The Role of C-terminal Tyrosine Phosphorylation in the Regulation of SHP-1 Explored via Expressed Protein Ligation*

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The protein-tyrosine phosphatase SHP-1 plays a variety of roles in the "negative" regulation of cell signaling. The molecular basis for the regulation of SHP-1 is incompletely understood. Whereas SHP-1 has previously been shown to be phosphorylated on two tail tyrosine residues (Tyr536 and Tyr564) by several protein-tyrosine kinases, the effects of these phosphorylation events have been difficult to address because of the intrinsic instability of the linkages within a protein-tyrosine phosphatase. Using expressed protein ligation, we have generated semisynthetic SHP-1 proteins containing phosphotyrosine mimetics at the Tyr536 and Tyr564 sites. Two phosphonate analogues were installed, phosphonomethylenephenylalanine (Pmp) and difluorophosphonomethylenephenylalanine (F2Pmp). Incorporation of Pmp at the 536 site led to 4-fold stimulation of the SHP-1 tyrosine phosphatase activity whereas incorporation at the 564 site led to no effect. Incorporation of F2Pmp at the 536 site led to 8-fold stimulation of the SHP-1 tyrosine phosphatase activity and 1.6-fold at the 564 site. A combination of size exclusion chromatography, phosphotyrosine peptide stimulation studies, and site-directed mutagenesis led to the structural model in which tyrosine phosphorylation at the 536 site engages the N-Src homology 2 domain in an intramolecular fashion relieving basal inhibition. In contrast, tyrosine phosphorylation at the 564 site has the potential to engage the C-Src homology 2 domain intramolecularly, which can modestly and indirectly influence catalytic activity. The finding that phosphonate modification at each of the 536 and 564 sites can promote interaction with the Grb2 adaptor protein indicates that the intramolecular interactions fostered by post-translational modifications of tyrosine are not energetically strong and susceptible to intermolecular competition.

The SHP-1 protein-tyrosine phosphatase (PTPase) (initially designated SHPTP-1, SHP, HCP, and PTP1C) is thought to play a role as a negative regulator of cell signaling in cells of hematopoietic lineage (1). Its involvement has been implicated in colony stimulating factor 1 receptor signaling pathways (2), B cell receptor-induced apoptosis and signaling (3, 4), Fcγ receptor-mediated phagocytosis (5), HoxA-mediated transcriptional repression (6), Abl-induced DNA damage response (7), erythropoietin receptor signaling (8), and T cell receptor signaling (9). Mice lacking wild-type SHP-1 show a “motheaten” phenotype characterized by patchy alopecia, severe combined immunodeficiency, and the lethal onset of hemorrhagic interstitial pneumonitis (1, 9). There has been a continuing interest in delineating the complete biological functions of SHP-1.

SHP-1 is a 68-kDa protein composed of two SH2 domains, a tyrosine phosphatase catalytic domain and a flexible C-terminal domain which has been proposed to play a regulatory role (Fig. 1A). There is a high resolution crystal structure of the SHP-1 catalytic domain indicating the classical PTPase fold (10). These enzymes have a highly conserved cysteine residue that serves as the catalytic nucleophile, generating a phosphocysteine covalent intermediate (11). The phospho-enzyme intermediate is then hydrolyzed producing inorganic phosphate. Whereas the authentic physiologic protein substrates of SHP1 have not been determined with certainty, several proposed phosphoprotein targets include Lyn, Syk, BLNK/SLP-65, Lck, ZAP-70, phosphatidylinositol 3-kinase, SLP-76, interleukin 2 receptor, IRF1, and the interferon consensus sequence-binding protein (1, 12, 13). Typically phosphopeptides or para-nitrophenol phosphate (pNPP) have been used to study the catalytic mechanism and regulation of SHP-1 in vitro (14).

The mechanisms of the regulation of SHP-1 are incompletely understood. No high resolution structure has been reported for the full-length protein. The two SH2 domains may be important in recruiting phosphotyrosine-containing proteins, controlling substrate specificity, and cellular localization of the enzyme. In addition, the N-terminal SH2 domain appears to play a role as a negative regulator of SHP-1 catalytic activity by directly binding to the SHP-1 catalytic domain (15). This inhibition can be relieved by phosphotyrosine containing peptides with appropriate sequences and possibly phospholipids (15, 16). Two C-terminal tyrosine phosphorylation sites at Tyr536 and Tyr564 have been mapped (Fig. 1A) and possible tyrosine kinases that catalyze these reactions include Lck, Abl, and the insulin receptor tyrosine kinase (7, 17–19). The function(s) of these phosphorylations have been difficult to address in detail because of the instability of these modifications because of the inherent catalytic nature of the phosphatase. Possible roles for the phosphotyrosines include the recruitment of phosphotyrosine-binding modules such as SH2 and PTB domain-containing proteins as well as activation of the SHP-1 catalytic activity. One or more of these phosphotyrosines could bind in an intramolecular or intermolecular fashion to the SH2 domains of SHP-1.

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The abbreviations used are: PTPase, protein-tyrosine phosphatase; SH2, Src homology 2; N-SH2, N-terminal SH2 domain; C-SH2, C-terminal SH2 domain; pY, phosphotyrosine residue; EpoR, erythropoietin receptor; pNPP, p-nitrophenyl phosphate; Pmp, phosphonomethylenephenylalanine; F2Pmp, difluorophosphonomethylenephenylalanine; Fmoc, N(9-fluorenylmethoxycarbonyl); MALDI, matrix-assisted laser desorption ionization; GST, glutathione S-transferase; ATPγS, adenosine 5′-O-(thiotriphosphate).
Recently, expressed protein ligation (20, 21) has been used to probe the function of phosphotyrosine modifications in the related protein-tyrosine phosphatase enzyme, SHP-2 (22). In this study, the phosphotyrosine mimetic phosphonomethylene-phenylalanine (Pmp) was incorporated into the C terminus of SHP-2 at two phosphorylation sites and the effects on SHP-2 catalytic behavior determined. Because the SHP-2 and SHP-1 tails show little sequence homology (Fig. 1B), and their SH2 domains appear to show different phosphotyrosine sequence preferences, it was not known whether the results with SHP-2 would extend to SHP-1. Here we use expressed protein ligation to probe the importance of C-terminal tyrosine phosphorylation of SHP-1 and find evidence both for stimulatory and adaptor functions.

EXPERIMENTAL PROCEDURES

Preparation of the Shp-1 Constructs—The full-length human shp-1 tyrosine phosphatase encoded in a DNA plasmid (gift of Dr. Jun Wang) was used to amplify DNA encoding amino acids 1–556 by use of primers containing NdeI and SmaI restriction digestion sites at the 5’ and 3’ ends, respectively. The PCR product was purified and ligated into the pTYB2 vector (New England Biolabs) in-frame with the intein and chitin binding domain open reading frames. The extra SmaI site at the 3’ end of the shp-1 gene was deleted using QuickChange site-directed mutagenesis (Stratagene) to give the plasmid pTYB2-shp-1-(1–556). This plasmid was used to generate 564-modified proteins (after ligation) and as a template to produce all other mutants by the QuickChange method including pTYB2-shp-1-(1–531), which was used to generate 536-modified proteins. All constructs were confirmed by DNA sequencing the entire open reading frames of the shp-1 gene.

Preparation of Phosphotyrosine (Phosphonate) Analogues—Fmoc-phosphonomethylene-L-phenylalanine (Fmoc-Pmp) was purchased from Advanced ChemTech and used without further purification. Fmoc-phosphonomethylene-L-phenylalanine (Fmoc-P,F,Pmp) was synthesized and characterized as described by Lawrence and colleagues (23).

Peptide Synthesis—Standard Fmoc-protected amino acids, peptide synthesis reagents, and Wang resins were purchased from Novabiochem. All peptides were synthesized on Wang resin using the standard Fmoc strategy on a Rainin PS-3 machine. Pmp, F2Pmp, or phosphotyrosine (Tyr(P)) containing peptides were prepared by incorporating the requisite synthetic protein catalytic behavior (14, 15), and this proved to be the physiologic sequences in these positions (Fig. 1C).

Grb2 Binding Studies—GST-Grb2 fusion protein was expressed in E. coli BL21(DE3) cells and purified by the use of glutathione-agarose (Sigma) as previously described (26, 27). The Grb2 binding studies were carried out as follows: 50 μl of GST-Grb2 beads slurry containing ~50 μg of fusion protein (estimated by 10% SDS-PAGE), was centrifuged at 2000 × g for 5 min in a small plastic (Eppendorf) tube. The supernatant was discarded and the pellet was washed twice with 0.1 ml of binding buffer (25 mM Na-Hepes, pH 7.5, 10% glycerol, and 100 mM NaCl), and then 10 μl of binding buffer was added to the pellet to resuspend the beads. The resuspended beads was added 10 μg of SHP-1 protein in 30 μl of binding buffer. After 4 min incubation at 16 °C, the reaction mixture was centrifuged at 2000 × g for 5 min. The pellets were then washed with 100 μl of times with 0.1 ml of binding buffer, finally mixed with 30 μl of 1× SDS loading dye at 95 °C for 5 min and the supernatant was loaded onto 10% SDS-PAGE. The dried gels were scanned and the amount of SHP-1 proteins bound to Grb2 was quantified using ImageQuant software.

RESULTS

Generation of Semisynthetic SHP-1—The N-terminal portion of SHP-1 including its SH2 domains and catalytic domain but lacking most of the C-terminal tail was subcloned into the pTYB2 vector in-frame with the intein-chitin binding domains. Two constructs were developed covering residues 1–531 and 1–556, which enable the ligation of 15- and 16-residue N-terminal cysteine containing peptides, respectively, to replace the physiologic sequences in these positions (Fig. 1C). Based on previously published studies, it was expected that partial deletions in the C-terminal sequence would resemble full-length protein catalytic behavior (14, 15), and this proved to be the case in our hands (data not shown). The requisite synthetic peptides containing either tyrosine or Pmp at the sites of phosphorylation were prepared by solid phase peptide synthesis. Cysteines were installed at the N terminus of these peptides replacing Gln (for 564-modified proteins) or Ser (for 536-modified proteins) in the natural sequences as required for the expressed protein ligation method. To facilitate ligation, the C-terminal residues of the recombinant protein were Gly, which was the natural residue of the 536-modified proteins but replaced a Ser in the 564-modified proteins. These mutations did not significantly affect the activities of the proteins as revealed by pNPP assay (data not shown).

Expressed protein ligation performed in the presence of thiophenol3 as previously described (20, 22) led to robust protein production (~2 mg/liter of E. coli cell culture), which appeared to be greater than 90% pure eluting from the chitin resin (Fig. 2A). Mass spectra supported the efficiency of the ligation process (Fig. 2B). It was also demonstrated that the phosphatase activity of standard

4 Whereas mercaptoethanesulfonate is a useful co-reagent for expressed protein ligation (21, 31), and was initially used for some of the experiments here, the semisynthetic SHP-1 proteins showed aberrant catalytic behavior compared with the fully recombinant proteins. In contrast, thiophenol in place of mercaptoethanesulfonate did not lead to this unnatural catalytic activity and was thus employed for all of the work described here.
SHP-1 recombinant protein identical in sequence length to the semisynthetic proteins showed similar catalytic behavior (data not shown).

Gel Filtration—There was a theoretical possibility that tail phosphorylation could promote intermolecular protein interactions via SH2 domain-phosphotyrosine interactions. This potential was assessed with the semisynthetic proteins by size exclusion chromatography (Fig. 3). A comparison of elution profiles of Pmp-containing versus tyrosine containing proteins showed no significant differences in the elution profiles (Fig. 3). Moreover, by comparison to the profiles of protein standards (25) it was shown that each of the semisynthetic proteins showed sizes consistent with monomers. Thus it can be concluded that at the concentration investigated (1–2 μg/ml), the Pmp groups do not promote protein dimerization or higher order oligomers.

Enzyme Activity of Pmp-substituted Semisynthetic SHP-1 Proteins—To address the possibility that the Pmp substitutions could modulate the enzyme activity of SHP-1, the relative activities of the semisynthetic proteins were studied with the well established substrate, pNPP. Because wild-type SHP-1 does not display Michaelis-Menten kinetic behavior, a fixed concentration of pNPP was employed. As can be seen, SHP-1/Pmp536 showed a substantial 4-fold increase in phosphatase activity compared with SHP-1/Tyr536 (Fig. 4). In contrast, SHP-1/Pmp564 showed very similar catalytic behavior compared with SHP-1/Tyr564. These results suggest site-selective activation of SHP-1 by tyrosine phosphorylation at Tyr 536 (Fig. 4). Analogous results were seen using phosphorylated RCM-lysozyme as substrate (data not shown).

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To probe the structural basis of Pmp536 activation, the phosphatase activity of the semisynthetic proteins was measured in the presence of phosphotyrosine peptide EpoR-pY-429 (AcHNLPHLKYLpYLVVSDF-CO2H). This peptide has been shown to stimulate the phosphatase activity of SHP-1 by relieving basal inhibition mediated by the N-terminal SH2 domain (15). As can be seen (Fig. 4B), 70 μM EpoR-pY-429 caused a similar 4-fold stimulation of SHP-1/Tyr536 and SHP-1/Pmp564. In contrast, whereas EpoR-pY-429 led to a 4-fold activation of SHP-1/Tyr536, it caused a more modest 1.5-fold activation of SHP-1/Pmp536. This is consistent with the possibility that Pmp536 can partially relieve the basal suppression mediated by the N-terminal SH2 domain.

Generation of a Semisynthetic SHP-1 with a Consensus Sequence—Because Pmp536 may be interacting with the N-termi-
nal SH2 domain of SHP-1 to enhance the phosphatase activity of SHP1, we considered the possibility that an amino acid sequence surrounding Pmp536, which was designed to interact with the N-SH2 domain, might show more robust stimulation of SHP-1. Based on the analysis of the affinity of the SHP-1 N-terminal SH2 domain using peptide libraries (28), a peptide containing the consensus sequence LHpYMNF (Fig. 5A) was synthesized for use in expressed protein ligation. This semi-synthetic protein (SHP-1/Pmp536con) was prepared as efficiently as SHP-1/Pmp536 and subjected to catalytic activity measurements. As shown in Fig. 5B, the activity of SHP-1/Pmp536con was greater than SHP-1/Pmp536 and minimally affected by the addition of EpoR-pY-429. This provides further evidence of an interaction between Tyr(P)536 and the N-terminal SH2 domain.

Effect of F2Pmp Incorporation on SHP-1—It has previously been shown by Burke and colleagues (29) that F2Pmp is a closer mimic of phosphotyrosine compared with Pmp. Because the consensus sequence for N-SH2 binding led to greater SHP-1 activation relative to the natural sequence, we pursued incorporation of F2Pmp at residues 536 and 564 of SHP-1, respectively. Synthesis of the difluoro analogue was carried out as described by Guo et al. (23) and incorporated into the same synthetic peptide sequences used with Pmp (Fig. 1C). These peptides were used to produce the desired semisynthetic proteins in similar efficiency compared with the Pmp derivatives. Strikingly, F2Pmp induced greater phosphatase activity at both positions 536 and 564 (Fig. 5C). An 8-fold activation in phosphatase activity was detected for SHP-1/F2Pmp536 compared with SHP-1/Tyr536, and SHP-1/F2Pmp536 did not show enhanced activity in the presence of EpoR-pY-429. Moreover, SHP-1/F2Pmp536 actually displayed a 1.6-fold increase in activity compared with SHP-1/Tyr564 or SHP-1/Pmp564. These results unmask a potential for tyrosine phosphorylation to provide modest stimulation of SHP-1 at the 564 position. This modest stimulation in the absence of peptide EpoR-pY-429 was less apparent in the presence of stimulatory peptide (Fig. 5C), presumably because the peptide effect was more dominant.

Effect of SH2 Mutations on the Activities of SHP-1—The structural basis of Pmp (and F2Pmp) activation of SHP-1 plau-
Regulation of SHP-1 by Phosphorylation

Fig. 6. Phosphatase activities of the SHP-1 proteins containing mutations in the N-SH2 (Arg30) or C-SH2 (Arg136) domains. A, phosphatase activities of semisynthetic SHP-1 proteins containing various SH2 domain point mutations; B, relative phosphatase activity of semisynthetic SHP-1 with Pmp536 substitution and SH2 mutations; C, relative phosphatase activity of semisynthetic SHP-1 with 564-F2Pmp substitution and C-SH2 mutation. The phosphatase activities of the SHP-1 semisynthetic proteins were measured in the presence of 2 mM pNPP, without (open bar) or with 70 μM EpoR-pY429 peptide (solid bar).

Fig. 7. Regulation of the SHP-1 protein activity by the phosphorylation of the C-terminal tyrosine residues. When both Tyr536 and Tyr564 are unphosphorylated, the N-SH2 domain interacts with the PTPase domain, inhibiting the phosphatase activity. Phosphorylation at Tyr536 leads to interaction with the N-SH2 domain, releasing the inhibitory effect of the N-SH2 domain on the PTPase domain. Phosphorylation of Tyr564 leads to interaction with the C-SH2 domain, partially and indirectly releasing the inhibitory effect of the N-SH2 domain, and modestly increasing the PTPase activity.

SHP-1 enzymes at a concentration identical to that used in the wild-type SHP-1 studies (Fig. 6A). Using the corresponding nonphosphorylated form of the peptide (EpoR-Y429) at the same concentration, no activation of the Arg30 mutants was detected (data not shown). Thus the putative interaction of phosphopeptide ligand with the N-SH2 domain of SHP-1 is unconventional compared with typical cases and the role of the FLVRES Arg is likely substituted by another residue in this case.

The effects of mutation of Arg30 on several of the phosphonate containing semisynthetic proteins were also examined (Fig. 6B and C). It can be seen that SHP-1/R30A-Pmp536 shows a 2-fold greater PTPase rate compared with SHP-1/R30A-Tyr564 (Fig. 6B). These results are consistent with the intermolecular studies with phosphopeptide EpoR-pY429 in which it was shown that the presence of Arg30 is not critical for PTPase stimulation. It was also shown that SHP-1/R136A-Pmp536 displays a 4-fold greater rate compared with SHP-1/R136A-Tyr536 arguing against the role of the C-SH2 domain in mediating PTPase activation by the Pmp536 modification. Finally, it was found that SHP-1/R136A-F2Pmp564 shows an identical rate to SHP-1/R136A-Tyr564 (Fig. 6C) suggesting that the C-SH2 domain is most likely responsible for the modest activation by the F2Pmp564 modification (Fig. 6C). Taken together, the data support a structural model of SHP-1 activation by phosphorylation shown in Fig. 7.

Grb2 Binding—The role of the phosphonate modifications of SHP-1 in mediating interactions with Grb2, an SH2 domain containing adaptor protein (22, 26), were studied by a variant of the GST pull-down assays. GST-Grb2 was immobilized on glutathione-agarose and incubated with SHP-1 semisynthetic proteins for 4 min prior to several brief buffer washes. It was found that both SHP-1/Pmp536 and SHP-1/Pmp564 proteins showed significantly greater binding than the unphosphorylated semisynthetic proteins (Fig. 8). Likewise, F2Pmp SHP-1 proteins showed greater binding to Grb2 than unmodified proteins. Interestingly, Pmp containing SHP-1 proteins showed somewhat more efficient binding (about 2-fold) to Grb2 compared with F2Pmp proteins. This could be the case because
The mean relative intensities from the three experiments are indicated.

F₂Pmp-modified SHP-1 semisynthetic proteins binding to Grb2; domains did not have a large effect on the relative proportion of argued against because mutations in the N-SH2 or C-SH2 SH2 domains, or a slower on-rate with respect to the Grb2 release from their intramolecular interactions with the SHP-1.

Acat enzymatic modification of recombinant proteins with ATP-blending phosphotyrosine. Thiophosphates, resistant to enzymatic used as mimics of phosphoserine and phosphothreonines, often used for the structure and function of a signaling protein technology can be fused with a readily prepared N-terminal SH2 domain (Fig. 7). Whereas mutation of the FLVRES Areg of the N-SH2 domain in the catalytic domain could even be contributing to binding in this peculiar case. Another explanation would be that the SHP-1 catalytic domain is perturbing the structure of the N-SH2 domain such that it behaves anomalously. Related to this idea, the catalytic domain could even be contributing residues that substitute for SH2 domain residues in the function of the domain. Finally, it should be mentioned that a loss of affinity of the N-SH2 domain for the catalytic domain may occur when Arg⁵⁰ is mutated, leading to PTPase activation. This weaker interaction may make the N-SH2 domain, even though damaged by Arg⁵⁰ mutation, more available for phosphotyrosine binding, which would offset the loss of affinity resulting from the Arg⁵⁰-phosphate interaction. Further structural studies will be needed to distinguish among these possibilities.

In contrast to the relatively large stimulation by phosphonates at the 536 position of SHP-1, the catalytic stimulation from F₂Pmp at the 564 site is rather modest at 1.6-fold. It is noteworthy that the effect seen with F₂Pmp was absent when the initial Pmp analogue was employed. This argues for the utility of the fluorophosphonate analogues bringing out more subtle structural effects because they are more faithful mimics. Whereas a small effect, the F₂Pmp effect can be structurally

Fig. 8. Grb-2 interactions with semisynthetic SHP-1 proteins. A relative level of Pmp⁵⁰⁶-modified SHP-1 semisynthetic proteins carrying SH2 mutations binding to Grb2. B, comparison of Pmp- and F₂Pmp-modified SHP-1 semisynthetic proteins binding to Grb2. C, relative levels of SHP-1/F₂Pmp⁵⁰⁶ carrying SH2 mutations binding to Grb2. GST-Grb2 was immobilized on glutathione resin and the level of SHP-1 protein ”pulled-down” was measured as described under “Experimental Procedures.” One SDS-PAGE, stained with Coomassie Blue, of three independent experiments, with good reproducibility, is shown. The mean relative intensities from the three experiments are indicated below each lane.

F₂Pmp have a lower relative affinity for Grb2, a slower rate of release from their intramolecular interactions with the SHP-1 SH2 domains, or a slower on-rate with respect to the Grb2 domains (these assays were designed to look at kinetic effects). However, the importance of slow intramolecular dissociation is argued against because mutations in the N-SH2 or C-SH2 domains did not have a large effect on the relative proportion of SHP-1 proteins bound to Grb2.

DISCUSSION

The value of expressed protein ligation in addressing structure-function relationships in reversible phosphorylation of signaling proteins is becoming increasingly well demonstrated (20–22, 31, 32). It is most conveniently applied when the region of the protein to be modified is in the C terminus of a signaling protein because the C-terminal thioester generated using intein technology can be fused with a readily prepared N-terminal cysteine containing peptide. Recently published studies on transforming growth factor-β-substrate interactions (31), Csk-Src regulation (20, 32), and SHP-2 tyrosine phosphatase (22) employing expressed protein ligation have illustrated the power and scope of the method. The recent work on SHP-2 illustrates the strength of the use of nonhydrolyzable phosphotyrosines to examine the structure and function of a signaling protein (22). Whereas for many years Glu and Asp have been used as mimics of phosphoserine and phosphothreonines, often successfully, no such encoded amino acid comes close to resembling phosphotyrosine. Thiophosphates, resistant to enzymatic hydrolysis, have been introduced into signaling proteins by enzymatic modification of recombinant proteins with ATP₈S. These are often sluggish reactions that generally afford low stoichiometries and often show poor regioselectivity (33). Moreover, thiophosphates can indeed be hydrolyzed chemically and enzymatically, by some phosphatases almost as rapidly as phosphate substrates (34), in contrast to the nonhydrolyzable analogues used here.

The poor homology of SHP-2 and SHP-1 C-terminal tail regions (Fig. 1B) made it difficult to predict based on the results of SHP-2 what to expect with SHP-1. It is now clear that phosphorylation at Tyr⁵³⁶ can activate the SHP-1 PTPase activity in analogy with Tyr⁴⁴² in SHP-2. However, the degree of activation, about 8-fold with F₂Pmp in SHP-1, is significantly greater than the 2–3-fold observed with Pmp and SHP-2. It remains to be seen if this difference is related to the choice of the phosphotyrosine analogue although given that only a 4-fold effect was seen with Pmp⁵³⁶ and SHP-1, this has to be considered a possibility. It should also be pointed out that a study with enzymatically phosphorylated SHP-1 at the 536 position also reports PTPase activation by this modification (18), although this was characterized with a less pure protein mixture and uncertain stoichiometry in which the SHP-1 undergoes dephosphorylation during the PTPase measurements.

The model of activation based on the findings with the consensus sequence engineered SHP-1, stimulation with EpoR-pY-429 peptide, and mutagenesis support the proposition that phosphoryl modification at 536 activates SHP-1 by interaction with the N-terminal SH2 domain (Fig. 7). Whereas mutation of the FLVRES Areg of the N-SH2 leads to somewhat unconventional behavior, it is clear that mutation of the C-SH2 domain does not play a role in activation by Pmp⁵³⁶ (Fig. 7). The gel filtration experiments showing that SHP-1/Pmp⁵³⁶ is monomeric support the likelihood that this interaction is intramolecular (Fig. 7). Furthermore, this activation model would be similar to the effect of phosphorylation of Tyr⁴⁴² in SHP-2 (22). Not addressed in these studies or in Fig. 7 is the interesting finding that deletion of the C-terminal tail 35 amino acids but not the final 60 amino acids appears to be activating for SHP-1 (15). The basis of catalytic activation by this truncation is not yet understood and may add complexity to the simple model in Fig. 7. It should be stated that deletion of the C-terminal 49 amino acid residues as present in SHP-1/Tyr⁵³⁶ or 23 amino acids in SHP-1/Tyr⁵⁶⁶ showed similar catalytic activity to full-length SHP-1 in our hands.

It is worthwhile to consider the reason why mutation of the FLVRES Arg itself leads to PTPase stimulation and yet these mutant proteins are still susceptible to phosphotyrosine activation. One possibility is that the SHP-1 N-SH2 domain is noncanonical and the FLVRES Arg contributes relatively little to binding in this peculiar case. Another explanation would be that the SHP-1 catalytic domain is disrupting the structure of the N-SH2 domain such that it behaves anomalously. Related to this idea, the catalytic domain could even be contributing residues that substitute for SH2 domain residues in the function of the domain. Finally, it should be mentioned that a loss of affinity of the N-SH2 domain for the catalytic domain may occur when Arg⁵⁰ is mutated, leading to PTPase activation. This weaker interaction may make the N-SH2 domain, even though damaged by Arg⁵⁰ mutation, more available for phosphotyrosine binding, which would offset the loss of affinity resulting from the Arg⁵⁰-phosphate interaction. Further structural studies will be needed to distinguish among these possibilities.
rationalized because it disappears upon mutation of the C-SH2 Arg<sup>136</sup> residue. Thus, it is likely that this phosphorylation of Tyr<sup>564</sup> can modestly enhance SHP-1 activity by intramolecular C-SH2 domain engagement, presumably by an indirect effect (Fig. 7). This is qualitatively similar to the behavior of Tyr<sup>580</sup> in SHP-2 although in that case the activation is more robust and observable with Pmp (2–3-fold), comparable with that of Tyr<sup>542</sup> phosphorylation in SHP-2 (22).

Interestingly, although phosphorylation of SHP-1 at 536 and 564 can allow for intramolecular interactions with the SH2 domain, they are still quite efficiently able to facilitate phosphorylation of SHP-1 by intramolecular transduction. The phosphorylation in SHP-2 although in that case the activation is more robust and observable with Pmp (2–3-fold), comparable with that of Tyr<sup>542</sup> phosphorylation in SHP-2 (22).

**Summary**—Expressed protein ligation has been used to generate site-specific and stoichiometric phosphate-modified forms of SHP-1 to simulate the effects of tyrosine phosphorylation of SHP-1. These studies describe the first comparative analysis of the effects of difluoromethylenephosphonate and methylenephosphonate as phosphotyrosine mimetics site-specifically incorporated within the context of a protein. It was shown that phosphate at the 536 position of SHP-1 is capable of up to 8-fold stimulation of the SHP-1 tyrosine phosphate activity, likely by intramolecular engagement of the N-SH2 domain, relieving basal inhibition. In contrast, phosphate modification of the 564 position of SHP-1 results in a smaller (1.6-fold) stimulation of the tyrosine phosphate activity, probably by an indirect effect via interaction with the C-terminal SH2 domain. The phosphate-modified SHP-1 proteins are readily able to recruit the SH2-containing adaptor protein Grb2 suggesting that the intramolecular interactions promoted by tyrosine phosphorylation are not highly favorable energetically. These studies suggest that tyrosine phosphorylation of SHP-1 could play distinct roles in cell signaling either by direct catalytic activation of the enzyme or by recruitment of other signaling molecules to a specific cellular location.

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