Phosphatase PPM1A Regulates Phosphorylation of Thr-186 in the Cdk9 T-loop

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Cdk9 is the catalytic subunit of a general RNA polymerase II elongation factor known as positive transcription elongation factor b (P-TEFb). The kinase function of P-TEFb requires phosphorylation of Thr-186 in the T-loop of Cdk9 to allow substrates to access the catalytic core of the enzyme. To identify human phosphatases that dephosphorylate the T-loop of Cdk9, we used a Thr-186-phosphospecific antiserum to screen a phosphatase expression library. Overexpression of PPM1A and the related PPM1B greatly reduced Cdk9 T-loop phosphorylation in vitro. PPM1A and Cdk9 appear to associate in vivo as the proteins could be co-immunoprecipitated. The short hairpin RNA depletion of PPM1A resulted in an increase in Cdk9 T-loop phosphorylation. In phosphatase reactions in vitro, purified PPM1A could dephosphorylate Thr-186 both with and without the association of 7SK RNA, a small nuclear RNA that is bound to ~50% of total cellular P-TEFb. PPM1B only efficiently dephosphorylated Cdk9 Thr-186 in vitro when 7SK RNA was depleted from P-TEFb. Taken together, our data indicate that PPM1A and to some extent PPM1B are important negative regulators of P-TEFb function.

The positive transcription elongation factor b (P-TEFb) is a cellular kinase complex that regulates elongation of most mammalian protein-coding genes transcribed by RNA polymerase II (1, 2). P-TEFb enhances the processivity of RNA polymerase II through the phosphorylation of the carboxyl-terminal domain (CTD) of the polymerase as well as antagonizing the actions of negative factors such as negative elongation factor and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole sensitivity-inducing factor. P-TEFb is comprised of cyclin-dependent kinase 9 (Cdk9), the kinase core, and its cyclin partner, either Cyclin T1, Cyclin T2, or Cyclin K. Two isoforms of Cdk9 exist, a major 42-kDa protein and a minor 55-kDa protein that contains 117 residues at the amino terminus that are absent in the 42-kDa protein (3). In most cells examined, Cyclin T1 is the predominant regulatory subunit for Cdk9. The Cyclin T1/P-TEFb complex has been studied extensively as this P-TEFb complex is targeted by the human immunodeficiency virus type 1 Tat protein to activate RNA polymerase II transcription of the integrated provirus, and this is essential for viral replication.

As a key transcription factor, the activity of P-TEFb is carefully regulated within the cell. The first level of control is mediated by the regulated expression of its components, namely Cdk9 and its Cyclin partners. In primary human CD4+ T lymphocytes and monocytes, Cdk9 and Cyclin T2 expression are relatively high, whereas that of Cyclin T1 is low. Upon T cell activation or monocyte differentiation, Cyclin T1 protein expression is strongly up-regulated by post-transcriptional mechanisms, whereas expression of Cdk9 and Cyclin T2 remains relatively constant (4–9). Low levels of Cyclin T1 in resting CD4+ T cells and freshly isolated blood monocytes may function as one of the rate-limiting factors not only for transcription of many cellular genes but also for transcription of the human immunodeficiency virus type 1 provirus (10, 11).

In cells where the overall amount of P-TEFb has reached its steady state, another level of control is used. At this level, P-TEFb is negatively regulated by its reversible association with a small nuclear RNA known as 7SK and the HEXIM proteins HEXIM1 and HEXIM2. In cells examined to date, the HEXIM1 protein is expressed at an approximate 10-fold higher level than the HEXIM2 protein (12). In HeLa cells, roughly 50% of cellular P-TEFb complexes are associated with 7SK/HEXIM and are catalytically repressed (13, 14). Previous studies have shown that 7SK RNA functions as a scaffold for the formation of the P-TEFb7SK-HEXIM1 complex. However, the association of 7SK is not sufficient to inactivate the kinase complex as HEXIM1 has been identified as the key suppressor for P-TEFb function both in vitro and in vivo (15, 16). Although the binding of 7SK to P-TEFb does not require HEXIM1, the binding of HEXIM1 to P-TEFb is strictly 7SK-dependent (16). It is currently believed that the existence of two pools of P-TEFb in the nucleus, an active 7SK/HEXIM-free and a repressed 7SK/HEXIM-bound pool, allows the cell to maintain P-TEFb activity at a regulated yet dynamic equilibrium (2). Recent work has also identified LARP7 (17) and PIP7S (18) as proteins associated in the P-TEFb-7SK small nuclear ribonucleoprotein complex.

Given the importance of P-TEFb regulation in RNA polymerase II transcription, we were interested in identifying cellular factors that regulate P-TEFb activation in addition to 7SK...
and the HEXIM1/2 proteins. A critical regulatory residue in Cdk9 is Thr-186 in a region of the protein termed the T-loop. Cyclin-dependent kinases contain a conserved threonine in their T-loop, and phosphorylation of this residue induces a conformational change that allows Cdk substrates to access the catalytic core of the enzyme (19, 20). In the case of Cdk9, the association of 7SK with P-TEFb has been shown to be dependent on Thr-186 phosphorylation (21). A recent study reported that under conditions of stress, either UV irradiation or treatment with high concentrations of hexamethylene bisacetamide, the phosphatases PP2B and PP1α act cooperatively to disrupt P-TEFb from the 7SK small nuclear ribonucleoprotein through the dephosphorylation of Thr-186 (22). However, kinases and phosphatases that regulate Cdk9 T-loop phosphorylation under non-stress conditions have not been identified.

Phosphatases that dephosphorylate Thr-186 in Cdk9 are likely to be protein-serine/threonine phosphatases. Cellular protein-serine/threonine phosphatases are classified into three families (23): the PPMs (protein phosphatases, magnesium-dependent), which are mostly monomeric enzymes requiring the presence of Mg2⁺ or Mn2⁺ for catalytic activities; PPPs (phosphoprotein phosphatases) such as PP1A and PP2A, which are mostly oligomeric holoenzymes with related catalytic subunits bound by distinct regulatory subunits; and the less well characterized FCP/SCP (transcription factor IIF-associating CTD phosphatase/small CTD phosphatase) family represented by FCP1 and SCPs that dephosphorylate the CTD of RNA polymerase II.

In the present study, we utilized a newly developed antiserum that recognizes phosphorylated Thr-186 in the Cdk9 T-loop. Using a phosphatase expression library, we identified two cellular phosphatases, PPM1A and the related PPM1B, that when overexpressed can dephosphorylate the Cdk9 T-loop in vivo. We found that PPM1A associates with Cdk9 in vivo and can directly dephosphorylate the Cdk9 T-loop in vitro. Depletion of PPM1A with shRNAs resulted in an increase in Cdk9 T-loop phosphorylation. Our data also suggest that although 7SK may protect Thr-186 from being dephosphorylated by PPM1B, PPM1A is able to counteract this function and dephosphorylate Cdk9 even in the presence of 7SK association. Our data indicate that PPM1A and PPM1B are negative regulators of P-TEFb function.

**MATERIALS AND METHODS**

**Cells and Reagents**—HeLa cells and human embryonic kidney 293T cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum plus 1% penicillin/streptomycin. Anti-phospho-Cdk9 (Thr-186) antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Anti-Cdk9 (C-20), anti-Lamin A/C, and anti-HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PPM1A monoclonal antibody was purchased from Abeam, Inc. (Cambridge, MA). Anti-FLAG® M2 affinity gel, anti-FLAG M2 monoclonal antibody from mouse, anti-FLAG antibody from rabbit, anti-β-actin monoclonal antibody from mouse, and protein A-Sepharose® 4B were all purchased from Sigma-Aldrich.

**Expression Plasmids**—To construct the expression plasmids for HA-tagged Cdk9, the open reading frame of Cdk9 42-kDa isoform or its T186A mutant were amplified by PCR from pFLAG-CMV2-Cdk9 or pFLAG-CMV2-Cdk9T186A (29) and inserted into a pCNA6/3HA vector that contained 3 copies of hemagglutinin epitope tag at the amino terminus of the expression cassette. Expression plasmids for PPM1A and PPM1B, namely pRK5/FLAG-PPM1A and pRK5/FLAG-PPM1B, and dominant negative PPM1A (pRK5/FLAG-PPM1AD239N) have been described previously (24). Expression plasmids were transfected into HeLa cells using Lipofectamine™ 2000 transfection reagent (Invitrogen) at 5 μg each for 10-cm tissue culture dishes or at 500 ng each for 6-well tissue culture plates. For shRNA depletion experiments, HeLa cell cultures (6-well dishes) were transfected with 4 μg of the mouse shPPM1A or human shPPM1A plasmids (24) using Lipofectamine 2000 (Invitrogen); cells were extracted 3 days post-transfection.

**Immunoprecipitation and Immunoblots**—Whole cell extracts were prepared by lysing cells with EBC buffer (50 mol of Tris, p H 8.0, 120 mol of NaCl, 1% Triton X-100) containing protease inhibitor mixture (Sigma-Aldrich) and 10 mM vanadyl ribonucleoside complex (New England Biolabs). Cytoplasmic and nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology) according to the manufacturer’s instructions. 500 μg of whole cell lysates were precleared for 30 min with protein A-Sepharose. Cleared lysates were then incubated with 200 ng of appropriate antibodies at 4 °C for 2 h before being immunoprecipitated by protein A-Sepharose (for immunoprecipitation of HA) or FLAG M2 affinity gel (for immunoprecipitation of FLAG). Precipitated beads were washed three times with buffer TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and split into different tubes for immunoblots or other analyses. Cell lysates or immunoprecipitates were separated on 10% SDS-polyacrylamide gels, and immunoblots were performed as described previously using SuperSignal West Pico Chemiluminescent Substrate (Pierce) for detection (30). Immunoblots were quantified with the Personal Densitometer (GE Healthcare) and Image Quant software.

**Immunofluorescence**—HeLa cells were grown on glass coverslips in 24-well dishes prior to plasmid transfections. The cells were fixed and processed for immunofluorescence as described previously (31) using phospho-Cdk9 (Thr-186) (Cell Signaling Technology, Inc.), Cyclin T1 (T-18; Santa Cruz Biotechnology), and FLAG (M2; Sigma-Aldrich) antisera for primary incubations and appropriate secondary antibodies conjugated to green, red, and far-red fluorochromes, respectively. Cells were counterstained with 4’,6-diamidino-2-phenylindole, and deconvolution microscopy was conducted as described previously (31). Images were deconvolved, and representative single z-sections from each series were prepared for presentation using Adobe Photoshop.

**In Vitro Phosphatase Assay**—HA-tagged Cdk9 and FLAG-tagged phosphatases were purified from cells lysates by immunoprecipitation. FLAG-phosphatases were eluted from FLAG M2 affinity gel by competition with 150 ng/μl 3× FLAG pep-
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tide (Sigma-Aldrich). In vitro phosphatase assays were carried out as described previously (24) by incubating FLAG-phosphatases with HA-Cdk9-containing protein A-Sepharose in a phosphatase buffer (7 mM imidazole, pH 7.6, 20 mM magnesium acetate, 300 μg/ml bovine serum albumin, 30 μM EDTA, 0.03% β-mercaptoethanol) at 30 °C for 30 min. Reactions were stopped by the addition of 4× Laemmli buffer and boiling at 90 °C for 5 min. Phosphorylation of Cdk9 at Thr-186 was then examined in immunoblots using anti-phospho-Cdk9 (Thr-186) antibody.

RNA Extraction and Northern Blots—P-TEFb complex was purified from 2 mg of HeLa cell nuclear extracts by the immunoprecipitation of HA-tagged Cdk9. 7SK small nuclear RNA was stripped from the complex as described above by first washing with TBS containing 0.3 M KCl and then washing with TBS containing 0.8 M KCl. One-tenth of the purified complex was used for immunoblots analysis, whereas RNA in the remaining portion was extracted with TRIzol reagent (Invitrogen) and resolved on a 10% urea-polyacrylamide gel. Northern blotting for the detection of 7SK and U1 small nuclear RNA was performed with the anti-FLAG antibody.

RESULTS

Specificity of Antiserum against Phosphorylated Cdk9 T-loop—To identify phosphatases that regulate phosphorylation of the Cdk9 T-loop, we first evaluated the specificity of an antiserum raised against a peptide sequence from Cdk9 containing a phosphorylated Thr-186. HeLa cells were transfected with plasmids that express either a FLAG-tagged wild type Cdk9 42-kDa protein or a mutant Cdk9 protein containing an alanine substitution for Thr-186 (T186A). Extracts were prepared, and expression of the wild type and mutant tagged Cdk9 proteins was examined in immunoblots (Fig. 1A). The FLAG antiserum demonstrated that both wild type and T186A Cdk9 proteins were expressed at similar levels. The antiserum against the phospho-T-loop recognized the overexpressed wild type FLAG-Cdk9 but not the FLAG-T186A protein. These data and results of in vitro dephosphorylation assays (see Figs. 4 and 5 below) indicate that the phospho-T-loop antiserum specifically recognizes the phosphorylated Cdk9 T-loop. This antiserum will henceforth be referred to as α-p-Cdk9 antiserum; the antiserum directed against a peptide at the carboxyl terminus of Cdk9 and that recognizes total Cdk9 will be referred to as α-Cdk9.

Overexpression of PPM1A or PPM1B Reduces Cdk9 Thr-186 Phosphorylation—Phosphatases that regulate Thr-186 phosphorylation in the Cdk9 T-loop should belong to the protein-serine/threonine phosphatase family. We therefore utilized a previously described phosphatase expression library (24) that contains the catalytic subunits of 39 protein-serine/threonine phosphatases, including 14 PPMs, 13 phosphoprotein phosphatases, five transcription factor IIF-associated CTD phosphatase/small CTD phosphatases, one PH domain phosphatase (PH domain and leucine-rich repeat protein phosphatase), four dual specificity protein phosphatases, and two pyrophosphatases. We used the α-p-Cdk9 antiserum to screen for phosphatases that reduce Cdk9 phosphorylation in overexpression experiments.

Cultures of 293T cells were co-transfected with FLAG-tagged Cdk9 and individual phosphatase expression plasmids, extracts were prepared, and the levels of Thr-186 phosphorylation were examined in immunoblots. Two strong candidate phosphatases were identified in this screen: the related PPM1A and PPM1B proteins. PPM1A and PPM1B (also called PP2Cα and PP2Cβ, respectively) are ubiquitously expressed monomeric enzymes and require Mg2+ or Mn2+ for catalytic activity (25). Of note, both PPM1A and PPM1B have been shown to dephosphorylate the T-loop of Cdk2 and Cdk6 (26, 27). To confirm that these phosphatases regulate Cdk9 T-loop phosphorylation, His-tagged expression plasmids for the two phosphatases and for a negative control SCP4 were co-transfected with a FLAG-tagged Cdk9 protein into 293T cells. Extracts were prepared and immunoprecipitated with the anti-FLAG antibody, and proteins present in immunoprecipitations were analyzed in immunoblots (Fig. 2). Overexpression of PPM1A, PPM1B, and SCP4 did not alter the overall expression level of the FLAG-Cdk9 protein present in immunoprecipitates as measured with the α-Cdk9 antibody. However, overexpression of either PPM1A or PPM1B, but not SCP4, greatly reduced Cdk9 T-loop phosphorylation. Overexpression of PPM1A and PPM1B also greatly reduced the amount of the HEXIM1 protein that co-immunoprecipitated with FLAG-Cdk9. The reduction in HEXIM1 associated with Cdk9 was expected as the association of HEXIM1 and 7SK RNA with P-TEFb requires phosphorylation of Thr-186 in the Cdk9 T-loop (21). We also observed in another co-immunoprecipitation experiment that overexpression of PPM1A reduced the association of 7SK RNA and HEXIM1 with a transfected FLAG-Cdk9 protein (data not shown).

PPM1A is localized both in the cytoplasm and nucleus, whereas PPM1B is found predominantly in the cytoplasm (25), suggesting that PPM1A is able to dephosphorylate Thr-186 throughout the cell, whereas the activity of PPM1B for Cdk9 may be restricted to the cytoplasm. Additionally our results from experiments described below indicated that PPM1A may play a greater role than PPM1B in regulation of Cdk9 T-loop phosphorylation (see Figs. 4 and 5 below). We therefore
focused on PPM1A and carried out an immunofluorescence analysis as an independent method to assess the consequence of overexpression on Cdk9 T-loop phosphorylation. A control experiment demonstrated that preincubation of fixed HeLa cells with a phosphorylated Thr-186 peptide abolished the immunofluorescence signal with the α-p-Cdk9 antiserum, demonstrating the specificity of the antiserum (data not shown). HeLa cells were transfected with a FLAG-PPM1A expression plasmid, and expression of endogenous Cyclin T1, endogenous Cdk9, and FLAG-PPM1A were examined (Fig. 3). Endogenous Cdk9 T-loop phosphorylation was high in cells not expressing the FLAG-PPM1A protein. In contrast, endogenous T-loop phosphorylation was significantly reduced in cells expressing the FLAG-PPM1A protein relative to non-transfected cells as indicated by the yellow signal in the merged images between Cyclin T1 and T-loop-phosphorylated Cdk9.

To further investigate the role of PPM1A in Cdk9 T-loop phosphorylation, HeLa cell cultures were transfected with a plasmid that expresses shRNA against human PPM1A or a negative control plasmid that expresses shRNA against mouse PPM1A; the human and mouse target sequences in PPM1A mRNAs are divergent, and the mouse shRNA cannot therefore deplete the human PPM1A protein (24). After 3 days of plasmid transfections, cell extracts were prepared, and immunoblots were performed to examine PPM1A, total Cdk9, and T-loop-phosphorylated Cdk9 (Fig. 4). The small interfering RNA against human PPM1A was able to deplete PPM1A levels about 7-fold relative to the negative control mouse PPM1A small interfering RNA. Although the overall level of Cdk9 was unaffected by the PPM1A depletion, T-loop phosphorylation was increased ~2.5-fold by the depletion, indicating that a reduction in PPM1A protein expression results in an elevated level of Cdk9 T-loop phosphorylation. We conclude from the data presented in Figs. 2–4 that PPM1A is a cellular phosphatase that can negatively regulate the phosphorylation of Thr-186 in the Cdk9 T-loop.

**FIGURE 2. Overexpression of PPM1A reduces Cdk9 Thr-186 phosphorylation.** 293T cells were co-transfected with FLAG-Cdk9 and His-tagged phosphatases. Cell extracts were prepared at 36 h post-transfection, and immunoprecipitations (IP) were performed with anti-FLAG antibody. The α-p-Cdk9 and α-HEXIM1 antisera were used in immunoblots (IB) to measure the level of Cdk9 Thr-186 phosphorylation and HEXIM1 protein in immunoprecipitates. The α-Cdk9 antiserum was used in the immunoblot to verify equal recoveries of total Cdk9 in immunoprecipitates. Whole cell extracts (WCL) were used in the immunoblot with α-His antiserum to monitor expression levels of His-tagged phosphatases.

**FIGURE 3. Immunofluorescence analysis of overexpressed PPM1A.** HeLa cells grown on coverslips were transfected with FLAG-PPM1A. At 36 h post-transfection, cells were fixed and stained with α-FLAG (1:20,000), α-p-Cdk9 (1:50), and α-Cyclin T1 (1:200) antisera. Lower panels show a different field of cells in enlarged form. The scale bar is 10 μm in all pictures. DAPI, 4',6-diamidino-2-phenylindole.
retained the ability to bind Cdk9, indicating that phosphatase activity is not required for the interaction. We note that the level of the D239N protein that was associated with Cdk9 was slightly higher than that of the wild type PPM1A protein, suggesting that the dephosphorylation of the Cdk9 T-loop by PPM1A may contribute to the disruption of the association between the phosphatase and Cdk9.

To determine whether PPM1A and PPM1B can directly dephosphorylate Thr-186 in the T-loop in Cdk9, we utilized an *in vitro* phosphatase assay system described previously (24). HA-tagged Cdk9 was expressed by plasmid transfection in HeLa cell cultures and purified by immunoprecipitation. The purified FLAG-PPM1A and FLAG-PPM1B from the experiment shown in Fig. 5A were incubated for 30 min under phosphatase reaction conditions with immobilized Cdk9-P-TEFb immunoprecipitation complexes. The products of dephosphorylation reactions were then examined in immunoblots using the α-p-Cdk9 antiserum (Fig. 5B). PPM1A was able to greatly dephosphorylate the Cdk9 T-loop, whereas PPM1B was able to dephosphorylate ~50% of Cdk9. These data suggest that PPM1A and to some extent PPM1B can directly dephosphorylate the Cdk9 T-loop.

Dependence of Mg²⁺ for T-loop Dephosphorylation and Effects of 7SK RNA—We wished to evaluate the effect of 7SK on Cdk9 T-loop dephosphorylation *in vitro*. We therefore evaluated the ability of the phosphatases to dephosphorylate Thr-186 with and without the deletion of 7SK RNA from Cdk9 immunoprecipitates. FLAG-tagged phosphatases were purified from transfected HeLa cells by immunoprecipitation and elution with free FLAG peptide. HA-Cdk9-P-TEFb complexes were immunoprecipitated from transfected HeLa cells, and these bead complexes were used for *in vitro* phosphatase assays. Depletions of 7SK were carried out by high salt washes of immunoprecipitates. The analysis of portions of salt-washed
characteristic of the PPM family members is the dependence on Mg$^{2+}$, presumably the portion associated with 7SK/HEXIM proteins. A mutation of Thr-186 was resistant to dephosphorylation by PPM1B, preceding the association of 7SK RNA with the Cdk9 T-loop and therefore is a negative regulator of P-TEFb function. The identification of PPM1A as a regulator of Cdk9 is not unexpected as PPM1A has been shown previously to dephosphorylate the T-loops of Cdk2 and Cdk6, two Cdk family members involved in cell cycle regulation (26, 27).

PPM1A is a key regulator of cell growth and signaling, and a number of its substrates have been identified. PPM1A regulates the cell cycle through the dephosphorylation of Cdk2 and Cdk6 (26, 27). PPM1A inhibits mitogen signaling through the c-Jun NH$_2$-terminal kinase (JNK) and p38 pathways through the dephosphorylation of factors that regulate these pathways (25). PPM1A has an important role in development and other processes by terminating transforming growth factor-β (TGF-β) signaling through the dephosphorylation of Smad 2/3 (24). Because P-TEFb is required for the expression of many if not most protein-coding genes, the identification of PPM1A as a negative regulator of Cdk9 provides insight into mechanisms that can control global gene expression. For example, in situations where the transcriptional needs of the cell are low, such as in resting lymphocytes or monocytes, dephosphorylation of Cdk9 by PPM1A may be involved in a general repression of transcription.

In this study, we also found that overexpression of PPM1B reduced Cdk9 Thr-186 phosphorylation in vivo. However, our data suggest that PPM1B may be a minor regulator of Cdk9 relative to the regulatory role of PPM1A. PPM1B is largely a cytoplasmic protein, indicating that PPM1B may have activity only for cytoplasmic Cdk9, whereas PPM1A is found in both the cytoplasm and nucleus and may have activity for all Cdk9 in the cell. PPM1B appears to efficiently dephosphorylate Cdk9 in vitro only after the depletion of 7SK RNA, suggesting that the association of Cdk9 with 7SK/HEXIM proteins protects it from the phosphatase activity of PPM1B. The association of Cdk9 with 7SK/HEXIM proteins is dynamic and shuttles between 7SK/HEXIM-free and 7SK/HEXIM-bound states (2). Because the association of Cdk9 with 7SK/HEXIM is dependent upon Thr-186 phosphorylation (21), the action of PPM1B on the 7SK/HEXIM-free Cdk9 may lead to a near complete dephosphorylation of the Thr-186 as no Cdk9 can associate with 7SK/HEXIM after the action of PPM1B.

Although we were able to co-immunoprecipitate endogenous Cdk9 and overexpressed PPM1A in multiple independent experiments such as the one shown in Fig. 5A, our experiments did not address the nature of the association between PPM1A with P-TEFb. It seems unlikely that PPM1A makes a direct protein-protein interaction with Cdk9 as we were unable to specifically co-immunoprecipitate either endogenous or overexpressed PPM1A with overexpressed Cdk9 in plasmid transfection experiments. However, we could detect Cyclin T1 in co-immunoprecipitations with overexpressed PPM1A (data not shown), suggesting that PPM1A associates with Cdk9 that is bound to a Cyclin subunit.

In our screen of a phosphatase expression library, we also observed that both PP1A and PP2B reduced Cdk9 T-loop phosphorylation but not to the extent of PPM1A and PPM1B (data not shown). Additionally PP1A and PP2B did not significantly reduce the association of HEXIM1 with Cdk9 unlike PPM1A and PPM1B, which largely abolished this association. A recent
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study reported that PP2B can act cooperatively with PP1α to release P-TEFb from the 7SK small nuclear ribonucleoprotein through dephosphorylation of Cdk Thr-186 when cells are subjected to stress by either UV irradiation or hexamethylene bisacetamide treatment (22). Although we did not carry out phosphatase reactions with purified PP1A or PP2B, it is possible that overexpression of these phosphatases under nonstressed conditions may indirectly lead to reduced Cdk9 T-loop dephosphorylation. PP2A has been shown previously to regulate phosphorylation of several serine and threonine residues in the carboxyl terminus of Cdk9 (28). It is possible that overexpression of PP1A or PP2B may result in dephosphorylation in Cdk9 serine/threonine residues outside the T-loop, and this may lead to forms of Cdk9 in which Thr-186 is susceptible to dephosphorylation by PPM1A or PPM1B.

PPM1A and PPM1B are monomeric enzymes and do not require a regulatory subunit for either catalytic function or substrate recognition. An important issue concerning PPM1A and PPM1B is whether the activities of the phosphatases for Cdk9 are regulated. It is possible that they are regulated through post-translational modifications or by unidentified regulatory partners. Alternatively the activities of PPM1A and PPM1B for Cdk9 may be constitutively active. Thr-186 phosphorylation may be regulated by the reversible association of 7SK RNA and HEXIM1/2 and perhaps other proteins with Cdk9. In this scenario, mechanisms and signals that regulate the association of Cdk9 with 7SK/HEXIM may confer to the cell the ability to adjust Thr-186 phosphorylation levels to meet varying transcriptional requirements.

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