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Recruitment of P-TEFb for Stimulation of Transcriptional Elongation by the Bromodomain Protein Brd4

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Summary

The cyclinT1/Cdk9 heterodimer that constitutes core P-TEFb is generally presumed to be the transcriptionally active form for stimulating RNA polymerase II elongation. About half of cellular P-TEFb also exists in an inactive complex with the 7SK snRNA and the HEXIM1 protein. Here, we show that the remaining half associates with the bromodomain protein Brd4. In stress-induced cells, the 7SK/HEXIM1-bound P-TEFb is quantitatively converted into the Brd4-associated form. The association with Brd4 is necessary to form the transcriptionally active P-TEFb, recruits P-TEFb to a promoter, and enables P-TEFb to contact the Mediator complex, a potential target for the Brd4-mediated recruitment. Although generally required for transcription, the P-TEFb-recruitment function of Brd4 can be substituted by that of HIV-1 Tat, which recruits P-TEFb directly for activated HIV-1 transcription. Brd4, HEXIM1, and 7SK are all implicated in regulating cell growth, which may result from their dynamic control of the general transcription factor P-TEFb.

Introduction

Transcriptional elongation by RNA polymerase (pol) II has been recognized as a highly regulated process capable of not only generating full-length RNA transcripts but also coordinating transcription with pre-mRNA processing (Sims et al., 2004). Several regulatory factors that specifically target the elongation stage have been identified. Among these, P-TEFb, containing Cdk9 and its regulatory cyclinT1 (CycT1) subunit (or the minor forms T2 or K) (Price, 2000), plays a critical role during the transition from abortive to productive elongation (Jones, 1997; Price, 2000). The transcriptional activity of P-TEFb depends on the Cdk9 kinase activity, which hyperphosphorylates the C-terminal domain (CTD) of the largest subunit of pol II to stimulate the processivity of elongation. Studies using either RNA interference or highly specific P-TEFb inhibitors have implicated P-TEFb as a global transcriptional elongation factor important for most pol II transcription (Chao and Price, 2001; Shim et al., 2002).

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Not only essential for general transcription, P-TEFb is also exquisitely required for HIV-1 gene expression. Tat, a viral-encoded regulatory protein, recruits host P-TEFb to the paused pol II through interacting with the TAR RNA structure located at the 5′ end of the nascent viral transcripts. Upon recruitment, the P-TEFb-associated Cdk9 phosphorylates the pol II CTD, leading to the generation of full-length HIV-1 transcripts that are essential for viral gene expression (Mancebo et al., 1997; Wei et al., 1998; Zhu et al., 1997).

In the nucleus, not every CycT1/Cdk9 heterodimer displays the P-TEFb transcriptional activity. In fact, about half of the heterodimers in HeLa cells are sequestered in a complex (termed the 7SK snRNP) with the 7SK snRNA and the HEXIM1 protein (Chen et al., 2004; Michels et al., 2003, 2004; Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003, 2004). Whereas 7SK serves as a molecular scaffold to mediate the interaction of HEXIM1 with CycT1/Cdk9, HEXIM1 inhibits the Cdk9 kinase activity and prevents P-TEFb from binding to transcriptional templates (Michels et al., 2004; Yang et al., 2001; Yik et al., 2003, 2004). The association of 7SK/HEXIM1 with CycT1/Cdk9 is a dynamic process and can be disrupted by certain stress-inducing agents such as the global transcriptional inhibitor actinomycin D, kinase/transcription-inhibitor DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), and DNA-damaging agent UV irradiation (Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003). At low dosages, these agents have been shown to stimulate the CTD phosphorylation and HIV-1 transcription (Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003), which could be attributable to the induced disruption of the 7SK snRNP and activation of P-TEFb. Moreover, in cardiac myocytes, hypertrophic signals also disrupt the 7SK snRNP, leading to a global increase in cellular RNA and protein contents and enlargement of heart cells, which is the cause of cardiac hypertrophy (Sano et al., 2002).

Besides 7SK and HEXIM1, a bromodomain protein, Brd4 has recently been identified as a major factor associated with the CycT1/Cdk9 heterodimer (Jang et al., 2005). Like all the components of the 7SK snRNP, Brd4 is also ubiquitously expressed (Shang et al., 2004). It belongs to the conserved BET family of proteins that carry two tandem bromodomains and an extra terminal (ET) domain (Jeanmougin et al., 1997). The bromodomain has been recognized as a functional module in helping decipher the histone code through interacting with acetylated histones (Zeng and Zhou, 2002). Consistent with this view, Brd4 has been shown to bind to acetylated euchromatin through acetylated histones H3 and H4 (Dey et al., 2003).

The demonstrated binding of Brd4 to CycT1/Cdk9 prompted us to investigate a potential role for Brd4 in P-TEFb-dependent transcription. Here, we show that Brd4 and HEXIM1/7SK existed in two mutually exclu-

sive CycT1/Cdk9-containing complexes and stress treatment caused a quantitative conversion of the 7SK snRNP into the complex containing Brd4 bound to CycT1/Cdk9. Importantly, the association with Brd4 was required to form the transcriptionally active P-TEFb for stimulation of pol II elongation. Moreover, Brd4 contributed to general transcription through its recruitment of P-TEFb to transcriptional templates in vivo and in vitro. The Mediator complex, which interacted with P-TEFb through Brd4, could potentially be the target for this recruitment. Interestingly, the P-TEFb-recruitment role of Brd4 could be functionally substituted by that of the HIV-1 Tat, which recruited CycT1/Cdk9 for activated HIV transcription.

Results

Salt-Sensitive Interaction of Brd4 with Core P-TEFb

We have previously performed immuno-affinity purification of Cdk9-associated factors from both HeLa nuclear extract (NE) and whole cell lysates (O'Keeffe et al., 2000; Yang et al., 2001). These experiments, however, did not identify Brd4 as a factor associated with the CycT1/Cdk9 heterodimer (referred to as core P-TEFb from here on). To investigate whether our conditions, which routinely used 0.5 M KCl in the washing buffer, may have disrupted the Brd4-core P-TEFb binding, we examined the stability of the interactions of Cdk9 with several protein factors under increasing KCl concentrations. FLAG-tagged Cdk9 (Cdk9-f) and its associated factors were affinity purified from NE of F1C2 cells, a HeLa-based cell line stably expressing Cdk9-f. The immunoprecipitates were washed with buffers containing increasing KCl concentrations and then eluted off the FLAG beads and analyzed by Western blotting (Figure 1A). Both CycT1 and HEXIM1 (HXM1) remained stably bound to Cdk9-f in buffers containing up to 0.3 M KCl (a slight decrease in their signals was due to a reduced association of Cdk9-f to FLAG beads by increasing salt concentrations). In contrast, the binding of Brd4 to Cdk9-f was significantly weakened by 0.25 M KCl and abolished by 0.3 M KCl (Figure 1A), revealing the salt-sensitive nature of the Brd4-core P-TEFb binding.

This phenomenon was conveniently exploited to affinity-purify FLAG-tagged wild-type (wt) Brd4-f and its mutant ΔBDI&II-f that lacks the two bromodomains from transiently transfected HeLa cells. Analysis by SDS-PAGE followed by silver staining indicates that the two Brd4 proteins were purified to homogeneity after washing with a buffer containing 0.5 M KCl (Figure 1B). These two proteins, together with the high salt-washed CycT1/Cdk9 heterodimer free of any associated Brd4, will be used in several subsequent experiments.

Brd4 and HEXIM1/7SK Exist in Two Mutually Exclusive CycT1/Cdk9-Containing Complexes

Approximately half of nuclear CycT1/Cdk9 have been shown to exist in a complex that also contains HEXIM1 and 7SK (Michels et al., 2003; Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003). To determine whether Brd4 and HEXIM1/7SK belong to the same or different P-TEFb-containing complexes, anti-FLAG immune complexes derived from NEs of two HeLa-based cell lines stably expressing either Brd4-f (MCAP) (Jang et al., 2005) or HEXIM-f (HH8) (Yik et al., 2003) were analyzed by Western and Northern blotting (Figure 2A). As expected, both complexes contained Cdk9 and CycT1. However, neither HEXIM1 nor 7SK was detected in the Brd4-containing complex. Likewise, only 7SK, but not Brd4, was present in the HEXIM1-containing complex. Thus, Brd4 and HEXIM1/7SK existed in two mutually exclusive CycT1/Cdk9-containing complexes.

Stress-Induced Quantitative Transfer of CycT1/Cdk9 from 7SK/HEXIM1 to Brd4

HEXIM1 and 7SK have been shown to dissociate from core P-TEFb in cells treated with certain stress-inducing agents such as actinomycin D, DRB, and UV irradiation (Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003, 2004). To investigate whether the Brd4-core P-TEFb binding could also be affected by these treatments, the interactions of Cdk9-f with CycT1, Brd4, HEXIM1, and 7SK present in the anti-FLAG immunoprecipitates derived from NEs of treated F1C2 cells were examined (Figure 2B). Compared to solvent DMSO (a negative control), actinomycin D, DRB, and UV all caused an almost complete dissociation of HEXIM1 and 7SK, but not CycT1, from Cdk9-f. In contrast, the same treatments consistently increased the Brd4-core P-TEFb binding by about 2-fold, even though the Brd4 level in NE remained unchanged before and after the treatments (Figure 2B, compare lane 2 with lanes 3–5). Given that under normal conditions only ~50% of the total nuclear core P-TEFb are in the 7SK snRNP (Yang et al., 2001; Yik et al., 2003) while the other 50% are in the Brd4-containing complex (Jang et al., 2005), the stress-induced 2-fold increase in the Brd4-core P-TEFb binding was significant and represented a quantitative conversion of the 7SK snRNP into the Brd4-containing complex.

This notion was further tested from a different angle. Like the situation involving F1C2 cells, treatment of MCAP cells, which stably expresses Brd4-f, with actinomycin D, DRB, or UV, also caused a ~2-fold increase in the level of Cdk9 associated with the immunoprecipitated Brd4-f (Figure 2C, compare lane 2 with lanes 3–5). Again, the treatments had no apparent effect on the Cdk9 level in these cells. Together, these results reveal a quantitative and dynamic exchange of partners by core P-TEFb in stress-treated cells, which may contribute to stress-induced gene expression.

Ser175 in Cdk9 Is Required for Binding of Brd4 to Core P-TEFb

A single amino acid, Thr186, located at the tip of the flexible T-loop in Cdk9, has been demonstrated as essential for the interaction of HEXIM1/7SK with core P-TEFb (Chen et al., 2004). To determine whether the same or different amino acids might be important for the Brd4-core P-TEFb binding, the abilities to associate with Brd4 by the transfected wt or mutant Cdk9-f were examined by anti-FLAG immunoprecipitation followed by Western blotting (Figure 3). None of the Cdk9 mutations affected the CycT1/Cdk9 heterodimer formation. As reported previously, both the T186A and T186E mutations significantly weakened the binding of HEXIM1

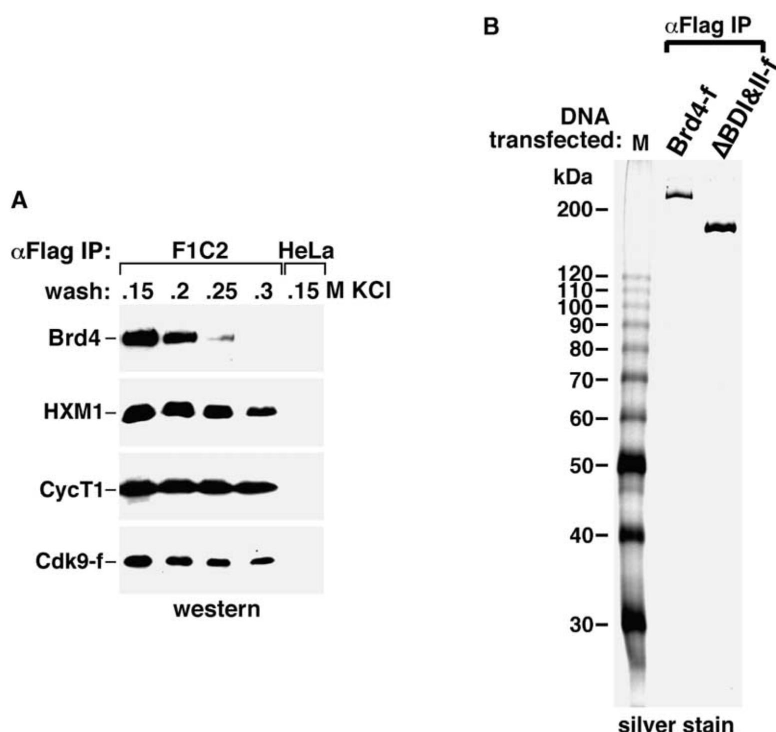

Figure 1. Salt-Sensitive Interaction of Brd4 with Core P-TEFb

(A) Nuclear extracts (NEs) in buffer D0.15M were prepared from HeLa or HeLa-based F1C2 cells stably expressing FLAG-tagged Cdk9 (Cdk9-f) and subjected to anti-FLAG immunoprecipitation followed by washing with D buffers containing the indicated amounts of KCl. The immunoprecipitates were then analyzed by Western blotting.

(B) Transiently transfected wild-type (wt) Brd4-f and its mutant ΔBDI&II-f were affinity purified from NEs by anti-FLAG immunoprecipitation and washed extensively with buffer D0.5M to remove all the associated proteins. The peptide-eluted Brd4 proteins were then analyzed by SDS-PAGE followed by silver staining to confirm their purity.

to core P-TEFb (Chen et al., 2004). These two mutations, however, did not affect the Brd4-P-TEFb binding. Rather, Ser175, which is conserved between CDK7 and Cdk9, was essential, as both the S175A and S175D mutations completely abolished the Brd4-core P-TEFb binding. In contrast, the D167N mutation, which destroys the Cdk9 kinase activity, and the alanine substitutions of either four or eight Ser/Thr residues near the C terminus of Cdk9, which disrupt the formation of the Tat-TAR-P-TEFb ternary complex (Fong and Zhou, 2000; Garber et al., 2000), had no detectable effect on the interaction between Brd4 and core P-TEFb.

It should be noted that the requirement of Ser175 for the Brd4-P-TEFb binding was demonstrated in the context of the CycT1/Cdk9 heterodimer, and it did not necessarily imply a direct contact of Brd4 with Cdk9 per se. In fact, a direct interaction of Brd4 with CycT1, but not Cdk9, has been demonstrated by using a GST pull-down assay (Jang et al., 2005). Combining these data, we speculate that the mutation of Ser175 in the Cdk9 T loop, which is well known to undergo major conformational changes in a phosphorylation-dependent manner, may induce a conformation in the CycT1/Cdk9 heterodimer that blocks the direct Brd4-CycT1 interaction.

S175D-Containing Core P-TEFb Is an Active CTD-Kinase but Defective Transcription Factor

Ser175 in Cdk9 is interesting in that a negatively charged residue at this position is important for the kinase activity of core P-TEFb toward the pol II CTD (Chen et al., 2004). The S175A mutation has been shown to abolish the kinase activity, whereas the S175D substitution completely restored the activity to that of wt (Chen et al. [2004] and also see Figures 4A and 4B). The fact that the S175D-containing core P-TEFb was able to phosphorylate the CTD but unable to bind to Brd4 offered a unique opportunity to examine the contribution of Brd4 to P-TEFb's transcriptional activity.

For this purpose, we performed an in vitro transcription assay with a pair of DNA templates (pHIV+TAR-G400 and pHIVΔTAR-G100) to detect the HIV-1 LTR-directed elongation of two G-less cassettes (G400 and G100) inserted at ∼1 kb downstream of the transcription start site (Zhou and Sharp, 1995). The reactions also contained HeLa NE, in which the CycT1/Cdk9 heterodimer was immuno-depleted with anti-Cdk9 antibodies under high-salt (0.5 M KCl) conditions that left Brd4 behind (Figure 4C). Compared to mock-depleted NE, depletion of core P-TEFb eliminated HIV-1 transcription (Figure 4D, lanes 1 and 2) as reported previously (Chen and Zhou, 1999; Zhou et al., 1998). Addition of wt, but not the S175A core P-TEFb, fully restored HIV-1 transcription (lanes 3 and 4). Interestingly, introduction of the S175D mutant, which was unable to bind to Brd4 in the reaction, largely failed to rescue transcription (lane 5), even though it had a comparable level of CTD-kinase activity as the wt core P-TEFb (Figure 4A).

Binding of Brd4 to Core P-TEFb Is Essential for Transcription

To further examine the role of Brd4 in P-TEFb-mediated transcription, we performed transcription reactions containing depleted HeLa NE in which both Brd4 and core P-TEFb were simultaneously removed under high-salt conditions. By using Cdc2 in NE as a control, the Western analysis confirmed the specificity and near completion of the double-depletion procedure (Figure

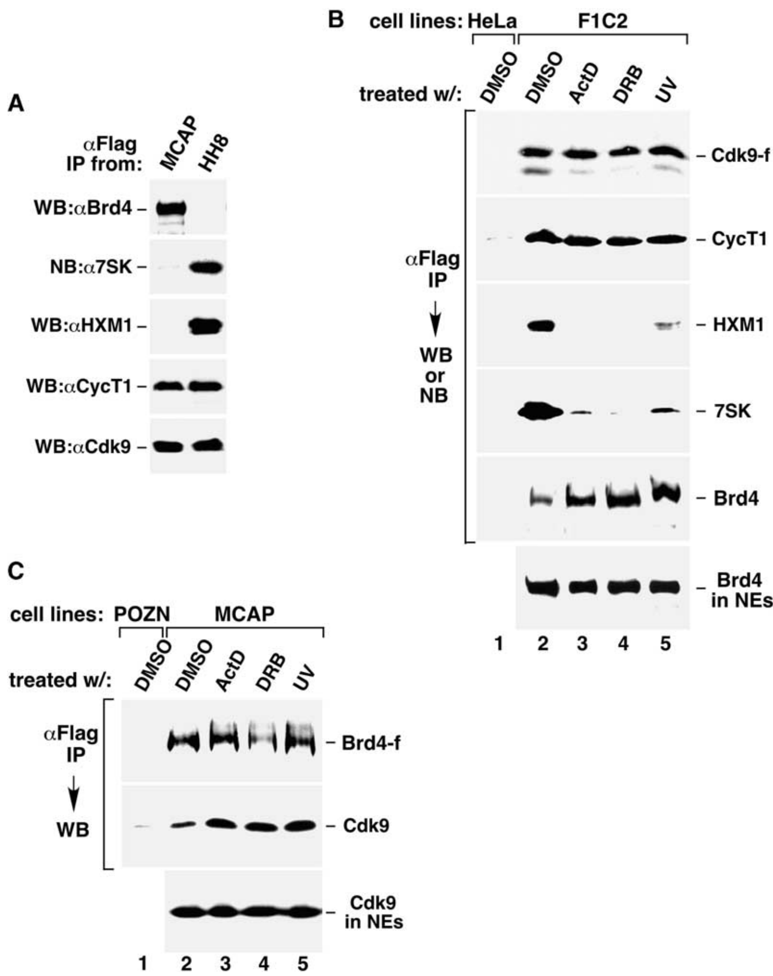

Figure 2. Dynamic Exchange of Brd4 and 7SK/HEXIM1 between Two Mutually Exclusive CycT1/Cdk9-Containing Complexes

(A) Brd4 and 7SK/HEXIM1 exist in two mutually exclusive CycT1/Cdk9-containing complexes. The CycT1/Cdk9 heterodimer and its associated factors were isolated by anti-FLAG immunoprecipitation (IP) from two HeLa-based cell lines expressing either FLAG-tagged Brd4 (MCAP) or HEXIM1 (HH8), followed by Western and Northern analyses.

(B and C) Quantitative transfer of CycT1/Cdk9 from 7SK/HEXIM1 to Brd4 in stress-induced cells. F1C2 and MCAP cells expressing FLAG-tagged Cdk9-f and Brd4-f, respectively, or their parental cell lines HeLa and POZN, were treated with the indicated stress-inducing agents or the solvent DMSO as a negative control. Cdk9-f, Brd4-f, and their associated factors were then purified by anti-FLAG IP from NEs and analyzed by Western and Northern blotting. The amounts of Brd4 and Cdk9 in NEs prior to IP were also shown.

5A). Whereas the addition of either core P-TEFb or Brd4-f alone into the reaction only slightly increased transcription, the presence of the two together fully restored transcription (Figure 5D, lanes 1–5). Furthermore, the ability of Brd4 and core P-TEFb to interact with each other was crucial to this restoration. This was illustrated by the failure to complement the double-depleted NE by the Brd4 mutant ΔBDI&II (lanes 6–11),

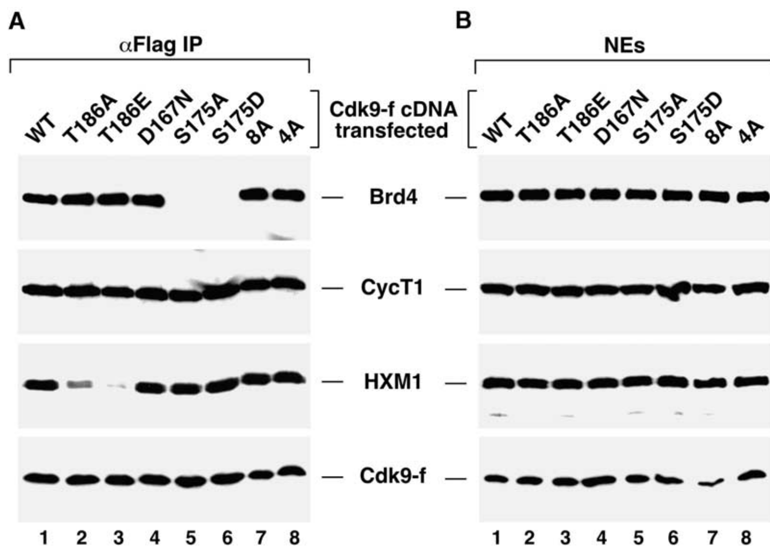

Figure 3. Serine 175 in Cdk9 Is Important for Binding of Brd4 to Core P-TEFb

HeLa cells were transfected with the indicated cDNA constructs expressing FLAG-tagged wt or mutant Cdk9-f. The levels of Brd4, CycT1, HEXIM1, and Cdk9-f in the anti-FLAG immunoprecipitates (A) and NEs (B) were detected by Western blotting.

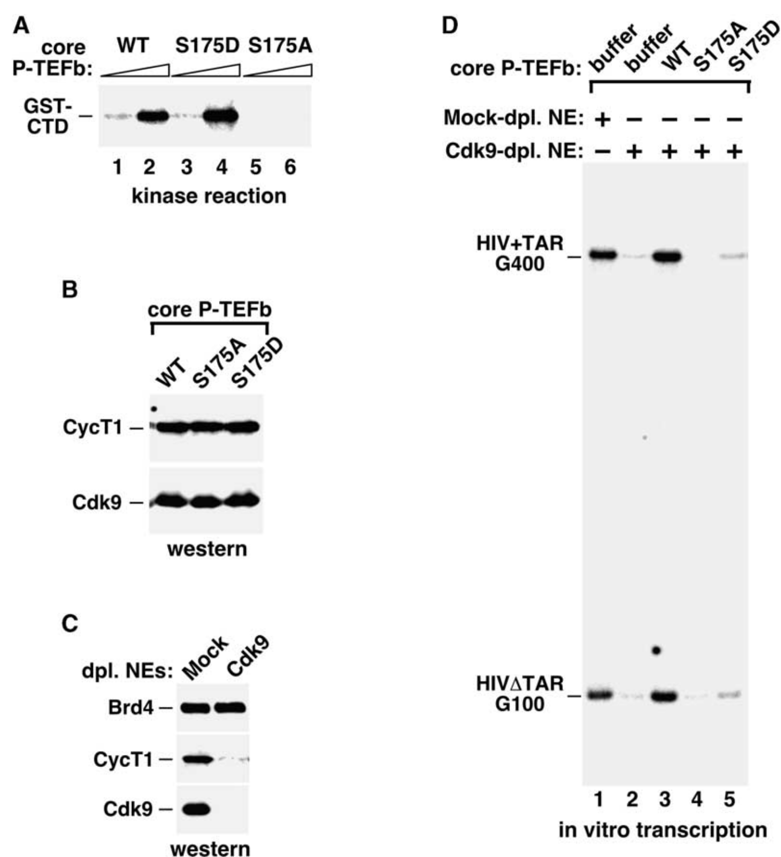

Figure 4. Core P-TEFb Containing Mutant Cdk9-S175D Is a Competent CTD-Kinase but Defective Transcription Factor

(A) A negatively charged residue at position 175 in Cdk9 is important for P-TEFb's kinase activity. In vitro kinase reactions containing the indicated wt or mutant core P-TEFb at two different concentrations (in 3-fold increment) and GST-CTD as a substrate were performed. Phosphorylated GST-CTD was detected by SDS-PAGE and autoradiography.

(B) The levels of Cdk9 and CycT1 in core P-TEFb analyzed in (A) and (D) were examined by Western blotting.

(C) Western analyses of Cdk9, CycT1, and Brd4 in HeLa NEs either mock depleted or depleted with the anti-Cdk9 antibodies under high-salt conditions.

(D) S175D-containing core P-TEFb is unable to restore HIV-1 transcriptional elongation to Cdk9-depleted NE. In vitro transcription reactions containing the indicated core P-TEFb, mock- or Cdk9-depleted HeLa NE, and two transcription templates, HIV+TAR-G400 and HIVΔTAR-G100, were performed. RNA fragments transcribed from two G-less cassettes inserted, respectively, into the two templates at a position ~1 kb downstream of the HIV-1 promoter were indicated.

which lacks the two bromodomains and is unable to bind P-TEFb (Jang et al., 2005), and the core P-TEFb mutant CycT1/S175D (lanes 12–15), which does not recognize Brd4. Taken together, these data support the model that the Brd4-core P-TEFb binding is required to form the transcriptionally active P-TEFb.

Brd4 Recruits P-TEFb to Transcriptional Template In Vivo and In Vitro

How did Brd4 contribute to P-TEFb's transcriptional activity? We have so far failed to detect a role for Brd4 to stimulate the catalytic activity of Cdk9 in vitro. However, a Brd4-dependent increase in the level of the CTD phosphorylation on Ser2 has been observed in vivo (Jang et al., 2005). Because the Ser2-phosphorylated pol II is known to be involved in elongation (Sims et al., 2004), the different effects of Brd4 on the P-TEFb-mediated Ser2 phosphorylation on the CTD in vivo and in vitro could be explained by a Brd4-mediated increase in P-TEFb binding to the transcriptional template.

To test this hypothesis, we performed a chromatin immunoprecipitation (ChIP) assay to investigate whether Brd4 may help recruit Cdk9 to the integrated HIV-1 LTR-luciferase reporter gene. Wt Cdk9 was clearly able to bind more strongly to the promoter, interior, and 3′ UTR regions of the chromatin template than S175D, which failed to bind to Brd4 and displayed signals only at the background level (Figure 6A).

To confirm the P-TEFb-recruitment function of Brd4 in vitro, we also examined the effect of Brd4 on the association of CycT1/Cdk9 with an immobilized 470 bp HIV-1 DNA template (–168 to +302) (Zhou and Sharp, 1995). The immobilized DNA fragment was incubated under transcription conditions with HeLa NE either mock depleted or depleted with the anti-Brd4 antibodies under high-salt conditions that left CycT1/Cdk9 behind. Although similar levels of Cdk9 and CycT1 were present in mock- and Brd4-depleted NEs (Figure 6B, right), a significantly reduced association of Cdk9 and CycT1 with the HIV-1 template was detected only when Brd4 was specifically removed from NE (Figure 6B, left). Taken together, our data, together with the observed decreased binding of Cdk9 to the chromatin template in cells expressing Brd4-siRNA (Jang et al., 2005), clearly indicate a role for Brd4 in recruiting P-TEFb to transcriptional template both in vivo and in vitro.

Brd4-Dependent Interaction of P-TEFb with the Mediator Complex

Because the effect of Brd4 on P-TEFb's transcriptional activity and association with the HIV-1 template could be detected in vitro, where naked DNA was used, we suspect that a nonchromatin-based mechanism could also be used to mediate Brd4's recruitment of P-TEFb to promoters. This mechanism may work in conjunction with the one described by Jang et al. (2005) that relies on the interaction of Brd4 with acetylated chromatin. Because Brd4 has been shown to associate with the Mediator complex (Jiang et al., 1998; Houzelstein et al.,

Figure 5. Interaction between Brd4 and Core P-TEFb Is Critical for Transcription

(A) Western analyses of Brd4, CycT1, Cdk9, and, as a negative control, Cdc2 in HeLa NE either mock depleted or double depleted with both the anti-Cdk9 and anti-Brd4 antibodies under high-salt conditions.

(B and C) Core P-TEFb, wt, and mutant Brd4-f affinity purified from NEs of transfected HeLa cells under high-salt conditions to remove their associated factors were examined by Western analyses.

(D) Restoration of transcription to NE depleted of both Brd4 and Cdk9 by Brd4 and core P-TEFb that are capable of mutual interactions. The indicated wt or mutant core P-TEFb and/or Brd4 were introduced into transcription reactions containing either mock- or Cdk9 and Brd4-depleted HeLa NE. G-less RNA transcripts derived from transcription templates HIV+TAR-G400 and HIVΔTAR-G100 were analyzed as described in Figure 4D.

2002; Jang et al., 2005), which is a multisubunit complex bridging the sequence-specific transcriptional activators with the basal transcriptional machinery (Myers and Kornberg, 2000), we investigated the possible role of the Mediator as a target for Brd4's recruitment of P-TEFb. First, our Western analysis confirmed the interaction of Brd4 with a core Mediator subunit, TRAP220, in MCAP cells, a stable cell line expressing Brd4-f (Figure 6C, lanes 1 and 2). Next, when wt and the S175D mutant P-TEFb, which is defective in Brd4 binding, were compared for their abilities to interact with the Mediator, only the wt complex coprecipitated TRAP220 (lanes 3–5), implicating a requirement for Brd4 to mediate the P-TEFb-Mediator interaction.

Brd4 Is Not Required for Tat-Activated HIV-1 Transcription

During HIV-1 transcriptional elongation, Tat is known to recruit the CycT1/Cdk9 heterodimer to the paused pol II through interacting with the TAR RNA element located at the 5′ end of the nascent viral transcripts. Given the data implicating Brd4 as a P-TEFb-recruitment factor important for cellular (Jang et al., 2005) as well as basal level HIV-1 transcription, we asked whether Brd4 is also required for Tat transactivation. First, we compared wt and the S175D core P-TEFb for their abilities to mediate Tat transactivation in Cdk9-depleted HeLa NE. As reported previously (Chen and Zhou, 1999; Zhou et al., 1998), addition of wt P-TEFb supported both basal and Tat-activated HIV-1 transcription (Figure 7A, lanes 1 and 2). Although the S175D P-TEFb functioned poorly in mediating basal HIV-1 transcription, it surprisingly restored transcription to approximately the same level as the wt when Tat was also in the same reaction (lanes 3 and 4). The S175D P-TEFb-mediated transcriptional activation was not only Tat dependent but also TAR specific, as the transcription from a mutant HIV-1 template with a defective TAR element (HIVΔTAR-G100) remained similarly low whether or not Tat was present (lanes 3 and 4). Thus, although the S175D P-TEFb was

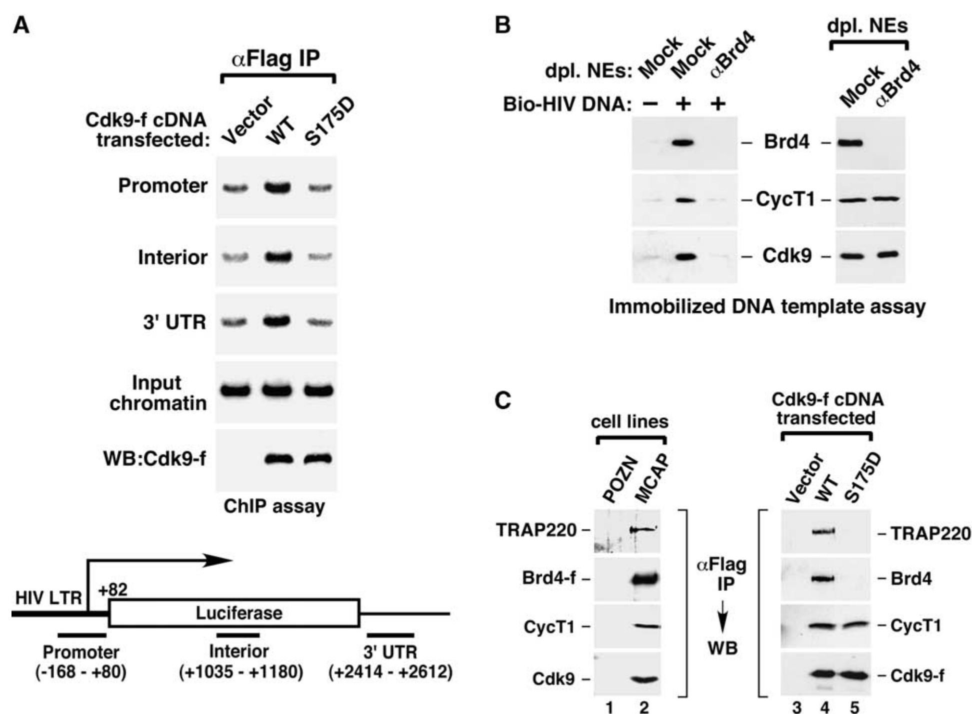

Figure 6. Brd4 Recruits P-TEFb to Transcriptional Template In Vivo and In Vitro

(A) Wt Cdk9 binds to HIV-1 chromatin template more efficiently than does Cdk9-S175D in vivo. A HeLa-based cell line containing the integrated luciferase reporter gene driven by the HIV-1 LTR (bottom) was transfected with plasmid expressing either FLAG-tagged wt Cdk9 or Cdk9-S175D, together with the CycT1- and Brd4-expressing constructs. ChIP was performed with the anti-FLAG beads. Three regions corresponding to the promoter, interior, and 3′ UTR of the integrated HIV-1 LTR-luciferase gene (bottom) were PCR amplified from the input chromatin. Wt or mutant Cdk9-f associated with the immunoprecipitated chromatin was detected by Western blotting.

(B) Brd4 is required for association of CycT1/Cdk9 with immobilized HIV-1 transcription template in vitro. HeLa NE was mock- or immunodepleted of the endogenous Brd4 under high-salt conditions and analyzed by Western blotting (right). The depleted NEs were then incubated with immobilized HIV-1 DNA template (from −168 to +302) under transcription conditions. Cdk9, CycT1, and Brd4 retained on the template were detected by Western blotting.

(C) Brd4-dependent interaction of P-TEFb with the Mediator. NEs from stable cell line MCAP that express Brd4-f, its control POZN or HeLa cells transfected with the indicated Cdk9-f cDNA constructs were subjected to anti-FLAG IP. The immune complexes were analyzed by Western blotting with the indicated antibodies.

a poor general transcription factor due to its inability to be recruited by Brd4 to the transcriptional template, it was fully capable of working with Tat and TAR in activating HIV-1 transcription, suggesting that the interaction between Brd4 and core P-TEFb was not essential for Tat transactivation.

To further test this notion in vivo, we examined the effect of the RNAi-mediated Brd4 depletion on basal and Tat-activated expression of a luciferase reporter gene under the control of the HIV-1 LTR (Figure 7B). The depletion of Brd4 was judged to be ∼70% efficient (Figure 7B, bottom) and caused a significant reduction in HIV-1 transcription as expected. However, a more pronounced reduction in basal than in the Tat-activated HIV-1 transcription was consistently observed (e.g., 4.1-fold versus 2-fold in Figure 7B, top), revealing a similar Brd4-independent Tat activation process in vivo. It should be pointed out that the small reduction in Tat activation by the Brd4 siRNA could be caused by some secondary events (e.g., reduced cellular levels of general transcription and translation factors or Tat expressed from the cotransfected plasmid), which may be Brd4 dependent and thus ultimately affect the luciferase production in the cell.

Finally, a stimulatory effect of Brd4 on Tat transactivation was also ruled out in HeLa cells that overexpressed Brd4 from a transfected plasmid. Overexpression of Brd4 caused a dose-dependent increase of only the basal level, but not the Tat-activated HIV-1 transcription (Figure 7C). In fact, the transfected Brd4 consistently caused a small and dose-dependent reduction in Tat-dependent HIV-1 transcription, suggesting that Tat and Brd4 may act competitively under these conditions.

Tat and Brd4 Compete for Binding to Core P-TEFb

Indeed, transfection into HeLa cells of wt, but not the C22G mutant Tat, which is defective in binding to P-TEFb, caused an efficient dissociation of Brd4 from core P-TEFb (Figure 7D). This occurred while the nuclear level of Brd4 was not affected by Tat expression. We speculate that the competition between Tat and Brd4 for the same core P-TEFb may result in less P-TEFb to be recruited via the highly effective Tat-TAR route

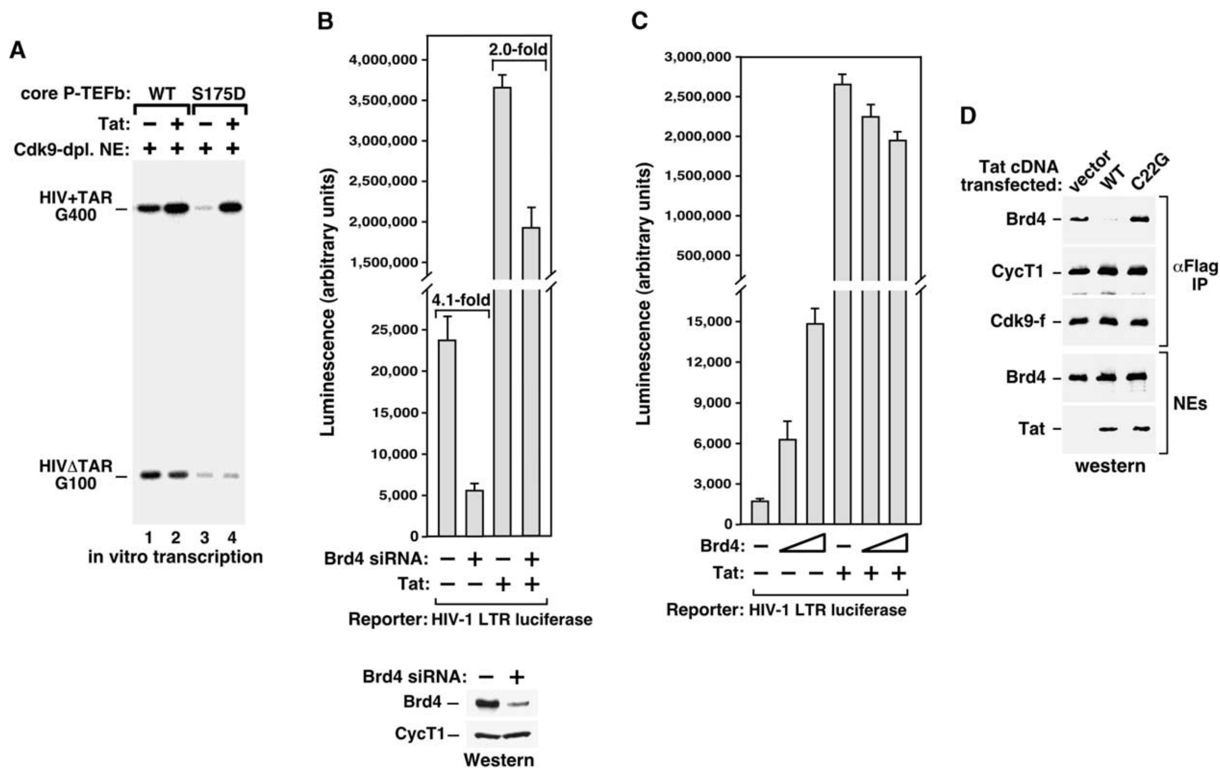

Figure 7. P-TEFb-Recruitment Role of Brd4 Can Be Functionally Substituted by that of Tat for Activated HIV-1 Transcription

(A) Brd4 is not required for Tat-activated HIV-1 transcription in vitro. In vitro reactions containing the Cdk9-depleted HeLa NE, wt, or mutant core P-TEFb, a pair of HIV-1 DNA templates containing either wt or mutant (Δ) TAR element, were performed in the presence (+) or absence (−) of Tat. The 400 and 100 base G-less RNA transcripts derived from the wt and mutant templates, respectively, were analyzed as described in Figure 4D.

(B) The Brd4 siRNA reduces basal level HIV-1 transcription more potently than it does to Tat transactivation. The Brd4 siRNA was introduced into HeLa cells via retroviral infection, and the Brd4 levels were examined by Western blotting (bottom). The infected cells containing the siRNA or an empty vector were cotransfected with an HIV-1 LTR-luciferase reporter gene and the Tat-producing plasmid. Luciferase activities were measured 48 hr later.

(C) Overexpression of Brd4 in vivo increases basal level but decreases Tat-activated HIV-1 transcription. HeLa cells with an integrated HIV-1 LTR-luciferase reporter gene were transfected with the vectors expressing Brd4 (in 3-fold increments) and/or Tat.

In (B) and (C), the results were normalized and are shown as an average of triplicate experiments. The error bars represent the mean ± SD.

(D) Competition between Tat and Brd4 for binding to P-TEFb. HeLa cells were cotransfected with a Cdk9-f cDNA together with either an empty vector (vec) or the vector producing wt or the C22G mutant Tat. Cdk9-f and its associated CycT1 and Brd4 in anti-FLAG immunoprecipitates were examined by Western blotting. The levels of Brd4 and Tat in NEs of transfected cells were also shown.

to the paused pol II and thus may explain the reduced Tat transactivation caused by Brd4 overexpression (Figure 7C).

Discussion

In HeLa cells, the population of CycT1/Cdk9 heterodimers sequestered in the 7SK snRNP, where the Cdk9 kinase activity is suppressed, has been studied extensively (Chen et al., 2004; Michels et al., 2003, 2004; Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003, 2004). In contrast, little is known about the state of active P-TEFb in vivo, especially whether there may exist an unknown factor(s) that can bind to CycT1/Cdk9 and mediate its transcriptional activity in general. In fact, it is widely presumed that the transcriptionally active P-TEFb exists and functions in the form of a free CycT1/Cdk9 heterodimer (Price, 2000). This paper and the accompanying one by Jang et al. (2005), however, have provided biochemical evidence challenging this conventional view. In these two studies, an interaction between Brd4 and core P-TEFb has been identified, which is essential for P-TEFb to stimulate pol II transcriptional elongation in general. Furthermore, Brd4 has been shown to recruit P-TEFb to transcriptional templates in vivo and in vitro. Thus, the complex containing Brd4 and the CycT1/Cdk9 heterodimer should now be viewed as the real active form of P-TEFb distinct from the 7SK:HEXIM1:P-TEFb snRNP, which undergoes quantitative conversion into the former under certain stress conditions for stress-induced gene expression.

The prevailing belief that the free CycT1/Cdk9 heterodimer represents the transcriptionally active form of P-TEFb came from studies performed by others and us that employed the recombinant heterodimer, which was analyzed in transcription reactions containing the Cdk9-depleted HeLa NE (O'Keeffe et al., 2000; Peng et al., 1998a, 1998b). In hindsight, Brd4, which was re-

leased from core P-TEFb during immunodepletion under high-salt conditions used in these studies and therefore still present in the reactions (see Figure 4C), very likely reconstituted with the exogenously added recombinant heterodimer (just like with the affinity-purified core P-TEFb, Figure 4D), leading to the restoration of transcription.

Although P-TEFb is required for efficient transcription of most cellular genes (Chao and Price, 2001; Shim et al., 2002), a long-standing question in the field concerns how it is recruited to all the different gene promoters. However, an exception can be found in the case of HIV, where P-TEFb is recruited by the HIV-1 Tat protein to stimulate pol II elongation (Price, 2000). In addition, several DNA sequence-specific transcription factors such as CIITA (Kanazawa et al., 2000), NF-κB (Barboric et al., 2001), Myc (Eberhardy and Farnham, 2001), STAT3 (Giraud et al., 2004), the androgen receptor (Lee et al., 2001), and the aryl hydrocarbon receptor (Tian et al., 2003) have also been identified as P-TEFb-associated factors that can potentially recruit CycT1/Cdk9 to their respective promoter targets. Because these are gene-specific transcription factors, their recruitment of P-TEFb would be expected to affect only a limited group of genes that bear their specific recognition sequences.

In contrast to these gene-specific transcription factors, Brd4 is not known to bind to any specific promoter sequences. Furthermore, unlike these previously identified P-TEFb-recruitment factors, whose gene-specific functions do not require them to bind to a substantial portion of cellular P-TEFb, Brd4 is able to associate with most, if not all, of the 7SK/HEXIM1-free CycT1/Cdk9 in vivo. This conclusion is based on the observations described in this and the accompanying paper (Jang et al., 2005) that about half of nuclear core P-TEFb was sequestered in the Brd4-containing complex and the other half in the inactive 7SK snRNP (Michels et al., 2003; Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003). In addition, the 7SK snRNP underwent quantitative conversion into the active Brd4-containing P-TEFb complex in stress-treated cells (Figure 2). Although only the HIV-1 LTR-based DNA/chromatin templates were used in the P-TEFb-recruitment assays performed under Tat-free conditions, the fact that Brd4 was able to increase the overall CTD phosphorylation in vivo and activate transcription from several cellular promoters (Jang et al., 2005) indicates a general role for this protein in transcription. Taken together, the abilities of Brd4 to associate stoichiometrically with the transcriptionally active P-TEFb, stimulate pol II phosphorylation and transcription in general, and recruit P-TEFb to DNA/chromatin templates in vitro and in vivo strongly implicate Brd4 as a general P-TEFb-recruitment factor for most pol II-dependent transcription.

It is interesting to note that although Brd4 enhances basal level transcription from the HIV-1 promoter, it is actually not required for Tat transactivation. In fact, excessive Brd4 interfered with Tat function (Figure 7C). The fact that Brd4 and Tat competed for binding to the same core P-TEFb (Figure 7D) indicates that the binding of one recruitment partner to P-TEFb may sterically prevent the binding of the other. Therefore, it appears that HIV-1 utilizes Tat as a highly efficient and specific P-TEFb-recruitment factor that can completely replace Brd4's analogous function on cellular genes. Besides Tat, it remains to be tested whether other known gene-specific P-TEFb-recruitment factors may also have a competitive relationship with Brd4.

What could be the target or targets for Brd4's recruitment of P-TEFb to cellular promoters? Besides binding to half of the cellular CycT1/Cdk9, Brd4 has also been shown to have high affinity for acetylated core histones H3 and H4, which are preferentially localized to the transcriptionally active euchromatin regions (Dey et al., 2003). Importantly, the bromodomains of Brd4 can apparently engage in simultaneous interactions with both acetylated histones and CycT1/Cdk9 (Jang et al., 2005). Furthermore, when the effect of the histone deacetylase inhibitor TSA on the binding of Brd4 and P-TEFb to the chromatin template was analyzed in cells expressing the Brd4-specific siRNA, the recruitment of P-TEFb to the chromatin template was found to depend on Brd4's binding to acetylated histones (Jang et al., 2005). This result implicates acetylated chromatin as a possible target for Brd4's recruitment of P-TEFb to transcriptional templates in vivo.

However, it is important to point out that both the transcriptional stimulation and P-TEFb-recruitment functions of Brd4 can also be detected in vitro in reactions containing simply naked DNA. These results suggest the existence of an alternative, nonchromatin-based mechanism for the Brd4-mediated recruitment of core P-TEFb. Indeed, data presented in this paper suggest that the human Mediator complex could potentially fit into such a role, as a mutant P-TEFb (S175D) incapable of binding to Brd4 lost the ability to associate with the Mediator (Figure 6C) and the HIV-1 DNA template in vitro (Figure 6B). This dependence on the Mediator for Brd4's recruitment of P-TEFb may also explain the observed stimulation of the CTD phosphorylation by the Mediator complex (Jiang et al., 1998).

Although both acetylated histones and the Mediator have been implicated to play a critical role in facilitating the recruitment of P-TEFb to transcriptional templates through Brd4, more structure-functional analyses are needed in the future to further characterize these interactions. We have noticed that the Brd4-mediated interaction of P-TEFb with the Mediator was apparently not stoichiometric (data not shown). Thus, besides the Mediator complex, there could be other components of the transcriptional machinery, especially those that can be modified through acetylation (Sterner and Berger, 2000), which may also help attract the Brd4-containing P-TEFb complex to cellular promoters. Finally, P-TEFb has been shown to interact directly with the CTD through a histidine-rich tract in CycT1 (Taube et al., 2002). Thus, Brd4 could conceivably facilitate the recruitment of P-TEFb to transcriptional templates through strengthening the P-TEFb-CTD interaction.

Brd4 has been implicated to play a growth-stimulatory role as suggested by the demonstration that the Brd4-heterozygotic mice display pre- and postnatal growth defects associated with a reduced proliferation rate (Houzelstein et al., 2002). On the other hand, several reports have documented the antigrowth functions of HEXIM1 and 7SK in cardiac myocytes and breast epithelial cells (Sano et al., 2002; Wittmann et al., 2003).

The opposing effects on cellular growth exerted by Brd4 and 7SK/HEXIM1, both of which target core P-TEFb but produce antagonizing results, support the idea that controlling the activity of the general transcription factor P-TEFb is central to the global regulation of cell growth and differentiation.

Experimental Procedures

Immuno-Affinity Purification of Cdk9-f and Brd4-f and Their Associated Factors

NEs were prepared from transfected HeLa or HeLa-based F1C2 and MCAP cells stably expressing Cdk9-f and Brd4-f, respectively. The extracts were dialyzed against buffer D (20 mM HEPES-KOH [pH 7.9], 15% glycerol, 0.2 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) containing 0.15 M KCl (D0.15M). The Cdk9 and Brd4-containing complexes were then affinity purified from NEs by incubating at 4°C for 2 hr with the anti-FLAG agarose beads (Sigma). After extensive washes with buffer D containing various amounts of KCl as indicated in the text, the purified proteins were eluted from the beads with the FLAG peptide prepared in buffer D0.1M.

Immunodepletion of Cdk9 and/or Brd4 from HeLa NE

Immunodepletion was performed by incubating 50 μl of HeLa NE (~7 mg/ml) containing 0.2% NP-40 and 0.5 M KCl with 3 mg of anti-Cdk9 and/or anti-Brd4 antibodies at 4°C for 30 min, followed by three rounds of incubation with 15 μl of protein A-Sepharose beads (Amersham Biosciences). The depleted extracts were dialyzed against buffer D0.1M prior to analysis in transcription and ChIP assays.

Transcription Assay

In vitro transcription reactions containing standard or immunodepleted HeLa NE and HIV-1 promoter templates were carried out as described previously (Zhou and Sharp, 1995). G-less RNA fragments derived from in vitro-transcribed HIV-1 transcripts were isolated after RNase T1 treatment and analyzed on 6% polyacrylamide sequencing gels.

ChIP Assay

HeLa cells (3×10^6) containing an integrated luciferase reporter gene under the control of the HIV-1 LTR (Yang et al., 2001) were seeded into a 15 cm dish one day before transfection. Cells were transfected with the vector expressing wt or mutant Cdk9-f (1 μg) together with constructs expressing untagged CycT1 (10 μg) and Brd4 (6 μg). At 36 hr posttransfection, cells were harvested and subjected to the ChIP assay based on a recently published protocol (Wang et al., 2005). After DNA purification, PCR reactions containing α-[^{32}P] dCTP (800 Ci/mmol) were carried out for 20 cycles, and the products were analyzed on a 6% polyacrylamide gel. Input and immunoprecipitated chromatin were analyzed first in pilot experiments to ensure that PCR reactions were occurring in the linear range of amplification.

In Vitro Kinase Assay

Core P-TEFb containing wt or mutant Cdk9-f were immunoprecipitated from NEs of transfected HeLa cells and washed with buffer D0.5M to strip away the associated factors. The kinase reactions contained core P-TEFb, 1 μg of immobilized GST-CTD, 5 mM $MgCl_2$, 50 μM cold ATP, and 1 μl of γ-[P^{32}]-ATP (3000 Ci/mmol) and were incubated at 30°C for 30 min. The phosphorylated GST-CTD was analyzed by SDS-PAGE followed by autoradiography.

Luciferase Assay

Retroviruses with or without the Brd4-specific siRNA (5′-GAACCTCCCTGATTACTATAA-3′) expression cassette cloned into the pSUPER vector (Oligoengine, WA) were produced in the GP2-293 packaging cell line (Clontech, CA). HeLa cells were then infected with the viral supernatants and selected with puromycin for 3 days. The selected cells in 6-well plates were cotransfected with 100 ng of the HIV-1 LTR luciferase reporter construct and 10 ng of a Tat-expressing plasmid. The total amount of plasmids (1.2 μg) was kept constant for each transfection by adjusting with the empty vector. Luciferase activity was measured 48 hr later. For the experiment measuring the effect of Brd4 overexpression on HIV-1 transcription, the cell line containing an integrated HIV-1 LTR-luciferase reporter gene was used.

Immobilized DNA Template Binding Assay

HeLa NE (50 μl at ~7 mg/ml) dialyzed against buffer D0.1M was incubated with 50 μl of IVT (in vitro transcription) buffer (50 mM KCl, 6.25 mM $MgCl_2$, 20 mM HEPES [pH 7.9], 2 mM DTT, 0.5 mM EDTA, 10 μM $ZnSO_4$, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and 8.5% glycerol), 1.0 μg of biotinylated HIV-1 LTR template (–168 to +302), and 1.0 μg of poly(dI-dC) at 30°C. After 30 min incubation, 400 μl of dilution buffer B (10 mM HEPES [pH 7.9], 100 mM KCl, 0.5% NP40, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF) and 30 μl of streptavidin-agarose beads (Upstate) preblocked in dilution buffer B plus 2 mg/ml BSA and 0.2 μg poly(dI-dC) were added to the reaction mixture and rotated at 4°C for 2 hr. The immobilized complexes were then washed extensively with dilution buffer B and eluted with 1× SDS-PAGE loading buffer at 95°C.

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