Two-pronged Binding with Bromodomain-containing Protein 4 Liberates Positive Transcription Elongation Factor b from Inactive Ribonucleoprotein Complexes*§

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The positive transcription elongation factor b (P-TEFb) exists in two forms in cells as follows: an inactive form where the core components cyclin T1 and CDK9 are incorporated in the 7SK small nuclear ribonucleoprotein complex containing the inhibitory molecule HEXIM1, and an active form, part of which associates with the bromodomain-containing protein BRD4. Here, we define a novel interaction between P-TEFb and BRD4 involving triacetylated cyclin T1 (acK380, acK386, and acK309) and the second bromodomain in BRD4. This interaction is observed with the short splice variant of BRD4 (amino acids 1–722) lacking a previously defined C-terminal P-TEFb-interacting domain (PID). Notably, P-TEFb complexes associated with short BRD4 contain HEXIM1 and 7SK snRNA, implicating the PID in the liberation of P-TEFb from the 7SK small nuclear ribonucleoprotein complex (7SK snPNP). Overexpression of the PID alone in cells dissociates HEXIM1 and 7SK snRNA from P-TEFb, but it is not sufficient to activate P-TEFb-dependent transcription of the HIV LTR. Our data support a model where two BRD4 domains, the second bromodomain and the PID, bind P-TEFb and are required for full transcriptional activation of P-TEFb response genes.

Transcription of eukaryotic genes by RNA polymerase II is tightly regulated at several levels, including preinitiation, initiation, promoter clearance, pausing, elongation, and termination (1, 2). The transition to productive elongation depends on the activity of the positive transcription elongation factor b (P-TEFb), which consists of a kinase subunit, the cyclin-dependent kinase 9 (CDK9), and one of three types of regulatory cyclin subunits, cyclin T1, cyclin T2a, and cyclin T2b (3). The kinase activity of CDK9 is required for phosphorylation of the negative transcription elongation factor and the C-terminal domain (CTD) of RNA polymerase II (on serine 2), a mechanism that dramatically enhances the elongation competence of RNA polymerase II complex (3).

P-TEFb exists in two major forms in cells. The catalytically active form consists of cyclin T proteins and CDK9 and is associated with bromodomain-containing protein 4 (BRD4), a member of the mammalian Mediator complex (4–6), or, as recently described, with a complex including mixed lineage leukemia fusion partners and the Paf1 elongation complex (7, 8). In contrast, catalytically inactive P-TEFb consists of cyclin T proteins and CDK9 associated with the 7SK snRNP, including 7SK snRNA and the inhibitory molecule HEXIM1 or in some tissues HEXIM2 (9, 10). Transition between inactive and active complexes is dynamic and tightly regulated by cell growth and stress responses (4, 11). Notably, post-translational modifications of components of P-TEFb are involved in regulating this transition. For example, dephosphorylation of threonine 186 in CDK9 by protein phosphatases PP1α and PP2B (12) or PPM1A and PPM1B (13) and phosphorylation of threonine 270 and serine 278 in HEXIM1 by phosphoinositide 3-kinase and protein kinase B (AKT) result in the dissociation of the 7SK snRNP and activation of P-TEFb (14, 15).

Recently, we showed that the acetylation of four lysines in the coiled-coil region of cyclin T1 liberates active P-TEFb from the 7SK snRNP (16). However, it remains unclear how acetylation of lysines in cyclin T1 contributes mechanistically to the activation of P-TEFb. We focused on BRD4 because it binds

**Significance:** BRD4 activates P-TEFb via the PID, and cyclin T1 acetylation may support this process.
P-TEFb and contains two tandem bromodomains, which represent bona fide binding domains for acetylated lysines (17). Other domains in BRD4 include an extra-terminal domain and the C-terminal helical PID conserved in other bromodomain and extra-terminal domain-containing (BET) proteins (18). The bromodomains in BRD4 (aa 58–169 and 349–461) were previously implicated in the interaction with P-TEFb (6) but also bind acetylated lysines in histones (19–21). A signature of acetylated Lys-9 in histone H3 and Lys-16 in H4 and phosphorylated serine 10 in histone H3 was identified in BRD4, previously implicated in the interaction with P-TEFb (6) but also bind acetylated lysines in histones (19–21). The PID in P-TEFb interacts. Our binding of P-TEFb to BRD4 and identify distinct roles of the C-terminal helical PID conserved in other bromodomain binding domains for acetylated lysines (17).

Here, we examined the role of cyclin T1 acetylation in the binding of P-TEFb to BRD4 and identify distinct roles of the BRD4 bromodomains and the PID in P-TEFb interactions. Our studies uncover that bromodomain 2 (BD2) in BRD4 binds tri-acetylated cyclin T1 and that the PID plays an active role in the dissociation of HEXIM1 from P-TEFb.

EXPERIMENTAL PROCEDURES

Materials—We purchased antibodies against CDK9, cyclin T1, cAMP-response element-binding-protein-binding protein (each from Santa Cruz Biotechnology, Santa Cruz, CA), HA (Roche Applied Science), tubulin, and FLAG (M2) (Sigma). Rabbit anti-HEXIM1 antibodies were a gift from Q. Zhou (University of California, Berkeley) and O. Bensaude (Ecole Normale Superieure, France). HA-cyclin T1 plasmids were previously described (16), and FLAG-tagged CDK9 and GST-CTD were provided by A. Rice (Baylor College of Medicine). Constructs encoding wild type and mutant FLAG-BRD4 expression vectors were previously described (24). The HIV LTR luciferase reporter construct was previously described (27).

The cDNA encoding each of the two bromodomains (BD1 and BD2) of human BRD4 (NP_4090597) consisting of residues 44–168 and 333–460, respectively, were received as a gift from Structural Genomics Consortium. The BD1 and BD2 cDNAs were cloned into the pNiC28-Bsa4 expression vector with a tobacco etch virus cleavage site. These recombinant constructs were transformed into Escherichia coli BL21 (DE3) cells for protein expression. Fusion proteins were purified using an NTA-agarose column and further purified after tobacco etch virus cleavage of the hexa-His tag by Superdex 75 size exclusion column. 15N-Labeled proteins for the NMR study were prepared in M9 minimal media using 15NH4Cl as the sole nitrogen source in H2O.

Transient Transfection and Luciferase Assays—HeLa cells were seeded into 6-well plates 12–24 h before transfection and were transfected using Lipofectamine reagent according to the manufacturer’s instructions (Invitrogen). In transactivation assays with the HIV LTR promoter reporters, HeLa cells were cotransfected with 200 ng of reporter construct and 800 ng of FLAG-tagged wild type and mutant BRD4 (ΔPID, PID, and PID-AAA). For PID competition assays, HeLa cells were cotransfected with HIV LTR-luciferase reporter plasmid (200 ng), wild type BRD4 (500 ng) or vector control, and PID- or PID-AAA-expressing constructs (500 ng). Luciferase activity was measured with the luciferase assay system kit from Promega (Madison, WI).

In Vitro Kinase Assay—293T cells were transfected using Lipofectamine as described above, and the assay was performed as described previously (16). Briefly, cells were cotransfected with HA-cyclin T1 and a construct expressing CDK9 together with the FLAG-tagged BRD4 constructs or a vector control. Total cell lysates were immunoprecipitated with HA antibodies. Immunoprecipitated material was subjected to in vitro kinase assays containing bead-bound P-TEFb, recombinant murine GST-CTD, and 10 µl of magnesium/ATP mixture (Millipore). Reactions were incubated at 30 °C for 20 min in a 50-µl volume. As a positive control, 10 units of recombinant CDK9/ cyclin T1 expressed in insect cells (Millipore) were incubated with recombinant GST-CTD. The complexes were resolved by SDS-PAGE and analyzed by Western blotting with anti-RNA polymerase II-CTD repeat YSPTSPS (phospho-Ser-2) antibodies (Abcam, Cambridge, MA) or anti-GST.

In Vitro Acetylation and Binding Assays—Plasmids expressing GST-cyclin T1 proteins were transformed into competent E. coli (strain BL21), and expression was induced by treatment with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. Solubilized proteins were purified with glutathione-coupled beads (Amersham Biosciences) and incubated with [acetyl-14C]coenzyme A (Amersham Biosciences) and purified histone acetyltransferase domain of p300 as described previously (28). After 30 min at 30 °C, beads were washed twice with 50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, and 0.075% SDS and once with 50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, and 1 mM DTT. Samples were denatured in Laemmli buffer and separated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue. The TnT-coupled transcription/translation reticulocyte lysate system (Promega) was used for BRD4 722 and BRD4 722ΔBD-expressing constructs containing Sp6 promoter in in vitro transcription/translation reactions following the manufacturer’s instructions. The translation product was confirmed by Western blotting.

NMR Spectroscopy Titration of Protein/Peptide Binding—Cyclin T1 peptides containing acetylated lysines were chemically synthesized by the Fmoc method. The cyclin T1 peptides are acK390 (VSLSacKEYRAC), ac390/386 (NSKSVPsAcKVS-LacKEYC), ac390/380 (NSacKVPsAKSLacKEYC), and ac380/386/390 (NSacKVPsAcKVS-LacKEYC). NMR samples containing recombinant BRD4-BD1 or BRD4-BD2 at 0.5 mM were prepared in 100 mM sodium phosphate buffer, pH 6.5, containing 100 mM NaCl and 1.0 mM DTT in 90% H2O, 10% 2H2O. NMR spectra were acquired at 25 °C on a Bruker 600 MHz spectrometer. BRD4-BD1 or BRD4-BD2 binding to lysine-acetylated cyclin T1 peptides was assessed by using two-dimensional 1H-15N-HSQC NMR spectra by titrating a stock solution of a lysine-acetylated cyclin T1 peptide into 15N-labeled BRD4-BD1 or BRD4-BD2 (0.1 mM) to a molar ratio of protein-to-peptide of 1:5. Binding affinity was determined monitoring changes of chemical shift perturbations of selected.
protein residues as a function of peptide concentration using the same procedure as reported previously (29).

RESULTS

Bromodomains and PID in BRD4 Interact with Distinct P-TEFb Complexes—To determine which domain in BRD4 mediates association to P-TEFb, we performed coimmunoprecipitation experiments with FLAG-tagged BRD4 proteins and endogenous P-TEFb. Experiments were performed in 293T cells with the long isoforms of BRD4 (BRD4 1362 aa), the short isoform of BRD4 (BRD4 722 aa), and the full-length BRD4 with deletions of both bromodomains (BRD4 1362ΔBD; Fig. 1A).

Following immunoprecipitation with FLAG antibodies, robust signals for cyclin T1 and CDK9 were detected in cells expressing full-length and 1362ΔBD BRD4, and weaker signals for both proteins were observed in cells expressing BRD4 722 (Fig. 1B, lanes 6–8). Western blotting with antibodies against FLAG and endogenous cyclin T1, CDK9, and HEXIM1 proteins. Note that the figure shows results from one gel and one exposure, and unrelated lanes loaded on the same gel were cut. WB, Western blot; ET, extra-terminal.
BRD4 with bromodomain deletions (BRD4 722ΔBD). Mutant BRD4, lacking both bromodomains, failed to bind endogenous cyclin T1 and CDK9 indicating that residual binding in the absence of the PID is mediated by the BRD4 bromodomains (Fig. 1C, lane 7). In this experiment, we also probed for HEXIM1 in the immunoprecipitated material. Surprisingly, we found that the short isoform of BRD4 bound HEXIM1 efficiently, whereas no HEXIM1 was found coimmunoprecipitating with full-length BRD4 despite the robust binding of cyclin T1 and CDK9 (Fig. 1C, lane 6 versus 8). Similar results were obtained when the immunoprecipitated material was analyzed by real-time RT-PCR for the presence of 7SK snRNA (supplemental Fig. 1). Importantly, binding of HEXIM1/7SK snRNA to the short BRD4 isoform also depended on the bromodomains in BRD4, suggesting that HEXIM1 and 7SK snRNA in complex with cyclin T1 and CDK9 associated with bromodomains in BRD4. These results demonstrate that BRD4 bromodomains and PID bind distinct P-TEFb complexes; the bromodomains interact with P-TEFb complexes bound to HEXIM1 and 7SK snRNA and are presumably inactive, whereas the PID binds complexes that lack HEXIM1 and 7SK snRNA and are considered active.

**BRD4 Bromodomain Interactions with P-TEFb Involve Cyclin T1 Acetylation**—To explore the role of cyclin T1 acetylation in the binding to the BRD4 bromodomains, we performed coimmunoprecipitation assays of cellular FLAG-tagged BRD4 722 proteins with an HA-tagged mutant of cyclin T1 in which all four acetylation sites were mutated to arginines (4KR (16)). After immunoprecipitation with α-FLAG antibodies, we found that binding of BRD4 722 to the 4KR mutant of cyclin T1 was 3-fold decreased implicating a role of cyclin T1 acetylation in the interaction with BRD4 (Fig. 2A, lanes 6 and 8). Importantly, a 6-fold decrease in binding was observed when wild-type cyclin T1 and the BRD4 722ΔBD mutant were tested, supporting a model where interactions between acetylated lysines and BRD4 bromodomains mediate the binding (Fig. 2A, lanes 6 and 7). Remarkably, a further decrease (10-fold) in binding was observed when mutant cyclin T1 and mutant BRD4 722 were tested pointing to additional effects of the BRD4 bromodomains or the 4KR mutant that are independent of cyclin T1 acetylation.
acetylation. The 4KR mutation may cause subtle structural changes or affect other post-translational modifications in P-TEFb.

We also performed experiments with BRD4 722 lacking only one bromodomain. Although we observed a decrease in binding to cyclin T1 with each of the individual bromodomain proteins as compared with wild type BRD4 722, we observed a striking difference in their binding behavior to the 4KR mutant cyclin T1. Although binding of BRD4 722/H9004 BD1 to the 4KR mutant was decreased (43%) to a similar degree as wild type BRD4 722 (57%), no decrease in binding of BRD4 722/H9004 BD2 to the 4KR mutant of cyclin T1 was observed indicating that the second bromodomain of BRD4 interacts with acetylated cyclin T1 (Fig. 2B).

To test whether direct interactions exist between acetylated lysines in cyclin T1 and the BRD4 bromodomains, bacterially expressed GST-cyclin T1 was purified, acetylated in vitro with recombinant p300, and incubated with in vitro transcribed/translated BRD4 722 or 722ΔBD mutant. After GST pulldown, we found that binding of BRD4 722 was enhanced when GST-cyclin T1 was acetylated as compared with nonacetylated cyclin T1 (Fig. 2C, lanes 3 and 4). However, significant binding to nonacetylated protein was also observed indicating that acetylation enhances but does not exclusively mediate the binding or that the recombinant cyclin T1 protein is already partially acetylated when isolated from bacteria. Binding was abrogated in the BRD4 722ΔBD mutant supporting the model that acetylated lysines in cyclin T1 bind to bromodomains in BRD4 (Fig. 2C, lanes 5 and 6).

Second Bromodomain in BRD4 Binds Tri-acetylated Cyclin T1—Next, we performed NMR binding studies of individual recombinant bromodomains of BRD4 and synthetic peptides covering the four acetylation sites in cyclin T1. In two-dimensional $^1$H-$^{15}$N heteronuclear single quantum coherence (HSQC) spectra, incubation of the second bromodomain (BD2) of BRD4 with a cyclin T1 peptide (aa 387–395) carrying an acetyl group at position 390 (acK390) caused weak chemical shift perturbations of protein amide resonance (Fig. 3, upper panel). A similar result was previously reported where the second bromodomain in BRD4 exhibited a $K_d$ of 110 μM for a Lys-390-acetylated peptide of cyclin T1, but it did not interact with a Lys-404-acetylated cyclin T1 peptide (30). This binding was as tight as the binding of BRD4 BD2 to histone H4 sequences (30). In our experiments, binding was enhanced when tri-acetylated cyclin T1 peptides (acK380, acK386, and acK390) were incubated with BRD4 BD2, causing more extensive chemical shift perturbations than singly or doubly acetylated peptides (Fig. 3, upper panel). No binding of any cyclin T1 peptide was observed to the first bromodomain of BRD4 supporting the data obtained in immunoprecipitation experiments showing binding of acetylated cyclin T1 to the second bromodomain in BRD4 (Fig. 3, lower panel). Similarly, nonacetylated cyclin T1 peptides did not bind to the BRD4 bromodomain proteins as expected (Fig. 3, 1st column, Non-Acetylated). Collectively, these results uncover novel binding properties of tri-acetylated cyclin T1 to the second bromodomain of BRD4.

**BRD4 PID Dissociates HEXIM1 and 7SK snRNA from P-TEFb**—Because we find that full-length BRD4 binds active P-TEFb in the absence of HEXIM1 and BRD4 lacking a PID binds P-TEFb complexes containing HEXIM1, we speculated that the PID may be involved in the dissociation of HEXIM1 from P-TEFb. To test this hypothesis, we coexpressed HA-tagged cyclin T1 and a FLAG-tagged version of the BRD4 PID (aa 1209–1362) in 293T cells, immunoprecipitated HA-cyclin T1, and blotted for endogenous HEXIM1. Coexpression of the PID, but not BRD4 722 or BRD4 722 lacking both bro-
modomains, dissociated HEXIM1 from cyclin T1, supporting the model that the PID not only binds P-TEFb but is also involved in its partitioning in active and inactive complexes (Fig. 4A). Similar results were observed when endogenous cyclin T1 was immunoprecipitated in 293T cells transfected with FLAG-tagged proteins corresponding to full-length BRD4/1362, a truncated BRD4 protein lacking the PID (aa 1–1208, BRD4ΔPID), the BRD4 PID alone, or a BRD4 PID mutant that no longer binds P-TEFb (PID-AAA, F1357A/E1358A/E1359A (24)). While in vector-transfected cells, HEXIM1 was readily detectable in the immunoprecipitated material, and no HEXIM1 was found associated with cyclin T1 in cells overexpressing the BRD4 PID but not the PID-AAA mutant (Fig. 4B, lanes 1, 4, and 5). Of note, the transfection efficiency of 293T cells with Lipofectamine reagent was >90%. Overexpression of full-length BRD4/1362 dissociated ~40% of endogenous HEXIM1 from cyclin T1, whereas BRD4 lacking the PID enforced the interaction by 1.5–2.5-fold (Fig. 4B, lanes 1, 2, and 3). The mechanism of this enhancement is unclear at this point. Immunoprecipitation of the BRD4 proteins with α-FLAG-agarose confirmed that endogenous P-TEFb associates strongly with the full-length BRD4 and the PID but only weakly with BRD4ΔPID; no binding was observed to the PID-AAA mutant or in vector-transfected cells as expected (Fig. 4B). These results are consistent with a model where the C-terminal PID in BRD4 dissociates HEXIM1 from P-TEFb.

To test whether 7SK snRNA is also dissociated from cyclin T1 in the presence of the BRD4 PID, we performed quantitative RT–PCR on the immunoprecipitated material. Overexpression of full-length BRD4 or the PID alone decreased levels of cyclin T1-associated 7SK snRNA by ~60 or ~90%, respectively, although expression of BRD4ΔPID or the PID-AAA mutants slightly enhanced the association (supplemental Fig. 2A). Similar results were observed in glycerol gradient centrifugation experiments where overexpression of the PID, but not the mutant PID-AAA, shifted endogenous cyclin T1 and CDK9 into the low molecular weight HEXIM1-free form supporting the model that binding of the PID to P-TEFb dissociates the 7SK snRNP (supplemental Fig. 2B).

As the dissociation of HEXIM1 and 7SK snRNA is linked to the activation of the P-TEFb kinase activity, we performed in vitro kinase assays of immunoprecipitated cyclin T1/CDK9 with a recombinant RNA polymerase II CTD protein as substrate (Fig. 4C). Coexpression of FLAG-PID induced hyperphosphorylation of the CTD by P-TEFb (Fig. 4C, lane 5) to similar levels as full-length FLAG-BRD4 (lane 4) as compared with vector-transfected cells (lane 1). Expression of the FLAG-PID AAA mutant or of the full-length BRD4 protein containing the AAA mutation within the PID induced less hyperphosphorylation (Fig. 4C, lanes 6 and 10) similar to the expression of the short BRD4/722 isoform lacking the entire PID (lane 11). Treatment with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, a known dissociator of the 7SK snRNP (4), also activated P-TEFb kinase activity as expected (Fig. 4C, lane 2). These results further support the model that expression of the PID alone or in context of full-length BRD4 dissociates HEXIM1 and 7SK snRNA from P-TEFb, thereby activating the P-TEFb kinase activity.

PID Is Not Sufficient to Activate P-TEFb-dependent Transcription—Next, we examined the effect of PID overexpression on the transcriptional activity of the HIV-1 long-terminal repeat (LTR). The transcriptional activity of the HIV LTR is strongly dependent on P-TEFb, and in the absence of the viral transactivator TAT relies on BRD4 to recruit P-TEFb to elongating transcripts (5, 6). Accordingly, we observed that coexpression of an HIV LTR luciferase reporter construct with full-length FLAG-BRD4 in HeLa cells increased luciferase activity by ~200-fold over activities in vector-transfected cells (Fig. 5A). In contrast, expression of the PID alone or the PID-AAA mutant did not activate HIV LTR activity, whereas expression of BRD4 lacking the PID (BRD4ΔPID) maintained ~30% of the activatory function of wild type BRD4 (Fig. 5A). These results indicate that dissociation of HEXIM1 and activation of the CDK9 kinase activity by the PID are not sufficient to activate P-TEFb-dependent gene expression in cells.

We speculated that overexpression of the PID alone may dominantly suppress the activity of wild type BRD4 in cells. Indeed, when we coexpressed the PID constructs with full-length BRD4 and the HIV luciferase construct, the transcriptional activity of full-length BRD4 was decreased by ~3-fold in cells coexpressing the PID but not the PID-AAA mutant (Fig. 5B). Collectively, these data demonstrate that the PID alone, despite effectively dissociating HEXIM1 from P-TEFb and activating the P-TEFb kinase activity, is not sufficient to activate P-TEFb-dependent transcription.

DISCUSSION

Our data uncover a new active role of the BRD4 PID in the dissociation of HEXIM1 from P-TEFb. We show that the two domains of BRD4 that were previously implicated in the binding to P-TEFb, bromodomains and the PID, interact with different P-TEFb complexes, those containing HEXIM1 and those that do not. The finding that a tri-acetylated cyclin T1 binds the second bromodomain of BRD4 is new and supports the notion that bromodomains in BET proteins preferentially bind multiply acetylated binding partners (17).

The question how three acetyl groups can be structurally accommodated in the second bromodomain of BRD4 is not clear at this point and deserves further study. The two bromodomains of BRD4 have previously been shown to bind to an H4 peptide di-acetylated at Lys-5/Lys-12 (31). Moreover, it was shown that an H4 peptide di-acetylated at Lys-5/Lys-8 binds to the first bromodomain in BRDT, a homologue of BRD4 mainly expressed in testis (32). Here, H4K5ac is bound to the canonical acetyl-lysine binding pocket of BRDT BD1, whereas acetylation of H4K8 enhances hydrophobic interactions with several hydrophobic residues outside the acetyl-lysine-binding site (32). A similar binding structure may exist with the tri-acetylated cyclin T1 peptide (K380ac/K386ac/K390ac) in association with the BRD4 BD2. Given that we did not observe multiplicility or sever exchange-induced line broadening effects of the NMR signals of the protein upon addition of the tri-acetylated peptide, it is unlikely that a single acetylated peptide would bind to multiple BRD4 proteins. The latter would also require a much longer distance between the acetylation sites.
FIGURE 4. PID in BRD4 dissociates HEXIM1 from P-TEFb. A, coimmunoprecipitation of endogenous HEXIM1 with HA-cyclin T1 in 293T cells cotransfected with indicated FLAG-BRD4 constructs. Immunoprecipitations (IP) were performed with HA antibodies, and coimmunoprecipitation of endogenous HEXIM1 or CDK9 was tested by Western blotting (WB). B, coimmunoprecipitation experiments of endogenous HEXIM1 with endogenous cyclin T1 in 293T cells transfected with FLAG-tagged proteins corresponding to the full-length BRD4 1362 aa, short BRD4 lacking the PID (aa 1–1208), the PID alone (aa 1209–1362), a PID with triple alanine mutations at position F1357A/E1358A/E1359A disrupting binding of the PID to P-TEFb (21). Immunoprecipitations were performed with endogenous cyclin T1 antibodies as well as with FLAG antibodies, and coimmunoprecipitation of endogenous HEXIM1, P-TEFb, or FLAG-BRD4 proteins was tested by Western blotting as indicated. Band intensities of immunoprecipitated HEXIM1 and CDK9 were analyzed by using the ImageJ software available at rsb.info.nih.gov and were expressed as HEXIM1/CDK9 ratio. Note that one exposure of one gel is shown, and unrelated lanes were cut. C, in vitro kinase assay of immunoprecipitated HA-cyclin T1 coexpressed in 293T cells together with indicated FLAG-BRD4 constructs and CDK9. Immunoprecipitated material was incubated with recombinant GST-CTD as a substrate in the presence of ATP, and the reaction was developed with Western blotting as indicated. Band intensities of immunoprecipitated HEXIM1 and CDK9 were analyzed by using the ImageJ software available at rsb.info.nih.gov and were expressed as HEXIM1/CDK9 ratio. Note that one exposure of one gel is shown, and unrelated lanes were cut. C, in vitro kinase assay of immunoprecipitated HA-cyclin T1 coexpressed in 293T cells together with indicated FLAG-BRD4 constructs and CDK9. Immunoprecipitated material was incubated with recombinant GST-CTD as a substrate in the presence of ATP, and the reaction was developed with Western blotting as indicated. Band intensities of immunoprecipitated HEXIM1 and CDK9 were analyzed by using the ImageJ software available at rsb.info.nih.gov and were expressed as HEXIM1/CDK9 ratio. Note that one exposure of one gel is shown, and unrelated lanes were cut.
The finding that the short isoform of BRD4 engages in complex formation with P-TEFb containing HEXIM1 is unexpected because binding of HEXIM1 or BRD4 to P-TEFb is considered mutually exclusive (Fig. 6). The function of the short isoform of BRD4 in cells is not clear at this point, although a protein corresponding to this isoform is detected in cells (33). The possibility exists that the short isoform can dimerize with the long isoform of BRD4 via BD1 as previously reported for BRD2 (34) and may thereby retain its ability to activate P-TEFb in cells. Notably, in midline carcinomas, the short BRD4 isoform is incorporated into a unique oncogenic fusion protein containing the nuclear protein in testis (NUT) (35). This BRD4-NUT oncoprotein resides in transcriptionally inactive chromatin domains consistent with the possibility that it binds inactive P-TEFb due to the lack of a PID (36).

Our data underscore the central role of the PID as a potent P-TEFb-binding domain in BRD4 and attribute a novel “dissociator” function to this domain in BRD4. In support of this new function, expression of BRD4 was recently linked to changes in the 7SK snRNA structure leading to 7SK snRNP disruption (37). In addition, binding of acetylated cyclin T1 to BD2 may also induce structural changes in the full-length BRD4 protein that may serve to fully expose the C-terminal PID. Our results that expression of full-length BRD4 is less efficient in dissociating HEXIM1 or 7SK snRNA from cyclin T1 than the PID alone support such a model where exposure of this domain may be regulated within the full-length BRD4 protein.

FIGURE 5. PID dominantly suppresses P-TEFb transcriptional activities. A, cotransfection of an HIV LTR luciferase reporter construct (200 ng) with wild type BRD4, BRD4 lacking the PID, the PID alone, or the PID-AAA mutant (800 ng) in HeLa cells. Relative luciferase activities of three independent experiments were averaged (± S.E.). B, cotransfection experiments of the HIV LTR luciferase reporter construct (200 ng) with wild type BRD4 (500 ng) together with the PID or the PID-AAA mutant (500 ng) in HeLa cells. Results (mean of three independent experiments ± S.E.) are expressed as relative luciferase activity. WB, Western blot.
Notably, in our experiments global dissociation of the 7SK RNP through expression of the BRD4 PID was not sufficient to activate P-TEFb-dependent transcription but suppressed dominantly the transcriptional activation induced by full-length BRD4. These data underscore the functional relevance of the bromodomains in BRD4 for active gene expression. A possibility is that the interaction between acetylated cyclin T1 and BRD4 BD2 may help recruit P-TEFb to active promoters by potentially strengthening BRD4/Mediator interactions (39–41) or interactions of acetylated histones with the first BRD4 bromodomain (31). Additional studies are under way to examine how the new dissociator function of the PID is controlled within the full-length BRD4 protein and what specific role BRD4 BD2 plays in regulating P-TEFb transcriptional activity.

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