Coordinate Regulation of STAT Signaling and c-fos Expression by the Tyrosine Phosphatase SHP-2*

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The src homology 2 (SH2) domain-containing protein-tyrosine phosphatase SHP-2 has been implicated as an important positive regulator of several mitogenic signaling pathways. SHP-2 has more recently been shown to be tyrosine phosphorylated and recruited to the gp130 component of the ciliary neurotrophic factor (CNTF) receptor complex upon stimulation with CNTF. CNTF does not, however, have a proliferative effect on response cells, but rather enhances the survival and differentiation of sympathetic, motor, and sensory neurons. In this study, expression of an interfering mutant of SHP-2 in the neuroblastoma cell line NBFL increased CNTF induction of a vasoactive intestinal peptide (VIP) reporter gene, and in cultures of sympathetic neurons, it resulted in an up-regulation of endogenous VIP and substance P (SP) gene expression. Members of the CNTF family of cytokines transmit their signal by activating associated JAK receptors, which in turn phosphorylate the receptor subunits, gp130 and LIFRβ, that lack any obvious catalytic motif in their cytoplasmic domains but are constitutively associated with protein-tyrosine kinases of the JAK (Janus kinase) family. Ligand-induced heterodimerization of gp130 and LIFRβ leads to tyrosine phosphorylation and activation of the associated JAKs, which in turn phosphorylate the β receptor subunits and downstream signaling molecules (8, 9). The phosphotyrosine residues on the β receptor subunits provide binding sites for SH2 domain-containing signaling molecules, and mutational analysis of the cytoplasmic domain of gp130 has identified the SH2 domain docking sites for Stat3 and SHP-2 (10).

Stat3 is a member of a family of transcription factors that, when activated by tyrosine phosphorylation, homo- or heterodimerize and translocate to the nucleus to direct transcriptional responses (see Ref. 11 for review). Many of the genes that are coordinately regulated by CNTF, including choline acetyltransferase (ChAT), VIP, and SP, contain consensus STAT-binding sites (12). In particular, within the 5′ end of the VIP gene is a 180-base pair cytokine response element (CyRE) that binds a complex composed of Stat1α and Stat3 and mediates CNTF-stimulated VIP expression (12).

SHP-2 (SHPTP2, PTP1D, PTP2C, or Syp) is a protein-tyrosine phosphatase that contains a C-terminal catalytic domain and a tandem array of two SH2 domains at its N terminus (see Ref.13 for review). SHP-2 has been implicated as an essential component in a variety of signaling cascades (13, 14). In EGF, PDGF, insulin, and FGF pathways, SHP-2 acts as a positive regulator in the activation of mitogen-activated protein kinase (MAPK) and mitogenic signaling (15–21). Recently, a positive role for SHP-2 has also been shown in JAK/STAT signal transduction pathways triggered by prolactin or IFN-α.
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or -β (22, 23). However, the finding that SHP-2 is a negative regulator of the RAS pathway in T cell receptor signaling (24) suggests that SHP-2 may have either a positive or negative role depending on the type of signaling pathway involved. The phosphotyrosine-dependent association SHP-2 displays with the gp130 component of the CNTF receptor complex suggests an important role for SHP-2 in this signaling pathway. We investigated this possibility by introducing an SHP-2 interfering mutant (N-P2) into NBFL cells and then examining CNTF-induced gene expression. We found that overexpression of the N-P2 mutant resulted in an up-regulation of CNTF-induced transcriptional activation of a VIP gene reporter plasmid, but a down-regulation of CNTF-induced c-fos expression. In NBFL cells that stably expressed the N-P2 mutant, gel shift analysis demonstrated that the N-P2 mutant prolongs STAT5 DNA binding activity while decreasing AP-1/DNA binding activity in response to CNTF. In sympathetic neurons, overexpression of the N-P2 mutant resulted in an increase in the CNTF-induced expression of endogenous VIP and SP genes. Together, these data indicate that in NBFL cells SHP-2 regulates CNTF-induced gene expression by controlling the signaling pathways that activate both STAT and AP-1 transcription factors. Hence, it is likely that SHP-2 controls the neuronal gene expression induced by CNTF in sympathetic neurons through a similar molecular mechanism.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The various SHP-2 and SHP-1 interfering mutants were constructed using the polymerase chain reaction (PCR) and primers that resulted in PCR amplification of either the N-terminal SH2 domain or the carboxyl N- and C-terminal SH2 domains. For all interfering mutants, the 5′ primer included sequences encoding the Flag epitope (DYKDDDDK). All mutants were cloned into the pRK5 mammalian expression vector (kindly provided by R. Derynck, University of California San Francisco, CA) under the control of the cytomegalovirus promoter, and pHSV-1 ampiclon vector under the control of the HSV-1 IE 4/5 promoter (25) (kindly provided by R. Neve, McLean Hospital, Belmont, MA). All PCR-amplified DNAs were sequenced and found not to contain nonspecific Tag polymerase-induced mutations. The full-length SHP-2 CS mutant (kindly provided by B. Neel, Beth Israel Hospital, Boston, MA) was also cloned into the pRK5 vector.

Cell Culture and Transfection—NBFL cells were maintained as described previously (6). Cells were seeded at 1 × 10⁶/10-cm², and 24 h later were cotransfected using the calcium phosphate precipitation technique (12). For VIP promoter activation studies, cells were cotransfected with 10 μg of VIP-luc (luciferase) plasmid, 10 μg of pRK5 expression vector encoding one of the SHP-2 or SHP-1 mutants, and 3 μg of pHSV-CAT to control for transfection efficiency. For c-fos promoter activation studies, cells were cotransfected with 10 μg of c-fos-CAT plasmid, 10 μg of pRK5 expression vector encoding the N-P2 mutant, and 1 μg of pHSV-luc to control for transfection efficiency. Transfected cells were plated in serum-free medium for 12-24 h before treatment with CNTF (25 ng/ml). Twelve hours later (VIP-luc) or 36 h later (c-fos-CAT), the cells were harvested, and luciferase (26) and CAT (27) activities were assayed. For stable transfectants, NBFL cells were cotransfected with 12 μg of pRK5 expression vector encoding the N-P2 mutant and 2 μg of puromycin resistance plasmid (pPD). Puromycin-resistant colonies were selected in 1 μg of drug/ml for 3 weeks. Single colonies expressing high levels of the N-P2 mutant were grown in 0.5 μg/ml of drug/ml for 3 weeks. Single colonies expressing high levels of the N-P2 mutant were grown in a C-terminal SHP-2 polyclonal antibody (Santa Cruz Biotechnology) had no overhang and were labeled with γ-32P ATP using T4 polynucleotide kinase. Nuclear extracts were prepared, and binding reactions were carried out as described previously (12). For supershift experiments, nuclear extracts were preincubated with 2 μg of poly(dI-dC) and anti-Stat1a (Transduction Laboratories), or anti-Stat3 (C-20; Santa Cruz Biotechnologies) for 15 min on ice. After the addition of the probe (~20,000 cpm), the binding reactions were continued for 20 min at room temperature. Samples were then resolved on a 5% non-denaturing polyacrylamide gel in 0.5 × Tris borate/EDTA buffer at 200 V. The sequences of the complementary nucleotides used (SIE [SIEm67], G3, and noncanonical AP-1 (m3CyB)) have been reported (12, 31).

Immunochemistry—Sympathetic neurons were fixed with 4% paraformaldehyde for 30 min. The Flag epitope-tagged mutants were detected by M5 antibody (25 μg/ml; Kodak) followed by the APAAP immunoalkaline phosphatase procedure (APAAP Kit; DAKO).

Packaging of HSV-1 Amplicon Vectors and Infection of Neuronal Cultures—pHSV-N-P2, pHSV-N-P1, and pHSV-LacZ amplicon DNAs were packaged into HSV-1 particles as described previously (25) with the following modifications. Plasmid DNAs were transfected into permissive Vero 2–2 cells using LipofectAMINE (Life Technologies, Inc.). One day later, cells were infected with δI5.2 helper virus (32). Virus were passaged and amplified three times to optimize helper:amplicon ratios. Amplified virus were purified and concentrated on sucrose gradients. pHSV-N-P2 and pHSV-N-P1 amplicon titers were determined by each well using the TF1Zol method for anti-Flag antibody (Rockland BHK cells. Helper virus titers were determined by plaque formation on Vero 2–2 cells. Titers of the viral stocks were 2 × 10⁶ infectious amplicon particles/μl and 1 × 10⁵ δI5.2 helper particles/μl, with an ampiclon to helper ratio of 2:10. The pHSV-LacZ virus was used at the same helper virus titer as the helper virus titer of the other two stocks to control for viral cytotoxicity. Sympathetic neurons were infected on the 3rd day of culture with 0.5 μl of each virus stock for 3 h. Twenty-four h after infection, the neurons were stimulated with CNTF (10 ng/ml) for 12 h.

Quantitative Reverse Transcriptase-PCR—To obtain a quantitative estimate of SP and VIP gene transcripts, we performed a quantitative RT-PCR assay (33), using neurofilament M (NF-M) as an internal standard. Total RNA was isolated from sympathetic neurons cultured in each well using the TRIzol reagent (Life Technologies, Inc.). Contaminating genomic DNA was removed by incubation with 10 units of DNase for 30 min at 37 °C in the presence of 40 units of RNasin (Promega). RNA was phenol/chloroform extracted and ethanol-precipitated. An average of 1 μg of RNA was obtained from each cell sample; half was used as control for genomic DNA contamination during PCR reactions and half was reverse transcribed as follows. Total RNA was mixed with 0.5 μg of oligo(dT) (Life Technologies, Inc.), 0.5 μM dNTP, and 1 × RT buffer (Promega) in a final volume of 19 μl and incubated at 65 °C for 5 min. After 10 min at 37 °C, 200 units of MMLV reverse transcriptase were added. The solution was incubated for 50 min at 37 °C, and the RT was then inactivated by heating to 95 °C for 5 min. The consistency of reverse transcription was validated by comparing the results of PCR analyses of 0.2, 0.5, and 1 μg of total RNA; reverse transcriptase activity was found to be quantitative because the PCR amplification products of SP, VIP, and NF-M were directly proportional to the amounts of input RNA. The cDNAs obtained from the reverse transcription reaction were used as template for PCR amplification. The sequence of each pair of primers has been reported (34). Each cDNA was amplified in the presence of [α-32P]dCTP to quantify incorporation into the specific DNA.
cDNA products after increasing numbers of cycles (Molecular Dynamics PhosphorImager®). A semi-logarithmic plot of the radioactivity incorporated versus the number of cycles was obtained for each cDNA. For SP and VIP, semi-logarithmic plots were obtained with cDNAs from both unstimulated and CNTF-stimulated sympathetic neurons. The number of cycles used to amplify each cDNA was chosen so that each PCR amplification proceeded in a linear range. The efficiency of amplification for each cDNA was deduced from the slope of the semi-logarithmic plot (33) and was constant in different runs. In our hands, the method resolves 2-fold differences in input cDNA over a range of 2 orders of magnitude. PCR reactions were carried out in a final volume of 10 μl consisting of cDNA, 1× PCR buffer (Promega), 1 unit of Taq polymerase, (Promega), 0.2 μM dNTPs, 1.5 mM MgCl₂, 0.5 μM each primer, 1.5 x 10⁶ cpm [α-³²P]dCTP. The number of cycles for each cDNA was as follows: SP, 24; VIP, 26; and NF-M, 27. The amount of cDNA (μl of the reverse transcription reaction) was as follows: VIP and NF, 0.5 μl; and SP, 2 μl. The typical amplification profile was 95°C for 1 min, annealing temperature for 1 min, and 72°C for 90 s. The annealing temperature for SP and VIP was 53°C and for NF-M was 65°C. PCR amplified cDNAs were separated by agarose gel electrophoresis on 2% agarose gels, visualized by ethidium bromide staining and quantified by measuring the incorporated radioactivity using a PhosphoImager®. Amplified PCR products of VIP and SP in each sample were normalized for binding to the SHP-2 phosphotyrosine containing binding site on gp130. NBFL cells were cotransfected with SHP-2 mutants. Further experiments were performed using the N-P2 interfering mutant because its effect on SHP-2 binding was comparable to that observed with endogenous SHP-2, tyrosine-phosphorylated gp130 and LIFRβ coimmunoprecipitated with the Flag-tagged N-P2 interfering mutant after CNTF stimulation (Fig. 1C; see Flag lanes). The 90-kDa phosphoprotein, however, was not detected in the immunoprecipitates, indicating that the 90-kDa phosphoprotein does not associate with SHP-2 through the N-terminal SH2 domain.

RESULTS

SHP-2 Is a Negative Signal Regulator in CNTF-induced VIP Gene Transcription in NBFL Cells—To determine whether SHP-2 may play a role in CNTF-induced gene expression, we first used the human neuroblastoma cell line, NBFL, as a model system. NBFL cells respond to CNTF by coordinately inducing multiple neuropeptide genes, in a similar way to sympathetic neurons (6). To remove endogenous SHP-2 activity from the CNTF signaling cascade in NBFL cells, we used SHP-2 interfering mutants that lack the catalytic domain (Fig. 1A). SHP-2 mutants should compete with endogenous SHP-2 for binding to the SHP-2 phosphotyrosine containing binding site on gp130. NBFL cells were cotransfected with SHP-2 mutants (N-P2, NC-P2, or CS-P2) and a CNTF-responsive luciferase reporter (VIP-luc) (6), which contains 1929 base pairs upstream of the transcription start site of the VIP gene. As a control for specificity of the SHP-2 mutants, we used the corresponding mutant forms of the related tyrosine phosphatase SHP-1 (N-P1 and NC-P1; Fig. 1A). While SHP-2 is broadly expressed, SHP-1 is mainly expressed in hematopoietic cells (see Ref. 13 for review). CNTF-stimulated VIP-luc reporter activity was ~11-fold in NBFL cells transfected with the parental expression vector alone (Fig. 1B, CT). Transfection of either the colinear N- and C-terminal SH2 domains (NC-P2) or the N-terminal SH2 domain (N-P2) of SHP-2 enhanced CNTF-stimulated VIP-luc activity ~2-fold over the CT value, without affecting the basal level. The effect of the SHP-2 mutants was specific, because overexpression of the corresponding mutant forms (N-P1 and NC-P1) of SHP-1 did not significantly alter basal or CNTF-induced VIP-luc transcription over the CT value. Transfection of the full-length but catalytically inactive dominant negative SHP-2 mutant (CS-P2), in which the essential catalytic cysteine residue has been replaced with serine (21), enhanced CNTF-stimulated VIP-luc activity ~3-fold over the CT value. The stronger effect of CS-P2 compared with the other two SHP-2 mutants could reflect the ability of the CS mutant to tightly bind and “trap” substrates in addition to competing for binding to gp130 (21, 35). These transfection studies using catalytically inactive SHP-2 mutants indicate that endogenous SHP-2 plays a negative role in the transmission of the CNTF signal in NBFL cells.

The N-P2 Interfering Mutant Specifically Associates with the CNTF Receptor Complex—Further experiments were performed using the N-P2 interfering mutant because its effect on CNTF-induced VIP expression in NBFL cells is substantial and comparable to that observed with the other mutants. To confirm that the N-P2 mutant interacts specifically with the CNTF receptor complex, we compared the pattern of phosphotyrosine-containing proteins that coimmunoprecipitate with endogenous SHP-2 with those that coimmunoprecipitate with the N-P2 interfering mutant following CNTF stimulation. We found that tyrosine-phosphorylated bands of ~190 and ~130 kDa, which correspond to LIFRβ and gp130 (36), coimmunoprecipitated with endogenous SHP-2 in CNTF-treated NBFL cells (Fig. 1C). Two additional tyrosine-phosphorylated proteins of ~90 and ~68 kDa also coimmunoprecipitated with the CNTF receptor complex. The ~68-kDa phosphoprotein comigrated with SHP-2 (Fig. 1C); however, the ~90 kDa phosphoprotein is of unknown identity (the protein was not recognized by antibodies to Stat1α or Stat3; data not shown). Interestingly, a 90-kDa tyrosine-phosphorylated protein named SIRPα1 has recently been characterized, which associates with SHP-2 upon EGF, PDGF, or insulin stimulation (37). Whether the 90-kDa protein that coimmunoprecipitates with SHP-2 upon CNTF stimulation might be SIRPα1 still remains to be determined. Similar to that observed with endogenous SHP-2, tyrosine-phosphorylated gp130 and LIFRβ coimmunoprecipitated with the Flag-tagged N-P2 interfering mutant after CNTF stimulation (Fig. 1C; see Flag lanes). The 90-kDa phosphoprotein, however, was not detected in the immunoprecipitates, indicating that the 90-kDa phosphoprotein does not associate with SHP-2 through the N-terminal SH2 domain.

The similar pattern of phosphotyrosine-containing proteins that coimmunoprecipitate with either the N-P2 interfering mutant or the endogenous SHP-2 after CNTF stimulation confirms that the N-terminal SH2 domain mutant of SHP-2 retains a high degree of specificity for tyrosine phosphorylated gp130. In addition, the substantial amounts of LIFRβ and gp130 that coimmunoprecipitate with the N-P2 mutant in the transient transfections (Fig. 1C; see Flag lanes) indicate that the overexpressed mutant binds with high affinity to tyrosine phosphorylated gp130.

N-P2 Mutant Prolongs DNA Binding of STAT Complexes in Response to CNTF.—The VIP gene contains a 180 base pairs -flanking sequence that binds activated STAT after CNTF stimulation and which mediates induction of the VIP gene (12, 31). To investigate how SHP-2 may regulate CNTF-dependent VIP transcriptional induction, we examined whether SHP-2 affects the ability of activated STAT to bind to elements within the CyRE using NBFL cDNA cell lines that stably express the N-P2 mutant. NBFL cells were transfected with an expression vector containing the N-P2 interfering mutant and puromycin-resistant clones were established and characterized for the level of expression of the mutant protein. Several clones were found that expressed the N-P2 mutant at levels comparable with that of the endogenous SHP-2 (Fig. 2A). In the following experiments, similar results were obtained with different clones; therefore, only data from one representative clone, NBFL-N2, are reported. Parental NBFL and NBFL-N2 cells were serum-starved for 12 h and then stimulated with CNTF for increasing times. Nuclear extracts were prepared before and after CNTF treatment, and the degree of STAT/DNA binding activity was assessed by EMSA using either the high-affinity variant of the c-fos sis-inducible element (SIE) sequence (38) or the acute phase response element (APRE)-like site of the CyRE (G3) (12) as a probe. In parental NBFL cells, CNTF treatment resulted in the stimulation of SIE binding activity of the STATs, which at 30 min was primarily represented by complex A (Fig. 2B). Supershift analysis confirmed this complex as being composed of Stat3 homodimers.
as described previously (38, 39). The two faster migrating complexes B and C were much less abundant. Complex C reacted almost quantitatively with Stat1α antibody, while complex B reacted with both anti-Stat1α and anti-Stat3 antibodies (Fig. 2B). None of the complexes were altered in the presence of irrelevant antibody. A small amount of complex A was still present 4 h after CNTF treatment, while complexes B and C were undetectable after 1 h. EMSA performed with NBFL-N2 nuclear extracts revealed an increase in the amount and duration of CNTF-induced binding of STAT proteins to the SIE probe (Fig. 2B); most notably in the Stat3-containing complex A, as shown by supershift analysis (Fig. 2B). When the G3
site within the CyRE was used as a probe in EMSA with nuclear extracts from NBFL or NBFL-N2 cells before and after CNTF stimulation (25 ng/ml) for the indicated times. EMSAs were performed with equal amounts of nuclear extract and labeled probe corresponding to the c-fos siis-inducible element (SIE) (B) or the APRE-like site of the CyRE (G3) (C). For supershift analysis (right panels), nuclear extracts from NBFL or NBFL-N2 cells treated for 30 min with CNTF (25 ng/ml) were incubated with SIE or G3 probes in the presence or absence of control antibody (Ab), anti-Stat1α, or anti-Stat3 and then subjected to EMSA. Complexes A, B, and C refer to Stat3 homodimers, Stat1α/Stat3 heterodimers, and Stat1α homodimers, respectively. S3-SS, Stat3 supershift; S1-SS, Stat1α supershift.

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Fig. 2. NBFL-N2 cells display increased formation of CNTF-inducible DNA/STAT complexes. A, cell extracts from parental cells (NBFL) or stable N-P2 transfected clones (NBFL-N2, NBFL-N6, and NBFL-N9) were separated on a 15% SDS gel, Western blotted, and then probed with a monoclonal antibody raised against the N-terminal region of SHP-2. The purified N-P2 fusion protein corresponds to the N-terminal SH2 domain of SHP-2. Endogenous SHP-2 and ectopically expressed N-P2 are indicated. B and C, nuclear extracts were prepared from NBFL or NBFL-N2 cells before and after CNTF stimulation (25 ng/ml) for the indicated times. EMSAs were performed with equal amounts of nuclear extract and labeled probe corresponding to the c-fos siis-inducible element (SIE) (B) or the APRE-like site of the CyRE (G3) (C). For supershift analysis (right panels), nuclear extracts from NBFL or NBFL-N2 cells treated for 30 min with CNTF (25 ng/ml) were incubated with SIE or G3 probes in the presence or absence of control antibody (Ab), anti-Stat1α, or anti-Stat3 and then subjected to EMSA. Complexes A, B, and C refer to Stat3 homodimers, Stat1α/Stat3 heterodimers, and Stat1α homodimers, respectively. S3-SS, Stat3 supershift; S1-SS, Stat1α supershift.
almost undetectable in NBFL-N2 cells.

To determine whether the N-P2 mutant down-regulates CNTF-induced c-fos expression (46, 47), NBFL cells were co-transfected with the N-P2 mutant and a mouse c-fos-CAT reporter, containing the 2356 to 1109 region of the c-fos promoter (48, 49). This sequence includes all elements known to be important for regulation, including the SIE and the serum response element (SRE). Consistent with the decrease in c-Fos levels observed in NBFL-N2 cells, the N-P2 mutant abolished the CNTF-induction of the c-fos-CAT reporter (Fig. 3D). This effect, although small because of the high basal activity of the c-fos-CAT reporter, was reproducible and statistically significant (comparison of bars 2 and 4; p < 0.02). Together, these data indicate that endogenous SHP-2 is a positive regulator of CNTF-induced c-fos expression.

SHP-2 Coordinately Down-regulates CNTF Induction of Endogenous VIP and SP Genes in Sympathetic Neurons—The VIP gene is one of several genes that are coregulated by CNTF in NBFL cells. The neurotransmitter gene expression induced by CNTF in NBFL cells mimics that which occurs in sympathetic neurons, suggesting that a common molecular mechanism mediates the response to CNTF in both NBFL cells and sympathetic neurons. Therefore, we examined whether SHP-2 may also play a role in CNTF-induced expression of the endogenous VIP gene in cultured sympathetic neurons. In addition, to address whether SHP-2 may control in a coordinate manner the transcriptional activation that occurs in CNTF-induced neuronal differentiation of sympathetic neurons, we also examined the role of SHP-2 in the expression of the SP gene.

Expression of SHP-2 in sympathetic neurons was confirmed by means of immunofluorescent staining and RT-PCR (data not shown). In time course (up to 72 h) and dose-response experiments (10–25 ng/ml CNTF), we found that 10 ng/ml CNTF induced a substantial and quantifiable increase of VIP and SP mRNA levels in as little as 12 h. VIP and SP gene expression was determined by a quantitative RT-PCR assay, using NF-M as an internal standard (Fig. 4A and B). Similar results were obtained when VIP and SP mRNA levels were normalized to constitutively expressed β-actin (data not shown).

To introduce the N-P2 mutant into sympathetic neurons, we
used an HSV-1 amplicon virus (HSV-N-P2). We also used an N-P1-containing virus (HSV-N-P1) to control for SH2 domain specificity, and a LacZ-containing virus (HSV-LacZ) to control for possible toxicity caused by the helper virus. Approximately 70% of the sympathetic neurons were infected with either HSV-N-P2 or HSV-N-P1 virus, demonstrating the efficiency at which the HSV-1 virus can transfer genes to postmitotic neurons (Fig. 5, B and C). The expression of the N-P1 and N-P2 interfering mutants increased up to 24 h and remained stable at least up to 72 h postinfection (data not show). A similar infection frequency and stability of expression was observed in cultures infected with HSV-LacZ and stained with X-gal (data not shown).

The effect of the SHP-2 mutant on VIP and SP gene expression was examined after 12 h of exposure to CNTF using the quantitative RT-PCR assay. In uninfected control cultures, CNTF stimulation resulted in VIP and SP mRNA inductions of 1.7- and 5.0-fold, respectively (Fig. 5D). In cultures infected with the HSV-N-P2 virus, however, CNTF induction of VIP and SP mRNAs was 4.3- and 11.2-fold, respectively. The effect was due to overexpression of the SHP-2 interfering mutant in HSV-N-P2 virus-infected cultures because CNTF induction of VIP and SP mRNAs in neurons infected with HSV-N-P1 or HSV-LacZ virus was similar to that observed in uninfected neurons. Normalization of VIP and SP mRNAs to β-actin as an internal standard gave similar results (data not shown). The down-regulation of VIP and SP expression in sympathetic neurons elicited by SHP-2 suggests that SHP-2 controls CNTF-induced expression of these genes in a coordinated manner and likely through a common molecular mechanism.

**DISCUSSION**

Our studies show that SHP-2 negatively regulates CNTF-induction of VIP and SP gene expression while at the same time positively regulating c-fos expression. These data indicate that SHP-2 has dual and opposing roles in a signaling cascade triggered by the same ligand and clearly demonstrate that SHP-2 is a multifunctional protein in the regulation of the pathways activated by cytokines or growth factors.

The enhancement of CNTF-induced STAT binding activity observed in NBFL cells that overexpress the SHP-2 interfering mutant suggests that the negative function of SHP-2 could be at the level of the STATs or the STAT activating JAK kinases. Possible targets for SHP-2 phosphatase activity may be Jak1 or Jak2 since these kinases are activated by tyrosine phosphorylation upon CNTF stimulation in NBFL cells (data not shown) and a direct association of SHP-2 with the JAKs has recently been shown (50–52). Inhibition of JAK-STAT signaling by SHP-2 through dephosphorylation of JAKs has been proposed in the interleukin-6 (IL-6)-triggered signaling pathway (51). In addition, a similar mechanism has been suggested for the related tyrosine phosphatase SHP-1, which plays a major role in terminating the erythropoietin- and α/β interferon-stimulated JAK-STAT pathway (23, 53). Alternatively, because SHP-2 associates directly with gp130, it may dephosphorylate phosphotyrosine docking sites on gp130. One obvious possibility is the Stat3 binding site on gp130 (10). Lastly, because maximal activation of Stat1α and Stat3 appears to be dependent on serine phosphorylation (54–56), it is possible that SHP-2 modulates STAT activation by controlling kinases of the MAPK
family. Cytokines of the CNTF family induce both tyrosine phosphorylation and H7 (a serine/threonine kinase inhibitor)-sensitive phosphorylation of Stat3 (57). Indeed, in NBFL cells, induction of VIP gene expression by CNTF is abolished by H7 (12, 42). A link between SHP-2 and MAPK activation has been shown in mitogenic signaling pathways, with SHP-2 being generally a positive, but sometimes a negative regulator of MAPK activity (58). MAPKs can be activated by cytokines in a cell-dependent manner (59, 60), and several lines of evidence suggest that JAKs may be required for activation of both STATs and ERK/MAPKs by cytokines, providing mechanisms for the optimization of gene induction (61–64).

Both the AP-1 and STAT binding elements present in the CyRE of the VIP gene cooperate to achieve maximal rates of transcription upon CNTF stimulation (12, 31). What is puzzling, however, is that while SHP-2 negatively regulates STAT binding activity, it in parallel positively regulates AP-1 binding activity by controlling levels of c-Fos. This observation raises the possibility that SHP-2 controls other regions, as yet unidentified, within the VIP CyRE, resulting in its down-regulation.

In the context of CNTF signaling, the down-regulation that SHP-2 exerts on VIP expression is in striking contrast with the up-regulation SHP-2 has on c-fos expression in NBFL cells. Multiple regulatory elements are present in the c-fos promoter, including the SIE and SRE. In our experiments, although over expression of the N-P2 interfering mutant significantly increased STAT binding to the SIE, the actual effect on c-fos expression of the N-P2 interfering mutant significantly increased SHP-2 has on CNTF induction of c-fos. Therefore, it appears that the positive effect SHP-2 has on CNTF induction of c-fos expression is not through STATs, but more likely through regulation of transcription factors that bind other regulatory elements in the c-fos promoter, such as the SRE. Indeed, a number of studies have indicated only a minor contribution of the SIE to induction of c-fos expression (see Ref. 63, and references within) but a major role for the SRE in a variety of physiological settings (38, 63, 65).

A requirement of SHP-2 in the activation of c-fos transcription has been reported previously in insulin (15), PDGF (66), and α-thrombin (20) triggered pathways. Expression of c-fos is rapidly increased in many cell types in response to several mitogens (67), and therefore the up-regulation of c-fos that is dependent on SHP-2 might be related to the mitogenic role that SHP-2 has on these cell types. However, because CNTF is not a mitogenic factor for either NBFL cells or progenitor sympathetic neurons (68, 69), but rather a factor that can induce differentiation, the induction of c-fos is likely to be needed in the context of neuronal differentiation.

The negative regulation that SHP-2 exerts on CNTF-induced expression of the SP and VIP genes is likely to be mediated through a common mechanism since STAT consensus binding sites are present in the promoter region of both genes (70, 71). It is conceivable then that SHP-2 may have a similar effect on the expression of other genes that contain STAT binding sites and that are coordinately regulated by CNTF. Indeed, STAT binding sites have also been identified within the promoter regions of the SOM, cholecystokinin, enkephalin, and ChAT genes. Together with VIP and SP, these neuropeptide and neurotransmitter synthetic enzyme genes define the cholinergic phenotype in sympathetic neurons (72). Therefore it is possible that SHP-2 is present in these neurons to monitor neuropeptide-induced cholinergic differentiation.

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