PTPH1 is a human protein-tyrosine phosphatase with homology to the band 4.1 superfamily of cytoskeletal-associated proteins. PTPH1 was found to associate with 14-3-3β using a yeast two-hybrid screen, and its interaction could be reconstituted in vitro using recombinant proteins. Examination of the interaction between 14-3-3β and various deletion mutants of PTPH1 by two-hybrid tests suggested that the integrity of the PTP is important for this binding. Although both PTPH1 and Raf-1 form complexes with 14-3-3β, they appear to do so independently. Binding of 14-3-3β to PTPH1 in vitro was abolished by pretreating PTPH1 with potato acid phosphatase and was greatly enhanced by pretreating with Cdc25C-associated protein kinase. Thus the association between PTPH1 and 14-3-3β is phosphorylation-dependent. Two novel motifs RSLS^359VE and RVDS^853EP in PTPH1 were identified as major 14-3-3β-binding sites, both of which are distinct from the consensus binding motif RSXKX^φ recently found in Raf-1. Mutation of Ser^359 and Ser^853 to alanine significantly reduced the association between 14-3-3β and PTPH1. Furthermore, association of PTPH1 with 14-3-3β was detected in several cell lines and was regulated in response to extracellular signals. These results raise the possibility that 14-3-3β may function as an adaptor molecule in the regulation of PTPH1 and may provide a link between serine/threonine and tyrosine phosphorylation-dependent signaling pathways.

Protein-tyrosine kinases and protein-tyrosine phosphatases (PTP) are the counterparts that orchestrate many aspects of cellular signaling events during such fundamental processes as cell growth and differentiation (1). PTPH1 (2) and PTPEGM1 (3) are the founding members of an expanding group of PTPs characterized by N-terminal segments of homology to cytoskeletal-associated proteins of the band 4.1 superfamily that includes band 4.1, ezrin, radixin, moesin, and merlin (4). Newly identified members of this group of PTPs include PTP-BAS/E/L1 (5–7), PTPD1/2E/RL10 (8–10), and PTP36/PEZ (11, 12). In these enzymes, the band 4.1 domain and PTP domain are separated by a central segment that contains one to five (PTPH1 has one) PDZ domains (also known as DHR or GLOF domains) (13). PDZ domains are involved in regulating protein-protein interactions, as illustrated in the interactions of postsynaptic density protein PSD-95 with neuronal type nitric-oxide synthase N-methyl-d-aspartic acid receptors, and Shaker-type potassium channels (14). PTPBAS (also known as FAP-1) with cell surface receptor FAS (15), and the human homologue of Drosophila discs-large tumor suppressor with band 4.1 protein (16). Band 4.1 homology domains themselves are also responsible for targeting cytoskeletal-associated proteins to cytoskeleton-membrane interfaces (4).

We have previously shown that the N-terminal segment of PTPH1 can regulate its enzymatic function (17). Tryptic removal of the N-terminal segment of PTPH1 activates the enzyme up to 10-fold. The observation of this intramolecular regulation of PTPH1 activity, and the possibility that the regulation of PTPH1 in vivo may be achieved by interaction with multiple proteins through the various binding domains discussed above, stimulated our interest in searching for proteins that interact with PTPH1. As described in the following study, 14-3-3β was identified as a PTPH1 interacting protein. 14-3-3s are a family of highly conserved acidic proteins, with molecular masses around 30 kDa, that are involved in cell cycle control, transformation, mitogenic signaling pathways (18–20), apoptosis (21), and learning (22). Their ability to form homotypic or heterotypic dimers (23) allows 14-3-3 to perform a role as a coordinator, adapter/linker, and scaffold in assembling signaling complexes. Furthermore, crystallographic studies indicate that each monomer in a 14-3-3 dimer contains a large groove that can dock another molecule (24, 25). In fact, 14-3-3 proteins have been found to interact with a number of signaling proteins, including Raf (26–30), polyomavirus MT antigen (31), Ber and Bcr/Abl (32, 33), phosphatidylinositol 3-kinase (34), Cdc25 (35), Cbl (36), tryptophan hydroxylase (37), platelet glycoprotein Iβ (38), and protein kinase C (39).

Our initial approach involved a yeast two-hybrid screening strategy. Using full-length PTPH1 as a bait, we have isolated 14-3-3β protein from a HeLa cell cDNA library. Stable complexes between PTPH1 and 14-3-3β were reconstituted in insect Sf9 cells. We have demonstrated that PTPH1 binds 14-3-3β in vitro in a serine phosphorylation-dependent manner. Two motifs in PTPH1 were shown to be involved in 14-3-3β binding, one of them is phosphorylated in vitro by C-TAK1 (cdc25C-associated protein kinase). Furthermore, we have shown that PTPH1 and 14-3-3 associate in vitro in several human cell lines and the complex may be regulated in response...
to extracellular signals. These results raise the possibility that 14-3-3β may function as an adaptor molecule in the regulation of PTPH1 and may provide a link between serine/threonine and tyrosine phosphorylation-dependent signaling pathways.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Yeast Two-hybrid Screen**—Full-length and truncated PTPH1 cDNAs were fused to the GAL4 DNA binding domain in vector pGBT9 to produce baits for yeast two-hybrid screens (see Fig. 1). All constructs were generated by convenient restriction enzyme sites or a polymerase chain reaction. Site-directed mutagenesis was performed using the Muta-Gen wild (Bio-Rad) and confirmed by double strand DNA sequencing. A HeLa cell two-hybrid cDNA library (fused to the GAL4 activation domain) in vector pGADGH and yeast host Saccharomyces cerevisiae strain HF7c were kindly provided by G. Hannon (Cold Spring Harbor Laboratory). Other plasmids used in control experiments were generous gifts of our colleagues at Cold Spring Harbor Laboratory: pGIPZs-TPCTP (Y.-F. Hu), pGIPZs-PTPH1 (J. Stolarov), and pGADGH-Ste4 (H. Tu). Yeast two-hybrid screening techniques and medium compositions were as described by CLONTECH. Initially, HF7c yeast cells were cotransformed with the full-length PTPH1 construct and the HeLa cDNA library. Interaction between PTPH1 and proteins encoded by the cDNA library will reconstitute the GAL4 activation domain (HIS3 and LacZ as reporter genes. HIS3 encodes a protein that represses yeast the ability to grow on histidine-free medium, whereas LacZ produces β-galactosidase that can be detected colorimetrically by filter assays. 3-Aminotriazole was included at 10 mM in all selection media to suppress leaky growth from HIS3.

**Antibodies and Immunoblot Analysis**—Mouse anti-PTPH1 serum, pAb25, and monoclonal antibody, mAb22, were generated using purified full-length PTPH1 from Sf9 cells as an antigen (17). Rabbit polyclonal antibodies against 14-3-3β (SC#628) and Raf-1 (SC#227) were purchased from Santa Cruz Biotechnology. Monoclonal antibody against Raf-1 (R19120) was purchased from Transduction Laboratories. Enhanced chemiluminescence reagents and secondary antibodies or protein A that were coupled to horseradish peroxidase used in all immunoblot analyses were purchased from Amersham Corp. and used as described previously (17).

**Cell Culture, Transfection, in Vivo Labeling, and Immunoprecipitation**—Human 293, A431, HaCaT, and Saos-2 cells were cultured in Grace’s medium. Cell lysates were prepared in Nonidet P-40 buffer and clarified as described previously (17). Mouse anti-PTPH1 serum, (SC#628) and Raf-1 (SC#227) were kindly provided by G. Hannon (Cold Spring Harbor Laboratory). Other plasmids used in control experiments were generous gifts of our colleagues at Cold Spring Harbor Laboratory: pGADGH-Ste4 (H. Tu). Yeast two-hybrid screening techniques and medium compositions were as described by CLONTECH. Initially, HF7c yeast cells were cotransformed with the full-length PTPH1 construct and the HeLa cDNA library. Interaction between PTPH1 and proteins encoded by the cDNA library will reconstitute the GAL4 activation domain (HIS3 and LacZ as reporter genes. HIS3 encodes a protein that confers upon yeast the ability to grow on histidine-free medium, whereas LacZ produces β-galactosidase that can be detected colorimetrically by filter assays. 3-Aminotriazole was included at 10 mM in all selection media to suppress leaky growth from HIS3.

**Yeast Two-hybrid Screen**—Full-length and truncated PTPH1 constructs were expressed in Sf9 cells and purified by fast protein liquid chromatography as described elsewhere (40).

**Phosphorylation of PTPH1 and Peptide Mapping**—Purified PTPH1 was phosphorylated for 30 min at 30 °C by recombinant C-TAK1 (42) in the following reaction buffer: 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 2 mM dithiothreitol, 50 μM γ-[32P]ATP (8000 cpm/pmol). For phosphopeptide analysis, 10 μg of phosphorylated PTPH1 were purified by SDS-PAGE. The gel slice containing the PTPH1 was then digested overnight in 30 mM NH4HCO3 containing 1 μg of sequencing grade trypsin (Boehringer Mannheim). The supernatants containing tryptic peptides were subjected to reverse-phase HPLC. The fractions containing the highest radioactive material were processed to determine both amino acid sequence and identity of the phosphorylated residue as described previously (43).

**RESULTS**

**14-3-3β Was Isolated as a PTPH1-interacting Protein by Yeast Two-hybrid Screen**—Two hundred positives were isolated from an initial screening of 1 million HeLa cell cDNA clones using full-length PTPH1 as a probe. After subsequent purification and curing processes, plasmids of 30 strong interacting clones were isolated for sequence analysis. Ten of these clones encode 14-3-3β fused in-frame with the GAL4 DNA binding domain. As controls under the same conditions, 14-3-3β did not interact with another protein-tyrosine phosphatase, TCPTP, or nuclear lamin C (Fig. 1B).

A series of PTPH1 constructs and the 14-3-3β clone were cotransformed into yeast to test the specificity of the interaction. Immunoblot analyses of the cotransformed yeast with an anti-GAL4 antibody indicate that each of the PTPH1 constructs was expressed to a similar level (data not shown). As shown in Fig. 1B, PTPH1 interacts specifically with 14-3-3β, but not with the protein of an unrelated yeast gene Ste4 fused to the activation domain, as indicated by the growth on selection medium without histidine. Furthermore, 14-3-3β interacted strongly only with full-length PTPH1 (PTPH1-1) and less strongly with N-terminal two-thirds of PTPH1 (PTPH1-N). PTPH1-F expressors grew on histidine-free medium containing 30 mM 3-aminotriazole, whereas PTPH1-N expressors grew only on medium containing less than 10 mM 3-aminotriazole. However, 14-3-3β did not interact with the A or C domain alone (PTPH1-A and PTPH1-C), nor with full-length PTPH1 containing an internal deletion that removes half of the B domain and half of the C domain (PTPH1-NC, Fig. 1B). We attempted to determine whether the B domain alone interacts with 14-3-3, however, the B domain construct (PTPH1-B) supported growth on histidine-free medium by itself (data not shown). Furthermore, the isolated PDZ motif (PTPH1-PDZ) contained in the B domain did not interact with 14-3-3 (data not shown). These results indicate that PTPH1 interacts with 14-3-3β specifically in the yeast two-hybrid system and that the integrity of PTPH1 is important for optimal interaction.

**Stable Complexes of PTPH1 and 14-3-3β Were Reconstituted in Vivo and in Sf9 Cells and Were Detected in Various Human Cells**—To test whether 14-3-3β can bind to PTPH1 in vitro, purified PTPH1 from Sf9 cells was incubated with MBP or MBP-14-3-3β that was prebound to amylose beads. As shown in Fig. 2A, PTPH1 bound specifically to 14-3-3β, but not the MBP.
moiety, in a concentration-dependent manner. However, binding of 14-3-3b did not result in a significant change in the enzymatic activity of PTPH1, as measured with three different tyrosyl-phosphorylated substrates (RCML, myelin basic protein, and pGluTyr) (Fig. 2B).

To reconstitute the interaction between 14-3-3b and PTPH1 in a cellular context, Sf9 cells were coinfected with baculoviruses expressing PTPH1 and 14-3-3b, and the complexes formed between the two proteins were analyzed by immunoprecipitation. As shown in Fig. 3A, 14-3-3b was present in the anti-PTPH1 immune complexes from [35S]methionine-labeled Sf9 cells that were coinfected with PTPH1 and 14-3-3b baculoviruses. Similarly, PTPH1 was detected in the anti-14-3-3b complexes from the coinfected Sf9 cells. The stoichiometry of the association was estimated to be ~2 mol of 14-3-3b per mol of PTPH1 by densitometric analysis of Fig. 3A.

To confirm that 14-3-3b and PTPH1 associate at physiological levels of expression, anti-14-3-3b immune complexes from untransfected human 293, A431, HaCaT, and Saos-2 cells were analyzed. As shown in Fig. 3B, PTPH1 was found in anti-14-3-3b immune complexes from each cell line tested. Interestingly, HaCaT cells have more PTPH1 than 293 cells, but less PTPH1 was found in complexes with 14-3-3b, even though both cell lines have similar amounts of 14-3-3b protein. This suggests that the extent of association may be regulated by factors, such as differential phosphorylation or subcellular localization of either protein, in addition to the abundance of the two proteins. Furthermore, treatment of quiescent 293 cells with EGF resulted in a 50% reduction in the association between PTPH1 and 14-3-3b (Fig. 3C).

Both PTPH1 and Raf-1 Can Interact with 14-3-3b in 293 Cells, but They Are Recovered in Different Complexes—Since both PTPH1 and Raf bind to 14-3-3b, they may both be recruited into the same multiprotein complexes and function in the same signaling cascades. To address this, immune complexes were analyzed from [35S]methionine-labeled 293 cells that were cotransfected with PTPH1 and Raf-1. As shown in Fig. 4, [35S]-labeled endogenous 14-3-3 proteins were found in both anti-PTPH1 and anti-Raf immune complexes. The significance of the slightly different spectra of 14-3-3 proteins detected in the two immune complexes is currently unknown. Conversely, both PTPH1 and Raf were also detected in anti-14-3-3b immune complexes. However, under conditions in which both PTPH1 and Raf were quantitatively immunodepleted from cell lysates by their respective antibodies, PTPH1 was not detected in anti-Raf immune complexes and Raf was
not observed in anti-PTPH1 immune complexes. Trace amounts of PTPH1 were detected in Raf immune complexes only from 293 cells in which PTPH1 was grossly overexpressed from a stronger promoter (data not shown). This situation is distinct from the association of Bcr and Raf, in which 14-3-3 serves as a molecular bridge to direct the interaction (33). This suggests that PTPH1 and Raf do not function simultaneously in the same 14-3-3 complexes or signaling cascades.

PTPH1 is Phosphorylated on Seryl Residues in Both Insect and Mammalian Cells, and Phosphorylation Is Required for the Association between PTPH1 and 14-3-3β—In the initial binding assays, we were unable to detect binding between 14-3-3β and PTPH1 expressed in E. coli (data not shown). However, using PTPH1 expressed in Sf9 cells the association with 14-3-3β was successfully demonstrated in vitro (Fig. 2A). PTPH1 from A431, 293, and Sf9 cells was phosphorylated on seryl residues (Fig. 5A), and PTPH1 from Sf9 cells contained at least four major phosphorylation sites (data not shown). These findings suggest that eukaryotic modifications such as phosphorylation may play a role in regulating the binding interaction. As indicated in Fig. 5B, the specific interaction of PTPH1 and 14-3-3β from Sf9, 293, and A431 cells was phosphorylated on seryl residues (Fig. 5A), and PTPH1 from Sf9 cells contained at least four major phosphorylation sites (data not shown). These findings suggest that eukaryotic modifications such as phosphorylation may play a role in regulating the binding interaction. As indicated in Fig. 5B, the specific interaction of PTPH1 and 14-3-3β from Sf9, 293, and A431 cells was phosphorylated on seryl residues (Fig. 5A), and PTPH1 from Sf9 cells contained at least four major phosphorylation sites (data not shown). These findings suggest that eukaryotic modifications such as phosphorylation may play a role in regulating the binding interaction. As indicated in Fig. 5B, the specific interaction of PTPH1 and 14-3-3β from Sf9, 293, and A431 cells was phosphorylated on seryl residues (Fig. 5A), and PTPH1 from Sf9 cells contained at least four major phosphorylation sites (data not shown). These findings suggest that eukaryotic modifications such as phosphorylation may play a role in regulating the binding interaction. As indicated in Fig. 5B, the specific interaction of PTPH1 and 14-3-3β from Sf9, 293, and A431 cells was phosphorylated on seryl residues (Fig. 5A), and PTPH1 from Sf9 cells contained at least four major phosphorylation sites (data not shown). These findings suggest that eukaryotic modifications such as phosphorylation may play a role in regulating the binding interaction. As indicated in Fig. 5B, the specific interaction of PTPH1 and 14-3-3β from Sf9, 293, and A431 cells was phosphorylated on seryl residues (Fig. 5A), and PTPH1 from Sf9 cells contained at least four major phosphorylation sites (data not shown). These findings suggest that eukaryotic modifications such as phosphorylation may play a role in regulating the binding interaction.
14-3-3b was totally abolished by the pretreatment of PTPH1 with PAP. The disruption of the association was not due to proteolytic degradation of PTPH1, since the overall level and integrity of the protein remained constant through the PAP treatment (Fig. 5B). These results demonstrated that phosphorylation of PTPH1 is a prerequisite for the association between PTPH1 and 14-3-3b.

C-TAK1 Phosphorylates PTPH1 on a Single Site and Enhances Its Association with 14-3-3b—To identify specific 14-3-3 binding sites regulated by protein phosphorylation, protein serine/threonine kinases were tested for their ability to phosphorylate PTPH1. C-TAK1 was originally purified as a Cdc25C-associated protein kinase (42). It binds to Cdc25C and phosphorylates serine 216, which represents a perfect 14-3-3 binding motif.2 As shown in Fig. 6, C-TAK1 phosphorylated PTPH1 on a single site and enhanced 14-3-3b binding by 5-fold. When C-TAK1-phosphorylated PTPH1 was trypsinized, only two tryptic peptides (p27 and p28) contained significant radioactivity. Sequencing of these two peptides indicated that p27 and p28 differ by a single arginine at the N terminus. This is due to the trypsin cleavage at alternative arginines in the amino acid sequence of RRSLS359VEH in PTPH1. To identify which serine was phosphorylated by C-TAK1, we subjected p28 to Edman degradation and determined at which cycle a

2 H. Piwnica-Worms, unpublished data.
play some features of the motif. To test whether both Ser359 is the phosphorylated amino acid.
m mutants were incubated with 5
b in 14-3-3-
B

FIG. 7. Identification of the phosphorylation sites important for the binding of PTPH1 to 14-3-3β. A, phosphorylation site mutants. Serine residues 359 and 853 of PTPH1 were mutated to alanine individually (SA1 and SA2) or together (SA3) and expressed in Sf9 cells. B, Ser359 is the major C-TAK1 phosphorylation site in vitro. Purified-PTPH1 wild-type (WT) and mutant (SA1) were phosphorylated by C-TAK1 and then incubated with MBP and MBP14-3-3β bound to beads. Aliquots of the binding mixtures and the bound material were immunoblotted with anti-PTPH1 and subjected to autoradiography. C, mutations of Ser359 and Ser853 in PTPH1 significantly reduced their binding to 14-3-3β. Lysates (2 mg) of Sf9 cells expressing the PTPH1 mutants were incubated with 5 μg of MBP or MBP14-3-3β bound to beads. Starting lysates (10 μg/lane) and bound materials were analyzed by immunoblotting with anti-PTPH1.

burst of [32P]phosphate was recovered. As shown in Fig. 6C, the third sequencing cycle is the radioactive peak, indicating that Ser359 is the phosphorylated amino acid.

PTPH1 contains novel 14-3-3-binding motifs different from the Raf Binding Consensus—Although PTPH1 does not contain an exact copy of the 14-3-3 binding motif corresponding to that found in Raf (RSXSXP) (48), it does have two potential 14-3-3-binding sites (RSLS359VEH and RVDS853EP) that display some features of the motif. To test whether both RSLS359VEH and RVDS853EP are involved in binding to 14-3-3β, serine residues 359 and 853 of PTPH1 were changed to alanine by site-directed mutagenesis (Fig. 7A).

To confirm that 14-3-3β binds to Ser359, the major site of phosphorylation by C-TAK1, purified Ser359 to alanine mutant (SA1) was phosphorylated by C-TAK1 and used in binding assays. As demonstrated in Fig. 7B, this mutation diminished the phosphorylation by C-TAK1 by 90% and abolished the enhanced binding to 14-3-3β.

When lysates from Sf9 cells expressing the two single serine to alanine mutants SA1 and SA2 were incubated with 14-3-3β beads, the binding of both mutants to 14-3-3β was significantly reduced compared with wild-type PTPH1. The double serine to alanine mutation, SA3, reduced the binding by ~80% (Fig. 7C).

Thus in a cellular context phosphorylation of PTPH1 on at least two sites regulates its association with 14-3-3β.

**DISCUSSION**

PTPs have the potential to exert a considerable influence on tyrosine phosphorylation-dependent signaling pathways, both augmenting and antagonizing the function of protein tyrosine kinases. Therefore, it is important to understand the mechanism of regulation of PTP function. Both protein kinases and phosphatases have been shown to be regulated by association with anchor and scaffold proteins (44). In this study, we have demonstrated for the first time an association between PTPH1 and 14-3-3β both in vitro and in vivo. Interestingly, both 14-3-3βs (20) and PTPH1 are abundantly expressed in brain,3 and in the rat, PTPH1 is concentrated particularly in thalamus (45).

Since none of the individual domains of PTPH1 alone interacted with 14-3-3β in yeast two-hybrid tests, the higher order structure of PTPH1 appears very important for the optimal interaction. We demonstrated further that serine phosphorylation of PTPH1 is a prerequisite for this binding. Binding of 14-3-3β to PTPH1 in vitro was abolished by pretreating PTPH1 with PAP and was greatly enhanced by pretreating with C-TAK1. This is in agreement with previous findings of a requirement for phosphorylation in other proteins that interact with 14-3-3 (21, 37, 46, 47). Using in vitro binding assays, we have identified two major 14-3-3β-binding sites in PTPH1. Of six potential serine phosphorylation sites in PTPH1 with some features of the 14-3-3 consensus recognition site identified in Raf (48), RSLS359VEH at the junction of the band 4.1 domain and middle segment and RVDS853EP in the catalytic domain display the greatest similarity to the RSXSXP (underline indicates phosphoserine) motif. Mutation of Ser359 and Ser853 to alanine reduced the association of phosphatase with 14-3-3β in vitro by 80%, indicating that Ser359 and Ser853 are the major 14-3-3β-binding sites. These results indicate that the consensus motif RSXSXP found in Raf is not strictly required for interactions with 14-3-3. Platelet glycoprotein Ibα (38), Bcr (32), and tryptophan hydroxylase (37) are known binding proteins of 14-3-3; however, inspection of their sequences also revealed no motif identical to the consensus RSXSXP. Recently, new 14-3-3 binding sequences were found in protooncogene Cbl as RSLPPS and RLOSTFS (49), which do not fit exactly with the consensus RSXSXP. Taken together, it would appear that the recognition of 14-3-3 can be achieved by multiple proteins containing phosphoserine in motifs that diverge significantly from the one found in Raf.

14-3-3 proteins are a family of at least seven members that display distinct expression patterns in different cells or tissues (20). Similar to the way SH2 domains bind to phosphotyrosyl residues, different isoforms of 14-3-3 proteins may bind specifically to phosphoserine residues contained in different recognition motifs. The different recognition motifs may be phosphorylated in response to distinct signals, creating an additional level of complexity and specificity in the role of 14-3-3 proteins. There are multiple sites of serine phosphorylation in PTPH1 in vitro (data not shown), including the two sites identified here, raising the possibility that PTPH1 may associate with different spectra of 14-3-3 proteins in response to various signals. Preliminary data suggest that 20% of PTPH1 is associated with 14-3-3β protein in quiescent 293 cells (data not shown), whereas this association drops by 50% upon EGF stimulation (Fig. 3C). The differential association of PTPH1 and 14-3-3 in different cell lines may reflect a difference in phosphorylation status or subcellular localization of PTPH1. Through the action

3 S.-H. Zhang and N. K. Tonks, unpublished data.
of 14-3-3, PTPH1 may function in cross-talk between protein serine and tyrosine phosphorylation-dependent steps in signal transduction pathways. We are currently trying to identify proteins that become linked to PTPH1 through binding of 14-3-3.

We have shown previously that the sequences flanking the catalytic domain of PTPH1 have an inhibitory effect on activity (17). However, using artificial substrates we have not been able to detect a significant effect of 14-3-3 binding on the phosphatase activity of PTPH1. It is possible that significant effects may be demonstrated with the yet to be identified physiological substrate(s) of PTPH1 instead of the artificial substrates used in the PTP assays to date. Indeed, the direct effects of 14-3-3 binding on the enzymatic properties of associated proteins such as Raf and protein kinase C (20, 26–30, 47) remain controversial and unclear. It is also possible that significant effects on the PTP activity of PTPH1 may require a combined action of several regulators, such as proteins interacting with the band 4.1 and/or PDZ domain of PTPH1, possibly with 14-3-3 as a PTPH1-associated protein.

PTPH1 was phosphorylated exclusively on serine in either insect Sf9 or human 293 and A431 cells. C-TAK1 phosphorylates Ser^359 on PTPH1, one of the two major 14-3-3 binding sites, almost exclusively in vitro. Mutation of Ser^359 to alanine reduced by 90% the C-TAK1-mediated phosphorylation of PTPH1 and completely abolished the C-TAK1-mediated enhancement of binding to 14-3-3 in vitro. Protein kinase C also phosphorylates PTPH1 and enhances its binding to 14-3-3, but the phosphorylation is complex, involving multiple sites (data not shown). Apart from C-TAK1 and protein kinase C, we have failed to detect significant phosphorylation of PTPH1 and enhancement of 14-3-3 binding by protein kinase A and casein kinase II. It is currently unclear which protein kinase phosphorylates PTPH1 in vivo; however, C-TAK1 and members of the protein kinase C family certainly cannot be ruled out.

In conclusion, we have identified 14-3-3 as a PTPH1-associated protein. Our findings of 14-3-3 binding to novel phosphoserine motifs in PTPH1 further support the notion that protein-protein interaction may play a significant role in the regulation and function of PTPH1, as well as other PTPs, in cross-talk mediated by protein serine and tyrosine phosphorylation cascades.

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