Tyrosine phosphatase SHP-2 dephosphorylates the platelet-derived growth factor receptor but enhances its downstream signalling

Runxiang ZHAO and Zhizhuang Joe ZHAO¹

Hematology/Oncology Division, Department of Medicine, Vanderbilt Cancer Center, Vanderbilt University, 547 MRB II, 2220 Pierce Avenue, Nashville, TN 37232-6305, U.S.A.

SHP-2 is a widely distributed Src homology 2 (SH2) domain-containing tyrosine phosphatase that is recruited to growth factor receptors on stimulation. We have transiently co-expressed several catalytically active and inactive forms of the enzyme with the platelet-derived growth factor (PDGF) receptor in human embryonic kidney 293 cells. The catalytically active forms of SHP-2 decreased the tyrosine phosphorylation of the receptor, whereas the catalytically inactive forms increased the phosphorylation. However, PDGF-induced activation of the mitogen-activated protein (MAP) kinase pathway was enhanced by the

active forms of SHP-2 but decreased by the inactive forms. The results suggest that the PDGF receptor is a physiological substrate of SHP-2 and that SHP-2 has a positive role in the PDGF-stimulated activation of MAP kinase. The dissociation of the receptor phosphorylation from the activation of MAP kinase suggests that signalling through growth factor receptors does not depend merely on their tyrosine phosphorylation.

Key words: cell transfection, MAP kinase, phosphorylation, SH2 domain, signal transduction.

INTRODUCTION

SHP-2, originally known as SH-PTP2, PTP2C, PTP1D or Syp, is a widely distributed protein tyrosine phosphatase (PTP) containing Src homology 2 (SH2) domains [1,2]. It has a great similarity to *Drosophila* corkscrew, which has a positive role in the transduction of the *torso* signal by acting in concert with D-raf [3]. Studies indicate that SHP-2 has a similar positive role in signalling in mammalian cells. Microinjection of anti-(SHP-2) antibodies, or a glutathione S-transferase fusion protein encoding the SH2 domains of SHP-2, blocks insulin-stimulated DNA synthesis [4]. The expression of a catalytically inactive cysteine-to-serine mutant of SHP-2 inhibits the activation of mitogen-activated protein (MAP) kinase induced by insulin, epidermal growth factor, platelet-derived growth factor (PDGF) and α -thrombin. It also decreases the activation of signal transduction and activators of transcription (STAT) factors by interferon α/β , and suppresses early gene transcription, DNA synthesis and cell proliferation [5–11]. In *Xenopus* embryogenesis, a dominant-negative mutant of SHP-2 produced by an internal phosphatase domain deletion blocks the fibroblast growth factor- and activin-mediated induction of mesoderm and MAP kinase activation induced by fibroblast growth factor [12].

Although the association of SHP-2 with the PDGF receptor (PDGF-R) and the phosphorylation of SHP-2 after PDGF stimulation have been well documented [13–16], whether the enzyme dephosphorylates the receptor *in vivo* and how it affects the downstream signalling of the receptor are not well defined. In the present study we have transiently co-expressed PDGF-R with several active or inactive forms of SHP-2 in human embryonic kidney 293 cells. The results demonstrate that SHP-2 dephosphorylates PDGF-R but enhances the activation of the MAP kinase pathway induced by PDGF. To our knowledge, this

is the first example that tyrosine phosphorylation of a receptor tyrosine kinase is not correlated with activation of its downstream signalling pathway.

MATERIALS AND METHODS

Materials

Human embryonic kidney 293 cells were obtained from the American Type Culture Collection. PDGF, Dulbecco's modified Eagle's medium, fetal bovine serum and penicillin/streptomycin antibiotic mixture were from Gibco. Polyclonal anti-(PDGF-R) and monoclonal anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology and Upstate Biotechnology respectively. Polyclonal anti-(SHP-2) was raised in a rabbit against an SH2-domain-truncated form of SHP-2 expressed in *Escherichia coli* [7].

Constructs of cDNA species

Human PDGF-R cDNA was cloned as described previously [17]. The constructs of SHP-2 are illustrated in Figure 1. The native SHP-2 and the catalytically inactive cysteine-to-serine mutant SHP-2(C-S) were as used in a previous study [7]. The remaining three constructs with a Myr tag (a consensus myristoylation sequence corresponding to the 15 N-terminal amino acid residues, MGSNKSQPKDASQRR, of human c-Src [18]) were designed to target the enzyme to the plasma membrane. DNA oligonucleotides encoding for this sequence were synthesized and inserted in frame in front of the 5'-coding region of SHP-2, SHP-2(C-S) and SH2-domain-truncated SHP-2 to give rise to Myr-SHP-2, Myr-SHP-2(C-S) and Myr- Δ SHP-2 respectively. The sequences were confirmed by DNA sequencing. All these constructs were made in the pRc/CMV vector (Invitrogen).

Abbreviations used: MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; PDGF, platelet-derived growth factor; PDGF-R, PDGF receptor; PTP, protein tyrosine phosphatase; SH2, Src homology 2; SHP-2(C-S), catalytically inactive cysteine-to-serine mutant of SHP-2; GAP, GTPase-activating protein.

¹ To whom correspondence should be addressed (e-mail joe.zhao@mcmail.vanderbilt.edu).

Figure 1 Constructs of SHP-2

Cell transfection, stimulation and extraction

Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 50 µg/ml streptomycin and 50 µg/ml penicillin. Transfection of cells was performed by using a modified calcium phosphate co-precipitation protocol [19]. In brief, confluent cells were split 1:10 and cultured overnight to approx. 25% confluency with 10 ml of medium in 10 cm plates. This was followed by the addition of calcium precipitates containing 25 µg of total DNA species. Ratios of the PDGF-R construct to various SHP-2 constructs were 1:2. After 24 h of incubation, cells were washed with PBS and then cultured in fresh medium for another 28 h before being harvested. For cell stimulation, 48 h after transfection, cells (80–90% confluency) were starved at 0% serum for 4 h and were then stimulated with 5 ng/ml PDGF for 5 min. After being washed with ice-cold PBS, cells were lysed in a buffer containing 50 mM β-glycerophosphate, pH 7.3, 2 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, 1% (v/v) Triton X-100, 0.2 mM Na₃VO₄, 0.1 µM microcystin, 1.0 mM benzamidine, 0.1 mM PMSF, 20 µg/ml leupeptin, 1 µM pepstatin A and 1 µg/ml aprotinin. Extracts were cleared by centrifugation and used for Western-blotting analyses and for assays of MAP kinase/ERK kinase (MEK) activity.

Western blotting

For Western-blot analyses, samples were separated by SDS/PAGE [10% (w/v) gel] and transferred to PVDF membranes (Amersham). The membranes were probed with various primary antibodies and detected by using the ECL[®] system with horseradish peroxidase-conjugated secondary antibodies (Amersham), in accordance with the manufacturer's protocol.

Activity assays of MEK

The crude cell extracts made in the extract buffer described above were used directly for activity assays. MEK activity was determined by measuring the ability of the enzyme to stimulate the myelin basic protein kinase activity of recombinant ERK2 in a coupled assay system [7]. In brief, samples were incubated with 3.3 µg/ml recombinant ERK2 in the presence of unlabelled ATP for 10 min; myelin basic protein and [γ-³²P]ATP were then added. The incorporation of ³²P into myelin basic protein represented the activity.

RESULTS

Effects of altered SHP-2 expression on phosphorylation of PDGF-R

A total of five SHP-2 constructs were employed in this study (Figure 1). Two of them encoded catalytically inactive cysteine-to-serine mutants. Mutation of the highly conserved cysteine residue within the catalytic centre of PTPs to serine has been widely used to impair the catalytic activity of PTPs [5–11]. The catalytically inactive forms of SHP-2 presumably have a dominant negative effect because they can compete with the native enzyme for SH2-domain-binding sites and prevent the latter from accessing target proteins. Three of the constructs have a Myr tag. The Myr sequence, derived from the N-terminal myristoylation sequence of c-Src, has been used to target Raf-1 to the plasma membrane, which led to constitutive activation of the enzyme [20,21]. SHP-2 can be recruited by binding to tyrosine-phosphorylated receptors on the plasma membrane, where it finds its substrates. The binding itself results in the activation of enzyme [16,22], and phospholipids such as phosphatidic acid might further activate it [23,24]. Thus targeting SHP-2 to the plasma membrane was intended to increase the accessibility of the enzyme to its substrates and to activate the enzyme through interaction with anionic phospholipids.

Expressions of these five forms of SHP-2 together with PDGF-R are illustrated in Figure 2(A). The transfection efficiency of 293 cells with the modified calcium phosphate precipitation method was more than 50%, as indicated by immunofluorescent cell staining with the anti-(PDGF-R) antibody (results not shown). In comparison with the level of endogenous SHP-2 represented by the vector control, high overexpression of the exogenous enzymes was observed, as determined by Western-blot analyses with anti-(SHP-2) antibody (Figure 2A). Further Western blot analyses with a series of diluted samples followed by densitometric analyses revealed 10–15-fold overexpression of the exogenous enzymes over the endogenous SHP-2. The Myr-tagged full-length enzymes ran slightly further than the native SHP-2, and the Myr-tagged SH2 domain-truncated Myr-ΔSHP-2 ran at approx. 47 kDa. Furthermore, the Myr-tagged enzymes were indeed found only in the membrane fraction, as indicated by subcellular fractionation (results not shown). The expression of PDGF-R was apparent as analysed by the anti-(PDGF-R) antibody, although enhancement of expression was hard to ascertain because essentially no endogenous PDGF-R was expressed in the wild-type 293 cells. The exogenously expressed PDGF-R ran as a doublet, probably owing to different degrees of glycosylation. Note that a slightly higher level of PDGF-R was consistently expressed in cells co-expressing Myr-SHP-2 and Myr-ΔSHP-2.

We first analysed the tyrosine phosphorylation of cellular proteins in transfected cells at the basal level. Growth factor receptors such as PDGF-R form dimers when binding with their ligands. Dimerization presumably permits an efficient intermolecular reaction and allows the two subunits to phosphorylate each other [25]. Under normal conditions, tyrosine phosphorylation of endogenous receptors is barely detected without stimulation by their ligands. Interestingly, strong phosphorylation of PDGF-R was observed when the receptor was transiently expressed in 293 cells, as shown in Figure 2(A). This might be because the receptor was so highly expressed that it reached a point for efficient intermolecular reactions to occur. Furthermore, the phosphorylation of the overexpressed PDGF-R was significantly affected by expression of different forms of SHP-2. The phosphorylation of PDGF-R was decreased by at least 50% by overexpression of the native SHP-2 but enhanced more than

Figure 2 Effects of altered SHP-2 expression on tyrosine phosphorylation of PDGF-R and PDGF-induced MEK activation

Cell transfection was performed as described in the Materials and methods section. **(A)** Tyrosine phosphorylation of cellular proteins under the serum-fed condition and expression levels of PDGF-R and SHP-2 constructs. Transfected 293 cells were grown under the normal serum-fed condition. Cell extracts (25 μ g total proteins) were separated by SDS/PAGE [10% (w/v) gel] and transferred to a PVDF membrane, then blotted with monoclonal anti-phosphotyrosine, followed by reblotting with anti-(PDGF-R) and then with anti-(SHP-2). **(B, C)** Tyrosine phosphorylation of proteins and activation of MEK after stimulation with PDGF. The same transfected 293 cells as described for **(A)** were starved at 0% serum for 4 h and then treated with 5 ng/ml PDGF for 5 min [lanes marked + in **(B)**; open bar in **(C)**] or left untreated [lanes marked - in **(B)**; filled bars in **(C)**]. Cell extracts were subjected to anti-phosphotyrosine Western blotting **(B)** and MEK activity analyses **(C)** as described in the Materials and methods section. p43 denotes the tyrosine-phosphorylated protein of approx. 43 kDa. Representative data from three experiments with similar results is shown.

2-fold by expression of the inactive mutant SHP-2(C-S). Myr-SHP-2 did not show a significant effect on the phosphorylation of PDGF-R, suggesting that targeting SHP-2 to the plasma membrane by attaching a Myr tag did not enhance its activity towards PDGF-R. This might be due to a steric effect prohibiting the catalytic centre of SHP-2 from accessing PDGF-R. Never-

theless, Myr-SHP-2(C-S) increased the phosphorylation of PDGF-R, indicating that membrane localization did not affect its ability to prevent the dephosphorylation of PDGF-R. In addition, Myr-SHP-2(C-S) itself was heavily phosphorylated on tyrosine residues, suggesting that membrane localization makes it a better substrate for a tyrosine kinase, perhaps PDGF-R. This might be attributable to the enhanced accessibility of the C-terminal tail of SHP-2 that contains tyrosine phosphorylation sites to the receptor tyrosine kinase. It should be noted that expression of both SHP-2(C-S) and Myr-SHP-2(C-S) resulted in tyrosine phosphorylation of a 43 kDa protein, as described previously [7]. Finally, Myr-ΔSHP-2 resulted in a more extensive dephosphorylation of PDGF-R than did the native SHP-2. This was expected because the membrane targeting made the enzyme readily accessible to the receptor and because the truncation of SH2 domains results in the activation of SHP-2 [26].

We then analysed tyrosine phosphorylation of PDGF-R after stimulation with its ligand (Figure 2B). For this purpose the transfected cells were serum-starved for 4 h before the addition of PDGF. In comparison with the levels of tyrosine phosphorylation in the basal serum-fed condition, serum starvation caused a noticeable decrease in the tyrosine phosphorylation of PDGF-R in vector control cells but had no significant effect on other cells. This suggests that the withdrawal of serum caused either a decrease in tyrosine kinase activity or an increase in tyrosine phosphatase activity. In any case, the co-expression of SHP-2 in active or inactive forms overcame such effects, implying that SHP-2 is responsible for the regulation of PDGF-R phosphorylation. On stimulation of the serum-starved cells with PDGF, phosphorylation of PDGF-R was increased by approx. 2–3-fold over the levels observed with serum-starved cells in all the transfected cells. Compared with the vector control cells, PDGF-induced tyrosine phosphorylation of PDGF-R in cells co-expressing SHP-2 or Myr-ΔSHP-2 did not show a significant decrease, as observed in the serum-fed basal condition, suggesting that PDGF-induced kinase activity overcame the phosphatase activity of SHP-2 and/or that the PDGF-induced phosphorylation of PDGF-R was not affected by SHP-2. Nevertheless, SHP-2(C-S) and Myr-SHP-2(C-S) caused a marked increase in tyrosine phosphorylation of PDGF-R in the PDGF-stimulated cells. Furthermore, the tyrosine phosphorylation of p43, Myr-SHP-2(C-S) and SHP-2(C-S) was enhanced on stimulation with PDGF. SHP-2, Myr-SHP-2 and Myr-ΔSHP-2 were also phosphorylated but not to an extent that could be seen by direct Western blotting analyses of cell extracts; immunoprecipitation of the proteins was required before the phosphorylation could be seen (results not shown). The high degree of tyrosine phosphorylation observed in the mutant forms of SHP-2 suggests that auto-dephosphorylation is mainly responsible for the dephosphorylation of SHP-2. As expected, we also observed co-immunoprecipitation of SHP-2, SHP-2(C-S), Myr-SHP-2 and Myr-SHP-2(C-S) with PDGF-R after stimulation of cells with PDGF, but the proteins found in the complexes represented less than 1% of the enzyme and the receptor expressed in the cells (results not shown). Furthermore, the catalytically inactive forms of SHP-2 did not show any stronger binding to the receptor than the corresponding catalytically active forms. This therefore rules out the possibility that the catalytically inactive forms of SHP-2 would block signal transduction by binding to PDGF-R through the catalytic domain.

Although it is hard to exclude totally the possible non-specific effects caused by the overexpression of proteins in cells, the fact that catalytically active SHP-2 and Myr-ΔSHP-2 decreased the phosphorylation of PDGF-R, whereas catalytically inactive SHP-2(C-S) and Myr-SHP-2(C-S) enhanced the phosphoryl-

ation, argues strongly that SHP-2 can directly dephosphorylate PDGF-R. Therefore we conclude that PDGF-R is a physiological substrate of SHP-2.

Effects of altered SHP-2 expression on PDGF-induced activation of the MAP kinase cascade

Because tyrosine phosphorylation of PDGF-R is the initial event in transduction of the PDGF signal, by affecting the tyrosine phosphorylation of PDGF-R, SHP-2 should have a profound effect on signal transduction through the receptor. To access the cell signalling initiated by PDGF, we analysed the activity of MEK, an intermediate upstream of MAP kinase in the well-defined Ras/MAP kinase signalling pathway. MEK is a highly specific dual-specificity protein kinase [27]. The coupled analyses described in the Materials and methods section make the assay highly sensitive and specific, compared with the relatively non-specific assay for MAP kinase with myelin basic protein as a substrate. Because 293 cells express no endogenous PDGF-R, the stimulation with PDGF of wild-type cells or cells overexpressing SHP-2 constructs alone resulted in no activation of MEK (results not shown). When PDGF-R was overexpressed in the cells, high levels of tyrosine phosphorylation of the receptor were observed even without treatment with PDGF. However, activation of MEK was not observed in the serum-starved or serum-fed cells, suggesting that tyrosine phosphorylation at the basal level is not sufficient to activate the downstream signal transduction. On stimulation of PDGF-R-transfected cells with PDGF, despite only a moderate increase in tyrosine phosphorylation of the receptor (see Figure 2B), a strong activation of MEK was obtained (Figure 2C). The level of activation was comparable with that observed with epidermal growth factor, as reported previously [7]. Compared with the vector control, the native SHP-2, which decreased the tyrosine phosphorylation of PDGF-R under the basal conditions, displayed a 60 % increase in MEK activity. In contrast, the inactive mutant SHP-2(C-S), which increased the tyrosine phosphorylation under the basal and stimulated conditions, decreased the activity by 40 %. Myristoylated SHP-2 and SHP-2(C-S) displayed even greater effects. The former increased MEK activity by 100 %, whereas the latter decreased it by 60 %. This suggests that targeting SHP-2 to the membrane does enhance the effects of the enzyme in signal transduction, although Myr-SHP-2 did not enhance the dephosphorylation of PDGF-R. Myr-ΔSHP-2, which displayed the strongest dephosphorylation of PDGF-R, enhanced the MEK activity by only 40 %, suggesting that the activation of MEK is not directly correlated with the dephosphorylation of PDGF-R either. Despite different effects exhibited by different forms of SHP-2 on tyrosine phosphorylation of PDGF-R, the activation of MEK was not observed without the addition of PDGF, suggesting that SHP-2 might be necessary but not sufficient for turning on the PDGF-R-mediated signalling pathway. Taken together, these results indicate that the catalytic activity of SHP-2 is at least partly required for MAP kinase activation induced by PDGF, and that SHP-2 has a positive role in the process, which is consistent with previous reports [4–12]. However, the present results suggest that there is no direct correlation between total tyrosine phosphorylation of the receptor and the activation of downstream MEK.

DISCUSSION

By overexpressing various active and inactive forms of SHP-2, the present study demonstrates that active SHP-2 decreases the tyrosine phosphorylation of PDGF-R, whereas catalytically

inactive SHP-2 increases the phosphorylation of the receptor. This suggests that PDGF-R serves as a physiological substrate of SHP-2. Because autophosphorylation of PDGF-R is absolutely required for transduction of the PDGF signal, one might have predicted that SHP-2 has a negative role in PDGF signalling. However, the catalytically active forms of SHP-2 decrease the tyrosine phosphorylation of PDGF-R but increase the activation of MAP kinase, whereas the inactive mutant forms of SHP-2 enhance tyrosine phosphorylation of the receptor but suppress the activation of the MAP kinase pathway. These results thus dissociate the tyrosine phosphorylation of PDGF-R from its downstream signalling transduction. One explanation for this is that SHP-2 affects only certain sites that are not related to MAP kinase activation. PDGF-R was shown to contain at least nine autophosphorylated tyrosine residues [28]. Phosphorylation of Tyr-857 in the second part of the kinase domain is important for the catalytic activity of the kinase, whereas the others interact specifically with different signal-transduction molecules. The intensive phosphorylation of the receptor itself and Myr-SHP-2(C-S) observed in this study is indicative of the activation of the kinase, which presumably leads to the phosphorylation of most, if not all, of the sites. SHP-2, in contrast, might attack only certain sites as indicated by the incomplete dephosphorylation of PDGF-R. Studies *in vitro* do indeed suggest that SHP-2 has a clear preference for certain receptor phosphorylation sites [29]. The most efficiently dephosphorylated sites are Tyr-771 (a GTPase-activating protein (GAP)-binding site), Tyr-751 (a Nck-and phosphoinositide 3-kinase-binding site), followed by Tyr-740 (a phosphoinositide 3-kinase-binding site). These three sites also represent major binding sites for SHC, an adaptor protein that couples Grb2 to PDGF-R and leads to the activation of Ras/MAP kinase. Thus SHP-2 would terminate the Ras/MAP kinase pathway by dephosphorylating SHC-binding sites on PDGF-R. This possibility does not seem to stand. Of course there are many other phosphorylation sites on PDGF-R that have not been characterized, and some of the sites might have a negative role by recruiting inhibitory proteins or affecting the kinase activity of PDGF-R, thereby inhibiting signal transduction through the receptor. Identification of the phosphorylation sites of PDGF-R that are affected by SHP-2 *in vivo* would help to clarify this.

Our study also demonstrated that SHP-2 has a positive role in the PDGF-induced activation of the MAP kinase pathway. This is consistent with the previous reports [4–11]. The precise mechanism by which SHP-2 regulates the activation of MAP kinase is not well understood. There could be several explanations for the positive action of SHP-2 in growth factor signalling. First, SHP-2 can serve as an adaptor to couple Grb2 to PDGF-R by binding to the PDGF-R via its SH2 domains and to Grb2 via its tyrosine-phosphorylated C-terminal segment, thus transducing the signal to activation of Ras [15]. However, this does not explain the requirement for PTP activity, because the catalytically inactive mutants SHP-2(C-S) and Myr-SHP-2(C-S), which displayed a higher degree of tyrosine phosphorylation, did not enhance the activation of MAP kinase but instead suppressed it. Therefore the present results suggest a more complicated role for SHP-2 than serving as an adaptor for Grb2 binding. Secondly, SHP-2 might affect the MAP kinase activation pathway by down-regulating the Ras-GAP. As described above, SHP-2 preferentially dephosphorylates the GAP-binding site Tyr-771, which presumably blocks the association of GAP with PDGF-R. GAP would otherwise negatively regulate Ras by enhancing the hydrolysis to GDP of GTP bound to Ras. It has been reported that GAP inhibits the PDGF-dependent activation of phospholipase C- γ and DNA synthesis [30]. Whether GAP has a

similar role in MAP kinase activation has not been defined. Thirdly, SHP-2 might act on other inhibitory signalling proteins such as the 43 kDa hyperphosphorylated protein observed in this study and other putative substrates of SHP-2 that have been reported [5,7,31–34]. Although hyperphosphorylation of these proteins accompanying the expression of catalytically inactive mutant forms of SHP-2 is correlated with blocking of growth factor-induced signal transduction, the detailed mechanism remains to be defined.

Above all, the present study suggests that the induction of tyrosine phosphorylation of the receptor kinases is not the sole determinant of signal transduction. Engagement of growth factor receptors with their ligands stimulates both tyrosine kinases and phosphatases. The activated PTPs could dephosphorylate the proteins phosphorylated by the activated tyrosine kinases. However, the dephosphorylation should not be viewed as a simple signal turn-off event. Instead it might enhance the signal by keeping the tyrosine phosphorylation at a high rate of dynamic turnover. This is reminiscent of the action of G-proteins: the binding of GTP to G-proteins induces protein–protein interactions; the hydrolysis of GTP to GDP completes the reactions. In this regard, signal transduction induced by growth factors might depend not on the net level of tyrosine phosphorylation but on the rate of its dynamic turnover, which requires the positive involvement of both tyrosine kinases and phosphatases. This would explain the role of SHP-2 in PDGF signalling described in this study.

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