Positive Effects of SH2 Domain-containing Tyrosine Phosphatase SHP-1 on Epidermal Growth Factor- and Interferon-γ-stimulated Activation of STAT Transcription Factors in HeLa Cells

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SHP-1 (also known as PTP1C, SHPTP-1, SHP, and HCP) is an SH2 domain-containing protein-tyrosine phosphatase. We have stably overexpressed the native form and a catalytically inactive cysteine to serine mutant of the enzyme, SHP-1-(Cys → Ser), in human cervical carcinoma HeLa cells. Following stimulation of the cells with epidermal growth factor (EGF) and interferon-γ (INF-γ), signal transducers and activators of transcription (STATs) activity was analyzed by using two [32P]-labeled DNA probes, namely hSIE which is derived from a high affinity mutant form of the serum-inducible element in the c-fos promoter and GAS which resembles the INF-γ activation site. EGF induced hSIE binding activity only, and the activity was suppressed by ~70% when the inactive mutant form of SHP-1 was expressed but was essentially unaffected by expression of the native enzyme. INF-γ treatment resulted in appearance of both hSIE and GAS binding activities. While expression of the inactive mutant reduced the activities by 30–50%, the native enzyme caused a 20–30% increase. Consistent with effects on STAT activation, altered SHP-1 expression also affected EGF-induced activation of the mitogen-activated protein kinase pathway; expression of SHP-1-(Cys → Ser) inhibited activation of MBK by ~25%, whereas expression of SHP-1 resulted in a ~25% increase. Further studies revealed that overexpression of SHP-1 caused decreased tyrosine phosphorylation of the EGF receptor and that EGF induced phosphorylation and recruitment of SHP-1. Together, the data suggest that SHP-1 is positively involved in EGF- and INF-γ-induced STAT activation in non-hematopoietic HeLa cells and that, in the EGF signaling system, SHP-1 functions at least partially by modulating tyrosine phosphorylation of EGF receptor.

Signal transducers and activators of transcription (STATs) are transcription factors that are activated by tyrosine phosphorylation in response to growth factors and cytokines (1). Activation of STATs via the JAK/STAT pathway has been well defined (1–3). Engagement of growth factor or cytokine recept
of MAP kinase was almost totally impaired (25). On the other hand, evidence also exists suggesting a negative role of SHP-2 in signal transduction. For example, recruitment of SHP-2 to CTLA-4 leads to inactivation of T-cell activation (26). It seems that the functional nature of SHP-1 and SHP-2 depends on the systems involved. In this study, we demonstrate that, in non-hematopoietic HeLa cells, SHP-1 is positively involved in EGF- and INF-γ-induced STAT activation.

EXPERIMENTAL PROCEDURES

Materials—HeLa cells were obtained from the American Type Culture Collection. Recombinant human EGF was from Upstate Biotechnology Inc., and INF-γ was from Boehringer Mannheim. Polyclonal anti-SHP-1 serum 2303 was produced by immunization of rabbits with purified recombinant SHP-1 as described previously (27). Monoclonal anti-phosphotyrosine and polyclonal sheep anti-EGF-R antibodies were purchased from Upstate Biotechnology, Inc. Monoclonal antibody specific for tyrosine-phosphorylated EGF-R was obtained from Transduction Laboratory. γ-32P]ATP and [α-32P]dCTP were purchased from Amersham Corp.

Buffer A is composed of the following: 50 mM β-glycerophosphate (pH 7.3), 5 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 1% Triton X-100, 0.1 M NaCl. Buffer B is composed of the following: 20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM EDTA, and 1 mM dithiorthreitol. Both buffers were also supplemented with 0.2 mM Na3VO4, 0.1 μM microcystin, 1.0 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotilin.

Generation of Stably Transfected Cells—The catalytically inactive mutant, SHP-1-(Cys → Ser), in which Cys-455 was mutated to Ser, was generated by the polymerase chain reaction method as described previously (28). The native enzyme SHP-1 and the mutant SHP-1-(Cys → Ser) were constructed into the pReCMV vector (Invitrogen) and were used to transfet cells according to a calcium phosphate co-precipitation technique; the plasmid vector was also transfected as a control (29). To obtain stable cell lines, transfected cells were selected in medium containing 0.5 mg/ml genitc (G418 sulfate, Life Technologies, Inc.), and single colonies expressing high levels of SHP-1 or SHP-1-(Cys → Ser) were obtained after 2–3 weeks. Wild type HeLa cells were grown in minimum essential medium supplemented with 10% fetal calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin. The transfected cells were maintained in the same medium supplemented with 0.25 mg/ml G418 sulfate.

Stimulation and Extraction of Cells—HeLa cells (~80% confluency) were starved at 0% serum for 24 h before treatment with EGF or INF-γ. The stimulation reactions were stopped by washing with ice-cold phosphate-buffered saline, and cells were extracted in the following ways. Whole cell extracts were made by homogenization of the cells in buffer A followed by high speed centrifugation. For preparation of nuclear extracts, detergent-solubilized cells were resuspended in three packed cell volumes of buffer B, swollen for 10 min, and lysed by repeated passage through a 25-gauge needle. Nuclei were collected by centrifugation at 16,000 × g for 20 s and then extracted in 2.5 packed cell volumes of buffer B supplemented with 0.42 mM NaCl and 20% glycerol. The supernatant (referred to as the nuclear extract) was cleared by centrifugation at 16,000 × g for 20 min, dialyzed for 18 h in buffer B, and used for STAT activity analyses.

Gel Mobility Shift Assays of STATs—The activities of STATs were determined by analyzing the ability of the proteins to form complexes with specific DNA probes in gel mobility shift assays. The DNA probes used were hSIE and GAS. They were made by annealing 5′-GAGACTGAGGCTGAAGTACTTTCAGTTTCATATTA-3′ and 5′-AAGTACTTTAGTGTCTTATATAA-3′, respectively. These double-stranded DNA probes with staggered ended were labeled by the Klenow fragment in the presence of [α-32P]dCTP (3000 ci/mmol). Free nucleotides were removed by using a nucleotide removing kit from Qiagen. For STAT activity analyses, nuclear extracts containing 15 μg of total proteins were preincubated with 2 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia Biotech Inc.) for 10 min. This was followed by addition of 2 fmol of 32P-labeled DNA probes and 15 min further incubation at room temperature. The buffer system for the reactions was 20 mM HEPES (pH 7.9), 25 mM KCl, 4 mM MgCl2, 0.5 mM dithiorthreitol, 1 mM EDTA, and 10% glycerol. The protein-DNA complexes were resolved on 5% polyacrylamide gels (acrylamide:bisacrylamide = 39:1) containing 2.5% glycerol made in 0.5 × TBE (Tris borate/EDTA) buffer. Gels were dried and exposed to x-ray film. The activity was quantified by densitometric analyses.

FIG. 1. Overexpression of SHP-1 and SHP-1-(Cys → Ser) (SHP-1(C-S)) in HeLa cells. The cell extracts were immunoprecipitated with anti-SHP-1 antibody, and the PTP activity in the immunoprecipitates was analyzed with 1 μM 32P-ENDpYINASL peptide. Inset, whole cell extracts (25 μg) from cells expressing vector alone, SHP-1, and SHP-1-(Cys → Ser) were subject to SDS-polyacrylamide gel electrophoreses and Western blot analyses with anti-SHP-1.

Effects of Overexpressed SHP-1 and SHP-1-(Cys → Ser) on EGF- and INF-γ-stimulated STAT Activation—Upon treatment of the transfected HeLa cells with EGF and INF-γ, activity of STATs in the nuclear extracts was analyzed by using specific DNA probes, namely hSIE which is a high affinity mutant of the serum-inducible element in the c-fos promoter and GAS which is derived from the INF-γ-activated site. Both have been widely used as DNA probes for detecting STATs activation induced by a variety of stimuli (5–7). EGF induced...
binding of STATs to hSIE not to GAS (Fig. 2A, data not shown for GAS binding), and altered expression of SHP-1 had no significant effect on the time course of the activation. In all three cell lines, EGF treatment resulted in a transient activation STAT activity toward hSIE which peaked at around 15 min. However, the magnitude of the activation was significantly affected. Although overexpression of wild type SHP-1 displayed no significant effect, expression of mutant SHP-1-(Cys → Ser) resulted in a ∼70% decrease in the hSIE binding activity. Treatment of the cells with INF-γ resulted in appearance of hSIE and GAS binding activities, and both were affected by the alteration of SHP-1 expression (Fig. 2B). Compared with the vector control, wild type SHP-1 caused a 20–30% increase in hSIE and GAS binding activities, whereas the mutant SHP-1-(Cys → Ser) reduced the binding activities by approximately 30–50%. The time course of the activation was also shifted when GAS was used as a probe.

All these DNA-protein interactions were sequence-specific, as the shifted bands disappeared when an excess amount of unlabeled oligonucleotides with the same sequences was included in the binding reactions (data not shown). The radioactive bands below the STAT-DNA complexes indicated by arrows are not counted as specific STAT binding activity. This has been noted in previous studies by others (5–7).

**Effects of Overexpressed SHP-1 and SHP-1-(Cys → Ser) on Activation of MEK**—Since MAP kinase has been shown to regulate activation of STATs (32, 33), we further examined the effects of the altered SHP-1 expression on activation of the MAP kinase pathway. This was carried out by measuring the activity of MEK which is a kinase directly upstream MAP kinase and activated in parallel with MAP kinase. As illustrated in Fig. 3, EGF-induced transient activation of MEK peaked at 5 min of stimulation. While overexpression of the native SHP-1 increased the peak activity by ∼25%, overexpression of the catalytically inactive mutant SHP-1-(Cys → Ser) decreased it by a similar extent. This suggests that SHP-1 plays a positive role in EGF-induced activation of the MAP kinase cascade, which is consistent with the results obtained from 293 cells (24). In contrast, activation of MEK induced by phorbol 12-myristate 13-acetate was not affected by the altered expression of SHP-1, indicating that the phorbol ester bypasses SHP-1 to activate MEK. Furthermore, INF-γ failed to cause MEK activation in all three transfected HeLa cell lines (data not shown).

**Effects of Altered SHP-1 Expression on Tyrosine Phosphorylation of EGF-R**—It has been reported that SHP-1 is recruited to the tyrosine-phosphorylated EGF-R (34), but it is not known how this affects tyrosine phosphorylation of the receptor. To address the problem, tyrosine phosphorylation of EGF-R and cellular proteins in cells with altered SHP-1 expression, before and after stimulation with EGF, was analyzed by Western blot analyses with anti-phosphotyrosine. As shown in Fig. 4A, altered expression of SHP-1 had no significant effect on tyrosine phosphorylation of cellular proteins at the basal level. Upon stimulation with EGF, tyrosine phosphorylation of EGF-R and a 50-kDa protein characteristic of the EGF stimulation of HeLa cells became apparent. In comparison with the vector control, overexpression of wild-type SHP-1 caused a marked decrease in the phosphorylation of the receptor, and expression of the inactive mutant had no significant effect on phosphorylation of
the receptor. Interestingly, despite the changes in the phosphorylation of the receptor, EGF-induced tyrosine phosphorylation of the 50-kDa protein appeared to be equivalent in all three cell lines. The effects on tyrosine phosphorylation of EGF-R by altered expression of SHP-1 were also apparent when analyzed by using an antibody specific for phosphorylated EGF-R. By contrast, Western blot analyses with a regular anti-EGF-R antibody revealed equal levels of EGF-R, indicating that expression of the receptor in the cells was not affected by the altered SHP-1 expression. The decreased tyrosine phosphorylation of EGF-R by SHP-1 was further confirmed by the anti-phosphotyrosine blotting of immunoprecipitates of EGF-R. As shown in Fig. 4B, essentially equal amounts of EGF-R were precipitated by the anti-EGF-R antibody, whereas tyrosine phosphorylation of EGF-R in cells overexpressing the native form of SHP-1 was significantly lower. Interestingly, association of SHP-1 with EGF-R was apparently not affected by the decreased phosphorylation of the receptor. In fact, a much higher level of SHP-1 was co-immunoprecipitated with EGF-R in cell overexpressing SHP-1. By contrast, association of SHP-1-(Cys → Ser) was marginal despite high levels of tyrosine phosphorylation of EGF-R and higher expression of SHP-1-(Cys → Ser), suggesting that the phosphatase activity is required for efficient binding. The fact that SHP-1 associates with EGF-R and decreases its tyrosine phosphorylation seems to suggest that SHP-1 directly acts on EGF-R. However, it is intriguing that SHP-1-(Cys → Ser) showed no enhancing effect on the phosphorylation and that it failed to bind to the receptor. A possible explanation is that EGF-R is phosphorylated on different sites and/or that other molecules are involved. Taken together with the positive effects of SHP-1 on the activation of MEK and STATs activities described earlier, the data suggest that overexpression of SHP-1 caused dephosphorylation of EGF-R and did not inhibit, but instead enhanced, downstream signaling.

Phosphorylation of SHP-1 following EGF Stimulation—SHP-1 undergoes phosphorylation on tyrosine following growth factor stimulation, which is thought to be important for its function (13). In fact, it has been reported that tyrosine phosphorylation of SHP-1 results in slight activation of the enzyme (35) and provides binding site for Grb2 (19). To examine tyrosine phosphorylation of SHP-1, the enzyme was immunoprecipitated by using anti-SHP-1 antibody, and the extent of tyrosine phosphorylation was determined by Western blot analyses of the immunoprecipitates with anti-phosphotyrosine. As shown in Fig. 5, a basal tyrosine phosphorylation of both SHP-1 and SHP-1-(Cys → Ser) was detected, with the mutant showing a high degree of phosphorylation. Upon stimulation, tyrosine phosphorylation of both SHP-1 and the inactive mutant SHP-1-(Cys → Ser) significantly increased. Considering the comparable amounts of SHP-1 and SHP-1-(Cys → Ser) precipitated as shown by the anti-SHP-1 blots, the mutant SHP-1-(Cys → Ser) was much more extensively phosphorylated. This high level of tyrosine phosphorylation observed in the SHP-1 mutant likely resulted from the abolition of its auto-dephosphorylation, implying that auto-dephosphorylation is responsible for dephosphorylation of SHP-1. Unlike EGF, INF-γ did not induce detectable tyrosine phosphorylation of SHP-1 (data not shown). Together, these data suggest that tyrosine phosphorylation of SHP-1 is probably not the single determinant factor in the positive role of SHP-1.

**FIG. 4.** Effects of altered expression of SHP-1 on tyrosine phosphorylation of EGF-R. Serum-starved cells transfected with vector control, wild type SHP-1, and catalytically inactive mutant SHP-1-(Cys → Ser) (SHP-1-(C-S)) were left untreated (lanes) or stimulated with 100 ng/ml EGF for 5 min (lanes). Whole cell extracts (A) or anti-EGF-R immunoprecipitates (B) were subject to SDS-polyacrylamide gel electrophoresis and Western blot analyses with anti-phosphotyrosine, anti-phosphorylated EGF-R, anti-EGF-R, and anti-SHP-1 antibodies as indicated. Arrows denote the position of EGF-R, and p50 represents the 50-kDa protein that is phosphorylated in response to EGF stimulation.

**FIG. 5.** EGF-induced tyrosine phosphorylation of SHP-1 in HeLa cells. Cells transfected with vector control, wild type SHP-1, and catalytically inactive mutant SHP-1-(Cys → Ser) (SHP-1-(C-S)) were left untreated (lanes) or stimulated with 100 ng/ml EGF for 5 min (lanes). Whole cell extracts were immunoprecipitated with anti-SHP-1 antibody, and the immunoprecipitates were subject to SDS-polyacrylamide gel electrophoresis and Western blot analyses with anti-phosphotyrosine and anti-SHP-1 antibodies as indicated.

**DISCUSSION**

In the present study, we have demonstrated that, in non-hematopoietic HeLa cells, expression of a catalytically inactive mutant of SHP-1 resulted in a significant decrease in EGF- and INF-γ-induced STAT activity, whereas overexpression of the native form of enzyme had no effect on the former but had a moderate positive effect on the latter. This suggests that SHP-1, like SHP-2, can function as a positive regulator in the activation of the STAT transcription factors. These results are consistent with the recent reports on the positive role of SHP-1 in activation of the MAP kinase pathway in human embryonic kidney 293 cells induced by serum and EGF and in macrophages induced by CSF-1 (24, 25). However, the data are opposite to the reports on the negative role of SHP-1 observed in many hematopoietic cell systems (14–21).

The negative role of SHP-1 in hematopoietic cell signaling has been extensively studied. In fact, previous reports have demonstrated that SHP-1 plays a negative role in INF-α/β- and erythropoietin-induced cell signaling by specifically dephosphorylating JAK1 and JAK2, respectively (14, 15, 36). There are several explanations for the discrepancy between our present results and the previous reports by others. First, our study...
was performed by using stably transfected human cervical carcinoma HeLa cells with altered SHP-1 expression while almost all the other studies were performed by using mature hematopoietic cells (macrophages or lymphocytes) derived from moth-eaten or viable moth-eaten mice which have a natural defect in SHP-1 expression (14, 15, 17–21). Second, SHP-1 has two forms (13). One referred to as the hematopoietic form is expressed predominantly in hematopoietic cells, and the other designated as the regular form is widely distributed. These two forms of SHP-1 differ in their N termini, which might affect their functions. Our current study was performed by using the regular form of SHP-1. Third, EGF and ING-γ were used in our study to stimulate cell signaling while most prior studies concerned signal transduction initiated by other stimuli such as INF-α/β and erythropoietin (14, 15). These differences in experiment approaches and in signaling systems may account for the differences in the distinct functions of SHP-1 in signal transduction. Above all, our results presented here provide further evidence to suggest that SHP-1 can play either positive or negative roles in regulating signal transduction pathways depending on the cell types, signaling systems involved, position of the enzyme targeted within the cells, and the particular molecules with which it interacts.

Although our data provide strong evidence that SHP-1 plays a positive role in regulating STAT activation in HeLa cells, the precise mechanism by which SHP-1 functions is not clear. Tyrosine phosphorylation of STATs is absolutely required for their activation. It is unlikely that SHP-1 enhances activation of STATs by directly acting on them. Instead, it may act directly or indirectly on a component that is upstream of the pathway. Recent studies have demonstrated that serine phosphorylation of STAT1 and STAT3 by MAP kinase is required in addition to tyrosine phosphorylation for maximum transcriptional activation (32, 33). This establishes a direct link between the MAP kinase pathway and STATs. Therefore, SHP-1 could affect the activation of STATs via regulation of MAP kinase. In the present study, we have found that overexpression of a catalytically inactive mutant of SHP-1 modestly suppressed EGF-induced MAP kinase activation whereas that of the native form of the enzyme enhanced it. Moreover, the peak of EGF-induced MEK activation was observed at 5 min while maximum activation of STATs was at 15 min. The data suggest that SHP-1 may indeed affect the EGF-induced STAT activation indirectly by altering the activation of MAP kinase. However, for the ING-γ-induced STAT activation, this may not be the case since the present study indicated that activation of MAP kinase is not involved in ING-γ-induced signaling. SHP-1 may be directly involved in the pathway leading to activation of STATs.

As an SH2 domain-containing protein, SHP-1 is among the signaling molecules that are recruited to tyrosine-phosphorylated receptors upon stimulation. In fact, binding of SHP-1 to EGF-R has been reported previously (34), and our results further verified it in EGF-treated HeLa cells. More importantly, our results indicated that active SHP-1 markedly reduced tyrosine phosphorylation of the receptor. However, rather than decreasing the downstream signal transduction of the receptor as one might expect, it enhanced it. This suggests that transduction of signals via EGF-R is not proportional to the level of its tyrosine phosphorylation and that dephosphorylation of the receptors, perhaps, at certain sites which otherwise negatively regulate the activity of the receptor or serve as docking sites for proteins with inhibitory functions, may be essential. This would explain the positive role of the native form of SHP-1. For the catalytically inactive mutant SHP-1-(Cys → Ser), one trivial explanation is that SHP-1-(Cys → Ser) may bind EGF-R via the inactive catalytic domain, thus preventing other signaling molecules from accessing the receptor and blocking their signal. This has been proven to be unlikely, since the mutant SHP-1-(Cys → Ser) was shown to be less efficient in binding to EGF-R than the native SHP-1. In fact, the inability of SHP-1-(Cys → Ser) to bind EGF-R might suggest that the binding alone requires PTP catalytic activity. Another consequence of the EGF stimulation of cells is the tyrosine phosphorylation of SHP-1 (Fig. 5). Previous studies have demonstrated that tyrosine phosphorylation of SHP-1 can provide binding sites for GRB2 (19), which presumably could lead to activation of the Ras/MAP kinase signaling pathway. However, since the Cys to Ser mutation of SHP-1, which diminishes auto-dephosphorylation of the enzyme, displayed an even stronger phosphorylation but inhibited downstream signaling, the present results suggest that tyrosine phosphorylation per se is probably not a key factor in the process. Further support for this is that tyrosine phosphorylation of SHP-1 was not observed in response to ING-γ stimulation although it had similar effects on STAT activation. Tyrosine phosphorylation also results in activation of SHP-1 (35). This is meaningful to the native form of SHP-1 but not to the dead mutant SHP-1-(Cys → Ser). Above all, the catalytic activity of SHP-1 has a major role in signaling transduction initiated by growth factors and cytokines. At present, we can only speculate that, as a positive regulator, SHP-1 functions by dephosphorylating the receptors at certain tyrosine phosphorylation sites that have inhibitory roles or by dephosphorylating other intermediate signaling proteins that would be activated.

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