and MAPKK construct were transfected along with 2 μg of CMV-β-gal for the differentiation assays or 2 μg of HA-ERK1 for the MAPK assays.

Differentiation Assays

Twenty-four hours after transfection, cells were diluted and transferred to plates coated with substrate and stimulated with 50 ng/ml NGF (2.5S, Promega, Madison, WI). Plates were coated with substrate by incubation with 20 μg/ml polylysine (Sigma, St. Louis, MO), dried, and then incubated with medium containing 15% serum (10% horse serum and 5% fetal calf serum). Medium and NGF were replenished every 48 h. After 72 h in the presence of NGF, cells were fixed with gluteraldehyde and formaldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal, Life Technologies), which generates a blue color in cells that contain the transfected β-gal DNA. Blue cells were scored for neurite length, and uncolored cells were also scored as an untransfected control in each experiment. A cell was judged to be differentiated if it had at least one neurite that was greater than two cell bodies in diameters. Two hundred cells were scored per transfection by two people in a double-blind format.

Immunoprecipitation and Western Blotting

Cellular extracts were made from transfected cells at various times after transfection in lysis buffer (50 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotnin, 10 μg/ml leupeptin). Extracts were centrifuged, and supernatants were incubated with anti-HA monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) and protein A-Sepharose beads (Sigma). Immunoprecipitates were washed in lysis buffer and boiled in SDS sample buffer, and proteins were separated by SDS-PAGE. Proteins in the gel were transferred to Immobilon membrane (Millipore, Bedford, MA) and probed with a anti-HA polyclonal antibody (Babco, Richmond, CA). Antigens were detected with an ECL chemiluminescent system (Dupont-New England Nuclear, Boston, MA). The relative amount of antigen was quantified by scanning densitometry (Bio-Rad, Hercules, CA). Levels of overexpression were estimated by densitometric quantification of blots containing a serial dilution of crude extracts along with quantitative immunoprecipitations of HA-tagged SHP1, SHP2, and MAPKK1 with anti-HA (Boehringer Mannheim) from transfected cells 48 h after transfection. Blots were probed with either polyclonal anti-SHP-1 (serum 237, raised in rabbit against *Escherichia coli*-produced SHP-1); polyclonal anti-SHP-2 (serum 1263, raised in rabbit against *E. coli*-expressed SHP-2), or monoclonal anti-MAPKK1 (no. M17020, Transduction Labs, Lexington, KY). The percentage of transfection efficiency was calculated these experiments by X-gal staining for the activity of transfected β-gal (an equivalent amount of CMV-β-gal plasmid was used as for the HA-tagged constructs).

MAPK Assays

At 48 h after transfection, extracts were prepared from transfected cells with and without a 3-min NGF stimulation in 50 mM β-glycerol phosphate, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 1% Triton X-100, 1 mM EGTA, pH 7.5, 10 μg/ml leupeptin, 10 μg/ml aprotnin, 2 μg/ml pepstatin A, and 1 mM benzamidine and 1 mM [4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride] (AEBSF) as protease inhibitors. Equivalent amounts of protein extract were immunoprecipitated with anti-HA monoclonal antibody (Boehringer Mannheim) and protein A-Sepharose beads (Sigma). Immunoprecipitates were washed in lysis buffer and assayed for MAPK activity by using myelin basic protein (Life Technologies) as a substrate in the presence of 25 mM β-glycerol phosphate, pH 7.3, 1.25 mM EGTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.15 mM Na_3VO_4, 2 mM protein kinase A inhibitor (PKI) peptide, 10 mM Calmidizolium, 1 mg/ml bovine serum albumin, and 100 μM ATP ([γ-^{32}P]ATP used for detection; New England Nuclear, 700 cpm/pmol). All samples were analyzed in duplicate, and endogenous MAPK activation in the untransfected cells was measured as a control for stimulation for each sample. The fold stimulation of the HA-extracellular regulated kinase (ERK) was tabulated instead of absolute activity to account for experiment to experiment variability in expression levels and transfection efficiency.

RESULTS

Wild-type or catalytically inactive forms of the human SHP-1 or SHP-2 genes were transiently transfected

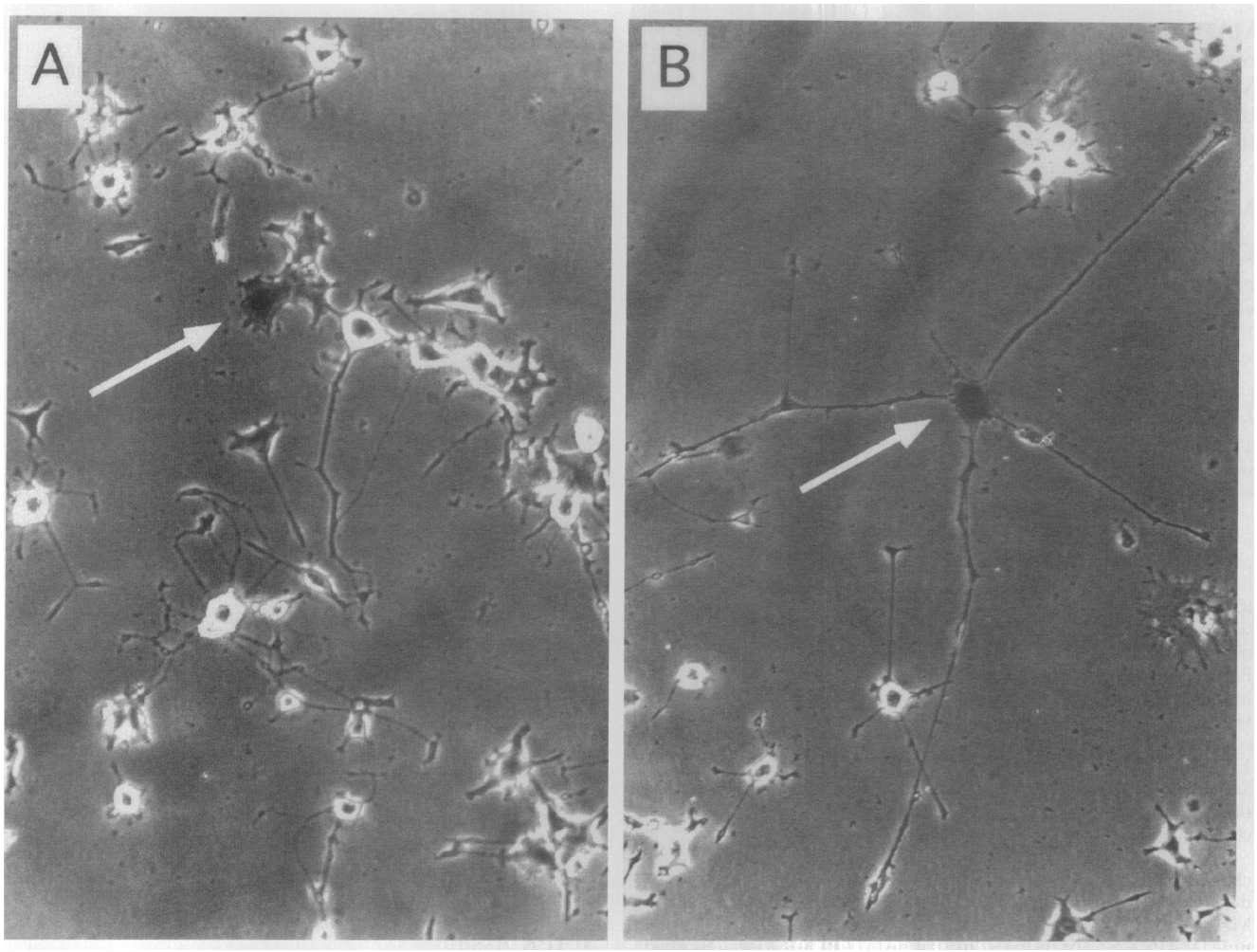

Figure 1. Examples of X-gal-stained transfected cells without neurites (A) and with long neurites (B). The criteria for scoring was that a cell was considered differentiated if it contained one or more neurite with a length greater that two cell bodies in diameters.

into PC12 cells. Previous studies have shown that when overexpressed in cells, catalytically inactive forms of SHP-1 or SHP-2 function as dominant negative mutants, interfering with the function of the endogenous enzymes (Zhao *et al.*, 1995b; Su *et al.*, 1996). After transfection, cells were stimulated with NGF and assayed for differentiation by using neurite outgrowth as a morphological marker (see MATERIALS AND METHODS for details). X-gal staining of cells for β-gal protein encoded by a cotransfected *lacZ* gene served as a marker for transfection. Examples of transfected cells stained with X-gal are shown in Figure 1 to illustrate the assay. An example of a cell scored negative for differentiation is shown in Figure 1A, and a cell scored positive is shown in Figure 1B.

To verify expression of exogenous copies of the mutant and wild-type forms of the phosphatases, extracts from the transfected cells were subject to Western blot analysis with an antibody against HA-tagged forms of the four proteins. Expression time courses of the transfected constructs are shown in Figure 2A. Expression of all four proteins can be seen from 24 h to 96 h after transfection. SHP-1 and SHP-2 phosphatases are both endogenously expressed in PC12 cells. The relative level of overexpression of the exogenous proteins as compared with the endogenous protein levels was assessed by Western blot analysis with polyclonal anti-SHP-1 and anti-SHP-2 antisera (Figure 2B). By using this method, the level of overexpression was estimated to be about twofold for the SHP-2 and threefold for the SHP-1 proteins in the transfected cells, with SHP-1 constructs being expressed at a consistently higher level than the SHP-2 constructs.

The percentage of cells that differentiated in the presence of the exogenous forms of the phosphatases was quantified by counting the number of transfected cells from each plate and scoring for neurite length. For each experiment, a mock transfection with the

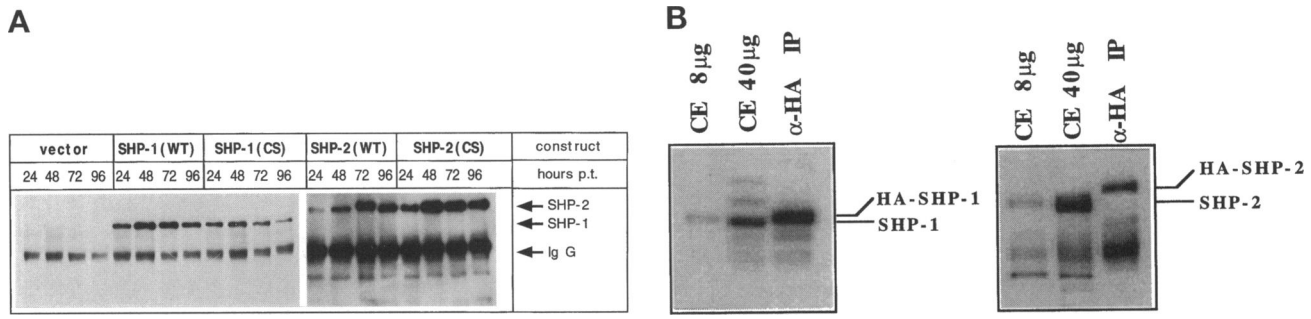

Figure 2. (A) Expression time courses of HA-tagged SHP-1 and SHP-2 constructs. Shown is a Western blot of exogenous phosphatase proteins immunoprecipitated from transfected cell extracts with an anti-HA monoclonal antibody, probed with an anti-HA polyclonal antiserum. Lanes are labeled from left to right as follows: Vector, a transfection with Rc/CMV; SHP1-WT, wild-type SHP-1; SHP1-CS, the dominant negative SHP-1 mutant; SHP2-WT, wild-type SHP-2; SHP2-CS, the dominant negative SHP-2 mutant. Extracts were prepared at 24, 48, 72, and 96 hours-post-transaction (hours p.t.). Arrows on the right indicate the SHP-1 and SHP-2 protein bands and the anti-HA IgG heavy chain band. A longer exposure of the SHP-2 blot compared with the SHP-1 blot is shown. Expression levels of the SHP-1 constructs appeared to be about threefold higher than that of the SHP-2 constructs as quantified by scanning densitometry of comparable exposures. The reason for this difference is unknown. All four proteins are detectable from 24 to 96 h after transfection (throughout the time course of the differentiation assay). (B) Expression levels of HA-tagged SHP-1 and SHP-2 proteins relative to levels of endogenous SHP-1 and SHP-2. Shown are Western blots of exogenous phosphatase proteins immunoprecipitated from 250 μg of transfected cell extracts with an anti-HA monoclonal antibody (α-HA IP) and probed with anti-SHP-1 (left) or anti-SHP-2 (right). Total cell extract (CE) was loaded in 8- and 40-μg aliquots for detection of endogenous SHP-1 and SHP-2. The level of overexpression in transfected cells was calculated by dividing the amount of HA-tagged antigen by the transfection frequency and then comparison of that value with amount of antigen in total CE. Transfection efficiency was estimated by cotransfection with an equivalent amount of plasmid encoding β-gal.

empty vector was included as a control. The graph shown in Figure 3 illustrates the percentage of cells with long neurites from the different transfections, along with untransfected controls. When the catalytically inactive form of SHP-2 was introduced into cells, it reduced the percentage of transfected cells with long neurites by approximately 50%. This reduction in the number of cells with neurites with SHP-2 was seen in seven independent experiments and was quantitatively similar to the reduction observed with a dominant negative MAPKK (Figure 3, discussed in the following section). The vector control and the wild-type SHP-2 demonstrated rates of differentiation not statistically different from the untransfected cells (Figure 3). Overexpression of wild-type and catalytically inactive form of SHP-1 also did not significantly affect the percentage of transfected cells that developed long neurites (Figure 3). These data indicate that SHP-2, but not SHP-1, activity functioned in the NGF-induced morphological differentiation.

Overexpressed SH2 domains could potentially disrupt signaling modules nonspecifically by competing for binding sites that are lower affinity for that particular SH2 domain and displacing other proteins that are normally bound. Overexpression of a catalytically inactive phosphatase could trap inappropriate substrates or mask important phosphotyrosine residues. The phenotype generated by the SHP-2 mutant protein appeared specific in that 1) transfections with wild-type SHP-1 and SHP-2 controlled for effects of overexpression of SH2 domains and tyrosine phosphatase domains and 2) the mutant form of SHP-1 controlled for overexpression of a receptor-associated catalytically inactive phosphatase. None of these control transfections had any significant effect on neurite outgrowth (Figure 3) even though their expression level was comparable to the SHP-2 mutant or for the SHP-1 proteins up to threefold higher (see Figure 2 legend).

The MAPK signaling cascade is activated by NGF stimulation of PC12 cells (Gomez *et al.*, 1990) and has been shown to be important for the differentiation response (Cowley *et al.*, 1994; Fukuda *et al.*, 1995). An inactivating form of MAPKK, the kinase just upstream of MAPK, blocked NGF-induced PC12 cell differentiation to the same extent as the SHP-2 mutant (Figures 3 and 4, compare lanes marked MKK SA and SHP-2 CS). To test whether SHP-2 and MAPKK functioned in the same pathway downstream of the NGF receptor, experiments were done wherein the dominant negative mutant of SHP-2 was cotransfected with a dominant negative mutant of MAPKK. If the SHP-2 phosphatase and the MAPKK kinase lie on the same pathway, no further reduction should be seen when both activities are blocked. As shown in Figure 4, the block to differentiation when the dominant negative mutant forms of SHP-2 and MAPKK were coexpressed was quantitatively similar to the block with either mutant alone, consistent with the hypothesis that they function in the same pathway.

When a constitutively active form of MAPKK is introduced into PC12 cells, it can induce neurite outgrowth, even in the absence of NGF (Cowley *et al.*, 1994). In our hands, an activated form of MAPKK not

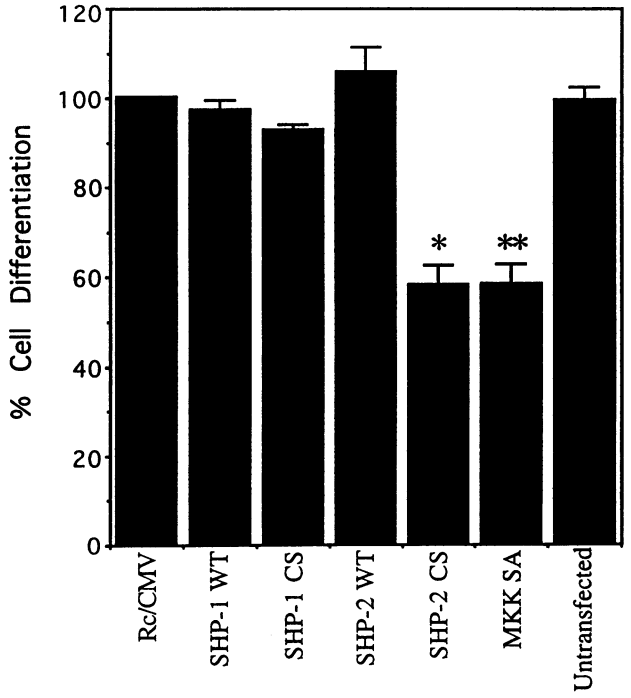

Figure 3. Effects of dominant-negative mutant forms of SHP-1 and SHP-2 on the number of NGF-stimulated transfected cells with long neurites. Cells were transfected with various constructs and assayed for differentiation with NGF. Bars: Rc/CMV, vector control; SHP-1 WT, wild-type SHP-1; SHP-1 CS, dominant-negative SHP-1 mutant; SHP-2 WT, wild-type SHP-2; SHP-2 CS, dominant-negative SHP-2 mutant; MKK-SA, dominant-negative MAPKK mutant; Untransfected, unstained cells from the same plates. Within each experiment, data for individual transfections were normalized to the vector control. The values are plotted as the means ± SE from three to seven experiments. *, $p < 0.0001$; **, $p < 0.0001$.

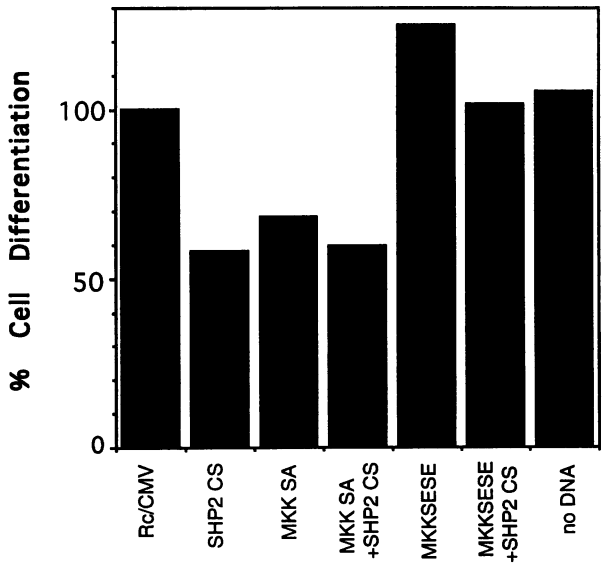

Figure 4. Effects of cotransfection of mutant forms of MAPKK and SHP-2 on PC12 cell differentiation. Bars: Rc-CMV, vector control; SHP-2 CS, dominant-negative SHP-2 mutant; MKK-SA, dominant-negative MAPKK mutant; MKK-SA + SHP-2 CS, cotransfection of the dominant-negative mutants forms of MAPKK and SHP-2 together; MKK SESE, constitutively active mutant of MAPKK; SHP-2 CS + MKK SESE, dominant-negative SHP-2 mutant cotransfected with the MAPKK constitutively active mutant; no DNA, untransfected control. The data shown are from one representative experiment. Similar results were obtained in two other experiments.

only induced neurite outgrowth in the absence of NGF but also accelerated the neurite outgrowth stimulated by NGF (Figure 4). Because these data were collected at a specific time point when not all cells have fully extended neurites (72 h in NGF), active MAPKK was observed to increase the percentage of differentiated cells (Figure 4, compare Rc/CMV with MKK SESE). To assess whether the block to differentiation observed with the SHP-2 mutant occurred upstream or downstream of MAPKK in the signaling pathway, we determined the effects of cotransfection of the constitutively active MAPKK gene along with the SHP-2 mutant. Coexpression of the activated form of MAPKK along with the SHP-2 dominant-negative mutant restored neurite outgrowth to levels comparable to the vector control (Figure 4). However, the increase in cells with long neurites seen with the activated MAPKK alone as compared with the vector control was no longer apparent in the presence of the SHP-2 mutant. This suggests that perhaps SHP-2 is not strictly upstream of MAPKK or that MAPKK is not completely sufficient for the differentiation at the levels expressed in these experiments. Thus, the data in Figure 4 suggest that SHP-2 is functioning, at least in part, upstream of MAPKK in the same signaling pathway downstream of the NGF receptor.

Both SHP-1 and SHP-2 have been shown to function in the activation of the MAPK isoforms ERK1 and ERK2 in other systems (see INTRODUCTION). To directly test the effects of the SHP-1 or SHP-2 phosphatase mutant proteins on the activation of MAPK in PC12 cells, cotransfection experiments were performed by using a gene encoding HA-tagged ERK1. Extracts were prepared from transfected cells in the presence and absence of stimulation with NGF. HA-ERK was immunoprecipitated from the extracts and assayed for activity with myelin basic protein as a substrate. Cotransfection of HA-ERK1 with a vector control plasmid was used as a positive control for stimulation in each experiment. In addition, cotransfection with a dominant negative MAPKK was used as a positive control for inhibition of the activation. As shown in Figure 5, the SHP-2 mutant significantly reduced ERK1 activation by NGF. The effects of the SHP-2 mutant on exogenous ERK1 were reproducible and statistically different than the vector control (Figure 5 legend). In contrast, the SHP-1 mutant construct did not affect or perhaps even slightly enhanced the

activation of HA-ERK1. These data indicate that although SHP-1 was found to act positively upstream of MAPK in other cell systems (Krautwald *et al.*, 1996; Su *et al.*, 1996), SHP-1 activity was apparently not required for the activation of MAPK in PC12 cells. Surprisingly, the wild-type SHP-1 protein interfered with the cotransfected HA-ERK1 activation when overexpressed (Figure 5). Unlike inhibition seen with the SHP-2 and MAPKK mutants, inhibition by wild-type SHP-1 was not observed consistently in all experiments. Given this caveat, these data suggest a possible negative role for SHP-1 in NGF signaling to MAPK.

DISCUSSION

In this report, we give evidence that the catalytic activity of the SHP-2 tyrosine phosphatase is required for the morphological differentiation of PC12 cells in response to stimulation with NGF. Introduction of a catalytically inactive mutant form of SHP-2 significantly blocked neurite outgrowth (Figures 3 and 4). In contrast, introduction of a catalytically inactive mutant of SHP-1 had no effect on induction of the morphological change (Figure 3). Thus, the cellular response to the NGF stimulation appeared to require SHP-2 to dephosphorylate an important substrate(s) not shared with SHP-1. The SHP-2 mutant protein was expressed throughout the time course of NGF stimulation (Figure 2A) so that essential functions of SHP-2 could be in early NGF signal transduction as well as later, acting directly in the process of neurite outgrowth. We present data indicating an important role for this phosphatase in early NGF signaling in the activation of MAPK (Figures 4 and 5). In support of an additional late function, SHP-2 has recently been shown to effect the phosphorylation of two proteins involved in cell adhesion (Ouwens *et al.*, 1996), which is an important component in the process of neurite extension. In addition, the role of SHP-2 in regulating growth factor-induced membrane ruffling (Cossette *et al.*, 1996) suggests a role for this phosphatase in cytoskeletal regulation, also intimately involved in neurite extension.

Activation of both MAPKK and MAPK has previously been shown to be required for NGF-induced neurite outgrowth in PC12 cells (Cowley *et al.*, 1994; Fukuda *et al.*, 1995). In *Xenopus* embryogenesis, as well as in several mammalian tissue culture systems, SHP-2 activity is required for MAPK activation (see INTRODUCTION). We suggest that SHP-2 also functions upstream of MAPKK in PC12 cells based on the facts that 1) no additive effect on inhibition of neurite outgrowth was observed when the activities of both the phosphatase and the kinase were blocked in cotransfection experiment with both dominant negative mutants and 2) neurite outgrowth could be restored to control levels in the presence of the mutant SHP-2 by an activating mutant of MAPKK (Figure 4). The level of overexpression of both the phosphatase mutant and the MAPKK mutants was moderate (less than twofold for both over endogenous enzyme levels; Figure 2B and our unpublished results), suggesting that these results were valid as "pseudoepistasis" tests in that exogenous proteins were being expressed at roughly physiological levels. Although the activated MAPKK could restore SHP-2 mutant cells to control levels of neurite outgrowth, the frequency of neurite outgrowth was still higher in the cells transfected with the activated MAPKK construct alone (Figure 4). This suggests, but does not prove, that SHP-2 may not be strictly upstream of MAPKK and may have an additional function in neurite outgrowth either downstream of, on a parallel pathway to, MAPKK.

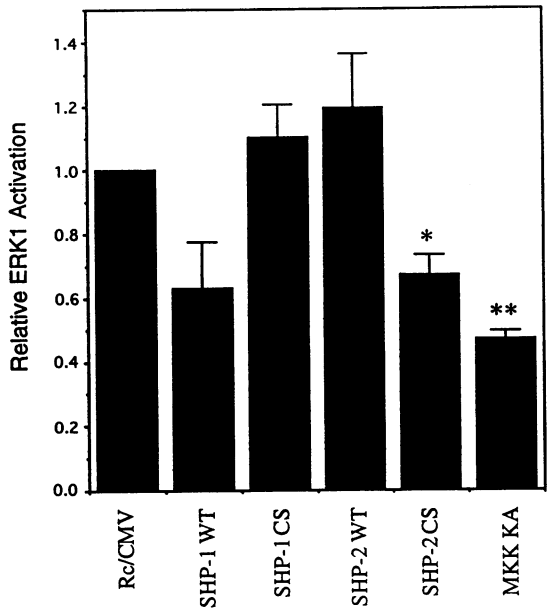

Figure 5. Effect of cotransfected SHP phosphatase constructs on HA-ERK1 activation by NGF. HA-ERK1 was immunoprecipitated from transfected PC12 extracts before and after stimulation with 50 ng/ml NGF for 5 min. Kinase assays were performed with myelin basic protein as a substrate. Fold stimulation of HA-ERK1 was calculated and normalized to the Rc/CMV vector control sample in each experiment. Shown are the relative stimulations (mean ± SE) from three to five experiments. Bars: Rc/CMV, vector control (set at 100%); SHP-1 WT, wild-type SHP-1 phosphatase gene; SHP-1 CS, dominant negative SHP-1 mutant; SHP-2 WT, wild-type SHP-2 phosphatase gene; SHP-2 CS, dominant negative SHP-2 mutant; MKK KA, dominant negative MAPKK mutant. *, $p = 0.01$; **, $p < 0.0001$.

Depending on the cell system and the receptor, both SHP-1 and SHP-2 phosphatase activities have been shown to be required for MAPK activation (Milarski and Saltiel, 1994; Noguchi *et al.*, 1994; Tang *et al.*, 1995; Zhao *et al.*, 1995b; Bennett *et al.*, 1996; Krautwald *et al.*, 1996; Su *et al.*, 1996). Both of these phosphatases are expressed in PC12 cells (Vambutas *et al.*, 1995), yet

only SHP-2 activity appeared to have a positive role in the activation of MAPK by NGF when tested directly by cotransfection of SHP mutants along with HA-ERK1 (Figure 5). This is in contrast to the embryonic carcinoma line 293, where introduction of either an SHP-1 or an SHP-2 mutant protein interferes with MAPK activation by EGF (Zhao *et al.*, 1995b; Su *et al.*, 1996). The effects of the SHP-2 mutant phosphatase on the activation of the cotransfected HA-ERK1 were consistent with our model that SHP-2 functions upstream of MAPKK in NGF-induced neurite outgrowth in PC12 cells.

The fact that SHP-1 is tyrosine phosphorylated and activated by NGF in PC12 cells suggests that this phosphatase is also playing some role in early NGF signaling (Vambutas *et al.*, 1995). Although we found no inhibitory effect of the inactive SHP-1 mutant on cotransfected HA-ERK1 activation or on the induction of neurite outgrowth, we did observe an attenuation of cotransfected HA-ERK1 activation with the wild-type SHP-1 protein, suggesting a possible negative role for this phosphatase in NGF signaling to MAPK. This negative role was not observed at the end point of neurite outgrowth, however (Figure 3). Even when both assays were performed in parallel on cells from the same transfection, overexpression of wild-type SHP-1 protein partially inhibited HA-ERK1 activation without significantly affecting neurite outgrowth. This discrepancy was unexpected considering data from us and others indicating an essential role for MAPKK/MAPK activation in the induction of neurite outgrowth. Either the HA-ERK cotransfection assay lacks the sensitivity needed to represent quantitative differences in endogenous MAPK or neurite outgrowth is not affected by the partial inhibition of early MAPK signaling. The fold stimulation of the HA-ERK1 in our cotransfection assay was low as compared with the fold stimulation of the endogenous MAPK from the same cells, likely due to the limited amount of HA-ERK obtainable from the ~5% of cells that were transfected. Perhaps the active SHP-1 dephosphorylated the HA-ERK1, bringing the activation below detectable levels in some experiments, while effects on endogenous MAPK were not significant enough to effect the subsequent neurite outgrowth. SHP-1 was expressed in this system at a consistently higher level than SHP-2 as well as being up to 15-fold more active as a tyrosine phosphatase than SHP-2 in vitro (Zhao *et al.*, 1995a). This could explain why SHP-2 wild-type did not give the same effect. An alternative explanation is that overexpressed wild-type SHP-1 only blocked MAPK activation at the very early time point (5 min), allowing essential signaling to occur later in the 72 h time course of the differentiation assay. This second alternative could not be addressed experimentally as, unlike endogenous MAPK, transfected HA-ERK1 activity was no longer detectable after 30 min of NGF stimulation. Our results also do not exclude a role for SHP-1 in other aspects of NGF-induced neuronal differentiation such as the induction of neuronal markers that are Ras independent (D'Arcangelo and Halegoua, 1993).

Although we saw a very reproducible inhibition of neurite outgrowth with the SHP-2 mutant phosphatase, the differentiation frequency was only reduced by about 50% (Figure 3). We also observed a ~50% reduction with the MAPKK dominant negative construct (Figures 3 and 4). A likely explanation for the incomplete inhibition we observed is that a small amount of residual phosphatase or kinase activity remained and was sufficient to transduce the signal in some cells. As compared with other cells systems more amenable to transient transfection, the level of overexpression we obtained in PC12 cells was very modest (estimated to be between 1.7- and 2.7-fold), and at these levels is not predicted to fully block the activity of the endogenous phosphatase (Bennett *et al.*, 1996). Alternatively, there may exist another pathway for differentiation in PC12 cells that does not require the activities of SHP-2 and MAPKK. In another report, microinjection of MAPKK dominant negative mutant constructs into PC12 cells only blocked NGF-induced differentiation in about 50% of the injected cells (Cowley *et al.*, 1994). Moreover, another study suggests that the pathway that leads to activation of the MAPK cascade is not sufficient to induce neurite outgrowth and that other signaling pathways are involved (Vaillancourt *et al.*, 1995).

The fact that SHP-2 appears to function in PC12 cells to induce the activation of the MAPK cascade (Figure 5) suggests that is does play an important role in early NGF signaling. Although SHP-2 might conceivably mediate activation of MAPK by acting as an adapter protein (Bennett *et al.*, 1994; Li *et al.*, 1994), it does not appear to be tyrosine phosphorylated in response to NGF (Vambutas *et al.*, 1995), and the mutant SHP-2 should have been as capable as the wild-type SHP-2 as an adapter. Similar to the scenario in insulin signaling (Noguchi *et al.*, 1994), SHP-2 must have an additional function in the induction of differentiation (and in activation of MAPK) that requires phosphatase activity.

The recent findings that SHP-2 interacts with Src and can dephosphorylate the C-terminal tyrosine that regulates Src tyrosine kinase activity suggest that Src could be an important substrate in PC12 cells (Peng and Cartwright, 1995). It has been shown that Src function is required for NGF-mediated differentiation in PC12 cells (Kremer *et al.*, 1991). In addition, an activated form of Src can induce the differentiation in the absence of NGF stimulation (Alema *et al.*, 1985). One model for how SHP-2 and Src may function in early NGF signaling is that SHP-2 serves to activate Src, which in turn phosphorylates Shc (McGlade *et al.*,

1992), allowing activation of the Grb2-Sos-Ras complex. Unlike other receptors, Grb2 does not bind directly to the NGF receptor and thus may require binding to Shc to be activated (Hashimoto *et al.*, 1994). Another possible mechanism involving Src as the relevant SHP-2 substrate is that SHP-2-activated Src phosphorylates and activates the MAPKK kinase, Raf (Marais *et al.*, 1995). Possible alternative pathways are suggested with the recent discovery of several SHP-2-specific substrates (Zhao *et al.*, 1995b; Fujioka *et al.*, 1996; Herbst *et al.*, 1996; Raabe *et al.*, 1996).

We have demonstrated a role for SHP-2, but not SHP-1, phosphatase activity in the process of neurite outgrowth in PC12 cells as induced by NGF receptor stimulation. We also have data consistent with the hypothesis that MAPKK functions downstream of SHP-2 in an NGF-mediated signal transduction pathway. In future experiments, we plan to exploit this system to better understand the interactions of the various proteins that have been shown to play a role in the NGF-induced differentiation in PC12 cells. By performing pseudoepistasis tests through cotransfection of multiple proteins known to function either positively or negatively in the differentiation process, we hope to elucidate the order of events in NGF signal transduction pathways.

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