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Cdk9 T-loop Phosphorylation is Regulated by the Calcium Signaling Pathway

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Abstract

Eukaryotic RNA polymerase II transcriptional elongation is a tightly regulated process and is dependent upon positive transcription elongation factor-b (P-TEFb). The core P-TEFb complex is composed of Cdk9 and Cyclin T and is essential for the expression of most protein coding genes. Cdk9 kinase function is dependent upon phosphorylation of Thr186 in its T-loop. In this study, we examined kinases and signaling pathways that influence Cdk9 T-loop phosphorylation. Using an RNAi screen in HeLa cells, we found that Cdk9 T-loop phosphorylation is regulated by Calcium/Calmodulin-dependent kinase 1D (CaMK1D). Using small molecule inhibitors in HeLa cells and primary CD4⁺ T lymphocytes, we found that the Ca²⁺ signaling pathway is required for Cdk9 T-loop phosphorylation. Inhibition of Ca²⁺ signaling led to dephosphorylation of Thr186 on Cdk9. In reporter plasmid assays, inhibition of the Ca²⁺ signaling pathway repressed the PCNA promoter and HIV-1 Tat transactivation of the HIV-1 LTR, but not HTLV-1 Tax transactivation of the HTLV-1 LTR, suggesting that perturbation of the Ca²⁺ pathway and reduction of Cdk9 T-loop phosphorylation inhibits transcription units that have a rigorous requirement for P-TEFb function.

Keywords

Cdk9; T-loop phosphorylation; Ca²⁺ signaling

Introduction

RNA polymerase II (RNAP II) transcription is comprised of multiple steps which are tightly regulated. The transcriptional elongation stage is critical for expression of most eukaryotic genes. A recent report examined whole genome occupancy of RNA polymerase II and found the enzyme to be paused in the promoter-proximal regions of most genes (Rahl et al., 2010). Recruitment of positive transcription elongation factor-b (P-TEFb) to these regions was essential for relieving the pause and ensuring transcript elongation (Rahl et al., 2010). This and other studies (Guenther et al., 2007; Hargreaves et al., 2009) reiterate the importance of P-TEFb and transcriptional elongation in cellular gene expression. P-TEFb is also involved in mRNA processing (Bres et al., 2008), histone modifications (Pirngruber et al., 2009) and is essential for HIV-1 gene expression (Mancebo et al., 1997; Yang et al., 1996; Zhu et al., 1997).

P-TEFb is a serine-threonine kinase complex with a catalytic subunit, Cdk9 and a regulatory subunit that is either Cyclin T1 or Cyclin T2. Cyclin T1 appears to be the major Cyclin partner of Cdk9 in most cell types. There are two isoforms of Cdk9: a major 42 kDa and a

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minor 55 kDa which are differentially expressed and localized (Liu and Herrmann, 2005; Liu et al., 2010; Shore et al., 2005). The primary function of P-TEFb is to hyperphosphorylate the C-terminal domain (CTD) of RNAP II and negative elongation factors, DSIF and NELF, resulting in mRNA elongation (Peterlin and Price, 2006). Cdk9 is regulated by post-translational modifications such as phosphorylation, dephosphorylation, ubiquitylation and acetylation (Barboric et al., 2005; Chen et al., 2008; Ramakrishnan et al., 2009; Sabo et al., 2008; Wang et al., 2008). Cdk9 kinase function is contingent on the phosphorylation status of Thr186 residue in the T-loop (Chen et al., 2004). This phosphorylation event changes the conformation of the T-loop allowing substrate and ATP into the Cdk9 catalytic site (Russo et al., 1996). A functional equilibrium of P-TEFb is maintained by its reciprocal association with positive and negative regulators. P-TEFb associates with HEXIM1 and other proteins within a scaffold generated by a small nuclear RNA, 7SK (Chen et al., 2004). This association in the 7SK snRNP complex requires that the Cdk9 T-loop is phosphorylated and serves to sequester the P-TEFb complex preventing aberrant transcription (Chen et al., 2004; Diribarne and Bensaude, 2009). P-TEFb is recruited to the HIV-1 promoter by viral transactivator, Tat (Karn, 2011). The requirement of P-TEFb for expression of most protein-coding genes is achieved by its recruitment to these promoters by Brd4 (Yang et al., 2008). We had recently reported that Cdk9 T-loop phosphorylation is very low in resting primary CD4⁺ T lymphocytes and is induced rapidly in a protein synthesis-independent manner when the cells are activated (Ramakrishnan et al., 2009). Although recombinant Cdk9 can phosphorylate its own T-loop by an autoactivation mechanism (Baumli et al., 2008), this auto-phosphorylation is relatively inefficient and it is possible, if not likely, that a yet unidentified activating kinase is responsible for T-loop phosphorylation. In resting CD4⁺ T lymphocytes where T-loop phosphorylation is low, Cdk9 is associated with its Cyclin partners, suggesting that either autophosphorylation is inhibited in resting cells or its activating kinase is not functional until T cell activation (Ramakrishnan et al., 2009).

In this study, we screened for kinases that regulate Cdk9 T-loop phosphorylation. We performed a siRNA screen of 78 ubiquitously expressed serine-threonine kinases and examined the effect on Cdk9 T-loop phosphorylation. We found that depletion of Ca²⁺/Calmodulin-dependent kinase 1D (CaMK1D) reduced Cdk9 T-loop phosphorylation. Furthermore, inhibition of Ca²⁺ signaling using small molecule inhibitors targeting different steps of the Ca²⁺ - Calmodulin pathway decreased Cdk9 T-loop phosphorylation. The inhibition of Ca²⁺ signaling also inhibited P-TEFb function in reporter plasmid assays and this effect could be partially rescued by a phosphomimetic Cdk9 mutant.

Materials and Methods

Cell culture

Peripheral blood mononuclear cells (PBMCs) from healthy blood donors (Gulf Coast Regional Blood Center, Houston) were purified by Ficoll-Hypaque (GE Healthcare) density gradient centrifugation. CD4⁺ T lymphocytes were isolated by negative selection using MACS CD4⁺ cell isolation kit II (Miltenyi Biotech). Activated cells were depleted from the CD4⁺ T lymphocytes using anti-CD30 microbeads to purify resting cells. The purity and resting status of these cells were determined to be >98% and >95% in G0/G1 cell cycle stage respectively, by flow cytometry as described previously (Ramakrishnan, 2009). HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Activation and treatment of cells

Resting CD4⁺ T lymphocytes at 10×10^6 cells/ml were activated with ionomycin (1 μ M) or phorbol 12-myristate 13-acetate (PMA) (1ng/ml) + ionomycin. Resting cells were treated with Thapsigargin (Sigma) (100 μ M); activated cells were treated with 50 μ M W-7 (N-(6-Aminoethyl)-5-chloro-1-naphthalenesulfonamide) (Calbiochem) for different time points as indicated in the text. In some experiments, HeLa cells were treated with W-7 in a dose-dependent manner for seven hours. In other experiments, HeLa cells were treated with 100 nM KN-93 or negative control KN-92 for different time points. MG101 (Sigma) was used at a final concentration of 50 μ M.

Immunoblots

Cells following treatment were lysed with EBCD buffer (50mM Tris-HCl, pH 8.0, 120mM NaCl, 0.5% NP-40, 5mM dithiothreitol) containing protease inhibitor cocktail (Sigma). Immunoblotting was performed as described previously (Ramakrishnan, 2009). T-loop phosphorylated Cdk9, T-loop phosphorylated Cdk2, Cdk9, Cdk7 and β -Actin were probed using antibodies against T-loop phosphorylated Cdk9 (p-Cdk9; Cell Signaling) (1:500), T-loop phosphorylated Cdk2 (p-Cdk2; Cell Signaling) (1: 250), Cdk9 and Cdk7 (Santa Cruz Biotech), and β -actin (Sigma) (1:5000), respectively. Immunoblots were quantified using Image J software (Abramoff, 2004).

RNAi screen

Seventy eight serine-threonine kinases that are constitutively expressed were selected by a literature search. For each gene in the screen, 3 siRNAs targeting different regions of the specific mRNA was designed and synthesized (1nM) by Ambion/Applied Biosystems. The list of genes and target sequences are shown in Supplemental Figure 1. The lyophilized siRNAs were reconstituted in nuclease-free water and frozen as aliquots at -80°C . Each siRNA (50 pmol) was delivered into HeLa cells by a reverse transfection method. Briefly, HeLa cells in DMEM with 10% FBS and no antibiotics were seeded the day before transfection in 10 cm culture plates. On the day of transfection, cells were collected by trypsinization and suspended in DMEM with 10% FBS and no antibiotics (80,000 cells/well/sample). siPORT neoFX transfection reagent (Ambion/Applied Biosystems) was diluted in Opti-MEM1 (Invitrogen) and incubated at room temperature (RT) for 10 minutes. SiRNAs were aliquoted into each well of 12-well tissue culture plates. The diluted transfection reagent was added to the siRNA and incubated for a further 10 minutes at RT for complex formation. Cells were added to this complex, mixed and incubated for 72 hours. Cell lysates were prepared in EBCD and immunoblotted for p-Cdk9, Cdk9 and β -actin. The criterion for calling a kinase a “hit” in the screen was that at least two of the three siRNAs against the protein reduced Cdk9 T-loop phosphorylation relative to control samples.

Plasmid transfection and Luciferase assay

HeLa cells in 12-well plates were transfected overnight with 1 μ g pNL4-3-Tat-Luc or PCNA-Luc or pHTLV-LTR-Luc (250ng) + pTax (50ng) using Lipofectamine 2000 (Invitrogen). pNL4-3-Tat-Luc contains Firefly Luciferase in place of Nef in a HIV-1 proviral plasmid (Connor et al., 1995). pPCNA-Luc containing the full-length (~1250) PCNA promoter and pHTLV-1-LTR-Luc and pHTLV-1Tax were kindly provided by Dr. S. Marriott (Baylor College of Medicine). To ensure equal amounts of DNA were transfected in experiments with pHTLV-1-LTR-Luc and pHTLV-1Tax, pcDNA empty vector was co-transfected with pNL4-3-Tat-Luc. In some experiments, HeLa cells were cotransfected with pNL4-3-Tat-Luc and Flag-wild type Cdk9 or a phosphomimetic Flag-T186D Cdk9 mutant. The Flag-T186D Cdk9 plasmid that contains a substitution of aspartic acid for threonine at residue 186 was generated by site-directed mutagenesis of Flag-wild type Cdk9. The

substitution was verified by DNA sequencing the mutated plasmid and its expression was confirmed by immunoblot (data not shown). The transfected cells were treated with 50 μ M W-7 for seven hours before harvesting. Cell lysates were analyzed for Luciferase activity using Luciferase Assay Kit (Promega) according to manufacturers' protocol. Total protein in the lysates was estimated using a Bradford assay (Bio-Rad).

Results

CamK1D regulates Cdk9 T-loop phosphorylation

To identify kinases that regulate Cdk9 T-loop phosphorylation, we carried out a siRNA library screen of 78 ubiquitously expressed serine-threonine kinases (Supplemental Figure 1). Three siRNAs were designed to target different regions of the each kinase transcript. SiRNAs were introduced into HeLa cells by reverse transfection, cells were lysed 72 hours post-transfection, and immunoblots performed to examine Cdk9 T-loop phosphorylation levels. The criterion for calling a kinase a "hit" in the screen was that at least two of the three siRNAs against the protein reduced Cdk9 T-loop phosphorylation relative to control samples. When two of the three siRNAs against the kinase failed to show an effect on Cdk9 T-loop phosphorylation, the third siRNA was not used in the screen. The summary of the siRNA screen results is shown in Supplementary Table 1. After completion of this screen and further analysis of positive hits in the screen, depletions of Ca^{2+} /Calmodulin-dependent kinase 1D (CaMK1D) were found to consistently reduce Cdk9 T-loop phosphorylation. The effects of siRNA depletion of CaMK1D, related calcium/Calmodulin-dependent kinases, and other kinases on Cdk9 T-loop phosphorylation are shown in Figure 1.

CaMK1D is localized to the cytoplasm and has no canonical nuclear localization signal, suggesting it is absent from the nucleus (Hook and Means, 2001) where the majority of Cdk9 localizes (Dow et al., 2010; Herrmann and Mancini, 2001). We over-expressed a HA-tagged CaMK1D in HeLa cells by plasmid transfection to investigate the kinase's role in Cdk9 T-loop phosphorylation. However, over-expression of CaMK1D did not result in elevated levels of Cdk9 T-loop phosphorylation in HeLa cells (data not shown). Increasing intracellular Ca^{2+} levels in HeLa cells by ionomycin treatment in conjunction with ectopic expression of CaMK1D also did not affect the level of Cdk9 T-loop phosphorylation (data not shown). Co-immunoprecipitation experiments with nuclear and cytoplasmic extracts in HeLa cells overexpressing CaMK1D failed to show any interaction of Cdk9 with CaMK1D (data not shown). Taken together, these negative data suggest that CaMK1D is likely to be an indirect regulator for the Cdk9 T-loop phosphorylation. However, as presented below, the induction of Cdk9 T-loop phosphorylation by PMA+ ionomycin in primary CD4^{+} T lymphocytes and the reduction of Cdk9 T-loop phosphorylation following CaMK1D depletion in HeLa cells indicate an important functional involvement of CaMK- Ca^{2+} signaling pathway in regulating Cdk9 T-loop phosphorylation.

Inhibitors of Ca^{2+} signaling pathway components reduce Cdk9 T-loop phosphorylation in HeLa cells

To determine whether the Ca^{2+} signaling pathway indeed regulates Cdk9 T-loop phosphorylation, we carried out experiments with small molecule inhibitors. Inhibition of CaMK has been reported to reduce Cdk2 activity (Kahl and Means, 2004). We verified the effect of treating HeLa cells with CaMK inhibitor KN-93 on Cdk2 T-loop phosphorylation. We found that inhibiting CaMK reduced the level of Cdk2 T-loop phosphorylation compared to cells treated with negative control, KN-92 (Figure 2A). HeLa cells were treated with the CaMK inhibitor KN-93 for one hour and four hours and Cdk9 T-loop phosphorylation was examined in immunoblots (Figure 2B). Inhibition of CaMK by KN-93 reduced the level of T-loop phosphorylation relative to the negative control structural

analog, KN-92. After one and four hours of KN-93 treatment, Cdk9 T-loop phosphorylation was reduced by 55% and 59%, respectively, relative to the negative control KN-92 when normalized to the loading control Cdk7 (Figure 2B). We also observed that total Cdk9 levels were reduced by approximately 15% by either one or four hours of KN-93 treatment. This observation of the reduction of total Cdk9 is discussed below.

We also treated HeLa cells with W-7, a Calmodulin (CaM) inhibitor and examined the effects on Cdk9 T-loop phosphorylation. We first confirmed the inhibitory effect of W-7 on Cdk2 T-loop phosphorylation as a positive control since CaMKs are regulated by Ca^{2+} /CaM binding (Corcoran and Means, 2001). Extracts were prepared after seven hours of either 20 μM or 50 μM W-7 treatment and Cdk2 T-loop phosphorylation and Cdk9 T-loop phosphorylation was examined in an immunoblot (Figure 2C and 2D). W-7 treatment of HeLa cells reduced the level of phosphorylated Cdk2 (Figure 2C). The inhibition of Calmodulin by 50 μM W-7 resulted in a 54% reduction in the level of T-loop phosphorylation. Additionally, 50 μM W-7 treatment resulted in a 29% reduction in total Cdk9 relative to the sample without W-7 treatment when normalized to loading control β -Actin (Figure 2D). We used propidium iodide staining and flow cytometry to examine apoptosis in this experiment, and no apoptosis was seen with the 50 μM W-7 treatment for seven hours (data not shown).

Inhibitors of Ca^{2+} signaling pathway components reduce Cdk9 T-loop phosphorylation in primary CD4^+ T lymphocytes

Resting primary CD4^+ T lymphocytes have low levels of Cdk9 T-loop phosphorylation that is rapidly induced independent of *de novo* protein synthesis (Ramakrishnan et al., 2009). We treated resting primary CD4^+ T lymphocytes with the Ca^{2+} signaling pathway inhibitor Thapsigargin (100 μM) and the cells were activated with either the Ca^{2+} ionophore, ionomycin, or PMA + ionomycin for one hour. When the level of Cdk9 T-loop phosphorylation was examined in immunoblots, we found that blocking Ca^{2+} signaling by Thapsigargin prevented the induction of T-loop phosphorylation compared to DMSO vehicle control treated cells (Figure 3A). Total Cdk9 levels were also reduced by Thapsigargin inhibition of the Ca^{2+} signaling pathway.

We also examined effects of W-7 on Cdk9 T-loop phosphorylation in primary CD4^+ T lymphocytes. The lymphocytes were first activated with PMA + ionomycin for 60 hours and strong induction of Cdk9 T-loop phosphorylation was observed as expected. The activated cells were then treated with 50 μM W-7 and cell extracts were prepared at 30 minutes, two hours, and four hours post W-7 treatment. Calmodulin inhibition by W-7 reduced the levels of T-loop phosphorylated Cdk9 by about 84% and total Cdk9 by about 38% after four hours of treatment (Figure 3B).

Treatment of resting primary CD4^+ T lymphocytes with inhibitors to other canonical T lymphocyte activation pathways (PKC, NF-AT, Lck, PI3K, PKA, MEK) did not appear to affect the level of Cdk9 T-loop phosphorylation (data not shown). These data from both HeLa cells and primary CD4^+ T lymphocytes indicate that inhibition of the Ca^{2+} -Calmodulin regulated pathway reduces Cdk9 T-loop phosphorylation.

Cdk9 protein stability appears to be mediated by the proteasomal pathway

We observed in the course of these experiments that inhibition of Cdk9 T-loop phosphorylation by siRNA to CaMK1D (Figure 1) or small molecule inhibitors (Figure 2 and 3) led to a reduction of total Cdk9 levels. This observation suggests that Cdk9 protein stability may be linked to T-loop phosphorylation. We explored if the proteasomal pathway was involved in Cdk9 protein turnover under these experimental conditions. HeLa cells were

treated with W-7 for seven hours and proteasomal-mediated proteolysis of Cdk9 was blocked by the inhibitor MG101. As a control, HeLa cells were treated with MG101 alone to rule out if inhibiting proteasomal pathway in the absence of W-7 treatment has an effect on phosphorylated and overall Cdk9 levels. The W-7 treatment reduced the levels of both phosphorylated Cdk9 and total Cdk9, and MG101 treatment for one hour partially rescued this reduction (Figure 4). These data suggest that Cdk9 protein turnover is mediated by the proteasomal pathway.

We also investigated in a detailed time course experiment if reduction in Cdk9 T-loop phosphorylation by CaM inhibition precedes Cdk9 protein degradation. HeLa cells were treated with 50 μ M W-7 and samples collected every hour for three hours. Cell lysates were prepared and immunoblot analysis carried out to probe for levels of Cdk9 T-loop phosphorylation, overall Cdk9 and Cyclin T1. We found that there was a decrease in the level of Cdk9 T-loop phosphorylation with no change in the overall Cdk9 protein level two hours after W-7 treatment (Figure 5). There was no change in the expression level of Cyclin T1. This suggests that dephosphorylation of Cdk9 T-loop by W-7 treatment precedes the degradation of total Cdk9 protein.

P-TEFb function is dependent on Ca^{2+} signaling

P-TEFb is required for RNA polymerase II elongation of the HIV-1 genome and most protein coding genes (Chao and Price, 2001). To assess the impact of Cdk9 T-loop phosphorylation status on its function in the P-TEFb complex, we carried out plasmid reporter assays. We used a HIV-1 reporter plasmid NL4-3-Tat that requires Tat to activate viral LTR-directed gene expression. We also used a Luciferase expression plasmid under control of the PCNA promoter, which appears to be dependent on P-TEFb as PCNA mRNA levels are reduced upon Cyclin T1 depletion with siRNA (Yu et al., 2006). Furthermore, to verify if the effect of inhibiting Ca^{2+} signaling is specific for promoters that have a strong requirement for P-TEFb function, we used a HTLV-1-LTR luciferase expression plasmid in conjunction with its transactivator Tax. We have previously shown that a dominant-negative Cdk9 protein does not inhibit Tax transactivation of the HTLV-1 LTR unlike its strong inhibition of Tat transactivation of the HIV-1 LTR, suggesting that Tax function may not be strictly dependent upon P-TEFb (Gold et al., 1998).

HeLa cells were transfected with pNL4-3-Tat-Luc, pPCNA-Luc, or pHTLV-1-LTR-Luc+ pTax and treated with 50 μ M W-7 for seven hours. An appropriate amount of plasmid pcDNA was transfected with pNL4-3-Tat-Luc in the experiment with HTLV-1-LTR+Tax to ensure that equivalent amount of DNA was introduced into HeLa cells. Cell lysates were prepared and Luciferase assays were performed. When normalized to total protein levels and compared to control treated cells, inhibition of Ca^{2+} signaling by W-7 reduced HIV-1 LTR gene expression by almost 52% and PCNA promoter expression by ~61% respectively (Figure 6A). W-7 treatment did not affect the expression of HTLV-1-LTR implying that the inhibition of Ca^{2+} signaling is specific to promoters that have a stringent requirement for P-TEFb (Figure 6B).

To verify if the reduction in HIV-1 LTR gene expression upon inhibition of Ca^{2+} signaling was mediated by the dephosphorylation of Cdk9 T-loop, we carried out additional plasmid cotransfection functional assays. HeLa cells were cotransfected with pNL4-3-Tat-Luc and pFlag-wild type Cdk9 or phosphomimetic mutant, pFlag-T186D Cdk9. The cells were treated with 50 μ M W-7 for seven hours and cell lysates analyzed for Luciferase activity. We found that there was a significant decrease in HIV-1 LTR expression when Ca^{2+} signaling was inhibited in cells transfected with wild type Cdk9 (~47%; Figure 7). However, in cells transfected with the phosphomimetic (T186D) Cdk9 mutant, the effect of inhibition of Ca^{2+} signaling on HIV-1 LTR expression was reduced relative to that seen with the wild

type Cdk9 (22% vs 47% inhibition) (Figure 7). This difference between the wild type and phosphomimetic mutant was observed in three independent experiments. Importantly, there was a statistically significant difference in the degree of HIV-1 LTR gene expression upon W-7 treatment in cells expressing wild type Cdk9 compared to phosphomimetic (T186D) Cdk9 mutant (Figure 7). This suggests that the phosphomimetic Cdk9 mutant is able to partially rescue the effect on HIV-1 LTR gene expression of inhibition of Ca^{2+} signaling by W-7 and this inhibition is likely mediated by the dephosphorylation of Cdk9 T-loop.

Discussion

Role of Ca^{2+} signaling pathway in regulating Cdk9 T-loop phosphorylation

T lymphocyte activation and differentiation is regulated by co-stimulatory molecules, cytokines and chemokines. These stimuli interact with the serine-threonine kinase network to control T lymphocyte differentiation and proliferation (Matthews and Cantrell, 2006). When T cell receptor (TCR) engages with antigen/major histocompatibility complex (MHC), intracellular stores of Ca^{2+} are mobilized. Although the initial engagement of antigen receptor-TCR triggers some cell membrane and cytoplasmic events within five minutes, a full commitment to T lymphocyte activation requires two hours (Crabtree, 1989). It has been proposed that this time requirement is essential for spatial and temporal movement of Ca^{2+} , a critical second messenger, amongst different cellular compartments to sustain T cell activation (Alkon and Rasmussen, 1988). In resting cells, cytoplasmic Ca^{2+} concentration is maintained at ~ 100 nM but upon cellular activation, mobilization of intra and extracellular Ca^{2+} can increase its levels to more than $1 \mu\text{M}$ (Berridge et al., 2000). Kinases such as Ca^{2+} -Calmodulin dependent kinases CaMKII and CaMKIV also modulate T lymphocyte cytokine production (Matthews and Cantrell, 2006).

We found in this study that Ca^{2+} -regulated pathways play a positive role in Cdk9 T-loop phosphorylation in primary CD4^{+} T lymphocytes and HeLa cells. We had earlier reported that activation of resting CD4^{+} T lymphocytes by the Ca^{2+} ionophore ionomycin leads to an induction of Cdk9 T-loop phosphorylation (Ramakrishnan et al., 2009). In this study, depletion of the kinase CaMK1D by siRNA reduced Cdk9 T-loop phosphorylation and inhibitors of Ca^{2+} -Calmodulin signaling pathway also decreased Cdk9 T-loop phosphorylation. While our results suggest that CaMK1D does not directly phosphorylate Cdk9, it is likely that CaMK1D acts upstream of a pathway that stimulates Cdk9 T-loop phosphorylation.

Cdk9 protein function is modulated by Cdk9 T-loop phosphorylation

We observed that perturbation of Cdk9 T-loop phosphorylation reduces the level of Cdk9 protein. Inhibition of Calmodulin with the small molecule W-7 reduced Cdk9 T-loop phosphorylation and decreased the overall Cdk9 protein level. This destabilization of Cdk9 protein upon W-7 treatment was mediated by the proteasomal degradation pathway. We speculate that the dephosphorylation of Cdk9 T-loop may be triggering Cdk9 protein turnover by the proteasomal pathway, although additional work will be required to establish this notion. Notably, dephosphorylation of Cdk9 T-loop precedes the degradation of total Cdk9 protein when Ca^{2+} signaling is inhibited. In this time course experiment we also observed that there was no change in the expression level of Cyclin T1. We also found in plasmid reporter assays that P-TEFb function is sensitive to Cdk9 T-loop phosphorylation status as expected, as W-7 treatment of cells inhibited the HIV-1 LTR and the cellular PCNA promoter. In addition to P-TEFb (Yu et al., 2008), CaM has been reported to regulate the expression of PCNA (Maga et al., 1997) and this may be the reason that W-7 treatment inhibited PCNA expression more than HIV-1 LTR expression. Although P-TEFb has been reported to be recruited by HTLV-1 Tax to activate HTLV-1-LTR (Cho et al., 2010; Zhou et

al., 2006), we have reported that a dominant-negative Cdk9 protein does not inhibit Tax transactivation (Gold et al., 1998). Our finding that W-7 reduces Cdk9 T-loop phosphorylation levels but not Tax activation function are consistent with our previous result with the dominant-negative Cdk9 protein and suggest that the mechanism of Tax transactivation is not as stringently dependent upon P-TEFb as that of the HIV-1 Tat protein. Finally, we found that the dependence of HIV-1 LTR expression on P-TEFb is likely mediated by the phosphorylation status of Cdk9 T-loop as a phosphomimetic (T186D) Cdk9 mutant was able to partially rescue the effect of W-7 treatment in plasmid cotransfection functional assays.

Possible role of phosphatases in response to Ca^{2+} signaling and maintaining Cdk9 T-loop phosphorylation status

Approximately 50% of Cdk9 in HeLa cells is found in a catalytically inactive complex with 7SK snRNA and several other proteins (Choo et al., 2010). Cdk9 T-loop phosphorylation is a prerequisite for this association with the 7SK RNP (Chen et al., 2004). This sequestration of Cdk9 can protect phosphorylated Cdk9 T-loop from the action of phosphatases (Chen et al., 2008). The subpopulation of activated P-TEFb not associated with the 7SK complex and involved in RNAP II transcription elongation can be functionally inhibited by the action of phosphatases. This pool of free P-TEFb may become unstable and may be degraded. When the calcium pathway is inhibited by using either RNAi or chemical inhibitors, two simultaneous effects can be envisaged: (i) Cdk9 in the small P-TEFb (Cdk9-Cyclin T) complex is dephosphorylated by phosphatases and degraded; (ii) the P-TEFb-7SK RNP may be disrupted and the released Cdk9 could be subject to the action of phosphatases before being degraded. PP1 α and PP2B have been identified as negative regulators of Cdk9 T-loop phosphorylation in response to Ca^{2+} signaling following cellular stress (Chen et al., 2008). The negative regulation of Cdk9 T-loop phosphorylation by these phosphatases in response to Ca^{2+} signaling is not necessarily inconsistent with our results, as PP1 α and PP2B act after cells are treated with hexamethylene bisacetamide (HMB) or UV that disrupts the association of Cdk9 with the 7SK RNP (Chen et al., 2008). It is not clear how these experimental conditions are related to those used in our study.

Cdk activating kinase and the regulation of Cdk9 T-loop phosphorylation

Cyclin-dependent kinases play important roles in two critical aspects of cell function; cell-cycle and transcription. While Cdk1, 2, 4 and 6 are involved in cell cycle control; Cdk9, 8 and 11 play a role in transcription. Activation of Cdks require the binding of its regulatory Cyclin subunit and the phosphorylation of the T-loop. Cdk7 is a component of general transcription factor TFIIF phosphorylating Ser5 residues of RNAP II CTD and acts as a Cdk activating kinase (CAK) by phosphorylating T-loop of cell-cycle Cdks (Fisher, 2005). Fission yeast Cdk9 is activated by one of the two CAKs, Csk1 but not Mcs6 which is the Cdk7 ortholog (Pei et al., 2006). In budding yeast, Cak1 phosphorylates the Cdk9 orthologs, Bur1 and Ctk1 (Ostapenko and Solomon, 2005; Yao and Prelich, 2002). Recombinant Cdk9 T-loop has been reported to be autophosphorylated (Baumli et al., 2008). This mechanism of T-loop phosphorylation is relatively inefficient suggestive of the involvement of an activating kinase. The activating kinase for Cdk9 has not yet been identified. While earlier studies to link Cdk9 phosphorylation *in vitro* with Cdk7 has not been successful (Chen et al., 2004; Kim and Sharp, 2001), development of reagents like an antiserum specific for phosphorylated T186 on Cdk9 (Wang et al., 2008) might be useful in resolving the question whether Cdk7 or another kinase is an activating kinase of Cdk9.

Implications of regulation of Cdk9 T-loop phosphorylation by Ca^{2+} signaling in cancer

The role of P-TEFb in HIV-1 replication and other diseases is well documented. Cardiac hypertrophy has been reported to be linked to an increase in Cdk9 activity (Sano et al.,

2002). Cancer is characterized by loss of control in proliferation and repression of apoptosis. Cdk9 is involved in DNA repair and has an anti-apoptotic effect, and dysregulation of Cdk9 may affect these processes and be involved in cellular transformation (Foskett et al., 2001; Liu et al., 2010). In mixed lineage leukemia, aberrant RNAP II transcriptional elongation has been implicated in the transformation process (Mueller et al., 2009). In this context, Cdk9 inhibitors are being investigated as potential cancer therapy. Flavopiridol and roscovitine are inhibitors of Cdk9 and are being evaluated for treatment of chronic lymphocytic leukemia (Alvi et al., 2005; Christian et al., 2007).

Ca^{2+} is a critical second messenger and cells need to maintain a fine threshold of intracellular Ca^{2+} levels for homeostasis (Berridge et al., 2000). The Ca^{2+} signaling apparatus is perturbed in transformed cells (Roderick and Cook, 2008). For instance, renal cell carcinomas display impaired Ca^{2+} intake, decreased transient receptor potential ion channel 4 (TRPC4) expression, and reduced secretion of thrombospondin-1 (THBS-1) resulting in a pro-angiogenic shift during carcinoma progression (Veliceasa et al., 2007). In a lung cancer model, the Ca^{2+} binding protein S100A13 has been reported to be associated with more aggressive and invasive phenotype (Pierce et al., 2008). Inhibition of Ca^{2+} -Calmodulin-dependent protein kinases, CaMK1 and CaMKII, by genetic or pharmacological means has been reported to reduce tumor cell proliferation in a breast cancer and osteosarcoma model respectively (Rodriguez-Mora et al., 2005; Yuan et al., 2007). Notably, CaMK1D has been found to be amplified in breast carcinomas (Bergamaschi et al., 2008). Our finding that inhibition of Ca^{2+} signaling reduces Cdk9 T-loop phosphorylation and Cdk9 function indicates that the relationship between Cdk9 and Ca^{2+} needs further investigation, as this may have implications for the development of new therapeutics for cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Depletion of CaMK1D reduces Cdk9 T-loop phosphorylation

SiRNAs (50 pmol) against CAMK1D and other indicated serine-threonine kinases were reverse transfected into HeLa cells. Cell lysates were prepared and analyzed in immunoblots for Cdk9 T-loop phosphorylation, total Cdk9 and β -actin (loading control).

Figure 2. CaMK- Ca^{2+} signaling pathway regulates Cdk9 T-loop phosphorylation

(A) HeLa cells were treated with 100 nM KN-93 or negative control, KN-92. Samples were collected at one hour and four hours post-treatment and cell lysates analyzed by immunoblots for level of Cdk2 T-loop phosphorylation. Inhibition of CamK by KN-93 reduces the level of phosphorylated Cdk2 compared to control KN-92 treated cells.

(B) HeLa cells were treated with 100 nM KN-93 (CaMK inhibitor) or KN-92 (negative control). Cell lysates prepared after one hour and four hours of treatment were analyzed in an immunoblot for effects on Cdk9 T-loop phosphorylation. After normalization to the Cdk7 loading control and compared to the negative control KN-92, quantification of the immunoblot showed a ~55% and ~59% reduction of Cdk9 T-loop phosphorylation at one and four hours, respectively, when CaMK was inhibited by KN-93. Total Cdk9 levels were reduced ~15% at one and four hours by treatment with KN-93.

(C) HeLa cells were treated with 20 μM and 50 μM Calmodulin inhibitor, W-7, for seven hours. Cell lysates were immunoblotted and probed for Cdk2 T-loop phosphorylation. There is a dose dependent reduction in the level of phosphorylated Cdk2 upon W-7 treatment compared to control untreated cells.

(D) HeLa cells were treated with the indicated concentration of W-7 for seven hours. Cell extracts were prepared and immunoblots were performed to examine Cdk9 T-loop phosphorylation. After normalization to the β -actin loading control, quantification of the immunoblots showed a ~ 54% reduction of Cdk9 T-loop phosphorylation and ~ 29% reduction in total Cdk9 levels after treatment with 50 μ M W-7 for seven hours relative to the untreated control.

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Figure 3. Small molecule inhibitors of Ca^{2+} signaling pathway affect Cdk9 T-loop phosphorylation

(A) Resting primary CD4^{+} T lymphocytes were treated with Thapsigargin ($100 \mu\text{M}$) or DMSO vehicle control and activated with either Ca^{2+} ionophore, ionomycin or PMA + ionomycin for one hour. Immunoblots were carried out on the cell lysates and examined for induction of Cdk9 T-loop phosphorylation. Immunoblots were quantified by normalizing to Cdk7 loading control and comparing to DMSO treated activated cells. In ionomycin and PMA + ionomycin treated cells, inhibition of Ca^{2+} signaling by Thapsigargin repressed Cdk9 T-loop phosphorylation by 80% and 60% respectively.

(B) Resting primary CD4^{+} T lymphocytes were activated with PMA + ionomycin for 60 hrs. The activated cells were treated with a final concentration of $50 \mu\text{M}$ W-7. Cells lysates were prepared at the indicated times and immunoblots were performed. Cdk7 was used as loading and specificity control. When compared to activated cells without W-7 treatment and normalized to loading control, quantification of immunoblots showed 84% reduction of Cdk9 T-loop phosphorylation in 4 hours and total Cdk9 levels were reduced by 38% in 4 hours.

Figure 4. Inhibition of the proteasomal pathway following W-7 treatment partially restores Cdk9 protein level

Two culture dishes of HeLa cells were treated with 50 μ M W-7 for seven hours. The proteasomal inhibitor MG101 (50 μ M) was then added to one culture dish for one hour. As an additional control, a culture dish of HeLa cells was treated with only MG101 for one hour. Cell lysates were prepared and immunoblots performed to examine Cdk9 T-loop phosphorylation and total Cdk9 protein levels. When compared to DMSO vehicle control treated cells and normalized to β -actin (loading control), quantification of the immunoblots showed ~ 50% decrease in Cdk9 T-loop phosphorylation and ~ 30% decrease in total Cdk9 levels upon W-7 treatment. Addition of MG101 resulted in significant increases of both phosphorylated and total Cdk9 protein level.

Figure 5. Dephosphorylation of Cdk9 T-loop precedes degradation of total Cdk9 following W-7 treatment

HeLa cells were treated with 50 μ M W-7. Samples were collected every hour for three hours. Cell lysates were prepared and immunoblots performed to examine levels of Cdk9 T-loop phosphorylation, overall Cdk9 and Cyclin T1. There was a reduction in the level of Cdk9 T-loop phosphorylation two hours post treatment with no change in the overall Cdk9 protein and Cyclin T1 expression.

A

B

Figure 6. P-TEFb function is dependent upon Ca^{2+} signaling pathway

(A) HeLa cells were transfected with either 1 μg pNL4-3-Tat-Luc or pPCNA-Luc overnight and treated with 50 μM W7 for eight hours. Cell lysates were prepared and Firefly Luciferase activities were measured and normalized to total protein amounts in cell extracts. The normalized Luciferase activity in untreated wells was 28,540 and 450 for pNL4-3-Tat-Luc and pPCNA-Luc, respectively, and was defined as 1.0 to calculate fold-change in the W7 treated wells. (** represents p value of < 0.005 and * represents p value of < 0.05 in a paired t-test).

(B) HeLa cells were transfected with either 250 ng pNL4-3-Tat-Luc + 50 ng pcDNA empty vector or 250 ng pHTLV-LTR-Luc + 50 ng pTax overnight and treated with 50 μM W-7 for seven hours. The cells were lysed and Firefly Luciferase assays were carried out. The Luciferase values were normalized to total protein in the cell lysates and set at 1.0 in untreated wells. (** represents p value of < 0.005 in a paired t-test).

Figure 7. Phosphomimetic Cdk9 mutant rescues P-TEFb function upon W-7 treatment

HeLa cells were cotransfected with either 250 ng pNL4-3-Tat-Luc + 500 ng Flag-wild type (Wt) Cdk9 or 250 ng pNL4-3-Tat-Luc + 500 ng Flag-T186D Cdk9 overnight and treated with 50 μ M W7 for seven hours. Cell lysates were prepared and Firefly Luciferase activities were measured and normalized to total protein amounts in cell extracts. The normalized Luciferase activity in untreated wells was defined as 1.0 to calculate fold-change in the W7 treated wells. Data shown is the average $\pm$ SD from three independent experiments (* represents p value of ≤ 0.05 in a paired t-test).