The CLK Family Kinases, CLK1 and CLK2, Phosphorylate and Activate the Tyrosine Phosphatase, PTP-1B*

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The protein-tyrosine phosphatase PTP-1B is an important regulator of intracellular protein tyrosine phosphorylation, and is itself regulated by phosphorylation. We report that PTP-1B and its yeast analog, YPTP, are phosphorylated and activated by members of the CLK family of dual specificity kinases. CLK1 and CLK2 phosphorylation of PTP-1B in vitro activated the phosphatase activity approximately 3–5-fold using either p-nitrophenyl phosphate, or tyrosine-phosphorylated myelin basic protein as substrates. Co-expression of CLK1 or CLK2 with PTP-1B in HEK 293 cells led to a 2-fold stimulation of phosphatase activity in vivo. Phosphorylation of PTP-1B at Ser50 by CLK1 or CLK2 is responsible for its enzymatic activation. These findings suggest that phosphorylation at Ser50 by serine threonine kinases may regulate the activation of PTP-1B in vivo. We also show that CLK1 and CLK2 phosphorylate and activate the S. cerevisiae PTP-1B family member, YPTP1. CLK1 phosphorylation of YPTP1 led to a 3-fold stimulation of phosphatase activity in vitro. We demonstrate that CLK phosphorylation of Ser83 on YPTP1 is responsible for the activation of this enzyme. These findings demonstrate that the CLK kinases activate PTP-1B family members, and this phosphatase may be an important cellular target for CLK action.

Modification of proteins by phosphorylation is a rapid and reversible mechanism to control their function, and is central to many signal transduction pathways. While serine/threonine phosphorylation of proteins is a common post-translational modification, only a small proportion of proteins are phosphorylated on tyrosine residues. Protein-tyrosine kinases, which include many growth factor receptors, are important regulators of cellular responses (1–4). Tyrosine phosphorylation may directly regulate enzyme activity, or it may direct the formation of large signaling complexes, which are essential for the transduction of signals throughout the cell. The levels of cellular protein tyrosine phosphorylation are governed by the combined actions of the tyrosine kinases and phosphatases. While the regulation of cellular tyrosine kinases has been extensively studied, comparatively little is known about the regulation of tyrosine phosphatases. Interestingly, like tyrosine kinases, the activity of tyrosine phosphatases is subject to regulation by both serine/threonine and tyrosine phosphorylation (5–13).

PTP-1B was the first tyrosine phosphatase to be isolated (14). While the regulation of PTP-1B activity in cells is poorly understood, it is known that phosphorylation of PTP-1B varies with the cell cycle and following treatment of cells with various stimuli, such as EGF, okadaic acid, and phorbol esters (5, 14–16). In vivo the phosphorylation of PTP-1B occurs on serine and tyrosine residues. In response to EGF stimulation of A431 cells, PTP-1B is phosphorylated at Tyr460 by the EGF receptor, which leads to a 3-fold activation of PTP-1B (15). Moreover, evidence that PTP-1B phosphatase activity is regulated by serine phosphorylation is mounting. Treatment of cells with cAMP analogs or okadaic acid resulted in the serine phosphorylation of PTP-1B and a 4-fold stimulation of PTP-1B phosphatase activity (14). Previous studies have identified several serine phosphorylation sites within the C-terminal regulatory domain of PTP-1B (5); however, phosphorylation at these sites does not lead to alterations in phosphatase activity. Therefore, it is likely that heretofore unrecognized phosphorylation sites within the catalytic domain of PTP-1B exist, and that these sites are important for the in vivo regulation of PTP-1B phosphatase activity.

The CLK family kinases are an evolutionarily conserved group of dual specificity kinases, capable of phosphorylating protein substrates on serine, threonine, and tyrosine residues. The prototypic CLK family kinase member, CLK1, was initially identified through its ability to autoprophosphorylate on tyrosine residues (17, 18). The family includes members from diverse species, including yeast, Drosophila, Arabidopsis, tobacco, mouse, rat, and human.

The biological functions of this family of proteins have remained elusive, but may play an important and evolutionarily conserved role in signal transduction within the cell. A critical role for the CLK family in development has been suggested by work on the Drosophila CLK homologue, DOA. Flies expressing low levels of the mutant DOA protein show marked neurologic abnormalities, and homozygosity for the DOA null allele is embryonically lethal (19).

Recent work on the murine CLK1 protein has begun to shed light on other physiological roles of the CLK family of kinases. Regulation of mRNA splicing is now recognized as a dynamic process, and one in which the CLK family of kinases may have an important function. CLK1 has been reported to bind to and phosphorylate serine/arginine-rich mRNA splicing factors on physiologically relevant sites in vitro (20, 21). Moreover, Colwill et al. (20, 22) demonstrated that overexpression of CLK1 in COS cells leads to the subcellular redistribution of serine/arginine-rich proteins, and to alterations in mRNA splicing in vivo.

The abbreviations used are: EGF, epidermal growth factor; BSA, bovine serum albumin; TBS, Tris-buffered saline; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; ERK, extracellular signal-regulated kinase; PNPP, p-nitrophenol phosphate; MBP, myelin basic protein.
vivo. Collectively, these data strongly suggest a role for the CLK family kinases in the regulation of mRNA splicing in vivo.

The CLK family kinases may also participate in intracellular signal transduction cascades. Myers et al. (25) showed that overexpression of CLK1 in the pheochromocytoma PC-12 cell line led to differentiation of these cells. Moreover, specific signal transduction intermediates were activated in these cells, including ERK1/2 and pp90Rsk. Furthermore, immunocytochemical staining of 3T3 cells expressing human CLK3 demonstrated that the majority of immunoreactivity was present within the cytoplasm, and was less abundant in the nucleus. Similarly, staining of endogenous CLK1 in PC12 cells found it to be mostly cytoplasmic as well. In agreement with a putative signaling role for the CLK kinases is the finding that ethylene stimulation of tobacco leaves stimulates the activity of the tobacco CLK family member, PK12 (24). These findings strongly suggest the existence of cytoplasmic targets for the CLK family kinases and their participation in intracellular signaling pathways.

We report here that PTP-1B and a yeast analog, YPTP1, are in vitro substrates for both CLK1 and CLK2. Moreover, phosphorylation of these two phosphatases by CLK1/CLK2 leads to their enzymatic activation in vitro. We have mapped the activation site within the catalytic domain of PTP-1B and show that it is important both for basal activity as well as enzymatic activation of PTP-1B. Furthermore, we show that co-expression of CLK1/CLK2 with PTP-1B leads to activation of PTP-1B in vivo.

**MATERIALS AND METHODS**

**Phosphatase Mutants**—Site-directed mutagenesis of hPTP-1B or YPTP1 was performed by polymerase chain reaction using 4-primer mutagenesis (25). The XL-1 Blue Escherichia coli strain was used as the host strain during mutagenesis. Two of the primers were anchored in the pGEX-KG sequence flanking the multi-cloning site: right primer, 5'-TCGTTTCCCAACAGTCAAAGGCG; left primer, 5'-CCCAATGT-GCTCGTATGTCCTCCC.

Primers overlapping the sites of mutagenesis were designed as follows, locations of the mutations are underlined: S50A Sense, 5'-GCTTAAAGAACAAAAACCGAAATAGGTACAGAGACGGCGCCCCC; S50A Antisense, 5'-CCGACTATGGTCAAAGGGGCGCCGTCTCTGTACC; S50T Sense, 5'-CCGACTAGTGGCTCAAGAGGGGGCGCCGTCTCTGTACC; S50T Antisense, 5'-CCGACTATGGTCAAAGGGTGTCACCTGTGTTACC.

**Protein Kinase Assays**—Bacterially expressed recombinant CLK protein was incubated with substrates in Kinase Reaction Buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM ATP, 10 mM MgCl2, 2 mM MnCl2). For assays requiring 32P incorporation, the Kinase Reaction Buffer was supplemented with 10 μCi [γ-32P]ATP. Reactions were carried out at room temperature for 20 min and were stopped by the addition of 3× Laemmli Sample Buffer, or diluted in Phosphatase Reaction Buffer and phosphatase activity monitored.

**Protein-tyrosine Phosphatase Assays**—The activity of YPTP1 and PTP-1B was assayed by hydrolysis of p-nitrophenol phosphate (PNPP). The phosphatases were incubated in Phosphatase Reaction Buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM dithiothreitol, 1 mM PNPP) for 2–20 min at 37 °C. The reaction was stopped with 0.2 v NaOH, and the absorbance at 410 nm was measured. The reactions were run in triplicate.

**RESULTS**

CLK1 and CLK2 Phosphorylate Human PTP-1B in Vitro—In experiments initially designed to test the phosphorylation dependence of CLK1 activity in vitro, we observed that when PTP-1B was incubated with CLK1, PTP-1B became highly phosphorylated. We subsequently found that recombinant, constitutively active CLK1 or CLK2 phosphorylated PTP-1B (Fig. 1A) in vitro. Phosphoamino acid analysis revealed that CLK1 and CLK2 phosphorylated PTP-1B exclusively on serine residues (Fig. 1B). In order to determine whether CLK phosho-

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2 H. Menegay, F. Moeslein, and G. Landreth, submitted for publication.
rylation altered PTP-1B activity, the phosphatase was preincubated in the presence or absence of either CLK1 or CLK2 for 20 min \textit{in vitro}. Subsequent \textit{in vitro} phosphatase assays demonstrated an approximate 5-fold activation of phosphatase activity of CLK1 or CLK2-treated PTP-1B (Fig. 2A). Incubation of PTP-1B with increasing amounts of CLK protein led to a corresponding increase in activation of PTP-1B (Fig. 2B). We conclude that phosphorylation of PTP-1B by CLK1 and CLK2 activates the phosphatase.

CLK1 and CLK2 Phosphorylate Serine 50 and Serines 242/243 on Human PTP-1B—

One-dimensional phosphopeptide mapping of PTP-1B was utilized to investigate which serine residues CLK1 and CLK2 phosphorylated \textit{in vitro}. The phosphopeptide maps demonstrate that CLK1 and CLK2 phosphorylated PTP-1B at similar sites, as evidenced by the detection of identical phosphopeptides (Fig. 3). Examination of the primary sequence of PTP-1B revealed multiple sites conforming to the CLK family consensus phosphorylation sequence (R/K-X-R/K-X-R/K-X-S-X-R).3 These data and the size of the phosphopeptides allowed identification of Ser50 as a likely site of CLK phosphorylation. Substitution of an alanine at the Ser50 site (S50A) by site-directed mutagenesis diminished CLK1 and CLK2 phosphorylation of PTP-1B by approximately 90%, indicating that this was the principal site of phosphorylation (Fig. 4A). To further establish that this residue was phosphorylated, we generated a threonine substitution at Ser50 (S50T). Following incubation of the S50T PTP-1B mutant with CLK1 or CLK2, phosphoamino acid analysis revealed the presence of phosphothreonine residues on the S50T mutant (Fig. 4B).

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\caption{CLK1 and CLK2 phosphorylate PTP-1B on serine \textit{in vitro}. \textit{A}, In \textit{vitro} kinase reactions were performed by incubating 2 \mu g of recombinant PTP-1B with 0.5 \mu g of the truncated constitutively active form of either CLK1 or CLK2 for 20 min. The 32P-labeled proteins were separated by SDS-PAGE, and visualized by autoradiography. The position of PTP-1B (indicated with arrowheads) was determined by Coomassie staining of the gels. \textit{B}, the labeled PTP-1B bands were subjected to phosphoamino acid analysis. The phosphoamino acids were separated by electrophoresis on TLC plates and visualized by autoradiography. The position of phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) as determined by ninhydrin staining of phosphoamino acid standards are indicated. The positions of partially hydrolyzed phosphopeptides (pPEP) are indicated.}
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\caption{CLK1 and CLK2 activate PTP-1B \textit{in vitro}. \textit{A}, Recombinant PTP-1B (0.1 \mu g) was preincubated in the absence or presence of (2 \mu g) constitutively active CLK1 or CLK2 in \textit{vitro} for 20 min in kinase reaction buffer. The PTP-1B or CLK1 or CLK2 was diluted in phosphatase reaction buffer and incubated another 2 min. Phosphatase activity was assayed by liberation of the PNPP cleavage product quantitated at OD410 by spectrophotometry. \textit{B}, a constant amount of recombinant PTP-1B (0.1 \mu g) was preincubated \textit{in vitro} with increasing amounts of constitutively active CLK1 or CLK2, or BSA for 20 min. \textit{In vitro} phosphatase reactions were then performed using PNPP as a substrate for PTP-1B. The results shown are the average (± S.D.) of three independent experiments.}
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\caption{CLK1 and CLK2 phosphorylate PTP-1B on identical peptides. Recombinant PTP-1B was phosphorylated \textit{in vitro} by either constitutively active CLK1 or CLK2. The 32P-labeled PTP-1B protein was separated by SDS-PAGE and transferred to nitrocellulose. The PTP-1B band was excised and subjected to digestion with trypsin, chymotrypsin, or a combination of trypsin and chymotrypsin. Tricine-SDS electrophoresis was used to separate the resulting peptides and the phosphopeptides visualized by autoradiography.}
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\begin{figure}
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\caption{CLK1 and CLK2 phosphorylate PTP-1B on serine 50 and serines 242/243 on Human PTP-1B—One-dimensional phosphopeptide mapping of PTP-1B was utilized to investigate which serine residues CLK1 and CLK2 phosphorylated \textit{in vitro}. The phosphopeptide maps demonstrate that CLK1 and CLK2 phosphorylated PTP-1B at similar sites, as evidenced by the detection of identical phosphopeptides (Fig. 3). Examination of the primary sequence of PTP-1B revealed multiple sites conforming to the CLK family consensus phosphorylation sequence (R/K-X-R/K-X-R/K-X-S-X-R).3 These data and the size of the phosphopeptides allowed identification of Ser50 as a likely site of CLK phosphorylation. Substitution of an alanine at the Ser50 site (S50A) by site-directed mutagenesis diminished CLK1 and CLK2 phosphorylation of PTP-1B by approximately 90%, indicating that this was the principal site of phosphorylation (Fig. 4A). To further establish that this residue was phosphorylated, we generated a threonine substitution at Ser50 (S50T). Following incubation of the S50T PTP-1B mutant with CLK1 or CLK2, phosphoamino acid analysis revealed the presence of phosphothreonine residues on the S50T mutant (Fig. 4B). Sub-
stitution of threonine for serine slightly decreases the affinity of the CLKs for this site compared with the native enzyme, as evidenced by the presence of equal amounts of phosphoserine and phosphothreonine on the S50T mutants. Moreover, proteolytic maps of the S50T mutant show increased phosphorylation on other phosphopeptides (see below), as well as on the phosphopeptide containing Thr50 (data not shown). We conclude that both CLK1 and CLK2 phosphorylate PTP-1B principally at Ser50.

The S50A mutant was phosphorylated by CLK1 and CLK2, albeit at lower levels than the wild-type enzyme, suggesting the existence of additional CLK phosphorylation sites on PTP-1B. Phosphopeptide maps of the S50T mutant show increased phosphorylation on other phosphopeptides (see below), as well as on the phosphopeptide containing Thr50 (data not shown). We conclude that both CLK1 and CLK2 phosphorylate PTP-1B principally at Ser50.

Serine 50 Is Important for Catalytic Activity of PTP-1B—We tested whether the mutation of Ser50 affected the phosphatase activity of PTP-1B. The basal activities of the wild-type, S50T, and S50A PTP-1B proteins were analyzed using in vitro phosphatase assays. The S50A mutants have significantly diminished basal phosphatase activity toward the PNPP substrate, compared with the wild-type enzyme, while the S50T mutants have wild-type level phosphatase activity (Fig. 5). CLK1 and CLK2 activated the phosphatase activity of S50T and wild-type PTP-1B (Fig. 6). However, the S50A mutant was resistant to activation by either CLK1 or CLK2 (Fig. 6). These data strongly suggest that Ser50 is the phosphorylation site on PTP-1B responsible for CLK-induced stimulation of phosphatase activity.

In order to determine whether the CLKs could activate PTP-1B toward a protein substrate, wild-type PTP-1B, the S50A mutant, and activated PTP-1B were assayed using tyrosine-phosphorylated MBP as a substrate. CLK2 phosphorylated PTP-1B exhibited a 3-fold increase in activity relative to the wild-type PTP-1B (Fig. 7). Furthermore, the S50A mutant possessed approximately 20% of the activity of the wild-type enzyme. Phosphorylation of PTP-1B by CLK2 led to a decrease in the $K_m$ by 3-fold (Table I). Interestingly, substitution of alanine for Ser50 led to 5-fold increase in the $K_m$ over wild-type; however, the $V_{max}$ for the S50A mutant was roughly half that
of the wild-type enzyme. The change in the \( \text{K}_m \) of the PTP-1B for substrate following phosphorylation by the CLKs is consistent with the observed increase in activity of PTP-1B for protein and synthetic substrates. The changes observed in the S50A mutant are concordant with the view that Ser50 is an important determinant of the substrate binding pocket conformation. Our results demonstrate that phosphorylation of PTP-1B at Ser50 enhances substrate binding to the enzyme.

CLK Kinases Activate PTP-1B

CLK1 and CLK2 Can Activate PTP-1B in Vivo—The effect of CLK1 and CLK2 on PTP-1B in vivo was investigated by over-expressing these proteins in HEK293 cells. GST-tagged full-length CLK1 or CLK2 were co-transfected with GST-tagged PTP-1B into HEK293 cells. Glutathione-Sepharose was used to precipitate the tagged proteins from the transfected cells. In vitro phosphatase assays were performed on the precipitated proteins. Co-expression of CLK1 with PTP-1B activated PTP-1B 2-fold in vivo (Fig. 8). Similar results have been obtained by co-expression of CLK2 with PTP-1B (data not shown).

CLK1 and CLK2 Phosphorylate YPTP1 on Ser 83—We investigated whether the CLKs could also activate other phosphatases related to PTP-1B. Yeast protein-tyrosine phosphatase, YPTP1, a \( \text{Saccharomyces cerevisiae} \) PTP-1B analog (30), was phosphorylated and enzymatically activated in vitro by CLK1 and CLK2 (Figs. 9A and 10). We therefore investigated which residues on YPTP1 the CLKs phosphorylated. We used the CLK consensus phosphorylation sequence to search the aligned sequences of YPTP1 and PTP-1B for potential phosphorylation sites. In YPTP1, Ser83 was found to closely conform to the consensus phosphorylation site of CLK1. This serine residue was mutated to alanine (S83A YPTP1) to test whether this serine was phosphorylated by the CLKs. We also substituted a serine for the invariant catalytic cysteine, Cys252, creating a catalytically inactive YPTP1 mutant (C252S YPTP1). Two-dimensional tryptic peptide mapping was performed on CLK1 phosphorylated wild-type, S83A, and C252S YPTP1. The tryptic maps of YPTP1 and C252S YPTP1 produced several major phosphopeptides and a number of minor phosphopeptides (Fig. 9B). Importantly, the tryptic map of S83A YPTP1 showed the

TABLE I

Kinetic analysis of PTP-1B proteins

| Protein               | \( \text{K}_m \) \( \mu \text{M} \) | \( \text{V}_{\text{max}} \) \( \text{nmol/min/ng} \) |
|-----------------------|-------------------------------|---------------------------------|
| Wild-type PTP-1B      | 11                            | 0.18 ± 0.01                     |
| Wild-type + CLK2      | 2.9                           | 0.19 ± 0.02                     |
| S50A mutant           | 53                            | 0.08 ± 0.01                     |

PTP-1B 2-fold in vivo (Fig. 8). Similar results have been obtained by co-expression of CLK2 with PTP-1B (data not shown). In a series of similar experiments, we co-expressed CLK1 or CLK2 with untagged-PTP-1B in NIH 3T3 cells and observed a 2–3-fold increase in phosphatase activity in immunoprecipitates of PTP-1B (data not shown). The 2-fold activation of PTP-1B in vivo by CLK1 is significantly lower than that observed in vitro. However, this is likely a consequence of the lower enzymatic activity of the full-length CLK1, relative to the constitutively active, truncated CLK1 employed in the in vitro studies. These data demonstrate that PTP-1B is regulated by the CLK family kinases in vivo as well as in vitro.
specific loss of a single phosphopeptide (Fig. 9B). A peptide map was produced from a mixture of all three phosphorylated forms of YPTP1 (wild-type, S83A, and C252S YPTP1), indicating that identical sites were phosphorylated in all three forms of YPTP1 (Fig. 9B). The loss of a single phosphopeptide in the S83A mutant demonstrates that Ser83 was phosphorylated by CLK1. The identities of the other major phosphorylation sites on YPTP1 are currently unknown.

Ser83 Is Essential for CLK1 Activation of YPTP1—We tested whether substitution of an alanine at Ser83 would affect the phosphatase activity of YPTP1. In vitro phosphatase assays were performed on YPTP1 proteins that had been incubated in the absence or presence of CLK1. Mutation of Ser83 to alanine resulted in a nearly 50% reduction in the basal activity of YPTP1 (Fig. 10). Moreover, S83A YPTP1 proteins are resistant to activation by CLK1 in vitro (Fig. 10). We conclude from these data that CLK phosphorylation of YPTP1 at Ser83 activates the phosphatase activity of YPTP1.

DISCUSSION

The CLK family kinases were initially identified on the basis of their ability to autophosphorylate on tyrosine residues. Subsequent analysis of the CLK kinases showed them to be members of the growing class of kinases termed dual-specificity kinases, capable of phosphorylating substrates on serine, threonine, and tyrosine residues. Work from several laboratories has suggested an important role for the CLK kinases in regulation of mRNA splicing in vivo (20–22, 31). Furthermore, the Arabidopsis CLK family member AFC1 is capable of regulating transcription in vivo (32). However, we have recently demonstrated that the majority of cellular CLK protein is located in the cytoplasm, suggesting the existence of non-nuclear targets for the CLK kinases. Moreover, a role for the CLK family kinases in signaling cascades has been suggested by several findings. Overexpression of CLK1 in PC12 cells caused the differentiation of these cells into a neuronal phenotype (23). Analysis of these cells showed that CLK1 expression activated elements of the mitogen-activated protein kinase signaling cascade, including ERK1/ERK2 and pp90RSK. Although CLK1 caused the activation of the ERKs and pp90RSK, the mechanism through which CLK stimulated these activities is unclear, as we have ruled out direct phosphorylation of these molecules by CLK1 (data not shown).

We report here the identification of a direct non-nuclear target of the CLKs, the tyrosine phosphatase, PTP-1B. Serendipitously, in the course of studying CLK1 activation in vitro, it was noted that CLK1 was capable of phosphorylating PTP-1B in vitro. We have subsequently demonstrated that both CLK1 and CLK2 are capable of activating PTP-1B in vitro and in vivo. Similarly, a yeast PTP-1B family member, YPTP1, was also
We observed that the phosphorylation of PTP-1B by CLK1 or CLK2 led to an approximately 5-fold stimulation of phosphatase activity. Phosphoamino acid analysis demonstrated that these enzymes phosphorylated PTP-1B on serine residues only. We determined that CLK1 and CLK2 phosphorylate PTP-1B on two sites within the catalytic domain, Ser\(^{50}\) and Ser\(^{242}/\)Ser\(^{243}\). Mutagenesis of Ser\(^{242}/\)Ser\(^{243}\) did not alter phosphatase activity nor did it affect the ability of CLK1 or CLK2 to activate PTP-1B, indicating this is not a regulatory phosphorylation site. However, substitution of an alanine at Ser\(^{50}\) significantly reduced the basal level of phosphatase activity of PTP-1B and the mutant phosphatase no longer activable by CLK1 or CLK2. Thus, phosphorylation of Ser\(^{50}\) is responsible for the observed activation of PTP-1B by CLK1 and CLK2.

Analysis of crystallographic data has shown that Ser\(^{50}\) lies near the substrate-binding pocket of PTP-1B (39). Furthermore, Sarmiento et al. (40) have recently shown that the three residues most responsible for determining the substrate specificity of PTP-1B are Tyr\(^{66}\), Arg\(^{17}\), and Asp\(^{48}\). The authors showed that mutation of these residues altered the \(K_{m}\) of these mutant enzymes. The dramatically reduced catalytic activity of the Ala\(^{50}\) mutant is consistent with the premise that the Ser\(^{50}\) residue is important for appropriate conformation of the substrate-binding pocket. Phosphorylation of this site may alter the characteristics of the binding pocket, and thereby lead to the activation of the phosphatase by shifting the binding pocket into a more open (active) state. Consistent with this hypothesis is the localization of Tyr\(^{66}\) in this same area of the protein. Moreover, alignment of YPTP1 with PTP-1B shows that the activating phosphorylation site on PTP-1B was Tyr\(^{66}\), which is directly phosphorylated by the enzyme, suggesting that an important regulatory serine phosphorylation site exists (5, 14). The previously identified sites of serine phosphorylation have been localized to the C-terminal regulatory domain of PTP-1B. Specifically, phosphorylation at these C-terminal sites has not been demonstrated to be responsible for enzyme activation, suggesting that the activating phosphorylation sites may lie within the catalytic domain (5).

PTP-1B has been shown to be one of the major tyrosine phosphatase activities within cells (14). Its activity and phosphorylation varies with the cell cycle and following stimulation with various cellular stimuli. However, the exact role of PTP-1B in the cell is not understood. Identified cellular targets for PTP-1B include the activated EGF receptor, the insulin receptor, and several integrins, suggesting that PTP-1B acts within cells to antagonize receptor driven signaling pathways (33–36). PTP-1B activity is at least partially controlled by regulation of intracellular compartmentalization, as it is localized to the endoplasmic reticulum by its C-terminal regulatory domain (37). Moreover, prolonged treatment of HeLa cells with insulin or 12-O-tetradecanoylphorbol-13-acetate leads to the alternative splicing of the PTP-1B mRNA, giving rise to a C-terminally truncated protein (38). This C-terminal truncation may be important in altering the subcellular localization of the enzyme. However, it is now apparent that phosphorylation of PTP-1B can directly control levels of activity of this phosphatase. PTP-1B activity is stimulated following EGF stimulation of A431 cells and phorbol ester treatment of HeLa cells (14, 15). PTP-1B appears to be regulated by multiple signaling pathways, as evidenced by discrete phosphorylation events following a variety of cellular stimuli. Treatment of cells with cAMP analogs leads to elevation in PTP-1B activity by 4-fold, while EGF stimulation of A431 cells leads to a 3-fold stimulation of phosphatase activity. However, these mechanisms drive this elevation in PTP-1B differentially, as cAMP promotes serine phosphorylation of PTP-1B while EGF stimulates the tyrosine phosphorylation of PTP-1B, suggesting the existence of multiple activating phosphorylation sites within the catalytic domain of PTP-1B. The first identified activating phosphorylation site on PTP-1B was Tyr\(^{66}\), which is directly phosphorylated by the EGF receptor (15). However, many cellular stimuli which activate PTP-1B lead only to serine phosphorylation of the enzyme, suggesting that an important regulatory serine phosphorylation site exists (5, 14). The previously identified sites of serine phosphorylation have been localized to the C-terminal regulatory domain of PTP-1B. Significantly, phosphorylation at these C-terminal sites has not been demonstrated to be responsible for enzyme activation, suggesting that the activating phosphorylation sites may lie within the catalytic domain (5).

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CLK Kinases Activate PTP-1B

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