Identification of a Putative Syp Substrate, the PDGF\(\beta\) Receptor*

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Richard A. Klinghoffer and Andrius Kazlauskas‡

From the National Jewish Center for Immunology and Respiratory Medicine, Division of Basic Sciences, Denver, Colorado 80206 and the University of Colorado Health Sciences Center, Department of Pharmacology, Denver, Colorado 80262

Because the protein-tyrosine phosphatase (PTP) Syp associates with the tyrosine-phosphorylated platelet-derived growth factor \(\beta\) receptor (\(\beta\)PDGFR), the \(\beta\)PDGFR is a likely Syp substrate. We tested this hypothesis by determining whether recombinant Syp (rSyp) and a control PTP, recombinant PTP1B (rPTP1B), were able to dephosphorylate the \(\beta\)PDGFR. The \(\beta\)PDGFR was phosphorylated at multiple tyrosine residues in an in vitro kinase assay and then incubated with increasing concentrations of rSyp or rPTP1B. While the receptor was nearly completely dephosphorylated by high concentrations of rPTP1B, receptor dephosphorylation by rSyp plateaued at approximately 50\%. Two-dimensional phosphopeptide maps of the \(\beta\)PDGFR demonstrated that rSyp displayed a clear preference for certain receptor phosphorylation sites; the most efficiently dephosphorylated sites were phosphotyrosines (Tyr(P))-771 and -751, followed by Tyr(P)-740, while Tyr(P)-1021 and Tyr(P)-1009 were very poor substrates. In contrast, rPTP1B displayed no selectivity for the various \(\beta\)PDGFR tyrosine phosphorylation sites and dephosphorylated all of them with comparable efficiency. A Syp construct that lacked the SH2 domains was still able to discriminate between the various receptor phosphorylation sites, although less effectively than full-length Syp.

These in vitro studies predicted that Syp can dephosphorylate the receptor in vivo. Indeed, we found that a \(\beta\)PDGFR mutant (F1009) that associates poorly with Syp, had a much slower in vivo rate of receptor dephosphorylation than the wild type receptor. In addition, the GTPase-activating protein of Ras (GAP) and phosphatidylinositol 3-kinase 3-kinase were less stably associated with the wild type \(\beta\)PDGFR than with the F1009 receptor. These findings are consistent with the in vitro experiments showing that Syt prefers to dephosphorylate sites on the \(\beta\)PDGFR, that are important for binding phosphatidylinositol 3-kinase (Tyr(P)-740 and Tyr(P)-751) and GAP (Tyr(P)-771). These studies reveal that Syp is a substrate-selective PTP and that both the catalytic domain and the SH2 domains contribute to Syp's ability to choose substrates. Furthermore, it appears that Syp plays a role in PDGF-dependent intracellular signal relay by selectively dephosphorylating the \(\beta\)PDGFR and thereby regulating the binding of a distinct group of receptor-associated signal relay enzymes.

Tyrosine phosphorylation modulates the activity of many enzymes involved in regulation of a wide variety of cellular events and is a reversible process governed by the opposing effects of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs)\(^1\) (1–3). In addition to a conserved catalytic domain, most PTPs also contain highly variable noncatalytic sequences, which can regulate the activity and/or subcellular location of the PTP (1, 3). The initially purified PTPs failed to show a high degree of substrate specificity (4), and this led to the hypothesis that subcellular targeting is the primary determinant in selection of a substrate. More recent evidence indicates that an interaction may exist between the amino acids surrounding the phosphotyrosine and the amino acids surrounding the mouth of the active site of the PTP (5–7). The relative strength of the interaction between a particular PTP and a tyrosine-phosphorylated protein may permit the PTP to discriminate between substrates. This possibility is supported by the identification of PTPs that show a high degree of substrate specificity; two different mammalian PTPs have been identified that appear specific for MAP kinases (8–12). Thus the choice of substrates not only depends on the subcellular location of a PTP, but at least in some instances on the subcellular specificity of the PTP.

Syp (also commonly called SH-PTP2 or PTP-1D) is one member of a small family of SH2 domain-containing PTPs, which also includes Corkscrew (Csw) and HCP (also called SH-PTP1 or PTP-1C) (13–14). All three of these PTPs appear to play important roles in signal transduction. Together with the Drosophila homologues of c-Raf and Ras, Csw acts downstream of the Torso receptor tyrosine kinase in a signaling cascade that is essential for normal development of the Drosophila embryo (15, 16). HCP plays a negative role in signaling of receptors such as the erythropoietin receptor and the B cell antigen receptor (17, 18). In contrast, Syp may function more like Csw, in that it appears to play a positive role in signaling. Catalytically inactive Syp constructs block fibroblast growth factor-driven mesoderm induction at a step prior to MAP kinase activation (19). Microinjection of neutralizing Syp antibodies or Syp SH2 domains severely inhibits growth factor-mediated DNA synthesis in mammalian cells (20, 21). These studies indicate that the SH2 domain-containing phosphatases play an important role in the regulation of intracellular signal relay cascades.

Recent studies have revealed that Syp can act as an adapter protein. In response to PDGF stimulation, Syp becomes phosphorylated at tyrosine 542 (Y542) (22), which is a good binding site for the Grb2 SH2 domain (23). Grb2 binds Syp at this phosphotyrosine site in vitro, and both Grb2 and the qua-

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‡ Established Investigator of the American Heart Association. To whom correspondence should be addressed. National Jewish Center for Immunology and Respiratory Medicine, Division of Basic Sciences, 1400 Jackson St., Denver, CO 80206. Tel.: 303-398-1454; Fax: 303-398-1225; E-mail: kazlauskasa@njc.org.

1 The abbreviations used are: PTP, protein-tyrosine phosphatase; MAP, mitogen-activated protein; GAP, GTPase-activating protein; PI, phosphatidylinositol; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; pNPP, para-nitrophenyl phosphate; WT, wild type; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor.
nidine nucleotide-releasing factor Sos communoprecipitated with Syt from lysates of PDGF-stimulated cells (22, 24). Moreover, studies with the add-back PDGFRβ receptor (βPDGFR) mutants indicate that binding of Syt is one of the mechanisms by which the βPDGFR is able to mediate activation of Ras (25). Thus, one of the apparent functions of Syt is to serve as an adapter protein in the process of PDGF-stimulated Ras activation.

Upon activation, the βPDGFR is phosphorylated at multiple tyrosine residues and thereby becomes a docking site for SH2-domain-containing signal transduction proteins. These proteins play important roles in the relay and control of mitogenic signals sent from the receptor to the nucleus. Some of the proteins that associate with the activated βPDGFR include phospholipase C-γ1, the GTPase-activating protein of Ras (GAP), phosphatidylinositol 3-kinase (PI-3 kinase), Nck, Shc, Grb2, pp60src, p85+, p59yck, an as yet unidentified 120-kDa protein, and Syt (26–28). Some of the receptor-associated proteins undergo tyrosine phosphorylation in a PDGF-stimulated cell, and this event affects their ability to send a biological signal. Given that Syt is brought into close proximity to these tyrosine-phosphorylated signaling molecules, it is likely that Syt regulates βPDGFR signal transduction by modulating the phosphorylation state of the members of the βPDGFR complex.

To test this hypothesis, we determined the in vitro ability of a recombinant form of Syt to dephosphorylate the βPDGFR. Our studies showed that Syt was capable of dephosphorylating the receptor and that there was a rank order for dephosphorylation of the various βPDGFR phosphorylation sites. Investigation of the basis for Syt's substrate specificity identified two main factors: 1) positioning of the Syt catalytic domain on the βPDGFR, as directed by the binding of Syt's SH2 domains to the receptor; and 2) an intrinsic preference of the catalytic domain for certain phosphotyrosine residues. To ascertain whether these in vitro studies reflected the in vivo situation, we compared the rank of receptor dephosphorylation of the wild type and the F1009 mutant, which binds Syt poorly. After stimulation with PDGF, the wild type receptor was dephosphorylated more rapidly than the F1009 mutant, suggesting that Syt functions to dephosphorylate the receptor in an intact cell. In addition, we found that the βS subunit of PI 3-kinase, and to a greater extent GAP, are less stably associated with the wild type receptor than the F1009 mutant, which is consistent with Syt's ability to dephosphorylate the PI 3-kinase and GAP binding sites. Our studies show that Syt displays a high degree of substrate specificity and that one of its substrates appears to be the activated βPDGFR.

EXPERIMENTAL PROCEDURES

Plasmid Construction—RNA was derived from Nu6 cells, a lung tumor cell line (55) as described (56). This RNA was utilized as a template in a reverse transcriptase reaction to synthesize Nu6 cDNA, which was used as the template in the polymerase chain reaction for amplification of Syt cDNA. A 1.85-kilobase PCR product containing the entire Syt coding region was amplified using the following primers, which were designed using the published human Syt DNA sequence (13) 5′-GCGGGGATCCATGACATCGCGGAGATGG-3′ (sense) and 5′-AAACCTGATCCGTCCTTCTTSCTTTAGGGAAGGGG-3′ (antisense). BamHI restriction sites were introduced during PCR amplification and are shown by the underlined characters. To generate the catalytic domain of Syt the same antisense primer was used as above, along with the following sense primer: 5′-TCACTGACTCGATTCCAGACCCTTACAGACTCCATG-3′. To generate the PTTP2 construct, the PT7-7 plasmid (57) was used as a PCR template with the following oligos: the 5′-oligo, 5′-ATCGAGGATTCCGAATGGAAGAACAGATCGAAGG-3′; and the 3′-oligo, 5′-TCAAGCCGAAGCCTTCTGAGAAACACTCACC-3′, introduced a HindIII restriction site (underlined). Following amplification, full-length PCR products were first subcloned into the pGEM T vector (Promega) and then into pRSET A (Invitrogen) using the restriction sites introduced during PCR amplification. The integrity of the front end of all clones was verified by dideoxy sequencing using the T7 promoter primer.

Expression and Purification of rSyt, rPTTP1B, and rCAT—Escherichia coli strain BL21(DE3) transformed with plasmid pRSET A-Syt, pRSET A-PTTP1B, or pRSETA-CAT was grown in 1 liter of LB medium containing 100 μg/ml carbenicillin to A600 = 0.7–0.9 and induced for 3 h at 30°C with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation and resuspended in 30 ml of sonication buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 20 μg/ml leupeptin, 1.0 μg/ml pepstatin A, 20 μg/ml aprotnin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml antipain). Cells were disrupted by one freeze thaw cycle followed by six 1-min rounds of sonication. Crude lysates were centrifuged at 10,000 × g, and the cleared supernatant was incubated at 4°C for 1 h with 1.5 ml of Ni2+–nitrilotriacetic acid resin in sonication buffer. Following the incubation, the Ni2+–nitrilotriacetic acid resin was collected by centrifugation and packed into a 10-ml column (Bio-Rad). The columns were washed with 25 ml of sonication buffer followed by 25 ml of wash buffer (50 mM sodium phosphate, 300 mM NaCl, and 10% glycerol, pH 6.0). The His-tagged fusion proteins were eluted from the column by applying wash buffer with increasing concentrations of imidazole. Fractions were collected, dialyzed against an SDS-PAGE gel, and the gel was stained with Coomassie Blue. rSyt and rCAT typically eluted at 100–150 mM imidazole, while rPTTP1B eluted at 250 mM imidazole. The desired column fractions were pooled, dialyzed against storage buffer (50 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, 80 mM NaCl, and 20% glycerol), aliquoted, and stored at −20°C. The rPTP preparations did not noticeably lose activity for 3–4 weeks.

Assays for PTP Activity—A series of three types of experiments were performed to characterize the rPTPs using para-nitrophenyl phosphate (pNPP) as a substrate. First, hydrolysis of 10 mM pNPP was determined as a function of enzyme concentration. Concentration values within the linear range for pNPP hydrolysis were then used to determine the hydrolysis of pNPP as a function of time. Again the reaction was noted. Working within the linear ranges for enzyme concentration and time, the Km and Vmax values for rSyt, rCAT, and rPTTP1B were obtained by determining the hydrolysis of pNPP as a function of substrate concentration. All reactions were performed in a total volume of 50 μl containing 50 mM sodium acetate (pH 5.5), 50 mM NaCl, 10 mM dithiothreitol, and 2 mM EDTA. Typically 2.5 μg of rSyt, 0.15 μg of rCAT, and 0.2 μg of rPTTP1B were used in the reactions. Reactions were allowed to proceed for 20 min at 24°C, quenched by adding 950 μl of 1 N NaOH, and then the A405 was determined. For each concentration of pNPP the A405 of a mock sample containing substrate and enzyme buffer was measured. The amount of para-nitrophenol liberated in the reaction was calculated by comparison with a para-nitrophenol standard curve.

To assay dephosphorylation of the βPDGFR and receptor-associated proteins, βPDGFR immunoprecipitates were prepared as follows. HepG2 cells expressing the wild type βPDGFR (25) were grown to near confluence in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, incubated overnight in Dulbecco’s modified Eagle’s medium, 0.1% calf serum, and were left resting or stimulated for 5 min at 37°C with 50 ng/ml PDGF-BB. The cells were lysed in EB (58) and the receptor was immunoprecipitated with the 30A polyclonal anti-βPDGFR antibody, as described previously (30). The βPDGFR immune complexes were radiolabeled in a standard in vitro kinase assay in the presence of γ32P-ATP as described previously (59) and washed 3 × in 2 × phosphate buffer. Washed immune complexes, representing an approximately 4.25 × 105 cells or 6.5 × 10−2 μg of βPDGFR, were resuspended in 20 μl of 2 × phosphate buffer and then added to microfuge tubes containing 20 μl of the appropriate amount of rPTP or rPTP storage buffer. Equivalent amounts of rSyt and rPTTP1B (standardized according to enzymatic activity toward pNPP) were used, and the reactions were allowed to proceed for 30 min at 30°C with 40 μl. Typically 1.0–5.0 μl of 5.0–20 μCi/ml γ32P-ATP (50 Cl/ml) were added to the appropriate amount of rPTP or rPTP storage buffer. Equivalents amounts of rSyt and rPTTP1B (standardized according to enzymatic activity toward pNPP) were used, and the reactions were allowed to proceed for 30 min at 30°C with 40 μl. Typically 0.5–5.0 μCl/ml of 5.0–20 μl rSyt or 0.05–0.3 μCl/ml of 5.0–20 μl rPTTP1B was used. Reactions were terminated by adding 40 μl of 2 × sample buffer (10 mM EDTA, 4% SDS, 5.6 μl β-mercaptoethanol, 20% glycerol, 200 μM Tris-HCl, pH 6.8, 2% bromphenol blue). The samples were boiled and resolved on a 7.5% SDS-PAGE gel. The gel was stained with Coomassie Blue and the gel was scanned using a phosphorimager (Molecular Dynamics) and the relative amount of isoelectrically resolved bands was quantitated using the ImageQuant program. The amount of the phosphatase activity present in each fraction was calculated. Phosphopeptide Maps—Following the standard Phosphopeptide assay.
described above, the receptor was subjected to two-dimensional phosphopeptide analysis as described previously (30). Briefly, the radiolabeled βPDGFR was resolved by SDS-PAGE, excised from the gel, and exhaustively digested with trypsin and then thermolysin. The resulting phosphopeptides were resolved by thin layer electrophoresis (pH 8.9) followed by ascending chromatography in a buffer containing isobutyric acid/dimethylformamide/glacial acetic acid/water/butanol (65:3:3:29:2).

In Vivo Receptor Dephosphorylation—To investigate whether Syp binding affects the in vivo rate of receptor dephosphorylation, we compared the time course of receptor dephosphorylation for the wild type and the F1009 βPDGFRs. The F1009 receptor is a tyrosine to phenylalanine substitution at tyrosine 1009 (35). HepG2 cells expressing either the wild type or F1009 βPDGFRs were left resting or were stimulated with 30 ng/ml PDGF-BB for the indicated period of time. The cells were lysed, and the βPDGFR was immunoprecipitated with the 3A1 anti-βPDGFR antibody as described above. Immunoprecipitates were resolved on a 7.5% SDS-PAGE gel, transferred to Immobilon, and subjected to antiphosphotyrosine (a mixture of 7F10:1:1000, Transduction Laboratories, and 4G10 1:1000, Upstate Biotechnology Inc.) or anti-PDGFR (3A1 1:1000) Western blot analysis. 80 and 20% of the receptor immunoprecipitate was analyzed by antiphosphotyrosine and anti-βPDGFR Western blotting, respectively. Immunoreactive proteins were detected by the ECL detection system (Amersham Corp.) and then quantitated by densitometry.

To investigate whether Syp-mediated receptor dephosphorylation affects receptor binding to signal transduction proteins, we compared the ability of the WT and F1009 βPDGFRs to bind to GAP and the p85 subunit of PI 3-kinase in vivo at 5 and 25 min post-PDGF stimulation. HepG2 cells expressing either the WT or F1009 receptor were left resting, were stimulated with 50 ng/ml PDGF-BB for 5 min, or were stimulated for 5 min, and then the medium was replaced with prewarmed Dulbecco's modified Eagle's medium containing 0.1% calf serum and allowed to incubate for another 20 min at 37°C. Finally, the cells were lysed as described above, the βPDGFR was immunoprecipitated, and immunoprecipitates were resolved on a 7.5% SDS-PAGE gel, transferred to Immobilon, and subjected to anti-PDGFR (3A1 1:1000), anti-p85 (1:500), or anti-GAP (69.3 1:5000) Western blot analysis. Immunoreactive proteins were detected and analyzed as described above.

RESULTS

Purification and Characterization of Recombinant PTPs—To investigate Syp's biochemical properties and substrate specificity, we produced and characterized recombinant Syp, and the well characterized protein, PTP1B, which was used as a control throughout these studies. Recombinant versions of Syp and PTP1B (rSyp and rPTP1B, respectively) were synthesized and purified as follows. DNA fragments corresponding to the full-length coding regions of human Syp and rat PTP1B were PCR-amplified and then subcloned into the pRSETA bacterial expression vector, which produces a fusion protein containing a hexahistidine leader (His tag). rSyp and rPTP1B were expressed in E. coli and purified to near homogeneity (approximately 80% pure) by nickel/nitrotriacetic acid affinity chromatography, as described under "Experimental Procedures." A Coomassie-stained gel (Fig. 1) shows the starting material and the purified enzymes. The typical yield of purified PTPs was 20 mg/liter for rSyp and 2 mg/liter for rPTP1B.

To characterize the recombinant enzymes, we determined their Km and kcat using the low molecular weight substrate pNPP. The linear range of PTP activity was determined for both enzymes as a function of time and enzyme concentration (data not shown). Staying within the linear range for time and enzyme concentration, the activity of each enzyme was measured as the concentration of pNPP was varied (Fig. 2). The phosphatase activity of Syp (kcat = 4.09 s-1) was 18.2-fold lower than that of PTP1B (kcat = 74.34 s-1). The Km values for Syp and PTP1B toward pNPP were 2.8 and 3.8 mM, respectively (Fig. 2). This standardization was necessary in order to meaningfully compare the ability of the two PTPs to dephosphorylate candidate physiological substrates described below.

rSyp Dephosphorylates the βPDGFR in Vitro—Activation of the βPDGFR results in tyrosine phosphorylation of the receptor itself and several SH2 domain-containing signal transduction proteins that associate with the receptor. Since Syp is recruited to this assembly of tyrosine-phosphorylated signaling proteins, it is possible that one or more of these proteins are Syp substrates. To investigate this possibility, we compared the ability of rSyp and rPTP1B to dephosphorylate the βPDGFR. The βPDGFR and the associated proteins in an in vitro assay. The βPDGFR was immunoprecipitated from resting or PDGF-stimulated HepG2 cells expressing the wild type receptor and subjected to an in vitro kinase assay, which results in the radiolabeling of the receptor itself as well as the following receptor-associated proteins: phospholipase Cγ1, GAP, p85, and an unidentified 120-kD protein. Phosphoamino acid analysis of each of these proteins showed that they were all primarily phosphorylated on tyrosine residues (data not shown). Previous studies have shown that the receptor labeled in such an in vitro kinase assay is phosphorylated at most of the in vivo sites (29, 30). The radiolabeled receptor complex was incubated with buffer or increasing amounts of PTP, the proteins were resolved by SDS-PAGE, the radiolabeled proteins were visualized by autoradiography, and the extent of dephosphorylation was determined by densitometric scanning of the autoradiogram. Both rSyp and rPTP1B were able to dephosphorylate a subset of the receptor-associated proteins, and the relative ability to dephosphorylate these proteins was comparable for the two PTPs (data not shown).

In contrast, the degree of dephosphorylation of the PDGFR differed for the two PTPs. Receptor dephosphorylation increased linearly as the amount of rPTP1B was increased, and at the highest dose approximately 70% of the receptor's phosphate was removed (Fig. 3B). Adding 5 times more rPTP1B...
resulted in the loss of more than 90% of the label from the receptor (data not shown). Incubation of receptor immunoprecipitates with increasing amounts of rSyp increased the degree of receptor dephosphorylation up to a maximum of approximately 50% (Fig. 3A, lanes 5 and 6). This appeared to be the maximal extent of dephosphorylation, since the receptor was not further dephosphorylated when 5 times more rSyp was added (Fig. 3A, lanes 7 and 8). Note that although a different number of moles of rPTP1B and rSyp were used in this experiment, the number of units of PTP activity for the two enzymes was identical. These experiments demonstrated that rPTP1B efficiently dephosphorylated the βPDGFR, and at high concentrations removed greater than 90% of the label. In contrast, rSyp-mediated βPDGFR dephosphorylation reached a plateau, and further dephosphorylation was not observed even with high concentrations of rSyp.

There are at least three explanations for why Syp was not able to efficiently dephosphorylate the receptor as did rPTP1B. First, rSyp may be a less stable enzyme than rPTP1B, so that over the time course of the phosphatase assay rSyp activity declines. We tested this possibility and found that both enzymes remain fully active during the entire duration of the assay (data not shown). Second, the SH2 domains of Syp may bind phosphorylated receptor tyrosine residues and thereby protect these sites from the PTPase activity of Syp. To test if Syp SH2 domains are able to protect the PDGFR from PTPs in our assay we added the SH2 domains of Syp to the reaction containing PTP1B. The amount of Syp SH2 fusion protein added to the phosphatase reaction corresponded to the molar concentration of rSyp that was used. Addition of Syp’s SH2 domains did not affect the ability of PTP1B to dephosphorylate the receptor (Fig. 3B and data not shown). This indicated that the inability of Syp to efficiently dephosphorylate the PDGFR is not merely due to protection of receptor phosphotyrosine residues by Syp’s SH2 domains. The third explanation for the difference observed between rSyp and rPTP1B is that rPTP1B is a very general phosphatase and acts indiscriminately, while rSyp is a selective phosphatase capable of dephosphorylating only a subset of the receptor’s phosphotyrosine residues.

Syp Specifically Dephosphorylates the βPDGFR—To address the possibility that rSyp dephosphorylates only a subset of receptor phosphorylation sites, we compared the βPDGFR phosphorylation sites that were dephosphorylated by rSyp and rPTP1B. The radiolabeled βPDGFR was incubated with buffer or with increasing concentration of rPTP, the phosphatase reaction was terminated, and the proteins were resolved by SDS-PAGE (as in Fig. 3, A and B). The receptor was detected by autoradiography, excised from the gel, exhaustively digested with trypsin and then thermolysin, and the resulting phosphopeptides were resolved in two dimensions. Fig. 4, panels A–C, shows that all sites on the βPDGFR were highly vulnerable to dephosphorylation by rPTP1B. Even
The low dose of rPTP1B (0.08 pmol) caused substantial dephosphorylation of most βPDGFR phosphotyrosine sites (Fig. 4, compare A and B). Adding 10 times more rPTP1B resulted in a severe reduction of the phosphotyrosine content of all peptide spots (Fig. 4C). A further 5-fold increase in the concentration of rPTP1B reduced the phosphotyrosine content of the βPDGFR to a level that was not detectable on two-dimensional phosphopeptide maps (data not shown). The progressive dephosphorylation of all sites on the βPDGFR by increasing concentrations of rPTP1B confirms the idea that PTP1B is a low specificity tyrosine phosphatase capable of dephosphorylating all available phosphotyrosine sites (4, 5, 31). These results also demonstrate that all of the phosphotyrosine sites on the βPDGFR are available for dephosphorylation in vitro.

While all phosphotyrosine sites on the βPDGFR are equally good targets for rPTP1B, maps of the βPDGFR dephosphorylated by rSyp showed that rSyp had a distinct preference for certain sites (Fig. 4, D–F). The low dose of rSyp primarily dephosphorylated spots 1, 6, 7, 9, and to a lesser extent 8a (compare E and D). A 10-fold increase in the amount of rSyp resulted in increased dephosphorylation of spot 1 as well as spots 6, 7, and 9, while other spots, 2, 3, 8a, and 8b, retained most if not all of their original phosphotyrosine (Fig. 4, compare D and F). Note that these Syp-resistant spots were effectively dephosphorylated by rPTP1B (compare A and C, with 4, D and F). The selective nature of rSyp’s activity on the βPDGFR is perhaps best appreciated by comparing the progressive dephosphorylation of spot 1 relative to spots 2 and 3 when the receptor is exposed to rPTP1B versus rSyp. The ratio of spots 1 to 2 or 1 to 3 remained relatively constant when the receptor was dephosphorylated by increasing concentrations of rPTP1B but became more dissimilar as increasing concentrations of rSyp were used (Fig. 4). These experiments indicate that the reason Syp is able to only partially dephosphorylate the receptor (Fig. 3A) is because only a subset of the βPDGFR’s phosphorylation sites are good Syp substrates.

While all sites on the βPDGFR were available for dephosphorylation (rPTP1B dephosphorylates them), the phosphotyrosine sites 1009 (spot 3) and 1021 (spot 8b and a portion of 8a) found within the carboxyl-terminal tail of the βPDGFR, appeared to be very resistant to dephosphorylation by rSyp. In contrast, the sites most affected by the activity of rSyp, Tyr(P)-771, and Tyr(P)-751 (spots 9 and 1, respectively) and to a lesser extent Tyr(P)-740 (spot 6), are found within the kinase insert domain of the βPDGFR. Thus it appears that this particular region of the βPDGFR is the primary target of rSyp phospho-
tase activity. We wanted to determine the mechanism behind rSyp’s preference for dephosphorylating sites in the kinase insert domain of the PDGFR over those found in the carboxy-terminal tail of the receptor. We previously determined that it is not merely due to the presence of the SH2 domains, since they do not protect the receptor from dephosphorylation by PTP1B (Fig. 3). Since Syp binds to the tail of the βPDGFR (32, 33), it may have poor access to the immediately proximal tail phosphorylation sites. Thus positioning of rSyp on the βPDGFR may mediate the ability of rSyp to dephosphorylate different sites on the receptor. Alternatively, the catalytic domain of Syp itself may be able to discriminate between the various phosphorylation sites.

To investigate the relative contribution of Syp’s SH2 and catalytic domains to Syp’s ability to act in a substrate-specific manner, we repeated our dephosphorylation assay using a recombinant protein encoding only the catalytic domain of Syp (rCAT). Kinetic analysis of rCAT demonstrated that deletion of Syp’s SH2 domains results in a dramatic increase in catalytic activity toward pNPP ($k_{cat} = 5, 700 \text{ s}^{-1}$) but no change in $K_m$ ($K_m = 3.1 \text{ mM}$). When the tyrosine-phosphorylated PDGFR was incubated with a concentration of rCAT containing approximately 4 × more PTPase activity than the high concentrations of rSyp or rPTP1B used in Fig. 3, spots 9 (site 771) and 1 (site 751) were selectively dephosphorylated (Fig. 5 compare A to B). In contrast spots 6 (site 740), 7 (a peptide mixture), and 8b (site 1021) were relatively untouched (Fig. 5 compare A to B). However, unlike full-length Syp, dephosphorylation of spots 2 (unidentified) and 3 (site 1009) is also observable at this low concentration of rCAT (Fig. 5, A and B). In the presence of 5 or 10 times more rCAT, both spots 3 and 8b (sites 1009 and 1021, respectively) were efficiently dephosphorylated (Fig. 5, compare C to D and E), indicating that the specificity for distinct autophosphorylation sites is greatly diminished when high concentrations of enzyme that lacks the SH2 domains is used. Therefore, rSyp’s selection of substrates is determined by at least two variables: 1) the intrinsic specificity of the catalytic domain for certain phosphotyrosine residues, and 2) the SH2 domains, which bind to the tail of the βPDGFR, and thereby may position Syp’s catalytic domain over the kinase insert domain of the βPDGFR.

The Contribution of Syp to Dephosphorylation of the βPDGFR in Vivo—The experiments described above demonstrated that rSyp dephosphorylates the βPDGFR in vitro and suggest that Syp may perform the same task in vivo. This possibility is supported by the observation that Syp binds to the βPDGFR in PDGF-stimulated cells (33, 34). Consequently, we investigated whether the βPDGFR undergoes receptor dephosphorylation and whether stable binding of Syp to the receptor is required for this event. For these studies we used the wild type βPDGFR, as well as the F1009 receptor, which has a tyrosine to phenylalanine substitution at tyrosine 1009 and binds Syp poorly in HepG2 cells or TRMP cells (33–35). HepG2 cells expressing the wild type or F1009 receptor were stimulated with PDGF and lysed after the appropriate time interval; the receptor was immunoprecipitated and the amount of recep-
FIG. 6. Comparison of the in vivo dephosphorylation rate of the wild type and F1009 βPDGFRs. HepG2 cells expressing the WT or F1009 βPDGFR were left unstimulated (−) or were exposed to 30 ng/ml PDGF (+) for the indicated length of time (in minutes). The cells were lysed, the receptor was immunoprecipitated and resolved by SDS-PAGE, and the region of the gel containing proteins of 120–240 kDa was subjected to antiphosphotyrosine (top portion of panel A) or anti-receptor (bottom portion of panel A) Western blot analysis. The extent of receptor tyrosine phosphorylation was determined by densitometry, normalized for the amount of receptor, and it is represented graphically as a percentage of receptor phosphorylation at the 4-min time point. Similar results were obtained in three different experiments. The difference in phosphorylation of the WT and F1009 receptors at the 40-min time point is statistically significant (p < 0.02).

To assess whether binding of Syp results in dephosphorylation of the receptor at key tyrosine residues in vivo, we performed the following experiments. First, phosphopeptide maps of the WT and F1009 βPDGFR labeled in vivo were compared at 5 and 25 min post-PDGF-stimulation, however no consistent differences were observed (data not shown). Consequently, we turned to a less direct method to examine receptor phosphorylation, namely by the ability to bind the various receptor-associated proteins. We speculated that Syp-mediated receptor dephosphorylation may affect the ability of some of the proteins to bind to the receptor. In our in vitro studies, Syp dephosphorylates Tyr(P)-771, and Tyr(P)-751, sites involved with the binding of GAP and PI 3-kinase, respectively, so we focused on these two receptor-associated proteins. HepG2 cells expressing both WT or F1009 PDGFRs were left unstimulated or exposed to PDGF for 5 or 25 min, the cells were lysed, the receptor was immunoprecipitated and the immunoprecipitates were analyzed for the presence of p85 and GAP. As expected, at 5 min post-PDGF stimulation, when the phosphotyrosine content of both the WT and F1009 mutant is high (Fig. 6A), both receptors are able to associate with comparable amounts of p85 (Fig. 7). The level of GAP that associated with the F1009 receptor was somewhat greater than the amount that coprecipitated with the WT receptor, and is consistent with our previous observations (35). At 25 min post-PDGF stimulation, when the phosphotyrosine content of the WT PDGFR is lower than that of the F1009 mutant, the amount of p85 bound to the WT receptor is noticeably diminished. In contrast, the amount of p85 bound to the F1009 receptor at 25 min remained similar to that observed at the 5-min time point (Fig. 7). This trend was even more striking when we compared GAP binding at the two time points. The amount of GAP that associated with the wild type receptor at 25 min was reproducibly reduced to nearly undetectable levels, whereas GAP binding to the F1009 receptor was only modestly reduced at the 25 min time point (Fig. 7). In contrast to p85 and GAP, binding of phospholipase C-γ1 to the WT and F1009 receptors was diminished to a similar extent at the 25-min time point (data not shown), and is consistent with the observation that the Syp does not efficiently dephosphorylate the tyrosine residue required for phospholipase C-γ1 binding (Y1021, spot 8b and a fraction of spot 8a). These results support the idea that binding of Syp to the activated PDGFR enhances the dephosphorylation of the receptor and regulates the duration of binding of a select group of SH2 domain-containing signal transduction proteins.
DISCUSSION

In this study we found that the βPDGFR is an in vitro substrate of rSyp and that rSyp dephosphorylated only a subset of the phosphorylation sites on the βPDGFR. In contrast, rPTP1B was able to dephosphorylate all of the βPDGFR sites indiscriminately. Syp's substrate specificity arises from both the catalytic domain and the SH2 domains. Furthermore, a mutant βPDGFR that does not efficiently associate with Syp loses its phosphotyrosine content more slowly than the wild-type receptor and displays a prolonged association with SH2-domain-containing signal transduction proteins, suggesting that in an intact cell, Syp functions to dephosphorylate the βPDGFR and thereby regulate the proteins that associate with the βPDGFR.

Syp is a Selective PTP—Investigation of PTP substrate specificity is essential for understanding the regulation of cellular function by tyrosine phosphorylation. Initial efforts to address the question of PTP substrate specificity suggested that PTPs were nonselective enzymes, since sufficiently high concentrations of PTP could dephosphorylate all tyrosine-phosphorylated substrate tested in an in vitro assay (31, 36). Other studies demonstrated that noncatalytic regions, found within most PTPs, functioned to target the PTP to a particular cellular location. These observations suggested that the cellular localization of the PTP, instead of the intrinsic properties of the PTP itself, defines its substrate specificity (37, 38). However, more recent evidence indicates that certain PTPs do exhibit selectivity toward distinct tyrosine-phosphorylated substrates, and that this selectivity is not controlled solely by cellular localization but may be defined directly by the compatibility of the catalytic site of the PTP with the amino acid context of the phosphorylated tyrosine residue (5–7). The selective dephosphorylation of MAP kinase by MKP-1 reinforces the idea that at least some members of the PTP family are capable of acting in a substrate-specific manner (8).

Our studies described herein indicate that Syp displays a discernible degree of substrate specificity by dephosphorylating distinct tyrosines residues found within the βPDGFR. The βPDGFR acted as an in vitro substrate of both rPTP1B and rSyp; however, the susceptibility of the receptor's phosphotyrosine residues to dephosphorylation by the two PTPs was dramatically different. While PTP1B dephosphorylated all of the receptor's phosphotyrosines with similar efficiency, rSyp was able to dephosphorylate only a subset of the available phosphorylation sites (Figs. 3–5). Tyr(P)-751 and Tyr(P)-771, and to a somewhat lesser extent Tyr(P)-740, were the primary targets on the βPDGFR for dephosphorylation by rSyp, and these three sites account for 45% of total phosphotyrosine in the βPDGFR (29). Interestingly, this is the extent of receptor dephosphorylation at 30 min post-PDGF stimulation in vivo (Fig. 6).

Other groups have also found that Syp displays a readily detectable degree of substrate specificity (39–41). Surprisingly, our findings that rSyp prefers the kinase insert phosphorylation sites over sites in the carboxyl-terminal tail, are opposite to those reported by Sugimoto et al. (39). It is possible that the differences arise from using intact proteins that contain multiple phosphorylation sites instead of peptides as a substrate. In addition, the apparent preference for the peptide-containing site 1009 by Syp, over the other phosphorylated peptides in the panel of phosphopeptide substrates, could be due to the activation of Syp by the Tyr(P)-1009 peptide (32).

The preferential dephosphorylation of phosphorylation sites residing in the kinase insert domain of the PDGFR by Syp seems to be directed by a combination of two main factors. The first is an intrinsic preference by the catalytic domain of Syp.

### PDGFR autophosphorylation sites

| Sequence |  |
|----------|---|
| 579      | S D G H E Y I Y V D P M |
| 581      | G H E Y I Y V D P M Q L |
| 740      | E S D G G Y M D M S K D |
| 751      | D E S V D Y V P M L D M |
| 771      | I E S S N Y M A P Y D N |
| 1009     | T S S V L Y T A V Q P N |
| 1021     | E G D N D Y I I I P L P D |

**Fig. 8. Alignment of the amino acid sequences surrounding βPDGFR tyrosine phosphorylation sites.** Boldface letters represent the phosphorylated tyrosine residue. Underlined letters represent amino acids that may contribute to a putative Syp active site recognition sequence.

Alignment of the sequences surrounding the PDGFR autophosphorylation sites provides some insight for the specificity displayed by the Syp catalytic domain (Fig. 8). Both sites 751 and 771 are found within the context of a possible consensus sequence for phosphotyrosine recognition by the Syp active site: ESXY(P)XXXXD. No other autophosphorylation site on the PDGFR corresponds to this sequence. This putative consensus sequence appears to be specific for dephosphorylation by Syp as shown by the inability of the low concentration of rCAT to dephosphorylate Tyr(P)-740, a site that does not correspond to the putative consensus sequence, whereas a concentration of PTP1B containing 20 times less phosphatase activity dephosphorylated this site to completion (compare Fig. 4, A and B, to Fig. 5, A and B). It is possible that the charged amino acids at positions −4 and +5, along with the hydrophilic serine at position −3 facilitate an interaction between the active site of the Syp catalytic domain and the phosphotyrosine residue targeted for dephosphorylation. Ongoing experiments are designed to investigate this possibility.

The second factor that contributes to Syp's ability to discriminate substrates is the SH2 domains. They could act by binding to the receptor's COOH-terminal phosphorylation sites and thus shielding them from dephosphorylation. However, Syp's SH2 domains do not protect the receptor from dephosphorylation by PTP1B (Figs. 3 and 4). Instead binding of Syp to the βPDGFR's COOH terminus may position Syp's catalytic domain over the receptor's kinase insert region and thereby greatly increase the effective concentration of Syp's catalytic domain in the vicinity of the kinase insert phosphorylation sites. If the SH2 domains do contribute to Syp's substrate specificity, then a PTP lacking the SH2 domains should have a diminished ability to choose its substrate. Indeed, removal of Syp's SH2 domains reduced Syp's specificity when sufficiently high concentrations of rCAT were used (Fig. 5). Interestingly, spots 3 and 8b, corresponding to sites 1009 and 1021 of the βPDGFR, COOH terminus respectively, appear to be dephosphorylated as well as or more efficiently than spot 6 (Tyr(P)-740), when Syp's SH2 domains have been removed but not when full length Syp is used as the source of phosphatase. Therefore, Syp's preference for kinase insert domain phosphorylation sites (particularly Tyr(P)-751 and Tyr(P)-771) appears to be mediated by both an enhanced interaction of phosphotyrosine residues found within a putative Syp recognition consensus sequence with the active site of Syp's catalytic domain, and the positioning of the Syp catalytic domain over the βPDGFR kinase insert domain as directed by the binding of Syp's SH2 domains to the COOH-terminal tail of the βPDGFR.

Syp May Regulate βPDGFR Signaling by Dephosphorylation of the βPDGFR—While Syp appears to contribute to dephosphorylation of the βPDGFR, it is curious that the receptor is not
notably dephosphorylated for the first 6 min after PDGF stimulation, even though Syp has associated with receptor at this time (33, 34, 42, 43). The steady state level of receptor tyrosine phosphorylation probably reflects a dynamic balance of receptor kinase activity and PTP activity. Perhaps Syp activity is inhibited immediately following receptor activation, by a mechanism such as threonine phosphorylation mediated by activated MAP kinase (44). Alternatively, tyrosine phosphorylation of Syp may act as a timer, so that Syp first dephosphorylates itself (43) and only then begins to dephosphorylate other proteins. The slow dephosphorylation of the βPDGF in vivo may be a necessary mechanism to facilitate the early signaling processes that involve receptor autophosphorylation and the binding of SH2 domain-containing signal transduction proteins.

The predominant dephosphorylation of βPDGF at Tyr(P)-751 and Tyr(P)-771 by Syp is intriguing, as it suggests a potential mechanism for regulating βPDGF-mediated mitogenic signaling. Tyrosine phosphorylation at 751 plays an important role in the binding and the activation of PI 3-kinase (26), and one group has found that Tyr(P)-751 is involved with Nck binding. In HepG2 cells we have found that Tyr-751 is not required for efficient binding of Nck to the βPDGF. Nck functions as an oncogenic protein as demonstrated by experiments in which overexpression of Nck led to cell transformation and tumor formation in nude mice (45, 46). Therefore, Nck binding to the βPDGF may engage signaling pathway(s) that promote(s) cellular proliferation, and in certain cell types Syp may be able to control the initiation of these events. βPDGF-mediated PI 3-kinase activation is also potentially regulated by dephosphorylation of tyrosine 751 by Syp. Evidence from several labs suggests that the increase in PI 3-kinase activity following growth factor stimulation is crucial for mitogenic signaling, and requires binding of PI 3-kinase to the tyrosine-phosphorylated βPDGF (47–50). Two adjacent sites on the βPDGF, site 740 and site 751, are capable of binding to the SH2 domains of the p85 subunit of PI 3-kinase (30, 51), and the binding to the SH2 domains of the p85 subunit of PI 3-kinase is necessary for efficient transduction of a mitogenic signal. Therefore, Syp's apparent ability to dephosphorylate the GAP binding site is a plausible mechanism by which Syp can enhance the βPDGF's mitogenic signal. This is consistent with a recent report that suggests that Syp's phosphatase activity is necessary upstream of PDGF-stimulated MAP kinase activation (53). A second route by which Syp enhances βPDGF signaling relates to Syp's ability to act as an adaptor protein that couples the receptor to proteins such as Grb2 (24) and thus to Ras activation (54). Given that Syp can regulate βPDGF signaling in both positive and negative ways, it is possible that Syp has a dual function in the context of βPDGF signaling. Immediately following βPDGF activation, Syp acts as a positive signal transducer, coupling the receptor to necessary downstream signaling proteins. Once the necessary events have occurred for efficient transduction of a mitogenic signal, Syp acts to turn off this signal by dephosphorylating key sites on the βPDGF and thus preventing uncontrolled cell proliferation.

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