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AFF1 is a ubiquitous P-TEFb partner to enable Tat extraction of P-TEFb from 7SK snRNP and formation of SECs for HIV transactivation

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The positive transcription elongation factor b (P-TEFb) stimulates RNA polymerase elongation by inducing the transition of promoter proximally paused polymerase II into a productively elongating state. P-TEFb itself is regulated by reversible association with various transcription factors/cofactors to form several multi-subunit complexes [e.g., the 7SK small nuclear ribonucleoprotein particle (7SK snRNP), the super elongation complexes (SECs), and the bromodomain protein 4 (Brd4)–P-TEFb complex] that constitute a P-TEFb network controlling cellular and HIV transcription. These complexes have been thought to share no components other than the core P-TEFb subunits cyclin-dependent kinase 9 (CDK9) and cyclin T (CycT, T1, T2a, and T2b). Here we show that the AF4/FMR2 family member 1 (AFF1) is bound to CDK9–CycT and is present in all major P-TEFb complexes and that the tripartite CDK9–CycT–AFF1 complex is transferred as a single unit within the P-TEFb network. By increasing the affinity of the HIV-encoded transactivating (Tat) protein for CycT1, AFF1 facilitates Tat's extraction of P-TEFb from 7SK snRNP and the formation of Tat–SECs for HIV transcription. Our data identify AFF1 as a ubiquitous P-TEFb partner and demonstrate that full Tat transactivation requires the complete SEC.

Transcription by RNA polymerase II (Pol II) is a dynamic process consisting of several distinct but interconnected stages. Over the past three decades, much attention has been focused on the initiation and preinitiation stages of the transcription cycle, because they had been thought to be the principal points at which transcription is controlled (1, 2). Since 2007, however, accumulating evidence has revealed that promoter-proximal pausing of Pol II during early elongation is much more prevalent than previously thought, suggesting that intricate control of gene expression can occur frequently at this stage also (3, 4). Indeed, the importance of controlled pause and release of Pol II is illustrated by the observations that this process plays a prominent role in regulating cell growth, renewal, and differentiation (5, 6).

Transcription of the integrated HIV-1 proviral genome is hypersensitive to elongation defects, thus making it an ideal model for elucidating the mechanism and factors that control elongation. It has long been known that in the absence of the HIV-encoded transactivating protein (Tat), Pol II can initiate transcription from the viral promoter efficiently but pauses soon after the synthesis of a short RNA segment that folds into a stem–loop structure termed the "transactivation-response" (TAR) element. Tat overcomes Pol II pausing by recruiting the host positive transcription elongation factor b (P-TEFb) to the newly formed TAR RNA to stimulate the production of full-length HIV transcripts (7, 8). Containing cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CycT1), P-TEFb triggers the release of paused Pol II by phosphorylating and thereby antagonizing the inhibitory actions of two negative elongation factors, DSIF and NELF (5, 6). P-TEFb also phosphorylates the C-terminal domain (CTD) of the largest subunit of Pol II, leading to the assembly of key transcription and RNA-processing factors on the CTD for stimulating elongation and the coupled mRNA processing (5).

Recently, studies using proteomics and biochemistry have demonstrated that, rather than the isolated P-TEFb, Tat recruits a family of closely related multisubunit complexes called the "super elongation complexes" (SECs), in which P-TEFb is a component, to the viral promoter to activate transcription (9, 10). Within the SEC, the AF4/FMR2 family proteins AFF1 and AFF4 are characterized as highly flexible scaffolding subunits that use short hydrophobic regions along their structurally disordered axis to bring two different transcription elongation factors, P-TEFb and ELL1 or 2, and members of the eleven-nineteen-leukemia/ALL1-fused gene from chromosome 9 protein (ENL/AF9) family into a single complex (10, 11). The recently solved AFF4–P-TEFb crystal structure further illustrates that a short sequence (amino acids 34–67) located near the AFF4 N terminus snakes across the surface of CycT1 and also may make direct contacts with Tat (12). When an SEC is recruited to the HIV promoter by Tat and TAR, the contained P-TEFb and ELL1/2 components potentially can act on the same polymerase enzyme to activate transcription synergistically (10). However, direct biochemical evidence still is needed to confirm that P-TEFb alone is not sufficient for full Tat activity and that efficient HIV transactivation must rely on the complete SEC.

In addition to residing in the SEC, P-TEFb also exists in several other complexes and shuttles between inactive and active states in response to different intracellular and extracellular signals (5, 7, 8). Under normal conditions, most nuclear P-TEFb

Significance

Transcriptional elongation by RNA polymerase II produces full-length RNA transcripts and plays a general and prominent role in regulating gene expression. The positive transcription elongation factor b (P-TEFb) is one of the most important transcription factors controlling this process. The core P-TEFb, consisting of cyclin-dependent kinase 9 and cyclin T (CycT), exists in a network of complexes that include the 7SK small nuclear ribonucleoprotein particle (7SK snRNP), the super elongation complexes (SECs), and the bromodomain protein 4 (Brd4)–P-TEFb complex. This study identifies AF4/FMR2 family member 1 (AFF1) as a ubiquitous binding partner of core P-TEFb throughout the entire P-TEFb network. By increasing the affinity of the HIV-encoded transactivating (Tat) protein for CycT1, AFF1 is required for Tat's extraction of P-TEFb from 7SK snRNP and the formation of SECs for maximal HIV transcriptional activation.

is sequestered in a catalytically inactive complex called "7SK small nuclear ribonucleic particles" (snRNP), which serves as a major cellular reservoir of uncommitted P-TEFb activity (13–16). When cells are exposed to a number of stress conditions that globally impact growth, the 7SK snRNP complex is disrupted, and P-TEFb is transferred to another complex containing bromodomain protein 4 (Brd4), which delivers P-TEFb to many primary-response genes to activate their expression (17–19). In HIV-infected cells, Tat also has been shown to target 7SK snRNP directly to extract P-TEFb (7), which eventually is converted to the SEC through a yet-to-be determined mechanism.

The core P-TEFb subunits CDK9 and CycT traditionally have been thought to be the only proteins shared among the various P-TEFb complexes, and AFF1 is considered a signature component of only the SEC. Here, we present evidence indicating that AFF1 also is bound to CDK9–CycT1 in 7SK snRNP and in the Brd4-containing complex. AFF1 is transferred together with CDK9–CycT1 from 7SK snRNP to the SEC by HIV Tat and from 7SK snRNP to Brd4 during the stress response. Previously, Tat and the 7SK snRNP component hexamethylene bis-acetamide–inducible 1 (HEXIM1) have been shown to bind competitively to the same region of CycT1 (20). Here we show that, by enhancing the interaction between Tat and CycT1, AFF1 facilitates Tat's extraction of P-TEFb from 7SK snRNP and the formation of the complete SEC. Together, our data identify AFF1 as a ubiquitous partner of core P-TEFb and provide definitive proof that full Tat activity requires the complete SEC.

Results

AFF1 Interacts with 7SK snRNP Components La Ribonucleoprotein Domain Family, Member 7 and HEXIM1. La ribonucleoprotein domain family, member 7 (LARP7) and HEXIM1 are two signature components of the 7SK snRNP (13, 21), whereas AFF1 has been identified as a scaffolding subunit of the SEC (10, 11). Surprisingly, when anti-AFF1 immunoprecipitates derived from HeLa nuclear extracts were examined by Western blotting, not only the conventional SEC subunits ELL2 and ENL but also LARP7 and HEXIM1 were readily detected (Fig. 1A, lanes 2 and 3). To rule out the possibility that the latter two proteins are bona fide but previously overlooked SEC subunits, the anti-LARP7 and anti-HEXIM1 immunoprecipitations also were examined (Fig. 1A, lanes 4–7). Indeed, both proteins failed to interact with the SEC subunits ELL2 and ENL but once again coprecipitated well with AFF1, CDK9, and CycT1 and also with each other. It is important to note that only endogenous but not transiently expressed proteins were analyzed in these coimmunoprecipitation (co-IP) experiments.

7SK snRNA-Dependent Interactions of AFF1 with HEXIM1 and LARP7. LARP7 and HEXIM1 can exist both inside and outside the 7SK snRNP (13, 21). To determine whether their interactions with AFF1 occur within the snRNP, HeLa nuclear extracts were incubated with RNase A to destroy 7SK snRNA, which functions as a scaffold to hold all the proteins together in the snRNP (15), before being subjected to anti-AFF1 immunoprecipitation. The destruction of 7SK snRNA removed all HEXIM1 and a significant portion of LARP7 but not CDK9 or CycT1 from the immunoprecipitated AFF1 (Fig. 1B), revealing the 7SK-dependent interactions of AFF1 with HEXIM1 and, to a lesser degree, LARP7. Consistently, the destruction of 7SK RNA by RNase A before anti-LARP7 immunoprecipitation also removed a significant portion of the associated AFF1 and P-TEFb and at the same time abolished the LARP7–HEXIM1 interaction (Fig. 1C).

It has been shown previously that mutant LARP7 with either its RNA-recognition motif (RRM) replaced by that of the prototypic human La or containing the Y127D substitution at a highly conserved position in the RRM displays a markedly decreased ability to bind to 7SK snRNA and other 7SK snRNP components (13). The data from immunoprecipitation and Western blotting in Fig. 1D indicate that these two mutants also showed significantly reduced interactions with AFF1, in agreement with
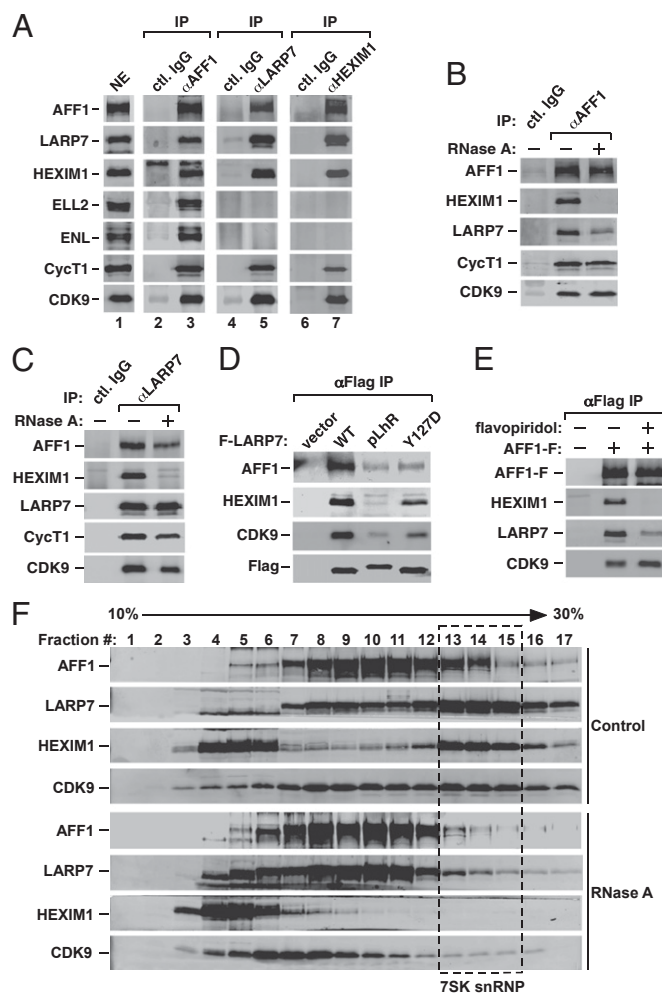

Fig. 1. AFF1 is a bona fide subunit of 7SK snRNP. (A) HeLa nuclear extracts (NE) were subjected to immunoprecipitation using the indicated specific antibodies or rabbit total IgG as a negative control (ctl). The immunoprecipitates (IP) and nuclear extracts were examined by Western blotting for the various proteins as marked. (B and C) HeLa nuclear extracts were incubated with RNase A before analysis by immunoprecipitation and Western blotting as in A. (D) HeLa cells were transfected with empty vector or with plasmid vectors expressing the indicated Flag-tagged WT or mutant LARP7 proteins. Anti-Flag immunoprecipitates derived from nuclear extracts were analyzed as in A. (E) HeLa cells expressing AFF1-Flag were treated with either flavopiridol or the vehicle control DMSO (–) and then were analyzed as in A. (F) HeLa nuclear extracts were incubated with or without RNase A and then were analyzed by glycerol gradient sedimentation. The indicated proteins in collected fractions were detected by Western blotting. The dashed box highlights the fractions that contain intact 7SK snRNP before RNase destruction.

the demonstration described above that the LARP7–AFF1 interaction is largely 7SK dependent.

AFF1 Is a Bona Fide Subunit of 7SK snRNP. Further evidence indicating that AFF1 is a component of 7SK snRNP came from an experiment that examines the effect of the CDK9 inhibitor flavopiridol on 7SK snRNP formation. It has been shown previously that stress-inducing agents such as flavopiridol can disrupt the RNP to release P-TEFb for stress-induced gene expression (19, 21–23). As is consistent with AFF1 being an integral part of 7SK snRNP, treating HeLa cells with flavopiridol caused the dissociation of all HEXIM1 and most LARP7 from immunoprecipitated Flag-tagged AFF1 (AFF1-Flag) (Fig. 1E). In contrast, the interaction of AFF1-Flag with CDK9 was largely unaffected.

Finally, the association of AFF1 with 7SK snRNP also was confirmed by a glycerol gradient analysis. The destruction of 7SK snRNA by RNase A caused all HEXIM1 and most LARP7 present in fractions 13–15, which sedimented near the bottom of the gradient and reflect the large-sized 7SK snRNP, to migrate toward the top of the gradient that corresponds to smaller-sized liberated proteins (Fig. 1F). The RNase A-induced disruption of 7SK snRNP also caused most CDK9 to disappear from fractions 13–15 and to reappear in fractions 6–8, a likely indication of the release of P-TEFb from the snRNP. Importantly, RNase A also markedly decreased the AFF1 levels in fractions 13–15 and shifted the position of AFF1 toward the top, suggesting that AFF1 was associated with 7SK snRNP before the latter's destruction. Taken together, these data indicate that AFF1 is a bona fide subunit of 7SK snRNP.

AFF1's Interaction with P-TEFb Is both Necessary and Sufficient for Its Sequestration in 7SK snRNP. AFF1 and its close homolog AFF4 are known to use dispersed short segments along their flexible axis to contact the various SEC subunits to assemble the complete SEC complexes (10, 11). To investigate whether AFF1 uses the same strategy to associate with 7SK snRNP, we performed co-IP experiments to examine the ability of three AFF1-deletion mutants to interact with components of the 7SK snRNP and the SEC. First, deletion of the AFF1 308 N-terminal amino acids, which correspond to the first 300 amino acids of AFF4 known to harbor the identified CycT1-binding site (CBS) (10, 11), caused AFF1 to lose the interactions with P-TEFb as well as with the 7SK snRNP subunits HEXIM1 and LARP7 (Fig. 2A). However, this mutant (Δ1–308), which still retains the regions homologous to those used by AFF4 to contact ELL2 and ENL (10, 11), displayed normal interactions with these two SEC components. In contrast to AFF1(Δ1–308), the other two AFF1 deletions, Δ358–378 and Δ757–776, showed drastically reduced binding to ELL2 and ENL, respectively, but still maintained WT interactions with HEXIM1 and LARP7 (Fig. 2A, lanes 4 and 5).

To demonstrate that the CBS is both necessary and sufficient for AFF1's association with 7SK snRNP, the isolated AFF1 N-terminal fragment [amino acids 1–308, hereafter, AFF1(1–308)] containing this segment was tested in another co-IP experiment (Fig. 2B). Moreover, upon fusion to GFP and the nuclear localization sequence, two shorter AFF1 fragments, amino acids

1–77 and 38–72, which correspond to the biochemically identified minimal CBS of AFF4 (amino acids 2–73) (11) and the ordered AFF4 segment resolved in the AFF4–P-TEFb crystal structure (12), respectively, were tested also (Fig. 2C). The data indicate that all three AFF1 fragments were capable of interacting with P-TEFb, HEXIM1, and LARP7 but not with the SEC subunits ELL2 and ENL; of the three, AFF1(38–72) displayed the weakest binding to 7SK snRNP. Taken together, these experiments indicate that the interaction with P-TEFb is both necessary and sufficient for AFF1's sequestration in the 7SK snRNP. AFF1 apparently uses only its N-terminal CBS to contact P-TEFb and then contacts the rest of the 7SK complex through P-TEFb and 7SK RNA.

Di-Alanine Substitutions in CBS Block AFF1's Interaction with 7SK snRNP. Several di-alanine substitutions introduced into the AFF4 CBS have been shown to decrease AFF4's binding to P-TEFb and to inhibit basal HIV transcription (11, 12). Because the CBS also is important for AFF1's interaction with 7SK snRNP, we generated and tested three AFF1 di-alanine mutations based on the effects of their corresponding mutations in AFF4. Of the three, M60A/L61A displayed the most drastic reduction in interaction with P-TEFb, and P39A/L40A had the smallest effect (Fig. 2D). The interactions of the three AFF1 mutants with HEXIM1 and LARP also showed the same or an even more exaggerated trend (Fig. 2D), as is consistent with the notion that the direct binding between the AFF1 CBS and P-TEFb enables AFF1 to associate with the rest of 7SK snRNP. It is interesting that the two AFF4 di-alanine substitutions that correspond to P39A/L40A and M60A/L61A in AFF1 were found to reduce P-TEFb binding equally well (11), revealing a difference between these two homologous proteins (Fig. 2E).

AFF1–P-TEFb Interaction Facilitates Tat's Extraction of P-TEFb from 7SK snRNP. With AFF1 shown to be a bona fide subunit of 7SK snRNP, it is important to determine the functional significance of this phenomenon. The recently solved AFF4–P-TEFb crystal structure reveals that the AFF1 homolog AFF4 is positioned to make direct contacts with HIV Tat on the surface of CycT1 (12). In light of this information and the previous demonstrations that Tat is able to extract P-TEFb directly from 7SK snRNP both in vitro and in HIV-infected cells (7), we examined the effect of AFF1 on Tat-induced disruption of 7SK snRNP.
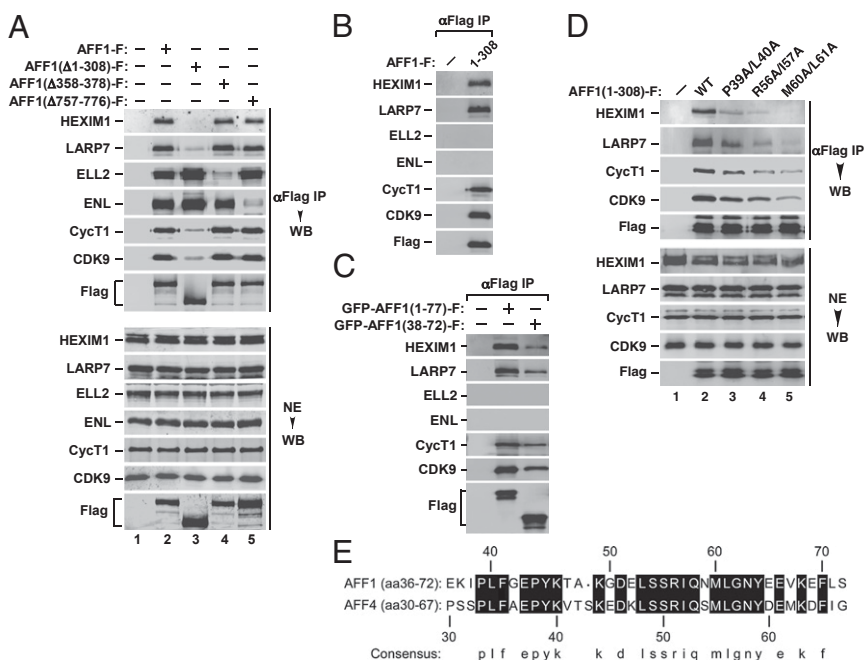

Fig. 2. AFF1's interaction with P-TEFb is both necessary and sufficient for its sequestration into 7SK snRNP. (A) Nuclear extracts were prepared from HeLa cells expressing the Flag-tagged full-length or truncated AFF1 proteins. Nuclear extracts and the anti-Flag immunoprecipitates derived from them were examined by Western blotting (WB) for the indicated proteins. (B–D) Anti-Flag immunoprecipitates from nuclear extracts of transfected HeLa cells were analyzed as in A. (E) The alignment of homologous AFF1 and AFF4 sequences encompassing the minimal CBS. The dark blocks identify amino acids that are identical in the two sequences.

Using the dissociation of 7SK snRNP components from immunoprecipitated Flag-tagged CDK9 (CDK9-Flag) as an indication of snRNP disruption, neither the expression of a relatively low level of Tat alone nor the introduction of extra AFF1 into cells produced much effect (Fig. 3A, compare lanes 4 and 5 with lane 2). However, coexpression of Tat and AFF1 caused a drastic reduction in the levels of HEXIM1 and methyl-phosphate-capping enzyme (MePCE) bound to P-TEFb and also partially decreased the interactions of 7SK snRNA and LARP7 with P-TEFb (Fig. 3A, lane 3). Meanwhile, more AFF1 became associated with P-TEFb in the presence of Tat than in its absence (Fig. 3A, compare lanes 3 and 5), as is consistent with the previous demonstration that Tat enhances the affinity of the AFF1 homolog AFF4 for P-TEFb (12).

The ability of AFF1 to promote Tat's extraction of P-TEFb from 7SK snRNP also was demonstrated from HEXIM1's perspective when the associations of CDK9 and CycT1 with immunoprecipitated HEXIM1 were analyzed. Again, the presence of extra AFF1 greatly facilitated Tat's disruption of the HEXIM1–P-TEFb interaction (Fig. 3B).

In contrast to the stimulatory effect caused by extra AFF1 in cells, reducing the AFF1 expression through shRNA (shAFF1)–mediated knockdown (KD) produced the opposite effect, interfering with Tat's extraction of P-TEFb from 7SK snRNP (Fig. 3C). Interestingly, AFF1 KD led to a general decrease in the nuclear level of HEXIM1, which in turn resulted in an overall reduction in the amounts of HEXIM1 bound to immunoprecipitated CDK9-Flag (Fig. 3C, compare lanes 4 and 5 with lanes 2 and 3). However, even though fewer 7SK snRNP were present in cells under these conditions, the loss of AFF1 still consistently decreased the ability of Tat to dissociate HEXIM1 from P-TEFb. In one representative experiment shown in Fig. 3C, Tat induced only 16% HEXIM1 dissociation in AFF1-KD cells, as compared with 53% in control cells. The residual Tat activity in the AFF1-KD cells could be caused by the presence of AFF4, which remained bound to P-TEFb and may have compensated for the loss of AFF1 (Fig. 3C, lanes 4 and 5).

Finally, we analyzed the effects of AFF1(1–308) containing either the WT or the M60A/L61A mutant CBS on Tat's dissociation of HEXIM1 from immunoprecipitated CDK9-Flag (Fig. 3D). Although WT AFF1(1–308) bound readily to P-TEFb and at the same time enabled Tat to dissociate HEXIM1, the mutant failed in both respects (Fig. 3D, compare lanes 4 and 5 with 3). This result indicates that the direct binding to CycT1 is required for AFF1 to facilitate Tat's extraction of P-TEFb from 7SK snRNP.
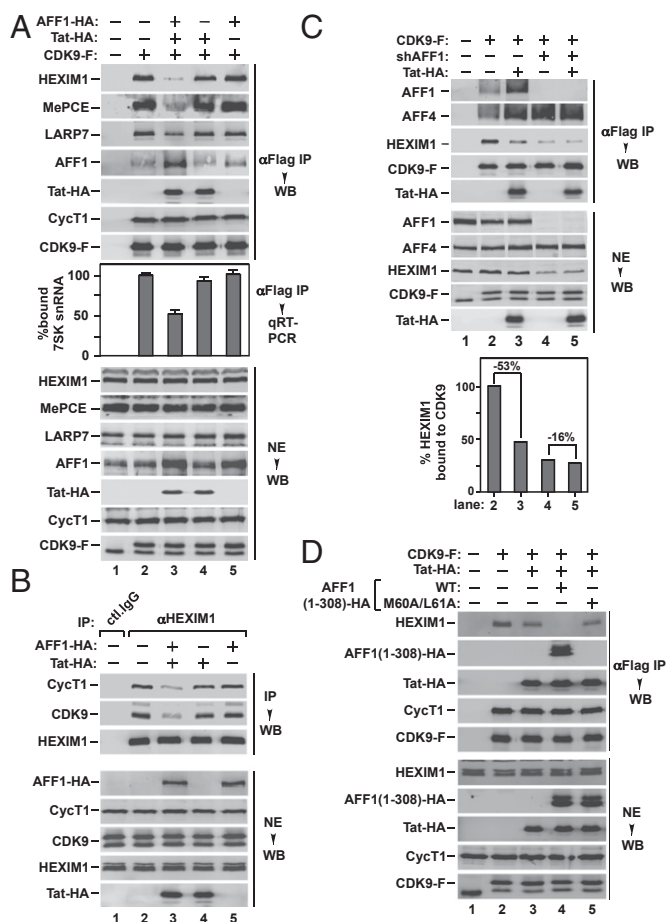
AFF1 Enhances the Affinity of Tat for P-TEFb. In an effort to probe the molecular basis underlying the AFF1-facilitated Tat's extraction of P-TEFb, we discovered that AFF1 significantly enhanced the binding to P-TEFb by the C22G mutant Tat protein (Fig. 4A, lanes 9 and 10). This mutation is located in the Cys-rich Tat-activation domain and has been shown to inhibit the Tat–CycT1 interaction severely, likely by abolishing an essential Cys–zinc bridge required for proper folding and function of Tat (24). The introduction of additional AFF1 into cells rescued the otherwise very weak interaction between Tat(C22G) and P-TEFb (Fig. 4A, lanes 9 and 10), although it did not affect the binding of WT Tat to P-TEFb significantly (Fig. 4A, lanes 7 and 8), probably because this binding already is quite strong under the current conditions involving overexpressed WT Tat (Fig. 4A, compare lanes 7 and 9).

The ability to rescue the binding of Tat(C22G) to P-TEFb was found to depend on AFF1's direct interaction with CycT1 through its N-terminal CBS sequence. Although AFF1(1–308) containing WT CBS significantly enhanced the Tat(C22G)–P-TEFb interaction, the fragment harboring the M60A/L61A substitution in CBS completely lacked this ability (Fig. 4B). These results, in combination with the recent demonstration that AFF4 and Tat likely make direct contact on the surface of CycT1 and that Tat enhances AFF4's affinity for P-TEFb (12), strongly support the notion that AFF4/1 and Tat bind cooperatively to CycT1.

AFF1 Enables Tat(C22G) to Extract P-TEFb from 7SK snRNP and Activate HIV LTR. HEXIM1 and Tat interact with CycT1 in a region that is immediately C-terminal to the cyclin box (24-26). Previous studies have shown that the interactions of Tat and HEXIM1 with CycT1 are mutually exclusive (20). Thus it is highly likely that the ability of AFF1 to increase the affinity of Tat for CycT1 is the principal driving force behind its promotion of Tat's extraction of P-TEFb from 7SK snRNP. In accordance with this idea, Tat(C22G), which by itself could barely associate with the immunoprecipitated CDK9 (Fig. 4C, lane 3), not only dramatically increased binding to CDK9 but also caused the dissociation of HEXIM1 and LARP7 from P-TEFb when expressed together with AFF1(1–308) (Fig. 4C, lane 4).

The functionality of the cooperative bindings of Tat(C22G) and AFF1 to CycT1 is illustrated further by the observation that the mutant Tat strongly synergized with AFF1 in stimulating the HIV LTR-driven luciferase expression from a stably integrated reporter construct in HeLa-based NH1 cells (Fig. 4D) (10). Although Tat(C22G) alone displayed no activation of HIV transcription (Fig. 4D, compare lanes 1 and 3), its cooperation with

Fig. 3. AFF1's interaction with P-TEFb promotes Tat's extraction of P-TEFb from 7SK snRNP. (A–D) Nuclear extracts were prepared from HeLa cells cotransfected with the indicated expression constructs and examined by Western blotting to detect the various proteins as marked. Nuclear extracts also were subjected to immunoprecipitation with the indicated antibodies, and the resultant immunoprecipitants were analyzed by Western blotting. The levels of 7SK snRNA bound to immunoprecipitated CDK9-Flag were examined by qRT-PCR (A, Middle). The levels of HEXIM1 associated with immunoprecipitated CDK9-Flag in lanes 2–4 in C were quantified, normalized to those of CDK9-Flag, and plotted (C, Lower) with the level detected in lane 2 set artificially to 100%.
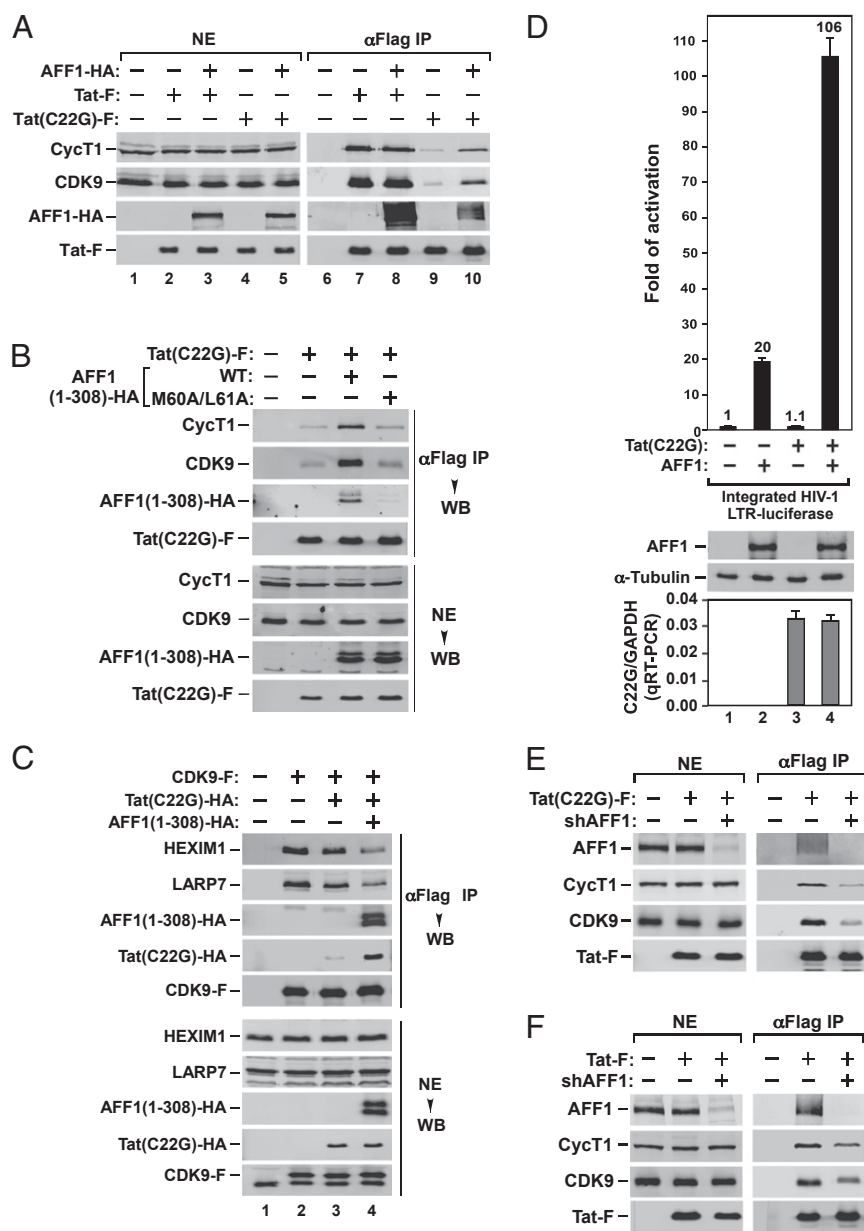
Fig. 4. AFF1 enhances the affinity of Tat for P-TEFb and enables Tat(C22G) to extract P-TEFb from 7SK snRNP and activate HIV transcription. (*A–C*) Nuclear extracts from HeLa cells cotransfected with the indicated expression constructs were subjected to anti-Flag immunoprecipitation, and the resultant immunoprecipitants were analyzed by Western blotting. (*D*) The HeLa-based NH1 cells containing the integrated HIV-1 LTR-luciferase reporter gene were transfected with the Tat(C22G)- and/or AFF1-expressing construct as labeled. Luciferase activities were measured in cell extracts, with the level of activity detected in cells transfected with an empty vector (–) set to 1. The error bars represent mean ± SD from three independent measurements. The cellular levels of AFF1 protein and the ratios of Tat (C22G) to GAPDH mRNAs were detected by Western blotting and qRT-PCR, respectively. (*E* and *F*) Nuclear extracts and anti-Flag immunoprecipitants derived from nuclear extracts of HeLa cells expressing either the indicated Tat proteins or shAFF1 were examined by Western blotting.

AFF1 enabled the viral LTR to be activated to a much higher level than that caused by AFF1 alone (Fig. 4*D*, compare lanes 2 and 4).

In contrast to the enhanced interaction between Tat(C22G) and P-TEFb as a result of extra AFF1 in the cells (Fig. 4*A*), the interaction was decreased markedly by the introduction of a specific shRNA, shAFF1, that knocked down AFF1 expression (Fig. 4*E*). Notably, the KD even reduced the interaction between WT Tat and P-TEFb (Fig. 4*F*), which was largely insensitive to overexpressed AFF1 (Fig. 4*A*). Taken together, these results are consistent with the notion that AFF1 enhances the affinity of Tat for CycT1, which competitively dissociates HEXIM1 and is responsible for AFF1's promotion of Tat's extraction of P-TEFb from 7SK snRNP.

AFF1(1–308) Is a Dominant-Negative Inhibitor of Tat's Activation of HIV Transcriptional Elongation. Based on our observations that the AFF1 CBS potently enhanced the Tat–CycT1 binding and strongly promoted Tat's extraction of P-TEFb from 7SK snRNP, one would expect this domain to be a powerful stimulator of Tat transactivation. To test this hypothesis, the effect of the CBS-containing AFF1(1–308) on Tat's activation of the integrated

HIV LTR-luciferase expression was analyzed in NH1 cells. Intriguingly, AFF1(1–308) not only failed to promote Tat transactivation; in fact, it strongly inhibited this process in the presence of endogenous AFF1 (Fig. 5*A*, compare lane 5 with lane 2, which harbors an empty vector). In contrast, expression of full-length AFF1 markedly enhanced Tat's activation of the LTR (Fig. 5*A*, compare lanes 3 and 2), indicating that the level and activity of AFF1 in these cells still was limiting. Finally, the N-terminally deleted AFF1 (Δ1–308) missing the CBS produced only a very minor effect on Tat transactivation (Fig. 5*A*, lane 4).

By conducting a quantitative RT-PCR (qRT-PCR)–based assay that measured the relative abundance of HIV mRNAs at several different locations downstream of the viral transcription start site, the inhibitory effect of AFF1(1–308) was found to occur largely at the transcription elongation level (Fig. 5*B*): AFF1(1–308) displayed the smallest inhibitory effect (31% reduction) on the Tat-induced production of very short HIV transcript from nucleotides 10–59 (nas), whereas its inhibition of the synthesis of longer transcripts at downstream locations (pro, int, and dis) was much more pronounced (90–94% reduction; Fig. 5*B*).
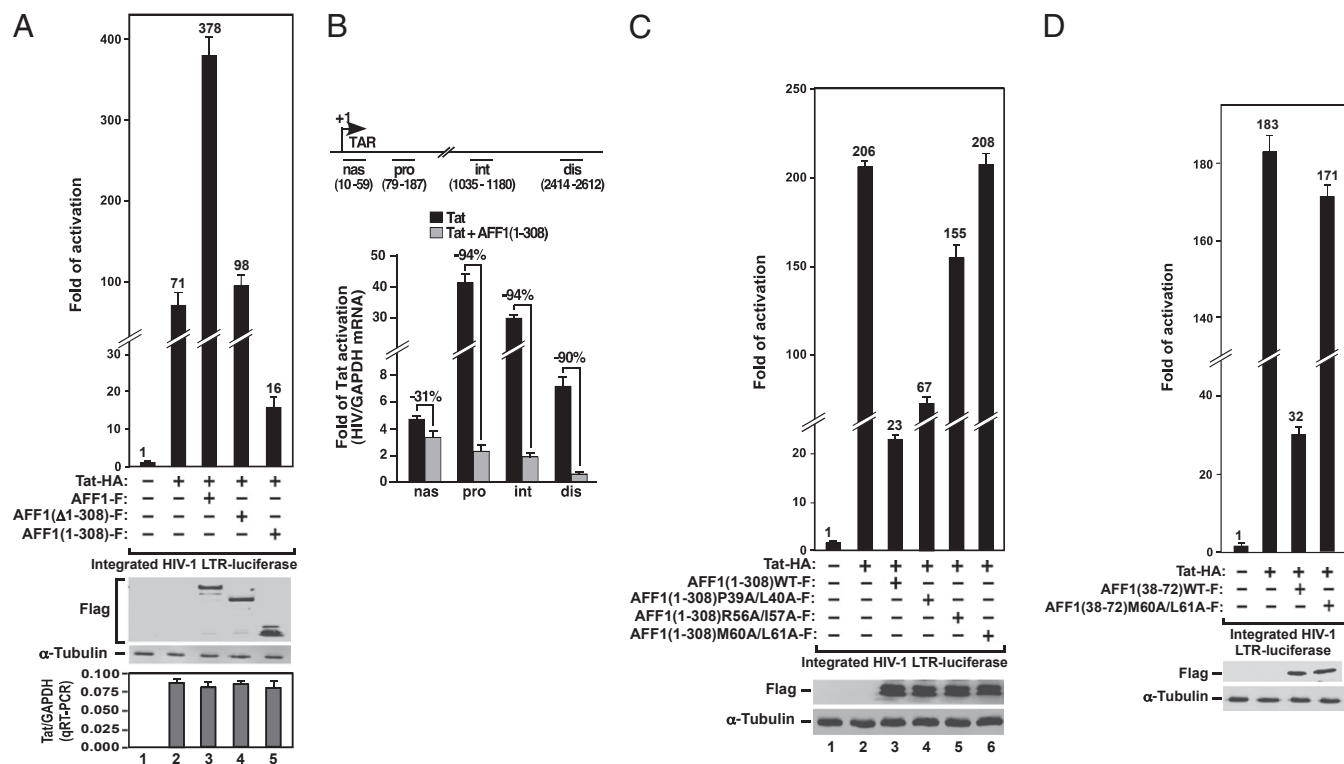
Fig. 5. AFF1(1–308) is a dominant-negative inhibitor of Tat-activated HIV transcriptional elongation, and the inhibition depends on AFF1's binding to P-TEFb through its N-terminal CBS sequence. (*A*) Luciferase activities were measured in extracts of NH1 cells containing the integrated HIV-1 LTR-luciferase reporter gene and expressing the indicated proteins. The activity detected in cells containing an empty vector (lane 1) was set to 1. The ratios of Tat to GAPDH mRNAs were determined by qRT-PCR and plotted (*A, Bottom*). The error bars represent mean ± SD from three independent experiments. (*B*) Total RNA was isolated from NH1 cells transfected with an empty vector or vectors expressing Tat alone or Tat plus AFF1(1–308). The mRNAs transcribed from the integrated HIV LTR-luciferase reporter gene were analyzed by qRT-PCR with primers that hybridize to the four different locations along the DNA template and were normalized to the GAPDH signal. The fold increase of Tat activation was calculated by comparison with signals obtained with an empty vector. The error bars represent the mean ± SD from three independent measurements. (*C* and *D*) Luciferase activities were measured and analyzed as in *A*. In *D*, the AFF1 fragments were expressed as GFP fusion proteins.

Binding of Isolated AFF1 CBS to P-TEFb Prevents Tat from Activating HIV Transcription. To confirm that the interaction with P-TEFb is required for the isolated AFF1 CBS to inhibit Tat's activation of HIV transcription, we compared the ability of WT CBS and the CBS di-alanine mutants described above to suppress the Tat-activated, HIV LTR-driven luciferase expression. Although AFF1 (1–308) containing WT CBS demonstrated strong suppression (Fig. 5*C*, lane 3 versus lane 2, showing a 9.0-fold reduction), the three mutants displayed no or partial suppressive activities (Fig. 5*C*, lanes 4–6). Importantly, the degree of suppression caused by the mutants correlated precisely with their levels of binding to P-TEFb (compare Figs. 5*C* and 2*D*). For instance, M60A/L61A and P39A/L40A displayed the weakest and strongest binding to P-TEFb, respectively, with R56A/I57A in the middle (Fig. 2*D*). Reflecting these differences, AFF1(1–308)M60A/L61A showed no suppression of Tat transactivation, whereas P39A/L40A displayed only mildly diminished suppressive ability as compared with WT, and R56A/I57A again had an intermediate effect (Fig. 5*C*).

Not only did the 308-aa AFF1 N-terminal fragment containing WT CBS demonstrate dominant-negative inhibition of Tat transactivation, a much shorter fragment, AFF1(38–72), corresponding to the ordered AFF4 segment resolved in the AFF4–P-TEFb crystal structure (12), also exerted efficient inhibition (Fig. 5*D*). Again, the M60A/L61A substitution introduced into this background largely abolished the inhibitory effect of this minimal CBS segment (Fig. 5*D*).

Binding of Isolated AFF1 CBS to P-TEFb Prevents Tat from Assembling Complete SECs. To investigate how the binding of the isolated AFF1 CBS to P-TEFb inhibits Tat transactivation, we examined the

ability of Tat to interact with the various SEC components in cells in which AFF1(1–308) containing either WT or mutant CBS sequences was overexpressed. In the absence of AFF1(1–308), Flag-tagged Tat (Tat-Flag) was able to coprecipitate with all the examined SEC subunits (Fig. 6*A*, lane 8). However, the presence of WT AFF1(1–308) caused Tat to bind readily to this AFF1 fragment but to lose the interactions with most SEC components (AFF1, AFF4, ELL2, and ENL) except CDK9 and CycT1 (Fig. 6*A*, lane 9). Thus, only a subcomplex containing Tat, P-TEFb, and AFF1(1–308) but not the complete Tat–SEC was able to form under these conditions. Given that AFF1 sequences C-terminal to the CBS are essential for recruiting components other than P-TEFb into the complete SEC (10, 11), it is likely that the AFF1 CBS in the subcomplex prevented full-length AFF1/4 from binding to P-TEFb and recruiting ELL2 and ENL into the complete SEC.

Again, correlating precisely with their different abilities to bind P-TEFb and inhibit Tat transactivation, the three di-alanine CBS mutants displayed a spectrum of activities in inhibiting the formation of the complete Tat–SEC. Specifically, from AFF1 (1–308)M60A/L61A to R56A/I57A and then on to P39A/L40A, there was a gradual increase in the abilities of these mutants to block the formation of Tat–SEC (Fig. 6*A*, lanes 10–12); this increase correlated well with their increasing ability to suppress Tat transactivation (Fig. 5*C*).

Full Tat Transactivation Requires Occupancy of the Complete Tat–SEC on HIV LTR. Combined, the data in Figs. 6*A* and 5*C* strongly suggest that full Tat transactivation as observed in Fig. 5*C* lane 2 requires the complete SEC. In contrast, the partial HIV LTR activation detected in Fig. 5*C* lane 3 very likely was mediated by the Tat–P-TEFb sub-
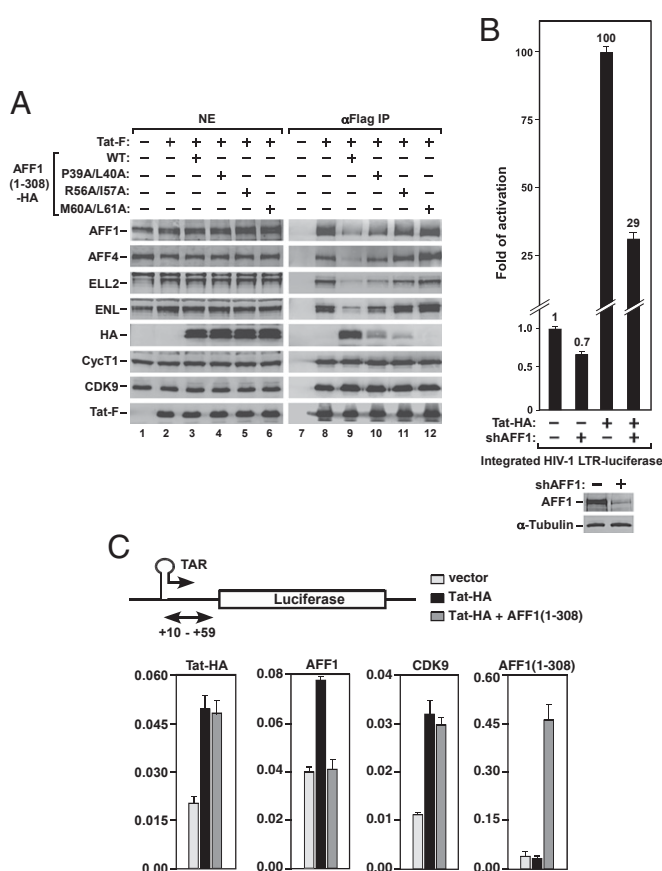
Fig. 6. Binding of AFF1 CBS to P-TEFb prevents Tat from assembling complete SECs on HIV LTR. (*A*) Nuclear extracts from HeLa cells expressing the indicated proteins and anti-Flag immunoprecipitant derived from the extracts were examined by Western blotting for the presence of the various proteins as marked. (*B*) (*Upper*) Luciferase activities were measured in extracts of NH1 cells expressing Tat-HA or/and shAFF1. The activity detected in cells containing an empty vector (lane 1) was set to 1. The error bars represent the mean ± SD from three independent experiments. (*Lower*) The efficiency of shAFF1-mediated AFF1 KD was determined by Western blotting using α-tubulin as a loading control. (*C*) NH1 cells transfected with an empty vector or vectors expressing Tat alone or Tat plus AFF1(1–308) were subjected to ChIP analysis to determine the levels of the indicated factors bound to the HIV promoter-proximal region from +10 to +59. The signals were normalized to those of input and were plotted. The error bars represent the mean ± SD from three independent measurements.

complex, which was the only part of the Tat–SEC left undisrupted by the dominant-negative CBS of AFF1 (Fig. 6*A*, lane 9).

To prove from a different perspective that maximal Tat activity truly depends on the complete SEC, shAFF1 was used to knock down expression of the SEC scaffolding subunit AFF1 (Fig. 6*B*). As did the inhibitory CBS, shAFF1 markedly suppressed Tat's activation of the HIV LTR (Fig. 6*B*). Its suppressive effect on Tat-independent viral transcription was comparatively less prominent.

The data presented so far indicate that, when expressed *in trans*, the AFF1 CBS prevents the formation of the complete, fully active Tat–SEC by inhibiting the scaffolding function of WT AFF1, and this inhibition in turn leads to the assembly of a less potent subcomplex consisting only of Tat, P-TEFb, and the bound CBS. To show that these CBS-induced events do occur on an integrated HIV chromatin template where they produce the expected functional consequence, quantitative ChIP analysis of the bindings of representative Tat–SEC components on an HIV promoter-proximal region (from +10 to +59) was conducted in NH1 cells containing an integrated HIV LTR-luciferase reporter construct (Fig. 6*C, Upper*).

As expected, the expression of Tat alone resulted in a significant increase in the occupancy not only by Tat but also by full-length AFF1 and CDK9 at the HIV promoter-proximal region (Fig. 6*C*), indicating the formation of Tat–SECs under these conditions. However, when Tat was coexpressed with WT AFF1 (1–308), a marked decrease in the level of AFF1 but little decrease in the levels of Tat and CDK9 was observed. Meanwhile, the coexpression also led to the detection of a high level of AFF1 (1–308), indicating the presence of the Tat–P-TEFb–AFF1(1–308) subcomplex at the promoter. Taken together, these results are consistent with a CBS-induced destruction of the complete Tat–SEC to create the Tat–P-TEF–CBS subcomplex on the viral LTR; this subcomplex is responsible for the significant decrease in Tat transactivation in CBS-expressing cells (Fig. 5*C*).

AFF1 also Is an Integral Component of the Brd4–P-TEFb Complex and Contacts Brd4 Through P-TEFb. Upon identification of AFF1 as a common subunit shared by 7SK snRNP and the SEC, we were curious to know whether this protein also could be found in the Brd4–P-TEFb complex, which is another major P-TEFb–containing complex known to exist in the nucleus (18, 19). The first piece of evidence supporting this possibility came from the identification by mass spectrometry of all the proteins that exist in the same complex(es) with stably expressed CDK9-Flag and Brd4-HA. These proteins were isolated through sequential affinity purifications using anti-Flag and then anti-HA beads in the same manner used to isolate the Tat–SEC (10). AFF4, AFF1, Brd4, and P-TEFb (CDK9, CycT1, and T2) but no other known components of 7SK snRNP or the SEC were found among the identified proteins (Table S1).

To verify the interaction between Brd4 and AFF1 and to rule out any potential artifact caused by ectopic expression of epitope-tagged proteins, coimmunoprecipitations followed by Western blotting were performed by targeting only the endogenous proteins. The experiments confirmed from both the AFF1 and Brd4 sides that Brd4 indeed interacts with AFF1 but not with the SEC component ELL2 (Fig. 7 *A* and *B*). In an effort to map the domains in AFF1 and Brd4 that are essential for AFF1's association with the Brd4–P-TEFb complex, we first found out that, just as in 7SK snRNP, AFF1(1–308) containing the CBS sequence was both necessary and sufficient for this association (Fig. 7*C*). Furthermore, the M60A/L61A substitution in CBS destroyed the interactions of AFF1 not only with CDK9–CycT1 but also with Brd4 (Fig. 7*D*). These results indicate that the association of AFF1 through its N-terminal CBS with CycT1 is sufficient for the AFF1–Brd4 interaction.

As for Brd4, the mutant lacking the C-terminal P-TEFb–interacting domain (ΔPID) (27) failed to bind to both P-TEFb and AFF1 (Fig. 7*E*), reinforcing the idea that no direct and stable interaction exists between AFF1 and Brd4 and that P-TEFb serves to connect the two proteins to form a larger complex. (This complex that contains P-TEFb, AFF1, Brd4, and perhaps other yet-to-be identified proteins henceforth is called the "PAB complex.") Finally, as is consistent with the autonomous nature of the Brd4–P-TEFb binding within this complex, the shRNA-mediated AFF1 KD did not affect the binding significantly (Fig. 7*F*).

CDK9–CycT1–AFF1 Is Transferred as a Single Unit to Brd4 upon Stress-Induced Disruption of AFF1-Containing 7SK snRNP. As indicated in Fig. 1*E*, when the CDK9 kinase inhibitor flavopiridol induced the disruption of 7SK snRNP, all HEXIM1 and most LARP7 were dissociated from AFF1. However, flavopiridol failed to disrupt the AFF1–P-TEFb interaction (Fig. 1*E*). It has been well documented that, when released from 7SK snRNP under many stressful conditions, including the inhibition of CDK9, P-TEFb is recaptured by Brd4, which delivers it to many primary-response genes for activation of stress-induced gene expression (17–19). Consistent with the idea that the tripartite CDK9–CycT1–AFF1 but not the CDK9–CycT1 dimer alone is transferred to Brd4 as a single unit from the disrupted 7SK snRNP, AFF1 was found to
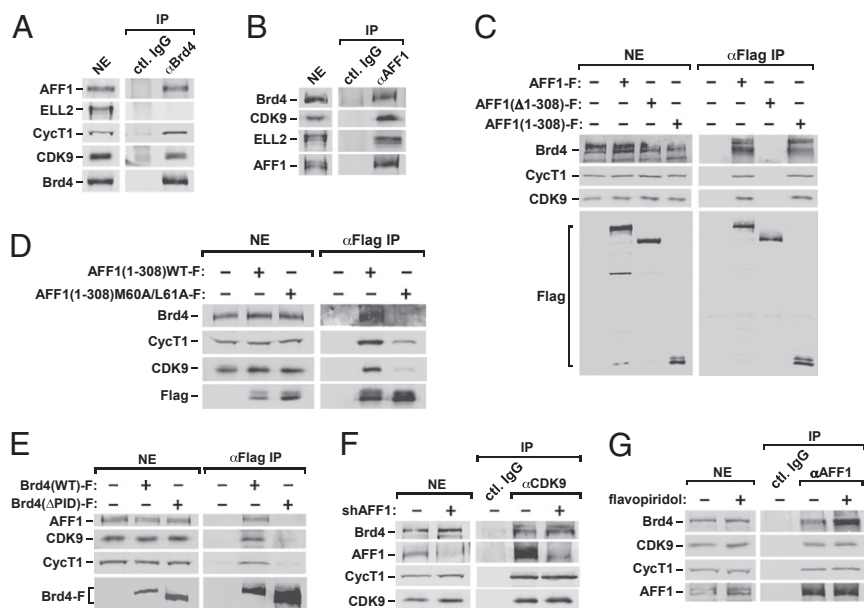
Fig. 7. AFF1 is an integral component of the Brd4–P-TEFb complex and arrives together with P-TEFb from stress-disrupted 7SK snRNP. (*A* and *B*) HeLa nuclear extracts were subjected to immunoprecipitation with the indicated specific antibodies or with rabbit total IgG as a negative control (ctl). The immunoprecipitants and nuclear extracts were analyzed by Western blotting for the various proteins as marked. (*C*–*E*) HeLa cells were transfected with the indicated expression constructs. Nuclear extracts and immunoprecipitants obtained with the indicated antibodies from the nuclear extracts were examined by Western blotting as in *A*. (*G*) HeLa cells were treated with flavopiridol or with the vehicle control DMSO (−) and then were analyzed as in *A*.

increase significantly the interaction with Brd4 but not with CDK9 and CycT1 in cells exposed to flavopiridol (Fig. 7*G*).

Discussion

The core P-TEFb consisting of CDK9 and CycT traditionally is viewed as the only invariant part of the entire network of P-TEFb complexes that include the 7SK snRNP, the Brd4-containing PAB complex, and the recently identified SEC. In this study, we present multiple lines of evidence to show that this popular assumption is incorrect for at least some fractions of cellular 7SK snRNP and PAB. Our data show that these two complexes clearly contain AFF1, which previously has been described only as a signature component of the SEC. Within these complexes, AFF1 is intimately associated with CycT1 through its N-terminal CBS sequence. When the AFF1-containing 7SK snRNP is targeted by HIV Tat or stress-inducing agents such as flavopiridol, the tripartite CDK9–CycT1–AFF1, but not the CDK9–CycT dimer, is released and transferred to Tat and Brd4, respectively (Fig. 8). Thus, instead of being merely a subunit of the SEC, AFF1 should be considered a ubiquitous partner of P-TEFb present in the entire network of P-TEFb complexes.

The trimeric composition of the CDK9–CycT1–AFF1 sub-complex existing as a single unit in larger macromolecular assemblies is reminiscent of the situation found in another multicomponent transcription-factor complex, TFIIH, which also contains a tripartite semiautonomous subcomplex (termed "CAK"), consisting of CDK7, CycH, and MAT1. MAT1 functions as an assembly factor for CAK and also can modulate CDK7 substrate specificity (28). Whether AFF1 may play a similar role in the network of P-TEFb complexes remains to be determined.

In addition to AFF1, AFF4 is another member of the AF4/FMR2 family that was indentified as a key component of the SEC (5, 6). The two proteins share ~37% identity over ~1,200 amino acids. Like AFF1, AFF4 has been found in all three major P-TEFb complexes in our study (Table S1 and Fig. 3*C*). In fact, AFF4 appears to be more predominant than AFF1 in PAB complexes, based on the number of unique peptides identified and the percentage of coverage by mass spectrometry (Table S1). Although both AFF1 and 4 exist in the major P-TEFb complexes, the two proteins show intriguing differences in their ability to mediate Tat transactivation, suggesting that the AFF1- and AFF4-containing complexes may have gene/activator-specific functions.
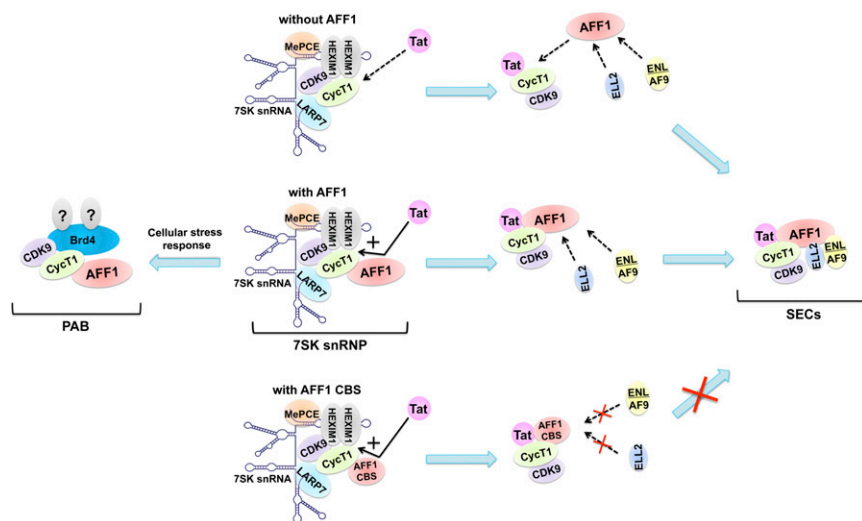

Fig. 8. AFF1 associates with P-TEFb to facilitate HIV Tat"s extraction of P-TEFb from 7SK snRNP and the formation of complete SECs for full activation of viral transcription. This model depicts AFF1 as a core P-TEFb subunit existing in all three major P-TEFb complexes and the role of AFF1 in facilitating Tat's disruption of 7SK snRNP and the formation of the SEC. Without AFF1 present in 7SK snRNP, Tat is much less efficient in extracting CDK9–CycT1 from 7SK snRNP and assembling the complete SEC. Although both full-length AFF1 and the AFF1 CBS can facilitate Tat's disruption of 7SK snRNP, only the former is capable of attracting other SEC components (ELL2 and ENL/AF9) into the complete SECs for efficient Tat transactivation.

These differences and their underling mechanisms and biological implications will be analyzed in detail in a separate study.

At this moment, we cannot tell whether AFF1/4 are present in all the 7SK snRNP and PAB complexes existing in a cell. That the introduction of extra AFF1 into HeLa cells further enhanced Tat's extraction of CDK9–CycT1 from 7SK snRNP (Fig. 3A) suggests that the 7SK snRNP population in this cell type has not been completely saturated with AFF1/4. Although not every 7SK snRNP may contain AFF1, our data indicate that the AFF1-containing subpopulation is preferentially targeted by Tat to release CDK9–CycT1. A likely explanation for this preference is that AFF1 existing in these snRNP significantly promotes Tat binding to CycT1, resulting in the dissociation of HEXIM1 that competes with Tat for binding to the same small region in CycT1 (20, 26). For example, AFF1 turns Tat mutant C22G, which by itself binds very poorly to CycT1, into a strong transactivator that can extract P-TEFb from 7SK snRNP efficiently and can activate HIV transcription (Fig. 4).

Recently, structural insights have provided an excellent explanation for AFF1's ability to enhance the Tat–CycT1 binding. It has been shown that Tat and the AFF1 homolog AFF4 not only are situated next to each other on the CycT1 surface but also bind cooperatively to CycT1 through their direct contact (12). The ability of AFF1/4 to increase the affinity of Tat for CycT1 can be explained by the observations that AFF4 provides additional binding surfaces for Tat and both AFF4 and CycT1 contribute to create a deep pocket which Tat occupies. This pocket is in contrast to the relatively flat and shallow Tat-binding grooves created by CycT1 alone (29), which result in a relatively weak interaction between Tat and CycT1.

When the tripartite CDK9–CycT1–AFF1 complex is transferred out of 7SK snRNP as a single unit, it enables Tat to assemble the complete SEC quickly without first having to find an AFF1 molecule to serve as the scaffold (Fig. 8). This process is blocked, and Tat transactivation is strongly suppressed, by the expression of the isolated AFF1 CBS in trans. Although the CBS can promote Tat's disruption of 7SK snRNP efficiently (Fig. 3D), the resultant CDK9–CycT1–CBS complex fails to attract the key SEC components ELL2 and ENL/AF9, and thus no functional SECs are formed on the viral LTR (Fig. 8).

Previously, anecdotal evidence has been reported supporting the idea that additional factors associated with the CDK9–CycT1 heterodimer are required to promote efficient HIV transcription. For example, the loss of Tat transactivation in CDK9-depleted nuclear extracts can be rescued by the addition of partially purified human P-TEFb, which may contain other associated factors, but not by the addition of recombinant CDK9–CycT1 (30). The results obtained with the AFF1 CBS in the current study have provided the strongest evidence so far in support of the idea that the P-TEFb subcomplex of the SEC is insufficient for Tat transactivation and that the complete SEC harboring two distinct classes of elongation factors within a single complex can lead to synergistic activation of HIV transcription (10). The minimal CBS peptide of AFF1(38–72) that possesses potent inhibitory activity could serve as the platform for developing small-molecule inhibitors of Tat transactivation that could suppress HIV replication therapeutically.

Within the SEC, AFF1/4 are known to be structural scaffolds that use their interspersed short hydrophobic regions to attract and coordinate other SEC components (7, 10, 11). However, AFF1does not seem to have such a role in either 7SK snRNP or the Brd4-containing PAB. In both complexes, AFF1 binds directly to CycT1 through its N-terminal CBS and does not appear to establish any independent and stable interactions with other components. In fact, CycT1 serves as a critical link connecting AFF1 to the rest of the two complexes.

Another intriguing observation is that the presence of AFF1 in 7SK snRNP and the PAB does not automatically result in the recruitment of the key SEC subunits ELL1/2, ENL, and AF9 into these two complexes. In the SEC, these proteins are known to bind to two distinct regions C-terminal to the CBS in AFF1 (10, 11). It is unclear how the binding of these signature SEC components to AFF1 is suppressed in 7SK snRNP and the PAB. Future studies are necessary to determine whether AFF1/4 can fold differently to mask the regions that normally are contacted by ELL1/2, ENL, and AF9 or whether differential post-translational modifications play a key role in allowing the binding to occur in one instance but not in others.

Experimental Procedures

Antibodies. Polyclonal antibodies against AFF1 (A302-344A; A302-345A), ELL2 (A302-505A-1), and ENL (A302-267A) were purchased from Bethyl Laboratories. The anti-AFF4 (ab57077) and anti-CycT1 (sc-10750) antibodies were from Abcam and Santa Cruz Biotechnology, respectively. The monoclonal antibodies against Flag (M2) and HA (3F10) were from Sigma-Aldrich and Roche, respectively. The antibodies against CDK9, LARP7, HEXIM1, and MePCE have been described previously (13, 15, 21, 31).

Co-IP. The co-IP assay was performed as described (10) with minor modifications. Briefly, for anti-Flag immunoprecipitation, nuclear extracts prepared from HeLa cells transfected with the indicated expressing constructs were incubated with anti-Flag agarose beads (Sigma) for 2 h before washing and elution. For precipitations of endogenous proteins, nuclear extracts were incubated overnight with the specific antibodies or total rabbit IgG and then with protein A beads (Invitrogen) for 1 h. After incubation, the immunoprecipitates were washed extensively with buffer D [20 mM Hepes-KOH (pH 7.9), 15% (vol/vol) glycerol, 0.2 mM EDTA, 0.2% Nonidet P-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 0.3 M KC] before elution with 0.1 M glycine (pH 2.0). The eluted materials were analyzed by Western blotting with the indicated antibodies.

Generation of AFF1-KD Cells. To silence AFF1 expression in HeLa cells, a specific shRNA sequence (shAFF1), 5′- CCGGGCCTCAAGTGAAGTTTGACAACTCG-AGTTGTCAAACTTCACTTGAGGCTTTTTG-3′, was cloned into the lentiviral vector pLKO.1. shRNA specific for GFP was used as a nontarget control. Lentivirus production and infection of HeLa cells were conducted as previously described (32).

Quantitative PCR. The reactions were performed with Applied Biosystems 7300 Real-Time PCR System and Finnyzme F-410L SYBR Green RT-PCR reagents according to the manufacturers' instructions. PCR primers were designed with Integrated DNA Technologies' Primer Quest. PCR conditions included an initial denaturing step at 92 °C for 2 min and then 40 (for qRT-PCR) or 50 (for ChIP-PCR) cycles of amplification. Each cycle consisted of 30 s at 92 °C, 30 s at 57 °C, and 30 s at 68 °C. For ChIP-PCR, threshold values (Ct) were calculated and normalized to the input. For qRT-PCR, the values were normalized to those of GAPDH to obtain the relative folds of induction. All reactions were run in triplicate.

ChIP Assay. The ChIP assay was performed as described (33) with some modifications. Briefly, NH1 cells containing the integrated HIV-1 LTR-luciferase reporter construct (34) and transfected with the indicated expression constructs were cross-linked with 2 mM disuccinimidyl glutarate for 45 min, followed by 1% formaldehyde for 10 min. Cross-linking was quenched by the addition of glycine (0.125 M for 5 min). Fixed cells were collected, resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1), and fragmented (30 s on/30 s off, for a total processing time of 30 min) using a Covaris-S2 sonicator (Covaris, Inc.). Sonicated lysates equivalent to 2×10^6 cells were incubated overnight with 3 μg antibodies per reaction, and the purified products were analyzed by qPCR. The sequences of the primers used for amplification of the HIV-1 promoter-proximal region are forward 5′-GTTAGACCAGATCTGAGCCT-3′ and reverse 5′-GTGGGTTCCCTAGTTAGCCA-3′. All signals were normalized to input DNA, and signals generated by nonspecific IgG in control immunoprecipitations were subtracted from the signals obtained with specific antibodies.

Glycerol Gradient Analysis. Glycerol gradients (10–30%) were established in modified buffer D [20 mM Hepes-KOH (pH 7.9), 0.3 M KCl, 0.2 mM EDTA, 0.1% Nonidet P-40] in 13.5-mL Beckman centrifugation tubes. HeLa cells were lysed in 0.5 mL of modified buffer D for 30 min at 4 °C. The lysates were centrifuged at 10,000 × g for 10 min, and the supernatants were loaded carefully over the top of the preformed glycerol gradients. Proteins then were fractionated at 4 °C by centrifugation in an SW 41Ti rotor (Beckman) at 38,000 rpm for 21 h. Fractions were collected, precipitated with trichloroacetic acid, and analyzed by immunoblotting with the appropriate antibodies.

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