Chemical Dissection of the Effects of Tyrosine Phosphorylation of SHP-2†

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ABSTRACT: The regulation of the protein tyrosine phosphatase (PTPase) SHP-2 by tyrosine phosphorylation has been difficult to elucidate because of the intrinsic instability of the phosphoprotein. In the past, expressed protein ligation has been used to site-specifically incorporate the phosphotyrosine mimic Pmp (phosphonomethylene phenylalanine) into the two tyrosine phosphorylation sites (542, 580) of SHP-2 one at a time to analyze the effects on catalytic behavior. In this study, we have incorporated two Pmps into the phosphorylation sites simultaneously and examined the effects of double SHP-2 tyrosine phosphorylation. We have found that the Pmp groups show close to additive effects on PTPase stimulation, suggesting dual SH2 domain occupancy. The relative effects of the phosphotyrosine analogue difluoromethylene phosphonophenylalanine (F_2Pmp) compared to those of Pmp were also examined. It was found that the F_2Pmp analogue showed slightly enhanced PTPase stimulation compared with the Pmp analogue, consistent with its higher affinity for SH2 domains. Taken together with the bis-Pmp studies, these data suggest that double phosphorylation of the SHP-2 C-terminus could give rise to a 9-fold overall PTPase activation, 30−50% of the value associated with deletion of the SH2 domains. Catalytically inactive forms of phosphorylated SHP-2 proteins were also produced by expressed protein ligation. This allowed for a systematic analysis of intermolecular autodephosphorylation of SHP-2, which revealed how conformational plasticity can modulate phosphotyrosine stability.

SHP-2 is a conserved cytoplasmic protein tyrosine phosphatase (PTPase) in many species, including *Drosophila*, *C. elegans*, *Xenopus*, and mammals, and is critical in the early development of these organisms (*1*). Mutations of SHP-2 have been linked with many cases of Noonan syndrome (*2*). Mammalian SHP-2 is a 68 kDa protein consisting of two SH2 domains: a catalytic PTPase domain and a C-terminal tail (Figure 1A). Although SHP-2 has been implicated in many cell signaling pathways elicited by growth factors, cytokines, and cell adhesion molecules, its regulatory mechanism is not completely understood (*3*−*15*). Biochemical studies have indicated that the N-terminal SH2 domain exerts an inhibitory effect on the basal activity of SHP-2, and certain phosphotyrosine-containing peptides can stimulate the PTPase activity of SHP-2 by selective interaction with its SH2 domains (*16*−*19*). The crystal structure of SHP-2 has revealed that the N−SH2 domain interacts with the catalytic domain, preventing the substrate from entering the active site, and the SHP-2 enzyme is maintained in an "inactive state" (*20*). The binding of the N−SH2 domain with phosphotyrosine-containing peptides presumably disrupts the intramolecular interaction between the N−SH2 domain and the PTPase domain, and the SHP-2 enzyme becomes more active.

The phosphorylation modification of two tail tyrosine residues (Tyr542 and Tyr580) has been proposed to be another possible regulatory mechanism for SHP-2 (*4*−*6*). The possible function of tail tyrosine phosphorylation includes the recruitment of SH2-domain- or phosphotyrosine-binding-domain-containing signaling molecules, such as Grb2, as well as the modulation of PTPase activity of SHP-2 (*4*−*6*). To clarify the effect of tail tyrosine phosphorylation on the function of SHP-2, we recently introduced a nonhydrolyzable phosphotyrosine analogue, phosphonomethylene phenylalanine (Pmp, Figure 1B), into the two known tyrosine phosphorylation sites individually by expressed protein

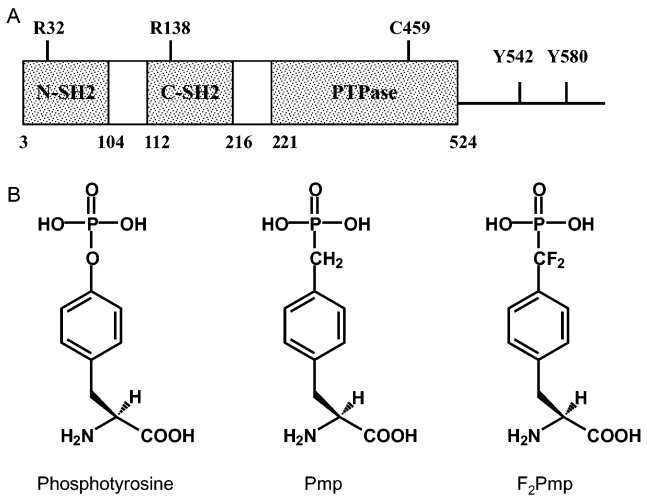

FIGURE 1: Architecture of SHP-2 and structures of phosphotyrosine analogues used in this study: (A) domain architecture of SHP-2 illustrating phosphorylation sites, key active site Cys, and key SH2 domain Arg sites; (B) chemical structure of phosphotyrosine and its nonhydrolyzable analogues Pmp and F_2Pmp.

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ligation (*21*). We found that each of the tail phosphonotyrosine modifications stimulate the PTPase activity via selective interactions with the individual SH2 domains (*21*). One phosphonate-modified enzyme, SHP-2-542-Pmp, can activate the MAP kinase pathway in living cells, indicating that tail tyrosine phosphorylation is likely to be important in this signaling cascade (*21*).

Although these initial studies provided new insights into the functions of SHP-2 phosphorylation, they left several important questions unanswered. First, since they appear to operate by distinctive mechanisms, do the tail phosphorylations synergize with each other? How ideal is the Pmp derivative for gauging phosphotyrosine effects compared to the difluoromethylene phosphonate (F_2Pmp)? What are the kinetics of intermolecular autodephosphorylation of tail-phosphorylated SHP-2? In this study, we use expressed protein ligation (*22*, *23*) to prepare the relevant semisynthetic proteins and have attempted to address these questions.

MATERIAL AND METHODS

Materials. Fmoc amino acids, HBTU, HOBT, and preloaded Fmoc-Wang-resin were purchased from Nova Biochem. Fmoc-Pmp-OH was commercially available from Advanced ChemTech. The QuikChange site-directed mutagenesis kit and *E. coli* BL21(DE3) competent cells were purchased from Stratagene. DNA primers were obtained from Intergrated DNA Technologies. RCM-lysozyme was purchased from Sigma. Chitin resins were purchased from New England BioLabs. The EnzChek phosphate assay kit was purchased from Molecular Probes. Full-length (1−593) recombinant SHP-2 (SHP-2-FL) was expressed and purified as described previously (*21*).

Preparation of SHP-2 Tail Peptides. Fmoc-difluoromethylene-L-phosphonophenylalanine (Fmoc-F_2Pmp) was prepared as described by Lawrence and colleagues (*24*). All peptides were synthesized using the Fmoc strategy with preloaded Wang resin on a Rainin PS-3 peptide synthesizer. Each peptide synthesis was performed using 0.1 mmol scale and 4 equiv of amino acid; HBTU and HOBT were employed for each coupling step. The coupling time for each cycle was 20 or 60 min depending on the sequence and length of the peptides. Double coupling was used for the coupling of difficult amino acids, including Val, Leu, Ile, Thr, and phosphotyrosine. To incorporate phosphonomethylene-L-phenylalanine (Pmp) or difluoromethylene-L-phosphonophenylalanine (F_2Pmp), double coupling (120 min for each cycle) was employed with 2 equiv of reagents used in each coupling cycle. Cleavage and deblocking of the peptide-immobilized resins were carried out by treatment with cleavage solution (10 mL of trifluoroacetic acid, 0.5 mL of thioanisole, 0.25 mL of ethanedithiol, 0.5 mL of H_2O, and 0.75 g of phenol) at room temperature for 3 h. The crude peptides were recovered by ether precipitation and further purified by reversed-phase HPLC using either a semipreparative or preparative C18 column. The masses of HPLC-purified peptides (>95%) were confirmed by electrospray ionization or MALDI mass spectrometry.

Plasmid Constructions. The plasmids PTYB2-SHP-2(1−539) and PTYB2-SHP-2(1−568) (*21*) were used as templates to generate the PTYB2-SHP-2(1−539, C459S) and PTYB2-SHP-2(1−569, C459S) constructs, respectively, by QuikChange site-directed mutagenesis (Stratagene) using the following primer pairs:

5′-CCGGTCGTGGTGCACTCCAGTGCTGGAATTG-GC-3′

and

5′-GCCAATTCCAGCACTGGAGTGCACCACCAC-GACCGG-3′

The resulting construct PTYB2-SHP-2(1−568, C459S) was used to generate PTYB2-SHP-2(1−568, R32A, C459S) and PTYB2-SHP-2(1−568, R138A, C459S) using QuikChange site-directed mutagenesis. Similarly, PTYB2-SHP-2(1−539, R32A, C459S) and PTYB2-SHP-2(1−539, R138A, C459S) were made from the PTYB2-SHP-2(1−539, C459S) plasmid. The primer pairs used for generating R32A mutants were

5′-GCAGTTTTTTGGCAGCGCCTAGTAAAAGTAA-CCC-3′

and

5′-GGGTTACTTTTACTAGGCGCTCCAAAAAACT-GC-3′

The primers used for generating the R138A mutants were

5′-GGTAGTTTTCTTGTAGCAG CGAGCCAGAG-CC-3′

and

5′-GGCTCTGGCTCTCTGCTACAAGAAAACTA-CC-3′

The PTYB2-SHP-2(191−568), PTYB2-SHP-2(191−568, C459S), and PTYB2-SHP-2(191−539, C459S) constructs were prepared from their full-length counterparts using the following primer pairs:

5′-CTTATAATGTTCCACAAGATCTGTCATATGT-ATATCTCCTTC-3′

and

5′-GAAGGAGATATACATATGACAGATCTTGTG-GAACATTATAAG-3′

The Quik Change reactions were carried out according to the conditions recommended in the supplier's brochure. The SHP-2 coding sequences of all constructs were confirmed by DNA sequencing.

Preparation of Semisynthetic SHP-2 Proteins by Expressed Protein Ligation. The semisynthetic SHP-2 proteins were generated by linking the recombinant SHP-2 N-terminal fragments with the requisite C-terminal tail peptides using expressed protein ligation as described previously (*21*). Briefly, *E. coli* BL21(DE3) cells transformed with individual SHP-2 plasmids were grown at 37 °C until the OD_{600} reached 0.6. The cell cultures were then induced with 0.3 mM IPTG at 25 °C for 5 h, pelleted, resuspended, and lysed by French press. The cell lysates were eluted over chitin resin and reacted with the corresponding C-terminal tail peptides in the presence of 2% thiophenol at room temperature for 24 h. The ligated SHP-2 proteins were dialyzed extensively to

remove the unreacted tail peptides and concentrated by Centriplus (10 kDa molecular weight cutoff, Amicon) to 1−2 mg/mL as determined by Bradford assay (*25*). The purities of the semisynthetic SHP-2 proteins were greater than 80% based on SDS−PAGE analysis and MALDI MS. The yields of each of the semisynthetic SHP-2 proteins were 2−3 mg per liter of *E. coli* culture. The following semisynthetic proteins were prepared as noted in the text and figures (see Figures 2−4): SHP-2^{586C}, SHP-2^{586G}, SHP-2^{586C}-542-Pmp, SHP-2^{586G}-580-Pmp, SHP-2^{586C}-542/580-bisPmp, SHP-2^{556C}, SHP-2^{556C}-542-Pmp, SHP-2^{556C}-542-F_2Pmp, SHP-2^{593G}, SHP-2^{593G}-580-Pmp, SHP-2^{593G}-580-F_2Pmp, SHP-2^{547CS}-542-pTyr, SHP-2^{547CS}-R32A-542-pTyr, SHP-2^{547CS}-R138A-542-pTyr, SHP-$2^{191-547CS}$-542-pTyr, SHP-2^{593GS}-580-pTyr, SHP-2^{593GS}-R32A-580-pTyr, SHP-2^{593GS}-R138A-580-pTyr, SHP-$2^{191-593GS}$-580-pTyr, SHP-$2^{191-593G}$. The nomenclature in the superscript refers to the final C-terminal amino acid (and N-terminal amino acid if other than 1), the presence of point mutations at residue 459 (S), 540 (C), or 568 (G). The hyphenated portion of the abbreviation defines the presence and site of SH2 domain Arg to Ala mutations and/or Pmp, F_2Pmp, or pTyr.

Phosphatase Assays Using Phospho-RCM-lysozyme as Substrate. RCM-lysozyme was tyrosine-phosphorylated using γ^{32}P-ATP by the recombinant catalytic domain of the insulin receptor kinase (provided by R. Kohanski) according to the method of Tonks et al. (*26*). To measure phosphatase activity, 2 μM of ^{32}P-labeled phosphotyrosine-RCM lysozyme was incubated with 0.8−1.6 μg/mL of SHP-2 proteins at 30 °C for 2 min in 25 μL of reaction buffer (25 mM Na-HEPES [pH 7.2], 1 mg/mL BSA, 5 mM EDTA, and 10 mM DTT). Reactions were quenched by transferring 24 μL of the reaction mixtures to 0.5 mL of activated charcoal suspension (100−400 mesh, Sigma) (*16*). The samples were centrifuged for 10 min at 13000*g*, and 0.3 mL aliquots of the supernatants were added to scintillation fluid and subjected to scintillation counting. Dephosphorylation rates were calculated on the basis of the fraction of radioactivity released into the supernatants.

Measurement of Dephosphorylation Rate by EnzChek Phosphate Assay. This was carried out on the basis of the method of Webb (*27*). Purified semisynthetic phospho-SHP-2 substrate (4−40 μM) was mixed with 200 μM methylthioguanosine (MESG) and 1 unit of purine nucleoside phosphorylase (PNP) in 55 μL of reaction buffer (20 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 50 mM NaCl, and 5 mM DTT). The reaction mixture was transferred to a quartz cuvette (60 μL minimum volume) and incubated at 25 °C for 2 min. A background scan was performed at 360 nm, and the readings were taken every 3 s. An aliquot of 5 μL of SHP-2 enzyme (25−500 nM final) was added to initiate the reaction. The absorbance at 360 nm was measured every 3 s for 2 min. The extinction coefficient of 2-amino-6-mercapto-7-methylpurine, determined using KH_2PO_4 as a standard inorganic phosphate source, was 0.010 139 μM^{-1} cm^{-1}. The phosphatase activity was linear with respect to time and enzyme concentration in the range used, and it was independent of the amount of coupling enzyme in the range of PNP used. All reactions were performed at least three times and showed good reproducibility (standard errors less than 20%).

RESULTS

Dual Phosphonate Incorporation and SHP-2 Enzymatic Activity. We recently found that tail Pmp modifications of SHP-2 at the two tyrosine phosphorylation sites Tyr542 and Tyr580 stimulate the PTPase activity of SHP-2 via selective intramolecular interactions with the N−SH2 and C−SH2 domains, respectively (*21*). It is in principle possible that these phosphonate interactions could show additive effects on PTPase activity. To investigate this possibility, we needed to generate the dual Pmp containing SHP-2, which would require the synthesis of a 54 amino acid peptide for expressed protein ligation. It proved to be difficult to generate this peptide, so we decided to make a slightly shorter peptide (47 residues) containing the two Pmp groups with a plan to make SHP-2 (amino acids 1−586). The 47 amino acid length peptide was selected because it contains six amino acid residues that are C-terminal to the 580-phophorylation site, which should be more than sufficient for canonical SH2 interaction.[2]

Syntheses of the singly (542) phosphonylated, unphosphonylated, and bisphosphonylated 47-mer peptides (NH_2−CEBTNIKYSLADQTSGDQSPLPPCTPTPPCAEMREDSARVBENVGLM−CO_2H, where B is either tyrosine or Pmp) proved to be feasible using the Fmoc strategy, and these were used in expressed protein ligation with SHP-2 (amino acids 1−539). The fourth pair of peptides (NH_2−CAEMREDSARVBENVGLM−CO_2H, where B is either tyrosine or Pmp) were used to ligate to SHP-2 (amino acids 1−568). The five semisynthetic proteins thus produced (SHP-2^{586C}, SHP-2^{586G}, SHP-2^{586C}-542-Pmp, SHP-2^{586G}-580-Pmp, SHP-2^{586C}-542/580-bisPmp, Figure 2A) were analyzed by MALDI MS (data not shown) and SDS−PAGE (Figure 2B), which showed that expressed protein ligation proceeded with approximately 85−90% efficiency and >80% overall purity.[3]

[1] Abrreviations: SH2 domain, Src homology 2 domain; SHP-2, SH2 domain containing phosphatase-2; Pmp, phosphonomethylene phenylalanine; F_2Pmp, difluoromethylene phosphonophenylalanine; Fmoc, fluorenylmethoxycarbonyl; pTyr, phosphotyrosine; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBT, 1-hydroxybenzotriazole; RCM-lysozyme, reduced carboxymethylated maleimidylated lysozyme; MESG, 2-amino-6-mercapto-7-methylpurine riboside; PNP, purine nucleoside phosphorylase; MALDI, matrix assisted laser desorption ionization; PTPase, protein tyrosine phosphatase; IPTG, isopropylthiogalactoside; SHP-2-FL, full-length recombinant SHP-2 protein; SHP-2-CAT, catalytic domain of SHP-2 prepared by semisynthesis (amino acids 191−593).

[2] Detailed analysis of phosphopeptide binding and X-ray structural studies have suggested that the SHP-2 SH2 domains may conceivably interact with peptide residues from pY-2 to pY+5 (*19*, *39*, *40*). As with most SH2 domain ligands, the principal interaction residues are pY+1, pY+2, and pY+3. The only altered residue that could potentially affect the SH2 domain interactions is His-540 (pY-2), which is changed to Cys-540 as required for expressed protein ligation. However, the precise effects of the pY-2 residue on selectivity or affinity have not been described. Indeed, at least seven different residues (S, Q, E, H, V, L, and G) at the pY-2 position have allowed for high-affinity peptide ligands of the N−SH2 domain (*39*).

[3] Nearly all of the semisynthetic proteins in this way contain 5−10% of a "nicked" form of the protein (a C-terminal fragment with about 20 kDa lower molecular mass compared to the full-length form and a stoichiometric amount of the N-terminal SH2 domain containing fragment [the latter not visible in these figures]), which is difficult to separate chromatographically. Because it is a consistent contaminant that in our experience does not significantly affect the overall activity measured with phosphorylated RCM lysozyme substrate, we have not carried out further purification to remove it from the semisynthetic proteins produced here.

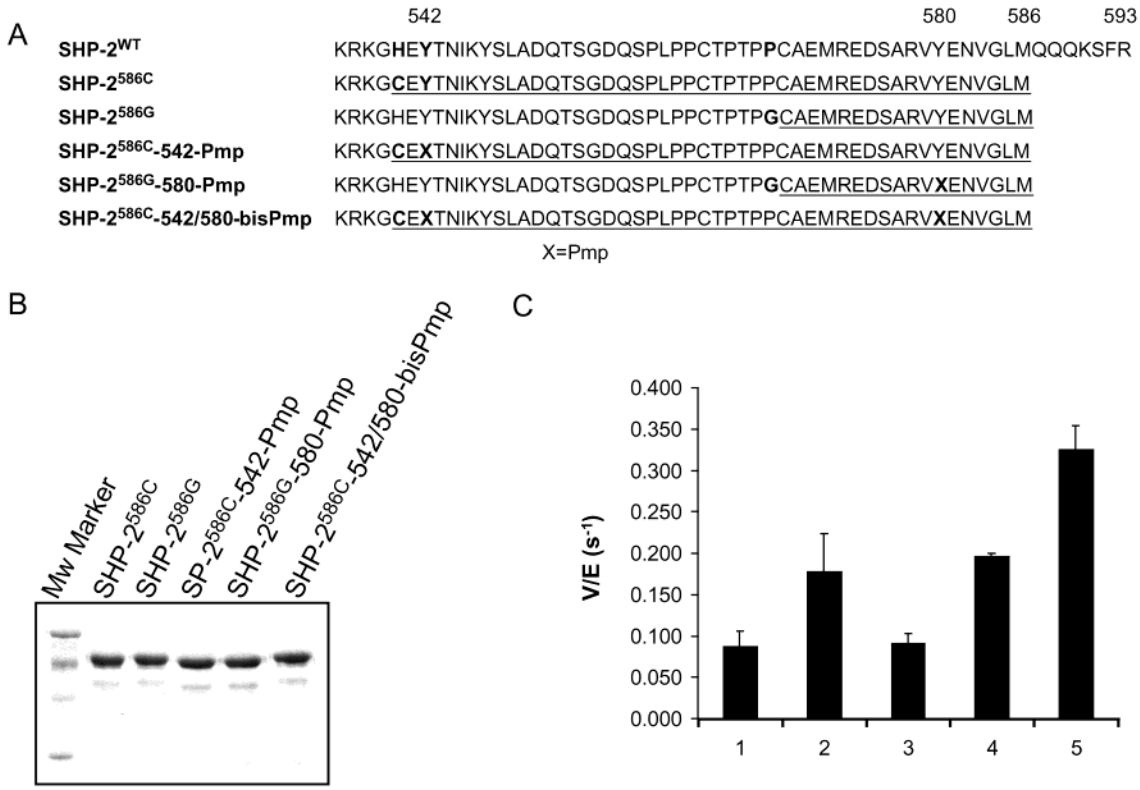

FIGURE 2: Construction and evaluation of semisynthetic SHP-2 containing two tail Pmp groups. (A) Incorporation of Pmps into known phosphorylation sites. The tyrosine-, mono-Pmp-, and dual-Pmp-containing semisynthetic SHP-2 proteins were made by ligating the chemically synthesized C-tail peptides (underlined) with N-terminal recombinant fragments. (B) A Coomassie stained 10% SDS−PAGE of tyrosine-, mono-Pmp-, and dual-Pmp-containing SHP-2 proteins. The far left lane contained molecular mass makers of 97, 66, 45, and 31 kDa, and samples in other lanes were labeled above each lane. (C) PTPase activity measurement of SHP-2 proteins using phospho-RCM-lysozyme as substrate. Bars 1−5 are respectively SHP-2^{586C}, SHP-2^{586C}-542-Pmp, SHP-2^{586G}, SHP-2^{586G}-580-Pmp, and SHP-2^{586C}-542/580-bisPmp. The rates are the average of three independent experiments, and the standard errors are shown.

Since the unligated proteins show similar PTPase activity compared to unphosphonylated semisynthetic SHP-2 proteins (data not shown), these were used in catalytic experiments without further purification.

The semisynthetic proteins were studied as catalysts with the tyrosine phosphorylated RCM-lysozyme substrate. As expected, the monophosphonylated proteins SHP-2^{586C}-542-Pmp and SHP-2^{586G}-580-Pmp each showed 2-fold higher PTPase activity than the unphosphonylated proteins SHP-2^{586C} and SHP-2^{586G} (Figure 2C). These results are similar to those reported previously for the related but nonidentical semisynthetic phosphonylated SHP-2 proteins SHP-2^{556C}-542-Pmp and SHP-2^{593G}-580-Pmp (*21*). The bisphosphonylated protein SHP-2^{586C}-542/580-bisPmp showed a 3.5-fold enhanced PTPase activity compared to the unphosphonylated proteins SHP-2^{586C} and SHP-2^{586G}, close to additivity for the singly phosphonylated proteins SHP-2^{586C}-542-Pmp and SHP-2^{586G}-580-Pmp. These results suggest that dual occupancy of the two SH2 domains by the two phosphates in doubly phosphorylated SHP-2 is favorable and that simultaneous engagement of both SH2 domains can synergize to activate the PTPase activity.

Preparation and Analysis of F_2Pmp Modified Semisynthetic SHP-2 Proteins. In the above and previous work, Pmp analogues have been employed as phosphotyrosine surrogates. While Pmps are highly structurally similar to phosphotyrosine, they show approximately 5-fold weaker affinity for SH2 domains compared to their phosphotyrosine counterparts (*28, 29*). In contrast, F_2Pmp analogues bind about as well as phosphotyrosines to SH2 domains (*28, 29*). Thus, the Pmp modifications may provide a lower limit to the degree of PTPase stimulation. To address this further, we synthesized the synthetic peptides CEZTNIKYSLADQTSGD and CAEMREDSARVZENVGLMQQQKSFR containing F_2Pmp (Z = F_2Pmp) for use in expressed protein ligation to generate 542-modified and 580-modified proteins SHP-2^{556C}-542-F_2Pmp and SHP-2^{593G}-580-F_2Pmp, respectively (parts A and B of Figure 3). In parallel, the Pmp and Tyr containing proteins SHP-2^{556C}, SHP-2^{556C}-542-Pmp, SHP-2^{593G}, and SHP-2^{593G}-580-Pmp were prepared as described previously (*21*).

The SHP-2^{556C}-542-F_2Pmp and SHP-2^{593G}-580-F_2Pmp proteins were about 3-fold more catalytically active than the corresponding Tyr containing proteins SHP-2^{556C} and SHP-2^{593G} and showed about 30−50% greater PTPase activity compared to SHP-2^{556C}-542-Pmp and SHP-2^{593G}-580-Pmp (Figure 3C). These results suggest that the fluorophosphonate analogues bring about modestly more activation than the methylene phosphonates by stronger engagement with the SH2 domains. Thus, the Pmp groups are likely to slightly underestimate the effects of the phosphotyrosines in this system.

Intermolecular Autodephosphorylation of Tyrosine Phosphorylated SHP-2. Given the known reduced stability of the phosphotyrosine modifications within SHP-2 because of autodephosphorylation, we decided to address this by examining intermolecular autodephosphorylation of the corresponding phosphorylated semisynthetic proteins. Thus, the

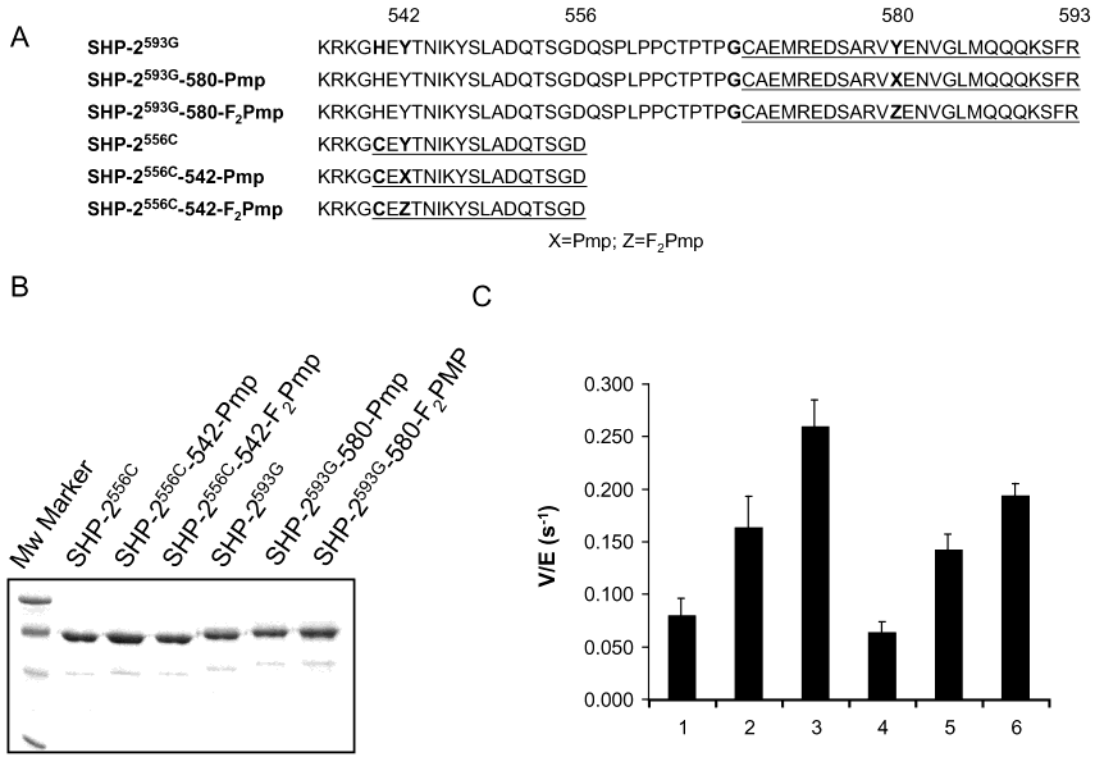

FIGURE 3: Effect of F_2Pmp modification on SHP-2 properties. (A) Incorporation of phosphotyrosine analogues into SHP-2 proteins. The C-terminal tail peptides (underlined) were chemically synthesized and then reacted with the appropriate N-terminal recombinant fragments to afford desired semisynthetic SHP-2. (B) Analysis of semisynthetic SHP-2 proteins by 10% SDS−PAGE stained with Coomassie blue. Molecular mass markers of 97, 66, 45, and 31 kDa were loaded in the far left lane, and the samples in the other lanes are indicated at the top of each lane. (C) PTPase activities of semisynthetic SHP-2 comparing Pmp and F_2Pmp modifications. The catalytic activities of semisynthetic SHP-2 proteins were measured using phospho-RCM-lysozyme as substrates. Bars 1−6 are respectively SHP-2^{556C}, SHP-2^{556C}-542-Pmp, SHP-2^{556C}-542-F_2Pmp, SHP-2^{593G}, SHP-2^{593G}-580-Pmp, and SHP-2^{593G}-580-F_2Pmp. The rates are the average of three independent experiments, and standard errors are shown.

proteins SHP-2^{547CS}-542-pTyr and SHP-2^{593GS}-580-pTyr were generated by expressed protein ligation with the appropriate phosphopeptides and recombinant SHP-2 fragments in which the active site Cys-459 to Ser mutation was present, rendering the proteins catalytically inactive. As controls, related phosphoproteins were also generated containing SH2 point mutations (SHP-2^{547CS}-R32A-542-pTyr, SHP-2^{547CS}-R138A-542-pTyr, SHP-2^{593GS}-R32A-580-pTyr, and SHP-2^{593GS}-R138A-580-pTyr) and lacking SH2 domains (SHP-$2^{191-547CS}$-542-pTyr and SHP-$2^{191-593GS}$-580-pTyr) that allow the importance of the intramolecular SH2 interactions to be examined (parts A and B of Figure 4). Finally, the unligated phosphopeptides pTyr-542 and pTyr-580 were also used for comparison in dephosphorylation studies.

Each of these peptides and proteins was investigated as a PTPase substrate with active full-length (SHP-2-FL) and catalytic domain fragments of SHP-2 (SHP-2-CAT) serving as catalysts (Figure 4C). As expected (*16*), SHP-2-CAT was considerably more efficient (20- to 30-fold) in dephosphorylation of all substrates examined compared to SHP-2-FL catalyst. Interestingly, the 580-phosphorylated peptide and proteins were significantly more efficient substrates for SHP-2 catalysts than the 542-modified substrates. Because of this increased efficiency, it was possible to do a more complete analysis with the 580-modified substrates. Several interesting features can be gleaned from Figure 5A. With SHP-2-CAT as catalyst, there was a relatively large inhibition (5-fold) of dephosphorylation when a functional C−SH2 domain in the substrate was present. When the substrate SH2 domains were deleted as in SHP-$2^{191-593GS}$-580-pTyr or when there was an inactivating mutation in the C−SH2 domain as in SHP-2^{593GS}-R138A-580-pTyr, the rate of dephosphorylation was accelerated. Interestingly, the full-length 580-modified SHP-2 substrate proteins (SHP-2^{593GS}-580-pTyr, SHP-2^{593GS}-R32A-580-pTyr, and SHP-2^{593GS}-R138A-580-pTyr) showed lower approximate K_m values than the peptide (pY-580) or SH2-domain-deleted SHP-2 substrate SHP-$2^{191-593GS}$-580-pTyr (Figure 5A).

The trends with the SHP-2-FL catalyst and 580-modified substrates were generally similar to those with SHP-2-CAT and 580-modified substrates (Figure 5B). However, the rate reduction associated with a functional C−SH2 domain was considerably smaller (<2-fold) than when the more active SHP-2 catalytic domain catalyst was used. Thus, SHP-2-FL catalyst showed relatively little discrimination between phosphopeptide and SHP-2 phosphoprotein substrates.

The 542-phosphorylated substrates were less efficiently processed compared to the 580-phosphorylated substrates, and only SHP-2-CAT gave a sufficient velocity to provide a reliable rate measurement with the assay employed. These experiments were also complicated because the 542-phosphorylated substrates were less soluble and it was only possible to employ up to 4.1 μM of these 542-modified substrates. However, it was observed that the full-length 542-phosphorylated SHP-2 proteins (SHP-2^{547CS}-542-pTyr, SHP-2^{547CS}-R32A-542-pTyr, and SHP-2^{547CS}-R138A-542-pTyr) were weaker substrates than the phosphopeptide pY-542 or the SH2-domain-deleted substrate SHP-$2^{191-547CS}$-542-pTyr.

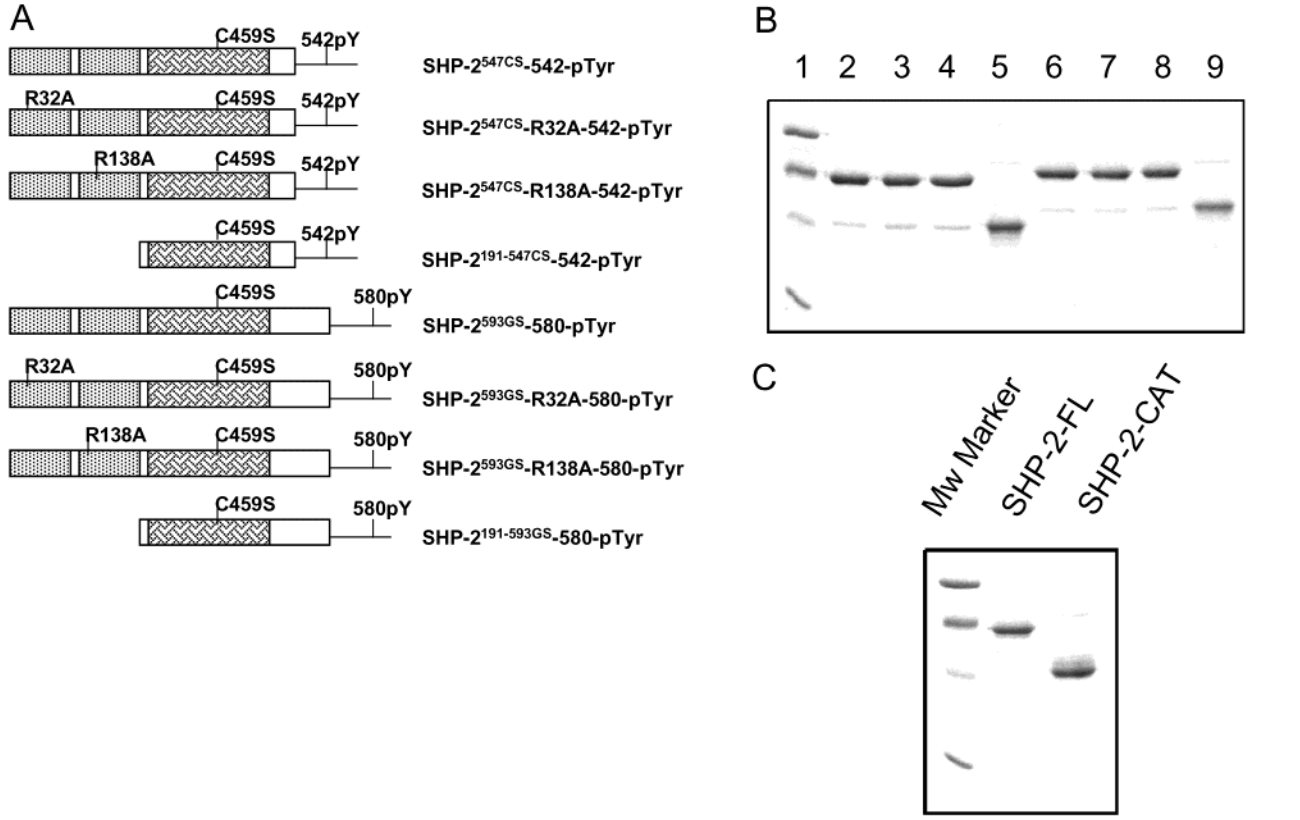

FIGURE 4: Preparation of phospho-SHP-2 substrates and SHP-2 enzymes for the characterization of the dephosphorylation reaction. (A) Schematic representation of phospho-SHP-2 proteins substrates used in this study. The phospho-SHP-2 protein substrates were prepared by ligating the monophosphotyrosine-containing tail peptides with the appropriate N-terminal recombinant fragments. To remove the intrinsic PTPase activity, the catalytic cysteine residue (C459) was mutated to serine. Two series of phospho-SHP-2 substrates were made for the two phosphotyrosine sites separately. Each series contains the full-length wild-type substrate, truncated substrate lacking two SH2 domains, and full-length mutant substrates containing functional mutations in either the N−SH2 domain (R32A) or the C−SH2 domain (R138A). (B) Coomassie contained 10% SDS−PAGE of phospho-SHP-2 proteins substrates. Lane 1 shows molecular mass makers of 97, 66, 45, and 31 kDa, and lanes 2−9 are respectively SHP-2^{547CS}-542-pTyr, SHP-2^{547CS}-R32A-542-pTyr, SHP-2^{547CS}-R138A-542-pTyr, SHP-$2^{191-547CS}$-542-pTyr, SHP-2^{593GS}-580-pTyr, SHP-2^{593GS}-R32A-580-pTyr, SHP-2^{593GS}-R138A-580-pTyr, SHP-$2^{191-593GS}$-580-pTyr. (C) 10% SDS−PAGE of SHP-2 proteins used as catalysts in autodephosphorylation studies stained by Coomassie blue (molecular mass markers from the top are 97, 66, 45, and 31 kDa). Both SHP-2-FL and SHP-2-CAT were prepared. SHP-2-FL was prepared as a standard recombinant protein as described previously (*21*). SHP-2-CAT was made by ligating the tyrosine containing tail peptide with the truncated N-terminal recombinant fragment (191−568).

There was a further 2-fold rate retardation observed when the full-length 542-modified substrate had a functional N−SH2 domain (SHP-2^{547CS}-542-pTyr and SHP-2^{547CS}-R138A-542-pTyr) compared to the one that did not (SHP-2^{547CS}-R32A-542-pTyr; Figure 5C).

DISCUSSION

Historically, detailed kinetic analysis of the regulation of proteins by phosphorylation has been a relatively challenging aspect of the cell signaling field. First, it is not always so easy to obtain the signaling protein of interest in purified form. Second, it is traditionally difficult to control the site selectivity and stoichiometry of post-translational modifications in signaling proteins. These modifications are generally introduced by enzymes that lack the efficiency and specificity to generate the quantities and purities of the target proteins desired. This problem is particularly apparent in the not uncommon cases where phosphatase enzymes are themselves modulated by phosphorylation on regulatory sites.

The methods of native chemical ligation (*30*, *31*) and expressed protein ligation (*22*, *23*) are valuable technologies to employ in settings where site-specific introduction of post-translational modifications is required. Thus, the chemically synthesized parts of the proteins can be decorated with whatever modifications are desired in complete stoichiometry to ensure biochemical purity. While these semisynthetic methods are most efficient in the modification of the C- or N-terminal regions of proteins, it is fortuitous that these are locations often targeted by protein kinases in signaling pathways. Moreover, it is possible to introduce nonhydrolyzable mimics of post-translational modifications, which is particularly valuable in settings where a phosphatase is modified by phosphorylation as is the case with SHP-2.

Work in this study has addressed several unresolved issues concerning the regulation of SHP-2 by tail tyrosine phosphorylation. First, it has been demonstrated that the two C-terminal phosphorylation events can cooperate in the activation of SHP-2. Since previous work has demonstrated that tail-phosphonylated SHP-2 exists as a monomer (*21*), these studies establish that intramolecular dual occupancy of the two SH2 domains by the 542-phosphate and 580-phosphate is feasible. Thus, as previously hypothesized, the spatial distance between these two phosphorylation sites is sufficient to allow energetically favorable interaction between

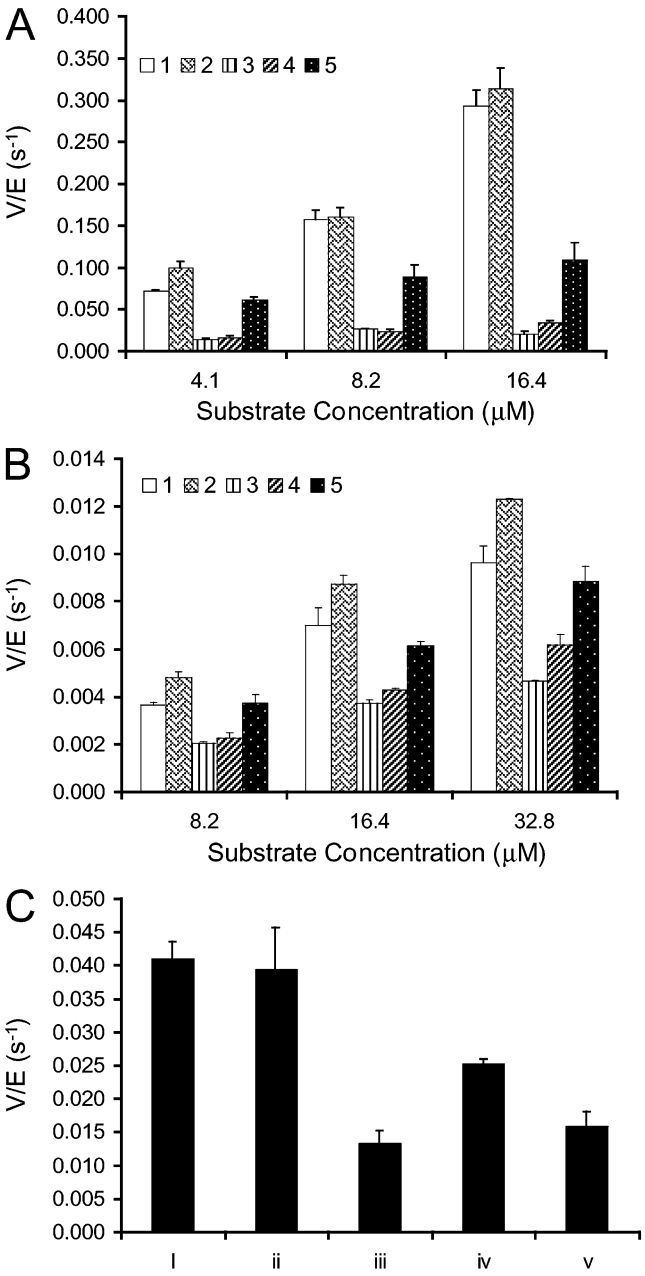

FIGURE 5: Intermolecular autodephosphorylation studies with SHP-2-FL and SHP-2-CAT as catalysts. (A) SHP-2-CAT as catalyst and 580-modified substrates. Bars 1−5 represent respectively substrates 580-pTyr peptide, SHP-$2^{191-593GS}$-580-pTyr, SHP-2^{593GS}-580-pTyr, SHP-2^{593GS}-R32A-580-pTyr, and SHP-2^{593GS}-R138A-580-pTyr. (B) SHP-2-FL as catalyst and 580-modified substrates. Bars 1−5 are respectively substrates 580-pTyr peptide, SHP-$2^{191-593GS}$-580-pTyr, SHP-2^{593GS}-580-pTyr, SHP-2^{593GS}-R32A-580-pTyr, and SHP-2^{593GS}-R138A-580-pTyr. (C) SHP-2-CAT as catalyst and 4.1 μM of 542-modified substrate. Bars i−v represent respectively substrates 542-pTyr peptide, SHP-$2^{191-547CS}$-542-pTyr, SHP-2^{547CS}-542-pTyr, SHP-2^{547CS}-R32A-542-pTyr, and SHP-2^{547CS}-R138A-542-pTyr. Rates measured as described in Materials and Methods represent the average of three runs with the standard errors shown.

these relatively widely spaced SH2 domains. Work of Shoelson, Eck, and colleagues has shown that >40 Å is required for the phosphates to extend into both SH2 domains (*18*−*20*). The 38 amino acid residues between positions 542 and 580 could provide well over 70 Å, assuming an extended conformation. Application of complex polymer statistical theory (*32*) would be necessary to more precisely define the subpopulations of the C-terminal tail conformations that are optimal for dual engagement of the SH2 domains, but the experimental results here confirm that such conformations are well-represented.

The implications for cell signaling are that dual phosphorylation of SHP-2 can lead to greater activation of the enzyme than single phosphorylation events. On the other hand, the opportunity for one of the vacant SH2 domains to engage with other phosphotyrosine recruiting proteins would be reduced when SHP-2 has been doubly phosphorylated. How this plays out in cellular function may be dependent on a given pathway.

The use of the more sophisticated phosphotyrosine analogue F_2Pmp has allowed a more precise estimate of activation of SHP-2 by C-terminal tyrosine phosphorylation. Each event is likely to lead to 3-fold activation of SHP-2 activity as assessed with a generic substrate. It should be pointed out that dual occupancy would be predicted to result in close to 9-fold activation based on the above findings with Pmp. This 9-fold activation would represent a substantial (30−45%) fraction of the maximal activation (20- to 30-fold) seen when the two SH2 domains are deleted.[4] Of course, the actual activation seen in vivo will likely depend on the substrate involved and the cellular context.

The modest (30−50%) enhanced stimulation by F_2Pmp versus Pmp is similar to that seen in the analogous system, SHP-1, where about 2-fold effects were observed (*33*). Taken together, these studies suggest that the commercially available Pmp analogues can provide good, but not perfect, estimates of the impact of phosphotyrosine in proteins. The better matched pK_a and hydrogen-bonding characteristics of the F_2Pmp make it the preferred analogue if feasible for a given study. However, the significant synthetic effort needed to produce this analogue will undoubtedly restrict its application to the biology community until it becomes more readily available.

Another feature of phospho-regulation of PTPases that has been understudied in the past has been the nature of the kinetics of dephosphorylation. This is a particularly challenging problem that has only been partially addressed here because only intermolecular reactions were looked at. The rate of intermolecular autodephosphorylation of SHP-2 was shown to be dependent on the surrounding sequence involved, with the 542-phosphate being more resistant to dephosphorylation. It was also demonstrated that the intermolecular autodephosphorylation could in all cases be inhibited in SHP-2 by the intramolecular interaction with an SH2 domain. This relative level of this inhibition varied from 1.5- to 5-fold with the form of the SHP-2 catalyst as well as the SHP-2 substrate phosphorylation site. However, the level of inhibition in all of these cases was considerably less than that observed in experiments involving phosphorylated Src and the PTPase CD45 where >20-fold rate reductions were seen, comparing the closed and open forms of the substrate (*34*). This suggests that the equilibrium constant governing the ratio of open versus closed forms of

[4] Previous studies have reported variable levels of SHP-2 activation by the addition of phosphopeptides and the deletion of SH2 domains (*16*−*19*). Part of the reason for discrepancies among studies could stem from the different choices and preparation of complex substrates, as well as from potential differences in assay conditions.

SHP-2 is smaller than that of tail-phosphorylated Src. Since tail-phosphorylated Src has additional intramolecular interactions involving its SH3 domain (*34*−*36*), such a difference would not be unexpected. In future studies, it would also be interesting to investigate the rate of dephosphorylation of doubly phosphorylated SHP-2, which might more closely approximate the equilibrium constant of tail-phosphorylated Src.

Not probed here is the potential importance of intramolecular SHP-2 autodephosphorylation. This technically challenging problem might be addressed in future experiments by using a caging-photodeprotection strategy of the catalytic cysteine nucleophile (*37*). Other PTPases in the cell could also modulate the stability of the phosphotyrosine linkages in SHP-2 and affect its function in complex signaling networks.

The biological importance of phosphorylation of these Tyr residues with regard to in vivo signaling is continuing to be addressed. Mutation of these residues to Phe does have significant implications on a number of pathways investigated including leptin and fibroblast growth factor signaling (*12*, *38*). Many of these and other cellular experiments have utilized overexpressed SHP-2, which might make it hard to see modest catalytic activation by phosphorylation. However, a "knock-in" experiment may ultimately be most revealing of subtle phenotypes induced by tyrosine phosphorylation.

SUMMARY

Expressed protein ligation has been used to dissect several of the subtle features of regulation of SHP-2 by tyrosine phosphorylation. The potential for multiple phosphorylation events giving rise to additive effects on the stimulation of SHP-2 activation was revealed. F_2Pmp was shown to be somewhat more effective in activating the catalytic activity of SHP-2 compared to the unfluorinated Pmp counterpart, presumably by more faithfully mimicking the authentic phosphotyrosine. The intermolecular autodephosphorylation of SHP-2 was investigated, which showed that intramolecular interactions could modulate the stability of phosphotyrosine linkages in these proteins. Overall, these studies underscore how the interplay of organic chemistry and enzymology can be used to clarify poorly understood aspects of cell signaling.

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