PSTPIP 2, a Second Tyrosine Phosphorylated, Cytoskeletal-associated Protein That Binds a PEST-type Protein-tyrosine Phosphatase*

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Although cytoskeletal regulation is critical to cell function during interphase and mitosis, the components of the cytoskeleton involved with its control are only beginning to be elucidated. Recently, we reported the identification of a cytoskeletal-associated protein, proline-serine-threonine phosphatase-interacting protein (PSTPIP), whose level of tyrosine phosphorylation was controlled by PEST-type protein-tyrosine phosphatases (PTPs) bound to a novel protein interaction site in the PSTPIP predicted coiled-coil domain. We also showed that the PSTPIP SH3 domain interacts with the Wiskott-Aldrich syndrome protein (WASP), a cytoskeletal regulatory protein, in a manner modulated by tyrosine phosphorylation. Here we describe the identification of PSTPIP 2, a widely expressed protein that is related to PSTPIP. PSTPIP 2 lacks an SH3 domain but contains a region predicted to bind to PEST-type PTPs, and structure-function analyses demonstrate that PSTPIP 2 interacts with the proline-rich C terminus of the PEST-type PTP hematopoietic stem cell factor in a manner similar to that previously demonstrated for PSTPIP. Confocal microscopy revealed that PSTPIP 2 colocalizes with PSTPIP in F actin-rich regions. PSTPIP 2 was found to be efficiently phosphorylated in v-Src-transfected or pervanadate-treated cells at two tyrosines conserved in PSTPIP, but in contrast to PSTPIP, tyrosine phosphorylated PSTPIP 2 was only weakly dephosphorylated in the presence of PTP HSCF. Finally, analysis of oligomer formation demonstrated that PSTPIP and PSTPIP 2 formed homo- but not heterodimers. These data suggest that a family of tyrosine phosphorylated, PEST PTP binding proteins may be implicated in cytoskeletal regulation.

The actin cytoskeleton is critically involved with a diversity of cell functions including cell morphology, motility, and cytokinesis (1). Although a number of structural and regulatory components of the cytoskeleton have been identified in both yeast and higher eukaryotic systems, the mechanisms by which the cell regulates the shape changes that occur during physiological responses remain to be determined. Of particular interest are the possible roles that tyrosine kinases and phosphatases may play in cytoskeletal regulation. Currently, there appears to be evidence for the involvement of at least three tyrosine kinases in cytoskeletal control. Early results suggested that Src family members regulated actin assembly and cell shape (2–10), and more recent studies have demonstrated that v-Src tyrosine kinase induces the tyrosine phosphorylation of a diversity of cytoskeletal-associated proteins including contactin, talin, paxillin, p130

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1 The abbreviations used are: PTP, protein-tyrosine phosphatase; HSCF, hematopoietic stem cell fraction; GST, glutathione S-transferase; HA, hemagglutinin.
The tyrosine phosphorylation states of Src and p125^Fak^ tyrosine kinases appeared to be modulated by PTPs in response to extracellular matrix binding to integrin receptors, again consistent with a role for these enzymes in cytoskeletal regulation (35, 37). The most convincing data regarding the participation of a known PTP in the regulation of a cytoskeletal-associated protein are found in the case of the regulation of p130^Cas^ tyrosine phosphorylation by PTP PEST (38, 39). As described above, p130^Cas^, which is a focal adhesion-associated protein containing an SH3 domain and a number of phosphotyrosines capable of potentially interacting with SH2 domains, is phosphorylated in response to integrin-mediated adhesion and in v-Src expressing cells. Analysis of substrates for PTP PEST using “substrate trap” catalytic domain mutants (40) demonstrated that p130^Cas^ was indeed a substrate for the tyrosine phosphatase activity of this PTP (38). The recognition of this substrate by PTP PEST appeared due to a combination of direct substrate binding to the catalytic domain as well as an interaction between the p130^Cas^ SH3 region and a proline-rich site in the phosphatase (39). Although the functional significance of p130^Cas^ dephosphorylation by PTP PEST remains to be determined, it is likely that the removal of phosphates results in the termination of downstream signaling events, some of which are likely to regulate the cytoskeleton (27).

Recently, we demonstrated yet another potential role for PEST-type PTPs in cytoskeletal control. In these studies, we showed that PTP HSCF, a PEST-type PTP expressed in hematopoietic progenitor cells, bound to a novel SH3 domain containing protein, termed PTPIP (proline-serine-threonine phosphatase-interacting protein) that is homologous to CDC15p, an Schizosaccharomyces pombe protein required for assembly of the cytokinetic cleavage furrow (41). PTPIP was tyrosine phosphorylated in pervanadate-treated or v-Src-transfected cells, and PTP HSCF dephosphorylated these tyrosines only when the two proteins interacted with each other through the C-terminal proline-rich domain of the PTP. Confocal microscopy revealed that PTPIP was localized to the cortical cytoskeleton, lamellipodia, and cytokinetic cleavage furrow, and overexpression of mammalian PTPIP in yeast S. pombe resulted in a dominant negative inhibition of cytokinesis. Analysis of the binding interaction between PTP HSCF and PTPIP suggested that the C-terminal proline-rich domain conserved in all PEST-type PTPs mediated binding to a tryptophan-containing site in the PTPIP potential coiled-coil domain, consistent with the proposal that PTPIP tyrosine phosphorylation is generally regulated by diverse PEST-type PTPs (42). The potential importance of PTPIP tyrosine phosphorylation was highlighted by studies aimed at determining the function of the SH3 domain of this protein (43). This work revealed that the SH3 domain of PTPIP interacted with two proline-rich regions in the Wiskott-Aldrich syndrome protein (WASP), an X-linked, cdc42 GTPase regulated cytoskeletal-associated protein whose mutation in humans results in immunodeficiency and thrombocytopenia by causing defects in the structure of the cortical actin cytoskeleton (44, 45). In addition, mutation of Bee-1, an Saccharomyces cerevisiae WASP homologue, resulted in defects in the cortical cytoskeleton and cytokinesis, consistent with roles for both the mammalian and yeast proteins in actin regulation (46). Analysis of the phosphorylated tyrosines in PTPIP demonstrated that one of two modified residues was found within the polyproline binding pocket of the SH3 domain (43). Mutation of this tyrosine to acidic residues (either glutamate or aspartate) to mimic the incorporation of a negatively charged phosphate resulted in an almost complete loss of WASP binding. These data suggested that tyrosine phosphorylation of PTPIP modulates its interaction with WASP and that one function of the bound PTP was to maintain PTPIP in a dephosphorylated and, hence, WASP-associated, state.

PTPIP is a tyrosine phosphorylated, cytoskeletal-associated protein that may be involved with the regulation of cytokinesis and the cortical cytoskeleton through its SH3-induced interactions with WASP. Here we describe a widely expressed, PTPIP-related protein, termed PTPIP 2, that is tyrosine phosphorylated but that lacks an SH3 domain. PTPIP 2 interacts with PTP HSCF in a manner reminiscent of PTPIP, but it is only weakly dephosphorylated by bound PTP HSCF. Both PTPIP and PTPIP 2 homo-oligomerize, and PTPIP 2, like PTPIP, is associated with the actin cytoskeleton. These data suggest that PEST-type PTPs interact with at least two tyrosine phosphorylated cytoskeletal-associated proteins, and these interactions may regulate diverse cytoskeletal functions.

MATERIALS AND METHODS
Cloning of a cDNA Encoding PTPIP 2—Small regions of homology with the PTPIP (protein tyrosine phosphatase-interacting protein) were identified by Blast scanning of the public data bases. Nucleotide probes derived from these homologous regions were used to screen several libraries using standard procedures. Full-length cDNA clones were isolated and sequenced using standard procedures.

Production of Polyclonal Antibodies—GST fusion proteins were constructed as described previously (41–43). These proteins were used to immunize rabbits, and the resultant antibodies were purified by affinity chromatography using the original GST fusion proteins as affinity matrices.

Coprecipitation Analyses—Coprecipitation was done as described previously (41–43). Briefly, nontransfected BaF3 or transfected COS cells were lysed in Nonidet P-40 buffer, and the resultant lysates were immunoprecipitated either with affinity purified polyclonal antibodies (BaF3) or anti-epitope tag monoclonal antibodies (transfected cells). The resultant precipitates were electrophoresed on SDS-polyacrylamide gels, electroethoretically blotted, and probed with either polyclonal or monoclonal antibodies.

GST Precipitation Analyses—GST constructs containing either wild type or mutant PTPIP 2 were produced as described previously (41–43). In vitro translation and GST precipitation were performed as described previously (41, 42). GST precipitation of proteins from transfected cell lysates was performed as described previously (41–43).

Phosphotyrosine Analyses—PTPIP 2 tyrosine residues were mutated to phenylalanine using polymerase chain reaction mutagenesis as described previously (41–43). Tyrosine phosphorylation of wild type and mutant PTPIP 2 was determined by immunoprecipitation of Myc epitope-tagged proteins from lysates of cells that were either incubated with the tyrosine phosphatase inhibitor pervanadate or transfected with the constitutively activated v-Src tyrosine kinase. The resultant precipitated proteins were analyzed by Western blotting with anti-phosphotyrosine antibody.

Oligomerization Analyses—Myc or FLAG epitope-tagged forms of PTPIP and PTPIP 2 were transfected in various combinations into COS cells, and lysates were immunoprecipitated with antibodies against either the Myc or FLAG epitopes and Western blotted with either antibody as described previously (41–43).

Confocal Microscopy—A form of PTPIP with the green fluorescent protein at its N terminus was constructed by polymerase chain reaction. This form of the protein shows an identical cortical distribution with the FLAG epitope-tagged form. The protein and Myc epitope-tagged PTPIP 2 were cotransfected into Chinese hamster ovary cells, and the cells were analyzed by confocal microscopy as described previously (41–43). In addition, Myc epitope-tagged PTPIP 2 was transfected into COS cells, and the colocalization of this protein with F-actin was determined using fluorescein isothiocyanate-labeled phalloidin as described previously Myc epitope-tagged PTPIP 2.

RESULTS
Isolation and Characterization of a PTPIP-related Sequence—In silico scanning of the public data bases with the

2 S. Spencer and L. Lasky, unpublished observations.
PSTPIP protein (41) resulted in the identification of several related sequences that were utilized to isolate cDNAs encoding PSTPIP-related proteins from human and murine sources. Fig. 1A illustrates that the murine sequence (predicted molecular mass of 38,948 Da) was approximately 41% similar to PSTPIP throughout the potential coiled-coil region of the protein, but the related protein lacked the SH3 domain that was previously shown to interact with the WASP (43). Analysis of two different human clones revealed approximately 41% similarity with PSTPIP and a much higher level of conservation with the murine PSTPIP-related sequence (87% sequence similarity). Comparison of the two human clones with PSTPIP and the murine PSTPIP-related sequence revealed that one human clone, 2A (predicted molecular mass of 36,097 Da), spliced out PSTPIP 2-Phosphatase Interactions 30489

![Sequence comparisons of various PSTPPIP](image)

**A** illustrates the sequences for PSTPIP (41), murine PSTPIP 2 (mupip2), and two alternately spliced forms of human PSTPIP 2 (hupip 2a and hupip 2b). The asterisk marks a tryptophan residue in PSTPIP that was previously shown to be critical for binding to PEST-type tyrosine phosphatases (42). The plus signs show two PSTPIP tyrosines previously shown to be phosphorylated in pervanadate-treated and v-Src-transfected cells (43).

**B** shows schematic structures of various PSTPPIP. SH3 corresponds to the PSTPIP SH3 domain, which has been found to interact with the WASP cytoskeletal regulatory protein. The potential coiled-coil domains as predicted by the coil program (see Fig. 7A) are also shown. Finally, the tryptophan residue (Trp-232 or Trp-206) found to be essential for PEST-type PTP binding is also illustrated.

**C** shows Northern blot analysis of murine PSTPIP 2 expression. Lane a, heart; lane b, brain; lane c, spleen; lane d, lung; lane e, liver; lane f, muscle; lane g, kidney; lane h, testis.

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26 residues near the N terminus, whereas a second clone, 2B (predicted molecular mass of 34,948 Da), spliced out 33 amino acids (Fig. 1B). Interestingly, the region spliced out from clone 2B contains a tryptophan residue (Trp-232 in the murine clone and Trp-206 in the human clone) that was previously shown to be critical for interaction with the PEST-type tyrosine phosphatase PTP HSCF and, likely, other PEST PTPs (see below) (42).

Examination of the region surrounding this tryptophan (murine residues 228–253) shows a high degree of conservation between PSTPIP and the murine and human PSTPIP-related proteins. Analysis of the two tyrosines (Tyr-344 and Tyr-367) in PSTPIP that were previously shown to be phosphorylated in v-Src-transfected or vanadate-treated cells showed that they were both conserved in the murine and human PSTPIP-related proteins (Fig. 1A) (43). Finally, two consensus SH3 binding sites (PXXP, murine PSTPIP residues 278–281 and 323–326) are conserved in all of these sequences (47). These data suggest that the murine and human clones encode PSTPIP-related proteins, and the molecules will hereafter be referred to as PSTPIP 2.

Previous Northern blot analysis demonstrated that PSTPIP 2 was expressed at relatively low levels in a restricted set of tissues including brain, spleen, lung, and testis (41). Fig. 1C illustrates that PSTPIP 2 is more broadly expressed in every tissue examined. In addition, it is likely that PSTPIP 2 is expressed at significantly higher levels than PSTPIP, because the blot shown here was exposed for a few hours versus the previously published Northern blot of PSTPIP expression, which was exposed for approximately 1 week.

**PSTPIP 2 Interacts with PTP HSCF—**Affinity-purified polyclonal antibodies against murine PSTPIP 2 and PTP HSCF (48) were produced against GST fusion proteins. These antibodies efficiently precipitated PSTPIP 2 and PTP HSCF from murine BaF3 hematopoietic progenitor cells (data not shown). To examine the interaction between endogenous PSTPIP 2 and PTP HSCF, coprecipitation studies were performed. Fig. 2 illustrates that anti-PSTPIP 2 antibodies efficiently coprecipitated PTP HSCF from BaF3 cells, consistent with an *in vivo* interaction between these proteins. Interestingly, although antibodies to PTP HSCF efficiently precipitated the phosphatase, the immunoprecipitate did not contain PSTPIP 2. Because the anti-PTP HSCF antibodies are directed against the C-terminal region of the PTP (48), including the C-terminal site that binds to PSTPIP 2 (see below), it is possible that the polyclonal antibodies disrupt the interaction between these two proteins (42). In summary, these data are consistent with an interaction between endogenous PTP HSCF and PSTPIP 2 *in vivo*.

Previous studies examined the interaction between PTP HSCF and PSTPIP in great detail (41, 42). These data demonstrated a high affinity interaction between the potential coiled-coil domain of PSTPIP and the 20-amino acid-long C-terminal proline-rich domain of all three PEST-type PTPs. In addition, a tryptophan residue at position 232 of PSTPIP was found to be critical for PTP HSCF binding, and an arginine (position 444) and a tryptophan (position 450) in the C-terminal proline-rich region of the phosphatase were also required for binding (42). To examine the mode of interaction between PSTPIP 2 and PTP HSCF, *in vitro* and *in vivo* interaction analyses were performed. Fig. 3 illustrates, using the previously described *in vitro* binding assay, that PSTPIP 2 interacts with PTP HSCF in a manner that is indistinguishable from PSTPIP 2 (42). For example, mutation of the tryptophan residue at position 232 in PSTPIP 2-Phosphatase Interactions

**FIG. 2.** Coprecipitation of endogenous PTP HSCF and PSTPIP 2 from BaF3 cells. BaF3 cell lysates from 5 × 10⁶ cells were immunoprecipitated (*IP*) with polyclonal antibody directed against either PSTPIP 2 (*α PIP-2*) or PTP HSCF (*α PTP HSCF*), and the immunoprecipitates were Western blotted with either antibody. As can be seen, α PIP-2 coprecipitates PSTPIP 2 as well as PTP HSCF. Antibody against PSTPIP 2 only precipitates PTP HSCF, possibly due to blocking of the phosphatase binding site. No reactivity was observed with preimmune serum.

**FIG. 3.** *In vitro* analysis of the interaction between PTP HSCF and PSTPIP 2. A, PSTPIP 2 (*PIP-2*), PSTPIP 2 (*PIP*), or proteins containing alanine substitutions at tryptophan residue 232 (PIP-2 W232-A or PIP W232-A) were translated *in vitro* with [35S]methionine and precipitated either with polyclonal antibodies (*α PIP-2* or *α PIP*) or with a GST fusion protein containing amino acids 305–453 including the C-terminal proline-rich binding site of PTP HSCF (GST-PST). Precipitated proteins were electrophoresed and analyzed on a FujiX™ scanner. B, *in vitro* translated PTP HSCF was precipitated with either polyclonal antibodies (*α PTP HSCF*) or GST fusion proteins containing the coiled-coil domains of PSTPIP 2 or PSTPIP 2 (GST-PST 2 or GST-PST) in the presence of no peptide (O) or 1 micromolar 20-mer peptides derived from the C-terminal proline-rich regions of the PEST-type PTPs HSCF (GFNLRIRPKGPGRDPP) or PTP HSCF (GFGNRCGKPKGPRDPP) and PEST (GFGNRCGKPKGPRDPP) or PSTPIP (PSTPIP 2 or PSTPIP 2) (*α PTP*). C, PTP HSCF with alanine substitutions at arginine residue 444 and tryptophan residue 450 was translated *in vitro* and immunoprecipitated with either polyclonal antibody (*α PTP HSCF*) or GST fusions derived from PSTPIP 2 or PSTPIP 2 (GST-PST 2 or GST-PST). *IP*, immunoprecipitation.
PSTPIP 2-Phosphatase Interactions

30491

**Fig. 4.** In vivo analysis of the interaction between PTP HSCF and PSTPIP 2. The top panel illustrates cotransfection analyses using Myc epitope-tagged PSTPIP 2 (PIP-2-MYC) and hemagglutinin epitope-tagged PTP HSCF (HSCF-HA). PIP-2 mutated to alanine at tryptophan residue 232 (PIP-2 W232-MYC) and PTP HSCF with the C-terminal proline-rich binding site deleted (HSCFΔpro-HA) were also tested. Transfected COS cell lysates were immunoprecipitated (IP) with antibody to Myc (α MYC) or hemagglutinin (α HA) and blots were probed with antibody to Myc. As can be seen, deletion of the C-terminal binding site or mutation of tryptophan 232 negatively affects the interaction between PTP HSCF and PSTPIP 2. The lower panel illustrates the effects of mutating the PTP HSCF C-terminal binding site residues arginine 444 and tryptophan 450 to alanine. As can be seen, mutation of the C-terminal tryptophan 450 to alanine has a partial inhibitory effect on binding, whereas mutation of both residues essentially abolishes binding.

Both PSTPIP and PSTPIP 2 results in a loss of binding to a GST fusion protein containing the C-terminal proline-rich binding domain of PTP HSCF. In addition, peptides derived from the C-terminal domain in PTP HSCF, as well as PTP PEP and PTP PEST, efficiently block the interaction between in vitro translated PTP HSCF and GST fusion proteins containing either the potential coiled-coil domain of PSTPIP or the entire PSTPIP 2 protein (Fig. 3B). These peptide blocking data suggest that all three PEST-type PTPs are likely to interact with PSTPIP 2 via their proline-rich C terminal. Finally, PTP HSCF with alanine substitutions in the critical arginine (position 444) and tryptophan (position 450) residues are unable to bind to GST fusion proteins containing PSTPIP or PSTPIP 2. These data suggest that the high degree of conservation surrounding tryptophan 232 is likely to be of functional importance to the binding of PTP HSCF by PSTPIP or PSTPIP 2 (42).

To examine the interactions between PSTPIP 2 and PTP HSCF under more physiological conditions, transfection studies were performed. Fig. 4 illustrates that although wild type PSTPIP is coprecipitated with HA-tagged PTP HSCF, mutation of the tryptophan residue at position 232 substantially decreases the interaction between the two proteins. Previously, we demonstrated that a form of PTP HSCF missing the C-terminal 20-amino acid proline-rich domain did not interact with PSTPIP (41), and cotransfection of this deleted form of the phosphatase with wild type PSTPIP 2 resulted in a lack of co-precipitation (also see below). In support of the importance of the proline-rich C terminus of the PTP, mutation of arginine residue 444 and tryptophan residue 450 together resulted in a clear loss of PSTPIP 2 coprecipitation. Together with the in vitro data, these in vivo studies support the conclusion that PSTPIP 2 interacts with PEST-type PTPs in a manner that is indistinguishable from PSTPIP 2 (41, 42).

**Tyrosine Phosphorylation of PSTPIP 2—**Previously, we demonstrated that PSTPIP was tyrosine phosphorylated in cells incubated with the pan-tyrosine phosphatase inhibitor, pervanadate, as well as in cells transfected with a plasmid encoding v-Src (41). In addition, we demonstrated that two tyrosines, at positions 344 and 367, were phosphorylated in both circumstances, and phosphorylation of residue 344 was required for phosphorylation of residue 367 (43). To analyze the tyrosine phosphorylation of PSTPIP 2, Myc-tagged protein was produced in cells incubated with pervanadate or co-transfected with v-Src. As Fig. 5 illustrates, PSTPIP 2 was tyrosine phosphorylated under both conditions. Comparison of the levels of tyrosine phosphorylation of PSTPIP 2 versus PSTPIP revealed that PSTPIP 2 was a significantly better (at least 5-fold higher levels of Tyr(P) on a molar basis) substrate for v-Src-induced phosphorylation (data not shown). To examine the possible phosphorylation of the two conserved PSTPIP 2 tyrosines found to be phosphorylated in PSTPIP, these residues in PSTPIP 2 were mutated to phenylalanine, expressed in cells treated with pervanadate or expressing v-Src, and analyzed by blotting with anti-phosphotyrosine antibody. Fig. 5 shows that mutation of PSTPIP 2 tyrosine residue 323 (analogous to residue 344 in PSTPIP) resulted in a small but significant decrease in phosphotyrosine levels, particularly in the pervanadate-treated samples. This result is in contrast to data obtained with PSTPIP, where mutation of this tyrosine resulted in a complete lack of tyrosine phosphorylation (43). Mutation of PSTPIP 2 tyrosine residue 333 (analogous to residue 367 in PSTPIP) appeared to give no significant decrease in phosphotyrosine levels. However, mutation of both PSTPIP 2 tyrosine residues to phenylalanine resulted in an almost complete loss of tyrosine phosphorylation. While differing somewhat from the previous results with PSTPIP, these data suggest that tyrosine residues 323 and 333 of PSTPIP 2 are both phosphorylated under pervanadate-treated and v-Src-transfected conditions.

Previous data demonstrated that PSTPIP was a substrate for dephosphorylation by the tyrosine phosphatase activity of PTP HSCF (41). In addition, we showed that this activity was dependent upon a physical interaction between the PTP and the PSTPIP substrate, because forms of the PTP lacking the C-terminal binding site did not modulate PSTPIP tyrosine...
phosphorylation. Because pervanadate incubation results in a high level of PTP HSCF 2 tyrosine phosphorylation (Fig. 5), it is likely that an endogenous PTP mediates the constitutive dephosphorylation of PTP HSCF 2 in vivo. To examine the potential role of PTP HSCF in PTP HSCF 2 tyrosine dephosphorylation, cotransfection studies were performed. Fig. 6 illustrates that cotransfection of wild type PTP HSCF resulted in only a minor reduction in the phosphotyrosine levels of PTP HSCF 2. This result is consistent with the suggestion that PTP HSCF 2 phosphotyrosines are not substrates for dephosphorylation by PTP HSCF. We also utilized substrate trapping mutants of PTP HSCF to examine whether PTP HSCF was a substrate for phosphatase-mediated dephosphorylation (38, 40, 41). In previous studies, mutation of two catalytically important residues (a cysteine or an aspartate) in the PTP resulted in the hyperphosphorylation of PTP HSCF by the substrate trapping effect (41, 42). In addition, we found that the PTP itself was hyperphosphorylated as well, suggesting that the enzyme regulated its own phosphotyrosine levels (41). In contrast to those studies, Fig. 6 shows that cotransfection of either the cysteine or aspartate mutated forms of PTP HSCF did not result in hyperphosphorylation of PTP HSCF 2. The evidence that substrate trapping did occur is shown by the finding that hyperphosphorylated PTP HSCF was found to coprecipitate with PTP HSCF 2, but only when the phosphatase contained the C-terminal PTP HSCF 2 binding domains (Fig. 6). Together with the wild type PTP dephosphorylation results, these data are inconsistent with a role for PTP HSCF in the regulation of PTP HSCF 2 tyrosine phosphorylation.

Oligomerization of PTP HSCF and PTP HSCF 2—Analysis of the PTP HSCF protein for potential coiled-coil regions using the coil or paircoil programs resulted in the identification of a domain between residues 160 and 210 with a high probability and a region between residues 78 and 130 with a much weaker propensity to form these structures (Fig. 7A) (49, 50). A similar analysis of PTP HSCF 2 revealed a region between residues 65 and 95 with a moderate propensity for forming coiled-coils. Because many coiled-coil-containing proteins, including cytoskeletal-associated proteins such as myosin, oligomerize, we tested the ability of PTP HSCF and PTP HSCF 2 to form homo- and hetero-oligomers in a coprecipitation assay. As Fig. 7B shows, PTP HSCF and PTP HSCF 2 are both able to form homo-oligomers, but they are incapable of forming hetero-oligomers. These data suggest that the potential coiled-coil domains of both proteins are likely involved with oligomerization.

Subcellular Localization of PTP HSCF 2—Previously, we demonstrated that PTP HSCF was preferentially localized to actin-rich regions of the cell including the cortex and lamellipodia during interphase and the cleavage furrow during cytokinesis (41–43). To examine the cellular localization of PTP HSCF 2, we transfected a Myc-tagged version of the protein either alone or together with a form of PTP HSCF containing the green fluorescent protein at its N terminus (GFP-PTP HSCF). Examination of Chinese hamster ovary cells transfected with Myc-PTP HSCF 2 by anti-Myc and fluorescein isothiocyanate-labeled phallolidin revealed that PTP HSCF 2 colocalized with actin in the cortical and lamellipodial regions of the cell. However, the degree of actin colocalization, particularly in the lamellipodia, was not as extensive as that previously observed for PTP HSCF (Fig. 8). Observation of transfected cytokinesing cells (Fig. 8) demonstrated that, in contrast to PTP HSCF, PTP HSCF 2 did not appear to localize preferentially to the actin-rich cleavage furrow (41). Instead, PTP HSCF 2 appeared to remain associated with the cell cortex during cytokinesis. Analysis of cells cotransfected with Myc-PTP HSCF 2 and GFP-PTP HSCF showed two results. In many cells, we observed colocalization of the two proteins at the cell cortex, consistent with the suggestion that both molecules are associated with the cortical cytoskeleton (Fig. 8). In transfected cells expressing high levels of both proteins, however, PTP HSCF and PTP HSCF 2 colocalized to an extensive meshwork-like structure throughout the cell cortex and cytoplasm. A similar type of structure was previously observed when PTP HSCF was cotransfected with a GFP-WASP protein (43). The nature of this extensive network is not clear, but it appears that the PTP HSCFs may induce these structures upon cellular overexpression.

Competition for PTP HSCF Binding in Vivo—One possible function for PTP HSCF 2 is to compete for PTP PEST binding to PTP HSCF, with the possible outcome that PTP HSCF would be more readily tyrosine phosphorylated by, for example, activated Src family members. To test for this possible mechanism, the levels of PTP HSCF associated with PTP HSCF were examined in the presence of increasing amounts of PTP HSCF 2. As Fig. 9 shows, PTP HSCF 2 was able to efficiently compete with PTP HSCF for binding to PTP HSCF, so that at high concentrations of PTP HSCF 2, less phosphatase was bound to PTP HSCF. Because all three PEST-type PTPs bind to PTP HSCF in a similar manner, it is likely that this competitive binding phenomenon is applicable to these other phosphatases as well. These data suggest that the differential expression of both forms of PTP HSCF may modulate PEST-type PTP binding and resultant phosphotyrosine levels of each of these cytoskeletal-associated proteins.

DISCUSSION

The roles of protein phosphorylation in the regulation of the complex physiology of the cytoskeleton are only beginning to be deciphered. Although the function of serine-threonine phosphorylation in, for example, the regulation of myosin function has been well characterized (51–56), the relationship of tyrosine phosphorylation to cytoskeletal control is less well understood.
We previously described PSTPIP, an SH3 domain containing cytoskeletal-associated protein whose tyrosine phosphorylation was regulated by a physical interaction with PEST family tyrosine phosphatases (41, 42). The functional significance of PSTPIP tyrosine phosphorylation was highlighted by the finding that the SH3 motif of this protein interacted with WASP, a protein whose mutation in humans and yeast induces cytoskeletal defects, and this physical interaction appeared to be modulated by phosphorylation of a tyrosine within the SH3 polyproline ligand binding region (43). Here we describe a second type of PSTPIP, termed PSTPIP 2, which lacks an SH3 domain, is tyrosine phosphorylated, associates with the cytoskeleton, and binds to PEST-type PTPs in a manner similar to PSTPIP. The discovery that PSTPIP 2 interacts with the PEST-type PTP HSCF suggests a novel mechanism for the regulation of tyrosine phosphorylation of the WASP-binding PSTPIP as well as other potential PEST PTP substrates.

PSTPIP 2 strongly interacted with PTP HSCF in in vitro GST fusion interaction and endogenous and transfected cell coprecipitation studies. Although the overall homology between PSTPIP and PSTPIP 2 was relatively low (~41%), the region surrounding tryptophan residue 232 showed a significantly higher degree of conservation (~73% over 30 residues). Because tryptophan 232 was previously shown to be critical for binding of PSTPIP to the C-terminal proline-rich domain of PTP HSCF (42), this result suggests that the extended region of homology surrounding this tryptophan is involved in the interaction of these two proteins. This proposal is supported by the studies showing that mutation of this tryptophan in PSTPIP 2 results in a loss of binding to the PTP, and either deletion of the C-terminal proline-rich region of the PTP or mutation of two residues (arginine residue 444 and tryptophan residue 450) within this region previously shown to be critical for PSTPIP binding results in deleterious effects on PSTPIP 2 binding as well. Further support for the interaction of PSTPIP 2 with all three PEST-type PTPs came from peptide blocking studies as well as the absolute conservation of the critical arginine and tryptophan residues in all PEST-type PTPs (57, 58). Together, these data suggest that PSTPIP 2 interacts with PEST-type PTPs in a manner that is similar to PSTPIP. In addition, the high degree of conservation between murine PSTPIP and human PSTPIP 2A supports the contention that the human protein also interacts with PEST-type PTPs in a similar manner. Interestingly, a large part of this region, including the critical tryptophan residue, is spliced out of human PSTPIP clone 2B, suggesting that this form of the protein would be unable to interact with phosphatases. In view of the model described below, the physiological significance of this non-PTP binding form of PSTPIP 2 remains to be investigated. Finally, one of the most highly conserved regions between PSTPIP, PSTPIP 2, and the S. pombe cytokinesis regulatory protein, CDC 15p (59), is in close proximity to tryptophan residue 232, suggesting that the yeast protein may also bind to tyrosine phosphatases.

Previous studies showed that two tyrosine residues at positions 344 and 367 of PSTPIP were phosphorylated in cells either treated with the phosphatase inhibitor pervanadate or transfected with the constitutively activated tyrosine kinase, v-Src (43). PSTPIP 2 appears to behave in a similar manner, with some notable exceptions. For example, PSTPIP 2 is highly tyrosine phosphorylated in pervanadate-treated and v-Src transfected cells, predominately at two tyrosines that are analogous to those phosphorylated in PSTPIP. However, in contrast to PSTPIP, inhibition of PSTPIP 2 tyrosine phosphorylation at residue 323, which corresponds to PSTPIP residue 344, does not inhibit tyrosine phosphorylation at other sites. PSTPIP 2 tyrosine residue 323 (and residue 344 in PSTPIP) are consensus Src family member phosphorylation sites, and we previously proposed that phosphorylation at this residue in PSTPIP allowed for the binding, perhaps via an SH2 or PTB domain, of either a Src-type or other tyrosine kinase that subsequently phosphorylated PSTPIP at position 367 (41). In addition, mutation of PSTPIP 2 tyrosine residue 333 did not effect a decrease in phosphotyrosine levels, whereas mutation of both residues resulted in a substantial loss of phosphotyrosine, suggesting that phosphorylation at residue 333 may inhibit phosphorylation at residue 323. In the case of PSTPIP 2, these residues are only separated by 9 amino acids, whereas in PSTPIP they are 22 residues apart. Thus, although the induction of phosphorylation for both PSTPIP and PSTPIP 2 seems to be similar, the regulation of this event appears divergent. Finally, the function of PSTPIP tyrosine phosphorylation is, at least in
part, to regulate the association of the protein with WASP through its SH3 domain (43). Because PSTPIP 2 lacks an SH3 domain and, hence, also lacks the ability to bind WASP (43), it is likely that the tyrosine phosphorylation of this protein has other functions.

Analysis of the regulation of PSTPIP tyrosine phosphorylation demonstrated that catalytically active PTP HSCF dephosphorylated the protein (41). In addition, these data demonstrated that catalytically inactive forms of the PTP predicted to mediate hyperphosphorylation through the substrate trapping effect were indeed able to enhance PSTPIP tyrosine phosphorylation, but only if there was a physical interaction between the two proteins (41). The fact that both PSTPIP and PSTPIP 2 are maintained in a constitutively dephosphorylated form in vivo suggests that both proteins may be maintained in this state by associated PEST-type PTPs. However, the data reported here clearly demonstrate that wild type PTP HSCF mediates a minor decrease in PSTPIP 2 tyrosine phosphorylation induced by v-Src. In agreement with these data, the cotransfection of two different substrate trapping mutants (CS HSCF and DA HSCF) did not induce PSTPIP 2 hyperphosphorylation, despite the fact that hyperphosphorylated (i.e. substrate trapped) PTP HSCF was coprecipitated with PSTPIP 2. In addition, we have recently found that PSTPIP is hyperphosphorylated in cell lines derived from PTP PEST knockout mice, whereas PSTPIP 2 is not. These data suggest that another PTP may regulate the phosphotyrosine levels of PSTPIP 2.

Cellular localization studies demonstrated that PSTPIP 2 associated with the F actin-containing cortical cytoskeleton in a manner reminiscent of the localization previously described for PSTPIP (41–43). In addition, cotransfection experiments demonstrated that both proteins exactly colocalized with each other in the cell cortex. Together, these data imply that PSTPIP and PSTPIP 2 may functionally interact in a manner that involves the cortical cytoskeleton. However, the mechanism by which these two proteins interact with the cell cortex remains to be determined. Although the mechanism by which these two proteins interact with the cell cortex remains to be determined. Although the colocalization of both proteins with F actin suggests the possibility of a direct molecular interaction, previous analysis of PSTPIP demonstrated that it does not directly bind to actin. In addition, because both proteins colocalize with each other in the cell cortex, this cellular localization must be due to the coiled-coil region, because PSTPIP 2 lacks an SH3 domain, and we have found that a form of PSTPIP lacking the SH3 domain colocalizes with cortical actin.

3 A. Angers-Loustau, J. Cote, A. Charest, D. Dowbenko, S. Spencer, L. Lasky, and M. Tremblay, submitted for publication.
4 Y. Wu and L. Lasky, unpublished observations.
late actin polymerization (43, 45, 60), and the filamentous structure previously observed in cells overexpressing PSTPIP and WASP are consistent with this proposal. The lack of hetero-oligomer formation between PSTPIP and PSTPIP 2 supports the suggestion that there are functional differences between the proteins, such as WASP binding specificity. Because both PSTPIP and PSTPIP 2 associate with an unknown component of the cortical cytoskeleton through an interaction with the coiled-coil region, it is also possible that this oligomerization event cross-links this uncharacterized cytoskeletal molecule. Further analysis of the mechanisms of oligomerization as well as the cytoskeletal component that binds to the PSTPIP and PSTPIP 2 coiled-coil regions may further illuminate the function of these domains.

PSTPIP and PSTPIP 2 are coexpressed in at least one cell line, BaF3, and the widespread expression of PSTPIP 2 suggests that this protein may be produced by a diversity of cell types. Because both proteins colocalize to the cortical cytoskeleton and efficiently bind to PEST-type PTPs, it is therefore possible that one of the functions of PSTPIP 2 is to modulate the levels of interaction between PSTPIP and the phosphatases, and this type of competition has been demonstrated here (Fig. 9). Although both PSTPIP and PSTPIP 2 are tyrosine phosphorylated, a functionally significant tyrosine modification was revealed with the demonstration that the interaction between the cytoskeletal regulatory protein WASP and the PSTPIP SH3 domain was modulated by this tyrosine modification (43). Because it has been shown that the levels of PSTPIP SH3 domain tyrosine phosphorylation are regulated by bound PEST-type PTP (41), inhibition of the binding of this enzyme would be expected to result in enhanced phosphorylation upon, for example, activation of Src family tyrosine kinases. Thus, in cells with high levels of PSTPIP 2, the levels of PEST-type PTPs bound to PSTPIP would be lower, as shown in this paper, and the potential levels of tyrosine phosphorylation of PSTPIP would therefore be higher. This would allow for an increased level of control over the amounts of PSTPIP tyrosine phosphorylation and WASP binding. Although we have attempted to demonstrate this type of regulation in cotransfection studies, it has been difficult to reveal the effects of PSTPIP 2 overexpression on the phosphotyrosine levels of PSTPIP due to the higher level of v-Src-mediated tyrosine phosphorylation of PSTPIP 2 (data not shown). An alternative possibility is that the concentrations of PSTPIP 2 are modulated during the cell cycle so that they are high during periods when PSTPIP is tyrosine phosphorylated, such as, for example, during cleavage furrow formation, and low when the protein must be dephosphorylated. Interestingly, a form of PSTPIP 2 was isolated that lacked the proposed PTP binding domain. Although the relative levels of this form of the protein have not been determined, the existence of this potentially non-PTP binding form of the protein suggests that it may be involved in activities differing from the one proposed here. Finally, because it has been shown that PTP PEST regulates the phosphotyrosine levels of p130Cas via both substrate recognition and SH3 binding (38, 39), it is also possible that either PSTPIP or the apparently more highly expressed PSTPIP 2 may sequester this phosphatase away from this focal adhesion-associated substrate.

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