Identification of a Novel Polyproline Recognition Site in the Cytoskeletal Associated Protein, Proline Serine Threonine Phosphatase Interacting Protein*

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Protein-protein interactions are often mediated by the recognition of proline-rich domains by SH3 or WW modules. Previously, we demonstrated that the PEST-type protein-tyrosine phosphatase, PTP HSCF (hematopoietic stem cell fraction), bound to a novel cytoskeletal associated protein, proline serine threonine phosphatase interacting protein (PST PIP), via an interaction between the proline-rich COOH terminus of the PTP and a site within the putative coiled-coil domain of PST PIP. Here we describe a more detailed analysis of this interaction. Earlier data suggested that the NH₂ terminus of PST PIP was important for binding to the phosphatase, and deletion of the NH₂-terminal 50 amino acids of the PST PIP resulted in an apparently misfolded protein that was incapable of binding PTP HSCF. To examine the region involved with binding to PTP HSCF, alanine-scanning mutants were produced at intervals throughout PST PIP. This analysis demonstrated that a tryptophan at position 232 was essential for binding in vitro. Transfection experiments demonstrated that the Trp²³² mutant protein was capable of association with the cortical cytoskeleton but was not bound to PTP HSCF in vivo. Alanine scanning of a peptide derived from the COOH-terminal proline-rich domain of PTP HSCF revealed that a subset of prolines, as well as other residues, was required for efficient binding to PST PIP, and introduction of alanines at some of these positions in the protein resulted in decreased binding to PST PIP in vitro and in vivo. Analysis of in vivo tryrosine phosphorylation of the Trp²³² mutant of PST PIP in the presence of v-Src revealed that this protein was phosphorylated more efficiently than the wild-type molecule. Thus, the interaction between PTP HSCF and PST PIP is mediated by a novel site in the cytoskeletal associated protein which interacts with residues within the proline-rich COOH terminus of the phosphatase.

The specific interactions of a large number of intracellular proteins are involved in a diversity of signaling pathways in eukaryotic cells. Although many of these interactions are mediated by, for example, the recognition of phosphotyrosines by SH2 domains, many important binding events are also generated by the recognition of proline-rich motifs (1). Two different domains, the SH3-type (2, 3) and the WW-type (4, 5), recognize proline-rich regions of proteins. SH3 domains are protein modules that are approximately 60 amino acid long which are found in a diversity of signaling and cytoskeletal proteins. An example of the importance of SH3 domain-mediated interactions is found in the GRB2-son of sevenless complex, where SH3-induced interactions are involved with growth factor receptor activation of the ras signaling pathway (6). SH3 domains recognize residues within type II polyproline helices with relatively low affinity (7–9). The interactions among residues conserved in a number of SH3 domains and both prolines as well as other residues within the polyproline helix of the binding partner can induce adhesion in either of two orientations, a result that enhances the combinatorial complexity of such binding events (7, 10). WW domains represent a second type of polyproline recognition motif which is approximately 38 amino acids long and contains two tryptophan residues, a COOH-terminal proline, and a high concentration of hydrophobic and aromatic amino acids (4, 5, 11, 12). As is the case with SH3 domains, WW modules recognize regions rich in proline, although the types of proline-containing sequences recognized by WW motifs are divergent from those that bind to SH3 domains (5, 13–15). The recognition pocket of the WW motif contains a hydrophobic binding site in part derived from the COOH-terminal tryptophan conserved in these modules (12), a configuration that differs substantially from the SH3 interaction region (7–9). The potential importance of WW domains is highlighted particularly in the case of the formins, polyproline-containing molecules involved with limb and kidney development which bind to proteins that contain WW motifs (16, 17). A direct demonstration of the physiological importance of WW-containing proteins has yet to be demonstrated, although recent data suggest a role for this type of interaction in retroviral budding from infected cells (18) and possibly in the regulation of the epithelial Na⁺ channel (19). Together, these results suggest that recognition of proline-rich domains by at least two divergent protein motifs may be a critical aspect of eukaryotic signaling.

We recently described the characterization of a novel, tyrosine-phosphorylated cytoskeletal associated protein, termed PST PIP,1 which is a substrate for one or more of the PEST family of protein-tyrosine phosphatases (PTPs) (20). PST PIP (proline serine threonine phosphatase interacting protein) contains a putative coiled-coil region as well as a COOH-terminal SH3 domain, and it is homologous to a Schizosaccharomyces pombe cytoskeletal associated protein termed CDC15p, which is involved with the assembly of the cytokinetic cleavage...
furrow during mitosis (21). Examination of the cellular localization of PST PIP during mitosis in 3T3 cells demonstrated that the mammalian protein was also associated with the cleavage furrow, consistent with a role for this protein in cytokinesis. In addition, overexpression of the mammalian protein in the yeast S. pombe resulted in localization of PST PIP to the cleavage furrow and a partial dominant negative effect on cytokinesis, again consistent with a role for PST PIP in cleavage furrow assembly.

Endogenous and transfected PST PIP was constitutively unphosphorylated on its tyrosine residues, and it appeared likely that this was in part due to an interaction with an endogenous PTP, since treatment of cells with the pan-PTP inhibitor pervanadate resulted in a high level of PST PIP tyrosine phosphorylation. PST PIP was also tyrosine phosphorylated in the presence of cotransfected v-Src, and these phosphoryrosines appeared to be substrates for dephosphorylation by the PEST-type PTP, PTP HSCF. Dephosphorylation of PST PIP phosphoryrosines by PTP HSCF required the presence of a proline-rich region that is ∼20 amino acids long in the COOH terminus of the phosphatase, and it was demonstrated that this region appeared to be necessary and sufficient to mediate binding between these two proteins. Examination of this COOH-terminal region revealed that it bore a striking resemblance to the proline-rich motifs recognized by SH3 domains, consistent with its interacting with the PST PIP SH3 module (7–9). However, analysis of the region of PST PIP which interacted with PTP HSCF revealed that the coiled-coil-containing domain was responsible for the binding of these two proteins. This surprising result suggested that a novel type of polyproline recognition motif is involved with the interaction between PST PIP and PST PIP because neither consensus SH3- nor WW-type modules were contained within the coiled-coil region.

Here we describe a detailed analysis of the interaction between the PTP HSCF COOH-terminal proline-rich region and the coiled-coil domain of PST PIP. Although a consensus WW-type motif is not found in the coiled-coil region of PST PIP, a tryptophan residue within this region appears to be critical for recognition of the proline-rich COOH terminus of PST PIP. In addition, a tryptophan in the PTP HSCF COOH terminus, together with a subset of prolines and glycine and arginine residues, also appears to be involved with this interaction. In vivo expression of the mutant form of PST PIP, which is incapable of binding PTP HSCF, results in enhanced tyrosine phosphorylation in the presence of the v-Src tyrosine kinase. Together, these data define a novel type of protein-protein interaction which appears to regulate the levels of tyrosine phosphorylation of a cytoskeletal associated protein.

MATERIALS AND METHODS

Mutagenesis of PST PIP and PTP HSCF—Deletions in the PST PIP molecule were made from both the amino and carboxyl termini. The deletions were constructed from Pfu polymerase chain reaction fragments that were ligated back into the original PST PIP expression vector (20). The polymerase chain reaction primers for the NH2-terminal deletions were (all 5′ to 3′) as follows: N-coil.1, CAGTTCGGAATCCATGATGATAGTTCGACATCGAC; N-coil.2, GGAGGATGGTGGCGGACGTCGAC; C-coil.1, TTGACCTCGACAGTGAATGATTGACGAC; C-coil.2, TTGACCTCGAGTCATACATCGACAGTGAATGATTGACGAC; C-coil.3, TTGACCTCGAGTCATACATCGACAGTGAATGATTGACGAC; C-coil.4, TTGACCTCGAGTCATACATCGACAGTGAATGATTGACGAC; C-coil.5, TTGACCTCGAGTCATACATCGACAGTGAATGATTGACGAC; C-coil.6, TTGACCTCGAGTCATACATCGACAGTGAATGATTGACGAC.

The common 3′-end primer was ACCTCAGTGTCATACATCGACAGTGAATGATTGACGAC.

The primers for the COOH-terminal deletions were as follows: C-coil.5, TTGACCTCGGATCATACATCGACAGTGAATGATTGACGAC; C-coil.6, TTGACCTCGGATCATACATCGACAGTGAATGATTGACGAC.

The primer N-coil.1 was used as the common 5′-polymerase chain reaction primer for the COOH-terminal deletions. The mutagenesis of PST PIP and PTP HSCF was accomplished using the QuikChange procedure (Bio-Rad). For PST PIP the mutagenesis primers were designed to change 3 contiguous amino acids to alanine. Mutations were spaced approximately 12 amino acids apart, with new restriction sites engineered in for identification of mutant clones. Primer annealing was carried out at 70 °C for 10 min, 57 °C for 10 min, room temperature for 5 min before then on ice for at least 2 h. The mutants were plated on LB agar plates with ampicillin and grown overnight at 37 °C. Single colonies were also mutated to alanine using the following primers (all 5′ to 3′): Cys256, GCCGAGGATGTTCTGAGTCGAC; Cys266, GCCGAGGATGTTCTGAGTCGAC; Cys276, GCCGAGGATGTTCTGAGTCGAC; Cys286, GCCGAGGATGTTCTGAGTCGAC.

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In vivo expression of the mutant form of PST PIP, which is incapable of binding PTP HSCF, results in enhanced tyrosine phosphorylation in the presence of the v-Src tyrosine kinase. Together, these data define a novel type of protein-protein interaction which appears to regulate the levels of tyrosine phosphorylation of a cytoskeletal associated protein.
suggest that the NH₂ terminus of PST PIP is required for the formation of a correctly folded protein that is capable of binding to PTP HSCF.

**Mutational Analysis of PST PIP**—To define more accurately the binding site within the PST PIP coiled-coil domain, a collection of cluster and point mutations was produced within this domain. Preliminary deletion mapping suggested that binding of PTP HSCF could be obtained with a form of PST PIP containing amino acids 1–264 of the coiled-coil domain, and the mutagenesis was thus confined to this region of the protein. Because protein folding appeared to be critical for the binding of PTP HSCF (Figs. 1 and 2), all 6 cysteine residues within this part of the protein were mutated to serines, and the resultant mutants were tested for interaction with the PTP HSCF GST fusion protein. The elimination of individual cysteine residues did not appear to affect the binding of these two proteins, suggesting that the protein could fold and function appropriately in the absence of individual cysteines (data not shown). Further mutational analysis of PST PIP was thus performed to identify a region(s) that was potentially directly involved with PTP HSCF binding. Clustered alanine substitutions were produced at approximately 12-amino acid intervals throughout the PST PIP coiled-coil domain, and each mutant was tested subsequently for binding to the GST-PTP HSCF fusion protein. Mutation of residue clusters L26Q, D238E, E50R, R52K, R73TS, NS0VG, R89EE, E110RQ, I122MD, L133YK, D144QK, E146R, Q158VE, E158S, and R158QN individually to alanine resulted in neither or a minor change in the binding activity of these two proteins in vitro (data not shown). However, Fig. 3 illustrates that the mutation of the tryptophan residue at position 232 of PST PIP to alanine resulted in a complete loss of binding to the GST-PTP HSCF fusion protein in vitro. Additionally, Fig. 4 shows that cotransfection of wild-type PST PIP together with PTP HSCF into COS cells resulted in an in vivo association of the proteins, as described previously (20), whereas cotransfection of the W232A mutant of PST PIP resulted in a complete lack of in vivo association, consistent with the in vitro binding studies. As expected, this mutant nonbinding form of PST PIP could no longer be “substrate trapped” (20, 22–24) by a dominant negative Cys-Ser mutant of PTP HSCF (Fig. 4), although it could clearly be tyrosine phosphorylated in the presence of v-Src (Fig. 4) or pervanadate (data not shown). Thus, whereas the wild-type PST PIP showed enhanced tyrosine phosphorylation in the presence of a dominant negative substrate trapping form of PTP HSCF (PTP HSCF C-S) (20, 22–24), the W232A mutant of PST PIP was not hyperphosphorylated in the presence of this mutant form of the enzyme (Fig. 4). Because previous results suggested that tropothen, aromatic, and hydrophobic residues are involved with the recognition of proline-rich domains when found appropriately spaced together in the context of other residues in WW-type domains (12, 14), we examined the PST PIP sequence for these residues near Trp232. This examination revealed that Trp232 is in 27 amino acids COOH-terminal to another tryptophan at position 205. In addition, a phenylalanine (Phe221) and leucine (Leu264) residue also occur near the Trp232 residue with a spacing that is reminiscent of the WW motif (11). However, when these residues were mutated to alanine, no effect on PTP HSCF binding was observed in vitro (Fig. 3). Thus, whereas the juxtaposition of these two tropothen residues, together with the involvement of Trp232 in the recognition of the PTP HSCF proline-rich motif, is reminiscent of the WW module, comparison of the region containing these nearby tryptophans to the consensus sequence described for WW-type domains (11) reveals that most

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2 Y. Wu and L. Lasky, unpublished observations.

3 D. Dowbenko and L. Lasky, unpublished data.
of the conserved residues within the WW module are not found in this region of PST PIP (data not shown). In addition, the spacing of the two tryptophan residues in PST PIP is somewhat longer than that found for typical WW-type motifs (27 amino acids for PST PIP versus ~22 amino acids for consensus WW domains). Finally, to ensure that mutation of the critical tryptophan residue did not result in a global effect on protein folding, as domains. Finally, to ensure that mutation of the critical tryptophan residue did not result in a global effect on protein folding, we expressed the W232A mutant in Chinese hamster ovary cells and analyzed by confocal microscopy. As Fig. 2 illustrates, the W232A mutant was transfected into Chinese hamster ovary cells and analyzed by confocal microscopy. As Fig. 2 illustrates, the W232A mutant was folded appropriately in vitro. These data thus suggest that tryptophan residue 232 may be directly involved with the interaction between PST PIP and PTP HSCF. In addition, because Trp232 does not appear to be embedded in a typical WW module (11), they also suggest that this region defines a novel type of protein-protein recognition motif.

**Residues Important for PST PIP Interaction in PTP HSCF**—To analyze the residues within the COOH-terminal 20 amino acid region of PTP HSCF which were critical for PST PIP binding, 20 amino acid peptides with alanines incorporated separately at each position were tested for blocking of the interaction in vitro. Previously we demonstrated that a 20-amino acid peptide derived from this region of three different PEST-type PTPs (25–27) was capable of efficiently blocking the binding of an in vitro translated form of PST PIP to a GST fusion of PTP HSCF containing the COOH-terminal 149 amino acids, including the COOH-terminal proline-rich binding site (GST-PTP HSCF) (20). Fig. 5 illustrates that alanine replacement of Arg436, Pro440, Gly442, Pro443, Arg444, Pro447, and Trp450 individually in a peptide derived from the COOH terminal of PTP HSCF resulted in a decreased inhibition of binding by the mutant peptides, whereas alanine replacement at the other sites within the peptide had little or no effect on the ability of these peptides to block the interaction in vitro. Importantly, these residues are conserved in all of the PEST-type PTPs (25–28), consistent with previous data (20) demonstrating that peptides derived from the other members of this family of phosphatases all effectively blocked this interaction (Fig. 5). To confirm the peptide mutation analysis, each residue found to be critical for PST PIP binding in the PTP HSCF COOH-terminal region was mutated to alanine in the context of the whole protein, and the ability of each mutant PTP to bind PST PIP was analyzed in vitro and in vivo. Fig. 6 illustrates that mutants of the phosphatase containing alanines at all but one of the positions predicted from the peptide mapping study (Pro443, Fig. 5) were substantially deficient in binding to GST-PST PIP in the in vitro binding assay, although 10-fold increased amounts of GST-PST PIP could interact with the mutant PTP HSCF proteins, suggesting only a partial loss of binding. In addition, production of a double mutation in two of the critical residues in this region of PTP HSCF (Arg444 and Trp450) resulted in a stronger inhibitory effect on binding to PST PIP (Fig. 6). In vivo analysis of these point mutants revealed only a modest effect on binding in the single mutants of PTP HSCF, consistent with the in vitro data suggesting that
sufficiently high levels of PST PIP could interact with the mutant proteins. However, as was observed in the in vitro experiments, the doubly mutated form of PTP HSCF (R444A, W450A) was as poor at interacting in vivo with PST PIP as the mutant that was missing the entire COOH-terminal proline-rich domain (PTP HSCF Asp24) (20). These data confirm the importance of these residues to the binding interaction, and they suggest that much of the COOH-terminal region of PTP HSCF may be required for highest affinity binding to PST PIP.

In Vivo Tyrosine Phosphorylation of PST PIP—Previously, we demonstrated that PST PIP was tyrosine phosphorylated when cotransfected with v-Src tyrosine kinase (20). In addition, we showed that this tyrosine-phosphorylated PST PIP was a substrate for dephosphorylation or substrate trapping by wild-type or dominant negative PTP HSCF, respectively, and that the substrate trapping activity required an interaction between the two proteins mediated by the COOH-terminal proline-rich region of the PTP (20). We also established that an endogenous tyrosine kinase(s) was capable of phosphorylating tyrosines within PST PIP in both BaF3 and transfected COS cells, and an endogenous tyrosine phosphatase(s) was capable of dephosphorylating these tyrosine residues.

In addition, preliminary evidence shown in Fig. 4 suggested that the W232A mutant was phosphorylated more efficiently than the wild-type PST PIP in the presence of v-Src. To examine more quantitatively the role of the Trp232 residue in v-Src-induced tyrosine phosphorylation, we transfected constant amounts of the wild-type and W232A mutant forms of PST PIP into COS cells together with increasing quantities of the v-Src expression plasmid and subsequently analyzed the levels of phosphotyrosine in immunoprecipitated PST PIP. Fig. 7 illustrates that the W232A mutant form of PST PIP, which was deficient in binding to PTP HSCF, whereas the wild-type (WT) form of the protein (PST PIP WT) is found in a complex with precipitated PTP HSCF and is hyperphosphorylated (substrate trapped; Refs. 20, 22–24) by dominant negative (C-S) PTP HSCF.

DISCUSSION

The modulation of the tyrosine phosphorylation of a diversity of cellular proteins by protein-tyrosine phosphatases is a critical aspect of cellular regulation (29). Many of these enzymatic dephosphorylations are mediated by the recognition of phosphotyrosine residues by SH2-type domains as well as direct recognition of the substrates by the catalytic domains of the enzymes (23, 30). Here we describe a novel mechanism for the regulation of tyrosine phosphorylation which involves the recognition of a proline-rich motif at the COOH-terminal region of the PTP by a tryptophan-containing site in the cytoskeletal associated protein, PST PIP, which is divergent from the previously described SH3- and WW-type polyproline binding modules.
Because this protein-protein interaction appears to be required for the dephosphorylation of PST PIP phosphotyrosines (20), it may be a potentially important new mechanism for the regulation of the cytoskeleton.

The mechanisms utilized by both SH3 and WW domains in recognizing proline-rich helices have been elucidated through structure-function analyses using x-ray crystallography, NMR, and site-directed mutagenesis. The SH3 domain consists of a highly structured module that is 60 amino acids long and appears to fold properly when expressed in the absence of other protein domains; this short motif is capable of binding to proline-rich peptides with relatively high affinity (7–9). The WW domain is also a relatively small (38 amino acids), highly structured motif that is capable of forming an active protein when expressed in the absence of other modules (12). This is in contrast to the polyproline recognition sequence found in PST PIP. In this case, deletion of the NH2-terminal 50 amino acids of the protein resulted in an apparently misfolded molecule that did not bind to the COOH-terminal proline-rich domain of PTP HSCF. These data are consistent with the possibility that this type of polyproline recognition domain may require a greater complexity of interactions than either the SH3 or WW

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**FIG. 6. In vitro and in vivo analysis of mutations of the COOH terminus of PTP HSCF.** Panel A, PTP HSCF constructs containing alanine substitutions at the indicated positions were transcribed and translated in vitro, and the resultant mutant proteins were precipitated (IP) with either 1 (top panel) or 10 (second panel) µg/ml GST-PST PIP. The third panel illustrates immunoprecipitations of the in vitro transcribed and translated PTP HSCF mutants with a monoclonal antibody directed against an NH2-terminal HA tag to ensure that all of the mutants were produced equally. Multiple bands precipitated with the GST-PST PIP fusion protein are apparently COOH-terminal proteolyzed products of the PTP (20). Also shown are GST-PST PIP precipitations (10 µg/ml) performed on a double mutant of PTP HSCF (Arg + Trp) with both residues Arg444 and Trp450 mutated to alanine (bottom panel). Panel B, COS cells were cotransfected with a 10:1 ratio of plasmids encoding HA-tagged PTP HSCF with the illustrated alanine substitutions or a mutant of PTP HSCF deleted for the COOH-terminal 24 amino acids (PTP HSCF Asp24) and wild-type PST PIP with a COOH-terminal FLAG epitope tag, respectively. Transfected cell lysates were immunoprecipitated with anti-HA monoclonal antibodies, and the precipitates were blot with anti-PST PIP polyclonal antibody to detect the relative amounts of PST PIP complexed with either wild-type or different mutant forms of PTP HSCF. Lysates were also immunoprecipitated with anti-HA antibody and blotted with the same antibody to ensure equal expression of PTP HSCF. Equal expression of PST PIP was determined by immunoprecipitating lysates with anti-FLAG monoclonal antibody and blotting with anti-PST PIP polyclonal antibody. Note the complete loss of coprecipitation in both the PTP HSCF Asp24 and PTP HSCF444 + 450 mutants.
modules. The importance of overall structure to ligand recognition by the WW domain is emphasized by mutation of the proline that is COOH-terminal to the critical tryptophan recognition residue (14). Mutation of this residue, which is conserved in all WW motifs, to alanine results in an inactive WW module, presumably because of a disruption in the fold of the domain. The PST PIP polyproline recognition sequence is missing this highly conserved proline (20), consistent with the possibility that other residues in the protein may be involved with the formation of the ligand binding site. Of potential importance is the finding that the region containing the polyproline recognition sequence in PST PIP is in a domain that is predicted to form a coiled-coil (31), and preliminary data suggest that this area of PST PIP mediates dimerization, a characteristic of coiled-coil-containing proteins. This, together with the results of the NH2-terminal deletion studies, suggests that the overall fold of this relatively extended domain might be critical for the formation of a correctly structured polyproline recognition site.

Although these results suggest that the PST PIP polyproline recognition domain is functionally and structurally divergent from the SH3 and WW modules, an interesting connection between these binding motifs is the inclusion of a critical tryptophan residue in all three domains. In both SH3 and WW motifs, these tryptophans are conserved in all of the modules that have thus far been identified. In the case of both the SH3 (10) and WW (14) motifs, the tryptophan appears to be critical for the interaction with the proline-rich peptide, as mutation of this residue results in diminished binding. Interestingly, this is also the case for the PST PIP proline-rich recognition site, consistent with the possibility that tryptophan residues are uniquely suited for the recognition of polyproline-rich domains. Structural data from both SH3 and WW domains confirm this hypothesis. In the case of the SH3 domain, the conserved tryptophan residue is found in the binding pocket, and this residue appears to interact by stacking with helically oriented tryptophans in the proline-rich ligand (8–10). NMR analysis of the WW domain from the Yes kinase-associated protein (32) likewise reveals an interaction between the conserved tryptophan residue and prolines in the proline helix recognized by this protein, although it is also possible that this conserved tryptophan is involved with the structure of the binding pocket (12, 14). The fact that mutation of a single tryptophan in the coiled-coil region of PST PIP abolishes both in vitro and in vivo binding to the phosphatase is consistent with the hypothesis that this tryptophan residue may interact similarly with potentially helically oriented prolines in the COOH terminus of the PEST PTPs. Alternatively, it is possible that conversion of this hydrophobic residue to alanine results in a misfolding of the protein. However, if the W232A mutant protein is folded improperly, it is likely that this is only a localized disruption, since it is still capable of associating with the cytoskeleton and is tyrosine phosphorylated in the presence of transfected v-Src or the PTP inhibitor, pervanadate. Interestingly, the tryptophan located NH2-terminal to the critical tryptophan involved with binding of PST PIP to PTP HSCF does not seem to be required for ligand recognition, a result similar to that found for the NH2-terminal tryptophan of the WW domain in Yes kinase-associated protein (14). Finally, although it appears that a number of other residues, particularly with hydrophobic and aromatic side chains, are involved with the recognition of the proline-rich ligand by both SH3 (8–10) and WW (12, 14) domains, mutation of two such residues in PST PIP (PHE221 and Leu224) does not have a significant effect on binding, consistent with the supposition that the polyproline recognition domain of PST PIP is divergent from the WW module.

Mutational analysis of the proline-rich domain of PTP HSCF is also compatible with the proposal that the PST PIP binding site is a novel polyproline recognition module. These data demonstrated that the binding site in the phosphatase appeared to stretch over a length of approximately 15 amino acids, from Arg436 to Trp450. This is in contrast to structural studies on SH3 and WW domain recognition sites, where mutagenesis, x-ray crystallography, and NMR analyses have demonstrated that stretches of 10–12 (8–10) or 6 (12, 14) residues, respectively, are required for the highest affinity interaction. In addition, although the PST PIP polyproline recognition domain appears most like the WW module in that it contains two relatively closely spaced tryptophans, the ligands recognized by the WW motif have been found to have the general structure X–X–X–X–X–X–X–X–X, with both prolines and the tyrosine performing critical recognition functions (5, 12, 13, 15). The PTP HSCF polyproline region contains two adjacent proline residues, one of which was found to be involved with binding, but there is not a tyrosine residue COOH-terminal to the second proline. Although these results suggest that the recognition of the PTP HSCF polyproline domain by PST PIP is quite different from the mechanisms utilized by SH3 and WW modules, a striking similarity is found in the involvement of closely spaced proline residues. Similar requirements have been found for the prolines in SH3 recognition sites (10), whereas mutation of these residues in WW recognition sites, although not quantitatively measured, also showed an effect on binding (14). Again, structural analysis of SH3 and WW modules bound to their cognate ligands illuminates the role of these prolines in binding. In the case of both of these motifs, the ligand adopts a type II polyproline helical conformation that allows for interactions between residues within the helical region and conserved side chains within the recognition modules (7, 10). Because mutation of the prolines in the PST PIP COOH-terminal region resulted in an effect on

\footnotesize{\textsuperscript{4} Y. Wu, S. Spencer, and L. Lasky, unpublished observations.}

\footnotesize{\textsuperscript{5} S. Spencer and L. Lasky, unpublished data.}
binding, it is likely that this region may also form a type II proline helix that disposes the relevant side chains in the appropriate conformation. In addition, and in contrast to the SH3 and WW recognition motifs, the glycine contained within this region also appears to be involved with binding to PST PIP. Because glycine residues are also mediators of peptide structure, it is possible that this residue may serve to fold this small region into an appropriate conformation, and it may be this high concentration of structure-inducing residues which allows this diminutive peptide to bind to PST PIP so efficiently (20). Significantly, mutagenesis studies have also revealed the importance of non-proline residues in the binding of both SH3 and WW motifs to polyproline ligands. In the case of the WW domain, the conserved tyrosine residue of the ligand is important for the interaction and makes a direct contact with the binding module (12), whereas amino acids in the NH2- or COOH-terminal regions of the SH3 recognition site can determine the orientation and affinity of binding of the peptide ligands (7). Because mutation of the arginines contained within the PTP HSCF COOH terminus had an effect on binding, it is possible that electrostatic interactions are involved with the binding event, as has been observed for SH3 recognition modules (7). The importance of the COOH-terminal tryptophan suggests the possibility of hydrophobic stacking interactions, perhaps with the important tryptophan residue in PST PIP. Finally, because the residues involved with binding are highly conserved (27), these data are completely consistent with previous studies demonstrating that COOH-terminal proline-rich peptides derived from the COOH termini of the related PTPs PEST, PEP (20), and BDP-1,4 effectively block the interaction between PTP HSCF and PST PIP.

The potential importance of Trp232 in the function of PST PIP is underlined by the finding that expression of the W232A mutant in COS cells together with the v-Src tyrosine kinase results in an enhanced tyrosine phosphorylation of the cytoskeletal associated protein. These data are consistent with the hypothesis that PST PIP interacts with endogenous PTPs in vivo, and this interaction mediates the removal of phosphates from tyrosine residues. Furthermore, because this mutation blocks the binding of the PEST-type PTP HSCF via the COOH-terminal proline-rich domain, these results suggest that it is probable that PST PIP interacts with one or more endogenous PEST-type tyrosine phosphatases in COS cells. However, the question remains as to why the W232A mutant is not constitutively tyrosine phosphorylated in the absence of v-Src, since it is likely that the protein is unable to bind endogenous PEST-type PTPs efficiently. Although it might be argued that the appropriate tyrosine kinase is not present in COS cells, we have demonstrated previously that the protein is tyrosine phosphorylated in the presence of vanadate, both in its endogenous state in BaF3 cells as well as when it is transfected into COS cells (20). A likely explanation for these data is that the kinase that phosphorylates PST PIP requires an activation event, such as tyrosine phosphorylation, to mediate this modification. Thus, v-Src, which is a constitutively activated tyrosine kinase (33), would be predicted to mediate the tyrosine phosphorylation of the W232A mutant in the absence of vanadate. In addition, the data suggest that vanadate must activate an endogenous tyrosine kinase(s), presumably by inhibiting an endogenous tyrosine phosphatase (22), which subsequently mediates the tyrosine phosphorylation of PST PIP. An understanding of the actual mechanism by which this activation event occurs, together with the nature of the tyrosine kinase(s) that mediate PST PIP tyrosine phosphorylation, should provide interesting insights into the function(s) of this cytoskeletal associated protein.

In conclusion, the data reported in this paper describe a novel protein-protein recognition system that is involved with the control of the tyrosine phosphorylation of a cytoskeletal associated protein. Further work will be directed toward refining the nature of this interaction, including a more detailed analysis of the PTP PIP residues involved with recognition of the proline-rich COOH terminus of the PTP as well as a structural elucidation of the complex. In addition, because PST PIP may be involved with the regulation of cytokinesis, an understanding of this interaction may allow for the development of peptidomimetic inhibitors of the interaction between the cytoskeletal protein and phosphatase which might prove useful for the development of drugs effective against rapidly dividing, transformed cells.

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