Abrogation of the Brd4-Positive Transcription Elongation Factor b Complex by Papillomavirus E2 Protein Contributes to Viral Oncogene Repression

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The cellular bromodomain protein Brd4 is a major interacting partner of the papillomavirus (PV) E2 protein. Interaction of E2 with Brd4 contributes to viral episome maintenance. The E2-Brd4 interaction also plays an important role in repressing viral oncogene expression from the integrated viral genome in human PV (HPV)-positive cancer cells. However, the underlying mechanism is not clearly understood. In host cells, Brd4 recruits positive transcription elongation factor b (P-TEFb) to stimulate RNA polymerase II phosphorylation during cellular and viral gene expression. P-TEFb associates with the C terminus of Brd4, which largely overlaps with the E2 binding site on Brd4. In this study, we demonstrate that E2 binding to Brd4 inhibits the interaction of endogenous Brd4 and P-TEFb. P-TEFb is essential for viral oncogene E6/E7 transcription in both HeLa and CaSkii cells that contain integrated HPV genomes. E2 binding to Brd4 abrogates the recruitment of P-TEFb to the integrated viral chromatin template, leading to inactivation of P-TEFb and repression of the viral oncogene E6/E7. Furthermore, dissociation of the Brd4–P-TEFb complex from the integrated viral chromatin template using a Brd4 bromodomain dominant-negative inhibitor also hampers HPV E6/E7 oncogene expression. Our data support that Brd4 recruitment of P-TEFb to the viral chromatin template is essential for viral oncogene expression. Abrogation of the interaction between P-TEFb and Brd4 thus provides a mechanism for E2-mediated repression of the viral oncogenes from the integrated viral genomes in cancer cells.

Papillomaviruses (PVs) are small DNA tumor viruses that induce a variety of benign and malignant epithelial lesions in the infected host (for a review, see reference 29). Over 140 human PV (HPV) types have been identified to date. Depending on the potential for induction of malignant transformation, these viruses are further classified into high-risk and low-risk HPVs. High-risk HPVs are strongly associated with cervical cancer (69), which is the second most common cancer among women worldwide and the leading cause of death from cancer among women in developing countries.

The PVs establish long-term, persistent infections. During latent infection, the viral genomes are maintained as extrachromosomal elements (episomes) that replicate along with host DNA. The PV E2 protein is a sequence-specific DNA binding protein involved in viral DNA replication, episome maintenance, and viral transcription (26). E2 consists of an N-terminal transcriptional activation domain linked to a C-terminal DNA binding/dimerization domain by a flexible hinge (39). The multiple functions of E2 rely on its sequence-specific recognition of a number of E2 binding sites within PV genomes. E2 initiates viral genome replication by loading the viral helicase E1 to the origin of replication (4, 43). It ensures the accurate partitioning of the replicated viral genomes to daughter cells by tethering them to host mitotic chromosomes (2, 31, 36, 48, 55). In addition, E2 can either activate or repress viral transcription, depending upon the sequence context of its cognate binding sites within the viral promoter region (56, 61). HPV E2 transactivates heterologous promoters containing multimerized E2 binding sites (27). On the other hand, E2 functions to repress the promoter directing the E6 and E7 viral oncogenes in the cancer-associated HPV type 16 (HPV16) and HPV18 genomes (5, 12, 50, 58, 60, 63).

During the malignant progression of high-risk HPV-positive lesions, the E2 gene is frequently disrupted as a consequence of integration of the viral genome (10, 29, 34). The loss of E2 expression derepresses viral E6 and E7 expression and, as such, has been mechanistically linked to the malignant progression of HPV-associated cancers (29). The dysregulated expression of E6 and E7 in turn inactivates the p53 and pRB tumor suppressors, respectively, by targeting them for degradation (19, 23, 44, 45, 51). Reexpression of E2 in HPV-positive cervical cancer cells represses the HPV E6/E7 expression and reverses cellular immortality (18, 21, 24, 30, 61). It has been suggested that E2 represses the E6/E7 promoter by binding its cognate sites proximal to the promoter and displacing other cellular transcription factors (13, 16, 59). In addition, E2 could function as an active repressor by preventing the assembly of a functional preinitiation complex (17).

Our previous proteomic analysis of E2 functional partners in host cells identified the cellular Brd4 protein as a major E2-interacting protein (67). Brd4 is a member of the BET family
proteins, which harbor double bromodomains implicated in chromatin targeting (15). It binds to acetylated histones H3 and H4 through its bromodomains and associates with both interphase chromatin and mitotic chromosomes (14). Brd4 plays a central role in host cellular growth control (28, 37) and cell cycle progression (15, 37, 42, 47, 65). We have shown that Brd4 functions to tether the bovine PV type 1 (BPV1) E2 protein and viral DNA to mitotic chromosomes (67). Through this tethering process, the Brd4-E2 complex participates in stable maintenance of viral genomes in dividing cells (1, 3, 8, 41, 67, 68).

E2 interacts with the C-terminal domain (CTD) of Brd4 through its N-terminal transactivation domain (67). The amino acids within the E2 transactivation domain involved in Brd4 binding are also required for both E2 transcriptional activation and repression of the viral E6/E7 promoter (25, 46, 53). This suggests that interaction with Brd4 is crucial for both the transactivation and repression functions of E2. Indeed, Brd4 mediates the transcriptional activation function of the E2 protein (32, 40, 53). Brd4 was also identified in the HPV11 E2 cellular protein complex implicated in repression of the E6/E7 promoter (64). It has been postulated that Brd4 accomplishes the coactivator and corepressor functions by recruiting E2 to its cognate binding sites within the viral promoter chromatin template in a bromodomain-dependent manner (35).

Brd4 plays an important role in cellular transcriptional regulation. Through interacting with the cyclin T1 and Cdk9 subunits of positive transcription elongation factor b (P-TEFb), Brd4 downregulates negative regulators, the HEXIM1 protein and 7SKsnRNA complex, from the P-TEFb complex, thereby transforming the P-TEFb into the transcriptionally active form (23, 45). Brd4-bound P-TEFb stimulates cellular gene transcriptional elongation through Cdk9-mediated phosphorylation of serine 2 (Ser 2) in the CTD of RNA polymerase II (RNAP II) (33, 46, 53). Brd4-bound P-TEFb stimulates cellular gene transcriptional elongation through Cdk9-mediated phosphorylation of serine 2 (Ser 2) in the CTD of RNA polymerase II (RNAP II) (33, 66). In addition, the Brd4-P-TEFb complex is essential for human immunodeficiency virus type 1 and human T-lymphotropic virus type 1 transcription (6, 9). Brd4-P-TEFb interaction increases dramatically in cells progressing from late mitotic to early G1 (65). This interaction recruits P-TEFb to mitotic chromosomes to stimulate the expression of key G1 and S phase cell cycle progression factors by recruiting E2 to its binding sites within the viral promoter chromatin template (67).

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**Immunoprecipitation.** For anti-FLAG Brd4 fragment coimmunoprecipitation of P-TEFb, 5 × 10⁶ exponentially growing C33A cells were transfected with 10 μg of either an empty vector or pOZN-TAP-hBrd4FL, pOZN-TAP-hBrd4L (LCTD), or pOZN-TAP-hBrd4FL constructs using the calcium phosphate method. At 48 hours posttransfection, the cells were lysed in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol [DTT], supplemented with protease inhibitors [Roche]). The cells were incubated on ice for 10 min, and NP-40 was added to a final concentration of 0.6%. After vortexing and centrifugation at 5,000 rpm for 5 min, the nuclear pellet was resuspended in ice-cold buffer B (20 mM HEPES [pH 7.9], 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT, supplemented with protease inhibitors [Roche]). To extract the nuclear proteins, the nuclei were passed through a 21-gauge needle five times and extracted at 4°C for 15 min. The nuclear pellets were isolated by centrifugation at 14,000 rpm for 15 min, and 2.4 mg nuclear proteins was measured using the bicinchoninic acid protein assay.

**Materials and Methods**

**Cell culture, cell lines, and transfection.** C33A, HEK293T, and HeLa cells were maintained in monolayer cultures in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (HyClone). The stable C33A/E2TA and C33A/E2TR cell lines have been described previously (67). CaSkii cells were maintained in monolayer cultures in RPMI medium 1640 (Invitrogen) containing 10% fetal calf serum. For transient transfection, 40% to 80% confluent cells growing in 10-cm dishes were transfected with 16 μg plasmid DNA using the FuGENE 6 transfection reagent (Roche). In some cases, cells were also transfected using the calcium phosphate method. Brd4 PtefB was mixed with 61 μl of 2 M CaCl₂ in a 500-μl final volume. After mixing with 500 μl 2× HBSS (0.4 M NaCl, 1.5 mM Na₂HPO₄, 55 mM HEpes, pH 7.0), the cell mixture was dropped onto ~40% confluent cells growing in 10-cm dishes.

**Recombinant plasmid construction.** pGEX2T-hBrd4(1313–1362) was kindly provided by Michael R. Botchan (University of California, Berkeley); plasmid pPOZN-E2TA has been described previously (67). For the pOZN-TAP-hBrd4FL, pOZN-TAP-hBrd4LCTD, and pOZN-TAP-hBrd4FL constructs, cDNAs encoding full-length human Brd4 (hBrd4), hBrd4 aa 1 to 1209 and hBrd4 aa 1 to 722 were first cloned individually into pOZN using the XhoI and NotI sites. A TEV protease-calcium binding protein-coding sequence was then inserted into the XhoI site of each construct. The bait vector pCLG is based on the previously described pCEL vector (20), where the extracellular and transmembrane domain of the Epo receptor have been replaced by those of the leptin receptor and the intracellular part of the XhoI site. The cDNAs encoding full-length human Brd4 (hBrd4), hBrd4 aa 1 to 722 were first cloned individually into pOZN using the XhoI and NotI sites. A TEV protease-calcium binding protein-coding sequence was then inserted into the XhoI site of each construct. The bait vector pCLG is based on the previously described pCEL vector (20), where the extracellular and transmembrane domain of the Epo receptor have been replaced by those of the leptin receptor and the intracellular part of the XhoI site.
temperature) and rotated at 4°C for 2 h. The beads were washed three times with 0.15 M KCl base buffer (0.15 M KCl, 20 mM Tris-Cl [pH 8.0], 10% glycerol, 5 mM MgCl$_2$, 0.1% Tween 20, and 0.2 mM phenylmethylsulfonyl fluoride [PMSF]) before elution with 40 μl sodium dodecyl sulfate (SDS) sample buffer.

For immunoprecipitation of E2, endogenous P-TEFb, and Brd4, 500 μg of nuclear extracts prepared from C33A cells and stable C33A/E2TA and C33A/E2TR cells were incubated with either 10 μg of anti-FLAG M2 beads preblocked with 3% BSA, 1 μg rabbit Brd4 antibody, or 1 μg rabbit Brd4 antibody, respectively, at 4°C for 1 h prior to mixing with 10 μl of protein A-Sepharose (GE Healthcare) (protein A Sepharose was omitted for M2 immunoprecipitation) for another hour. One microgram of normal rabbit immunoglobulin G (IgG) (Roche) (protein A Sepharose was omitted for M2 immunoprecipitation) for another hour. One microgram of normal rabbit immunoglobulin G (IgG) (Roche) (protein A Sepharose was omitted for M2 immunoprecipitation) for another hour. One microgram of normal rabbit immunoglobulin G (IgG) (Roche) was incubated with 10 μl of GST-CTD$_{aa}$ (Brd4 aa 1313 to 1362) expressed in Escherichia coli BL21(DE3) (pLysS) and immobilized on glutathione-Sepharose 4B following the manufacturer’s protocol (Sigma). For pulldowns, 10 μl of GST- or GST-CTD$_{aa}$-saturated glutathione-Sepharose 4B beads was preincubated with 0.15 M KCl HEGN buffer (0.15 M KCl, 20 mM HEPES [pH 7.6], 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, and 1 mM FMSF, supplemented with protease inhibitors) and incubated with 1 μg of C33A nuclear extract at 4°C for 4 h. Following three washes with 0.15 M KCl HEGN buffer, bound proteins were eluted from the beads by incubation with 40 μl of 2 M KCl HEGN at 4°C for 0.5 h with rotation. The eluted proteins were separated on a 10% SDS-polyacrylamide gel and analyzed by Western blotting using cyclin T1 and Cdk9 antibodies. Proteins eluted from 1 μl of the GST- or GST-CTD$_{aa}$-saturated glutathione beads were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining.

**MAPPIT assay.** The mammalian protein-protein interaction trap (MAPPIT) assay was performed as described previously (20). Briefly, 50% confluent HEK293T cells grown in a 24-well plate were cotransfected with 0.15 μg each of the pXP$_D_{pFu}$-pRIP-luci reporter construct and bait, prey, or control constructs as indicated in Fig. 3D. 0.15 μg pEGFP-C1 was used as a transfection efficiency marker. At 24 hours posttransfection, cells were either stimulated with 100 ng/ml leptin or left untreated. Twenty hours later, cells were lysed using reporter lysis buffer (Promega), and luciferase activities were determined according to the manufacturer’s manual (InovioGen). The samples were separated on a 5.5% SDS-polyacrylamide gel and analyzed by Western blotting using cyclin T1 and Cdk9 antibodies. Proteins eluted from 1 μl of the GST- or GST-CTD$_{aa}$-saturated glutathione beads were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining.

**RNAi II Ser 2 phosphorylation.** C33A and C33A/E2TA cells (1 × 10^5) were lysed directly in SDS sample buffer supplemented with phosphatase inhibitors (Sigma). The samples were separated on a 5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Western blotting was performed using antibodies recognizing RNAI II CTD Ser 2 phosphorylation and RNAI II (Covance).

**ChIP.** HeLa cells were transfected with either pOZN-E2TA or an empty vector using the calcium phosphate method. At 48 hours posttransfection, the chromatin was prepared as described previously (62). Chromatin from 1 × 10^5 cells was incubated with 5 μg of rabbit polyclonal Brd4 antibody (53), Cdk9 antibody (Santa Cruz Biotechnology), normal rabbit IgG (Upstate), or the antibody recognizing RNAI II CTD Ser 2 phosphorylation at 4°C for 12 h. After washing twice with dialysis buffer (2 mM EDTA, 50 mM Tris-Cl [pH 8.0], 0.2% Sarkosyl) and four times with immunoprecipitation wash buffer (100 mM Tris-Cl [pH 9.0], 500 mM LiCl, 1% NP-40, 1% deoxycholic acid), immune complexes were eluted by incubating in elution buffer (50 mM NaHCO$_3$, 1% SDS) for 15 min. Recovery of the immune complexes and de-cross-linking of chromatin were performed as described previously (62). After protease K digestion, phenol-chloroform extraction, and ethanol precipitation, the DNA was resuspended in 30 μl H$_2$O, and analyzed by PCR using primers specifically amplifying the HPV18 E6/E7 promoter-proximal region and the E6/E7 coding sequence. ChIP assay was also performed on HeLa cells transfected with either pOZN-HPV18 E2, pcDNA4C-Xpress-SV40NLS-hBrd4-BDI/II, or an empty vector using the antibodies recognizing RNAI II CTD Ser 2 phosphorylation and Cdk9.

To quantitate the ChIP signal, real-time PCR was performed using the Bio-Rad iQTM 5 multicolor real-time PCR detection system (Bio-Rad). Each reaction mixture contained 12.5 μl of iQTM SYBR green supermix (Bio-Rad), 1 μl of ChIP sample, and 0.4 μl of each primer in a total volume of 25 μl. PCR was carried out at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. The data were analyzed with Bio-Rad iQ5 software (Bio-Rad). Each assay was performed in triplicate, and the average specific signal for each ChIP sample was normalized to its input signal. The PCR primer sequences were 5’-GAG TAA CCG AAA AGC GTC GTG (HPV18 E6/E7 promoter, upstream), 5’-GGT CGC CGT GTT GGA GTC GTT C (AAG CTC TCA AAG CG (HPV18 E6/E7 promoter, downstream), 5’-CGG TGC CAG AAA CCG TGT TGC AA (E6/E7 coding sequence, upstream), and 5’-TG CTC GCT GTT GGA GTT GGT CTC (E6/E7 coding sequence, downstream).

**High-risk HPV E6/E7 repression assay.** To test the effect of P-TEFb knockdown and E2 expression on E6/E7 transcription, HeLa and Ca Ski cells were transfected with different combinations of small interfering RNA (siRNA) (Dharmacon) and recombinant constructs using the calcium phosphate method as indicated in Fig. 5. Cells were harvested at 72 h after transfection. To study the effect of the Brd4 double-bromodomain dominant-negative inhibitor on E6/E7 transcription, HeLa cells were transfected with different combinations of E6/E7 expression constructs and pcDNA4C-Xpress-SV40NLS-hBrd4-BDI/II as indicated in Fig. 7C. Cells were harvested at 48 h posttransfection.

For Northern blotting, total RNA was extracted using TRIzol reagent according to the manufacturer’s manual (Invitrogen). Ten micrograms of the total RNA were resolved on a formaldehyde agarose gel, transferred, and UV cross-linked to a Hybond N+ nylon membrane (GE Healthcare) for hybridization. The DNA probe used in the hybridization was prepared with a Prime It random labeling kit (Stratagene) using PCR-amplified actin gene, HPV18 E6/E7, or HPV16 E6/E7 DNA fragments as templates.

For protein analysis, cells were lysed in extraction buffer containing 10 mM HEPES (pH 7.9), 300 mM NaCl, 3 mM MgCl$_2$, 1 mM DTT, and 1 mM PMSF, supplemented with protease inhibitor cocktails (Roche) and Ser/Thr protein phosphatase inhibitor cocktails (Sigma). After 20 min of incubation on ice, lysates were clarified by centrifugation at 5,000 rpm for 5 min. Extracts (20 μg) were resolved on an SDS-polyacrylamide gel. Western blotting was performed using p53 antibody (Santa Cruz), Xpress antibody (Invitrogen), anti-HA–HRP antibody (Roche), and actin antibody (Chemicon).

**Immunofluorescence staining.** C33A cells were transfected with either an empty vector or pcDNA4C-Xpress-SV40NLS-hBrd4-BDI/II. At 24 hours posttransfection, cells were fixed with 3% paraformaldehyde in PBS. Cells were incubated in blocking/permeabilization buffer (0.5% Triton X-100 and 3% BSA in PBS) for 20 min at room temperature and stained with Brd4 rabbit polyclonal antibody (53) and Xpress mouse monoclonal antibody at room temperature for 60 min. After incubation, cells were washed three times with blocking/permeabilization buffer and incubated with Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes) and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) for an additional 60 min. Cells were counterstained with DAPI (4′-6′-diamidino-2-phenylindole) and examined with an Olympus IX81 inverted fluorescence microscope.

**RESULTS**

**P-TEFb binds to the C-terminal tail of Brd4.** Prior to the study published by Bisgrove et al. (6), the P-TEFb binding site on Brd4 was initially mapped to the bromodomains (33). In this study, we reexamined the P-TEFb binding site on Brd4 in a coimmunoprecipitation experiment. C33A cells were transfected with FLAG-tagged Brd4 constructs encoding full-length Brd4, a mutant lacking the CTD, or the short isoform of Brd4 that lacks the C-terminal half of the protein. In contrast, they were not coimmunoprecipitated by either the subunits of P-TEFb. Cdk9 and cyclin T1 were efficiently coimmunoprecipitated with Brd4, a mutant lacking the CTD, or the short isoform of Brd4 by its input signal. The PCR primer sequences were 5′-GGG TAA CCG AAA AGC GTC GTG (HPV18 E6/E7 coding sequence, upstream), and 5′-TG CTC GCT GTT GGA GTT GGT CTC (E6/E7 coding sequence, downstream).

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made by Bisgrove et al. and demonstrates that the major binding site of P-TEFb is present in the C-terminal tail of Brd4 (6). In addition, recombinant GST-CTD\textsubscript{50} encoding residues 1 to 1362 (full length [FL]), 1 to 1209 (CTD deletion [ΔCTD]), or 1 to 722 (short isoform [S]). Anti-FLAG immunoprecipitates were immunoblotted with HA (for Brd4), Cdk9, and cyclin T1 antibodies. (B) Immobilized GST-Brd4 CTD\textsubscript{50} (CTD) or GST (−) was incubated with C33A nuclear extracts. Interacting proteins were eluted, separated on an SDS-polyacrylamide gel, and immunoblotted with Cdk9 and cyclin T1 antibodies. The GST proteins eluted from the beads were visualized by Coomassie blue staining. Experiments were repeated three times, and similar results were obtained.

FIG. 1. P-TEFb binds to the CTD of Brd4 both in vivo and in vitro. (A) C33A cells were transfected with FLAG-HA-tagged Brd4 constructs encoding residues 1 to 1362 (full length [FL]), 1 to 1209 (CTD deletion [ΔCTD]), or 1 to 722 (short isoform [S]). Anti-FLAG immunoprecipitates were immunoblotted with HA (for Brd4), Cdk9, and cyclin T1 antibodies. (B) Immobilized GST-Brd4 CTD\textsubscript{50} (CTD) or GST (−) was incubated with C33A nuclear extracts. Interacting proteins were eluted, separated on an SDS-polyacrylamide gel, and immunoblotted with Cdk9 and cyclin T1 antibodies. The GST proteins eluted from the beads were visualized by Coomassie blue staining. Experiments were repeated three times, and similar results were obtained.

FIG. 2. The E2-Brd4 complex is less sensitive to salt than the Brd4–P-TEFb complex. Brd4 immunoprecipitation (Brd4 IP) was performed using nuclear extracts of stable C33A/E2TA cells to pull down E2 protein or using C33A nuclear extract to pull down P-TEFb. Normal rabbit IgG was used in the control immunoprecipitation (Con IP). The purified immune complexes were washed with buffers containing increasing concentrations of NaCl and eluted using SDS sample buffer. Bound proteins were immunoblotted using specific antibodies as indicated. Shown is a representative result from three independent experiments.

The E2-Brd4 complex is less sensitive to salt than the Brd4–P-TEFb complex. Since both the viral E2 protein and the cellular P-TEFb complex interact with the C-terminal tail of Brd4 and since a previous study showed that P-TEFb–Brd4 interaction is sensitive to salt (6), we examined whether the viral E2 protein and P-TEFb bind to Brd4 with different salt sensitivities. Brd4 coimmunoprecipitations were performed to pull down the E2 protein from C33A/E2TA nuclear extracts and cyclin T1 and Cdk9 from C33A nuclear extracts. As shown in Fig. 2, significant amounts of E2, Cdk9, and cyclin T1 were coimmunoprecipitated by the Brd4 antibody but not by the control normal rabbit IgG. More importantly, upon washing the immune complexes with buffers containing increasing concentrations of NaCl, E2 remained tightly associated with Brd4 even at 1 M NaCl. In contrast to this observation, P-TEFb subunits became dissociated from Brd4 as the salt concentration in the buffers increased. At NaCl concentrations of above 350 mM, most of Cdk9 and cyclin T1 were removed from the Brd4 immune complex. This result shows that the E2-Brd4 complex is much more resistant to increasing salt concentration than the Brd4–P-TEFb complex, suggesting that the E2-Brd4 interaction is stronger or that E2 and Brd4 have a different mode of interaction. Since both E2 and P-TEFb, on their own, remain associated with Brd4 in the presence of 150 mM NaCl, it is suggested that E2 and P-TEFb can bind to Brd4 either simultaneously or competitively under physiological condition.

E2 interacts with Brd4 but not P-TEFb. In order to determine if E2 is able to cocomplex with Brd4 and P-TEFb or to compete the P-TEFb and Brd4 interaction, we next examined whether E2 and P-TEFb interact with each other in cells. We first tested whether E2 could coimmunoprecipitate Cdk9 and cyclin T1 from cell lysates. As shown in Fig. 3A, anti-FLAG M2 beads were used to immunoprecipitate FLAG-tagged E2 proteins from the C33A/E2TA nuclear extract. C33A/E2TR cells stably expressing the E2TR mutant lacking the Brd4 interaction domain and C33A cells without E2 expression were used as negative controls. Western blotting shows that Brd4 is efficiently coimmunoprecipitated with E2TA but not E2TR. Neither is it present in the C33A coimmunoprecipitation sample (Fig. 3B). However, no P-TEFb was detectable in any of the coimmunoprecipitation samples (Fig. 3B). Consistently, in the reciprocal Cdk9 coimmunoprecipitation, no E2TA was coimmunoprecipitated (Fig. 4A, E2 blot, lane 8). These results suggest that, in cells, E2 binds efficiently to Brd4 but does not become associated with P-TEFb either directly or through Brd4, indicating that E2 is likely to compete with P-TEFb for Brd4 binding (Fig. 3A).

To confirm that E2 and P-TEFb do not interact directly in the absence of Brd4, we took advantage of the recently developed MAPPIT technique (20). The MAPPIT technique is a cytokine receptor-based two-hybrid technique for detecting protein-protein interactions in mammalian cells (20). In this system, the bait protein is fused to a kinase-active homodimeric cytokine receptor and the prey protein to gp130 that contains functional STAT recruitment sites (20). When the bait and prey constructs are coexpressed in mammalian cells, bait-prey interaction reconstitutes a functional receptor complex that, upon ligand binding, initiates the JAK-STAT signaling cascade, leading to transcription of a luciferase reporter gene under the control of the STAT3-responsive promoter, rPAP1 (Fig. 3C) (20). This approach has the advantage over yeast
FIG. 3. E2 binds to Brd4 but not P-TEFb. (A) Cartoon representation of the experiment design for testing E2, Brd4, and P-TEFb interaction. Anti-FLAG M2 immunoprecipitation was performed using C33A cells stably expressing FLAG-tagged E2 protein to determine whether E2 exists in a complex with Brd4 and/or P-TEFb. (B) Nuclear extracts from C33A cells (H11002), stable E2TA cells (TA), and stable E2TR cells (TR) were incubated with M2 beads to immunoprecipitate FLAG-tagged E2 proteins. Bound proteins were eluted and immunoblotted with HA (for E2), Brd4, Cdk9, and cyclin T1 antibodies. (C) Layout of a MAPPIT-based two-hybrid assay for detecting E2 interaction with the Brd4 CTD and P-TEFb. Cells expressing the chimeric cytokine receptor with the C-terminal baits and the gp130-prey chimeras were stimulated with leptin ligand. Upon cognate bait-prey interaction, which leads to recruitment of gp130 containing four STAT binding sites, leptin binding induces activation of ligand receptor-associated JAKs. Subsequent phosphorylation of the recruited STAT induces reporter expression from the rPAP1 promoter. The Brd4 CTD, Cdk9, and cyclin T1 (CycT1) were used as baits, and the E2 fused to gp130 was used as prey. Protein-protein interaction between E2 and the Brd4 CTD, but not cyclin T1 or Cdk9, was detected. (D) MAPPIT assay detects E2-CTD interaction but not E2-P-TEFb interaction. 293T cells were transiently cotransfected with the pXP.d3-rPAP1-luci reporter and bait, prey, or control constructs as indicated. Cells were stimulated with leptin (+) or left untreated (−). Luciferase activity is expressed as the mean ± standard deviation of measurements from triplicate transfections. SV40 large T antigen (SVT) prey served as a negative control. RNF41 prey, which binds to the bait receptor itself, served as a positive control.
activity, E2 prey and Cdk9 bait coexpression does not lead to significant stimulation of the reporter luciferase activity above the negative control level. Similar results were obtained when cyclin T1 was used as bait. This result further demonstrates that E2 does not interact directly with the P-TEFb subunits in cells.

E2 binding to Brd4 inhibits the P-TEFb and Brd4 interaction in vivo. Since both E2 and P-TEFb can bind to the C-terminal tail of Brd4, with the E2-Brd4 complex being more resistant to salt than the P-TEFb–Brd4 complex, we next tested how E2 affects P-TEFb and Brd4 interaction in vivo. To this end, we performed coimmunoprecipitation of endogenous Brd4 and P-TEFb subunits from nuclear extracts in the presence and absence of E2. Nuclear extracts isolated from C33A cells and stable E2TA and E2TR cells were immunoprecipitated using Brd4 antibody and Cdk9 antibody, respectively. As shown in Fig. 4A, Brd4 antibody efficiently coimmunoprecipitates both Cdk9 and cyclin T1 from the C33A nuclear extract (Fig. 4A, Cdk9 and cyclin T1 blots, compare lane 10 with lane 4). Brd4 antibody also coimmunoprecipitates a large amount of E2TA from the stable C33A/E2TA cells (Fig. 4A, E2 blot, lane 11). Compared to the case for C33A cells, P-TEFb coimmunoprecipitated by the Brd4 antibody was reduced to 52% for Cdk9 and to 45% for cyclin T1 in stable E2TA cells (Fig. 4A, Cdk9 and cyclin T1 blots, compare lane 11 with lane 10). In the reciprocal coimmunoprecipitation, Brd4 coimmunoprecipitated by Cdk9 antibody is also reduced to 37% in E2TA cells compared to C33A and E2TR cells (Fig. 4A, Brd4 blot, compare lane 8 with lanes 7 and 9). This inhibition is likely due to the E2-Brd4 interaction, as the P-TEFb and Brd4 coimmunoprecipitation was not affected very much in the stable C33A/E2TR cells expressing an E2 mutant that does not interact with Brd4 (Fig. 4A, lane 9 of Brd4 blot and lane 12 of Cdk9 and cyclin T1 blots). Besides E2TA, we also tested the high-risk HPV18 E2 for the ability to inhibit P-TEFb and Brd4 interaction. 293T cells were transfected with either the pOZN-HPV18 E2 construct (18E2) was coimmunoprecipitated with either normal rabbit IgG (Con IP) or Brd4 antibody (Brd4 IP). Nuclear extracts (input) and immunoprecipitates were immunoblotted for Brd4, E2 (HA), Cdk9, and cyclin T1 using specific antibodies. The relative band intensity of coimmunoprecipitated Brd4 and P-TEFb was quantified using IMAGE J software and is indicated under each lane. Experiments were repeated twice, and similar results were obtained. (B) Nuclear extract of 293T cells transfected with pOZN control vector (–) or pOZN-HPV18 E2 construct (18E2) was coimmunoprecipitated with either normal rabbit IgG (Con IP) or Brd4 antibody (Brd4 IP). Nuclear extracts (input) and immunoprecipitates were analyzed as for panel A.

FIG. 4. E2 inhibits P-TEFb and Brd4 interaction in vivo. (A) Nuclear extracts from C33A parental cells (–) or C33A cells stably expressing HA-tagged E2TA (TA) or E2TR (TR) were coimmunoprecipitated with either normal rabbit IgG (Con IP), Cdk9 antibody (Cdk9 IP), or Brd4 antibody (Brd4 IP). Nuclear extracts (input) and immunoprecipitates were immunoblotted for Brd4, E2 (HA), Cdk9, and cyclin T1 using specific antibodies. The relative band intensity of coimmunoprecipitated Brd4 and P-TEFb was quantified using IMAGE J software and is indicated under each lane. Experiments were repeated twice, and similar results were obtained. (B) Nuclear extract of 293T cells transfected with pOZN control vector (–) or pOZN-HPV18 E2 construct (18E2) was coimmunoprecipitated with either normal rabbit IgG (Con IP) or Brd4 antibody (Brd4 IP). Nuclear extracts (input) and immunoprecipitates were analyzed as for panel A.

To this end, we performed coimmunoprecipitation of endogenous Brd4 and P-TEFb subunits from nuclear extracts in the presence and absence of E2. Nuclear extracts isolated from C33A cells and stable E2TA and E2TR cells were immunoprecipitated using Brd4 antibody and Cdk9 antibody, respectively. As shown in Fig. 4A, Brd4 antibody efficiently coimmunoprecipitates both Cdk9 and cyclin T1 from the C33A nuclear extract (Fig. 4A, Cdk9 and cyclin T1 blots, compare lane 10 with lane 4). Brd4 antibody also coimmunoprecipitates a large amount of E2TA from the stable C33A/E2TA cells (Fig. 4A, E2 blot, lane 11). Compared to the case for C33A cells, P-TEFb coimmunoprecipitated by the Brd4 antibody was reduced to 52% for Cdk9 and to 45% for cyclin T1 in stable E2TA cells (Fig. 4A, Cdk9 and cyclin T1 blots, compare lane 11 with lane 10). In the reciprocal coimmunoprecipitation, Brd4 coimmunoprecipitated by Cdk9 antibody is also reduced to 37% in E2TA cells compared to C33A and E2TR cells (Fig. 4A, Brd4 blot, compare lane 8 with lanes 7 and 9). This inhibition is likely due to the E2-Brd4 interaction, as the P-TEFb and Brd4 coimmunoprecipitation was not affected very much in the stable C33A/E2TR cells expressing an E2 mutant that does not interact with Brd4 (Fig. 4A, lane 9 of Brd4 blot and lane 12 of Cdk9 and cyclin T1 blots). Besides E2TA, we also tested the high-risk HPV18 E2 for the ability to inhibit P-TEFb and Brd4 interaction. 293T cells were transfected with either the pOZN-HPV18 E2 construct or a control vector. Brd4 coimmunoprecipitation was performed to compare the amounts of P-TEFb associated with Brd4 in the presence and absence of HPV18 E2. The data show that HPV18 E2 also leads to dramatically reduced amounts of Cdk9 and cyclin T1 that are associated with Brd4 (Fig. 4B). In summary, our data demonstrate that E2 binding to Brd4 significantly interferes with the P-TEFb and Brd4 interaction in vivo.

P-TEFb is essential for HPV E6/E7 oncogene expression from the integrated viral genome. Brd4 recruitment of P-TEFb to chromatin template is crucial for stimulating the expression of its downstream genes (33, 42, 65). In HeLa cells containing an integrated HPV18 genome and a disrupted E2 gene, Brd4 localizes to the viral E6/E7 promoter independently of E2 (64), suggesting that it may recruit P-TEFb to stimulate viral E6/E7 transcription. Reexpression of E2 in HeLa cells efficiently represses HPV E6/E7 expression from the integrated HPV18 genome (18, 21, 30, 61), and the interaction of E2 with Brd4 has been implicated in this repression (64). Prior to determining whether E2’s competitive effect on P-TEFb and Brd4 association could contribute to its repression function, we tested whether P-TEFb is important for the E6/E7 oncogene transcription in cancer cell lines carrying the integrated viral genomes. Since the E2 repression function was best established...
in HeLa cells carrying the integrated HPV18 genome, we first examined this cell line in our study. The cells were cotransfected with P-TEFb siRNA (1:1 mixture of Cdk9 and cyclin T1 siRNA) or control siRNA in combination with pcDNA4C-E2TA, pcDNA4C-HPV18 E2, or an empty vector. The E6/E7 mRNAs expressed in the treated cells were analyzed by Northern blot hybridization. Because E6 targets p53 for ubiquitin/proteasome-mediated protein degradation, repression of E6 transcription also leads to accumulation of p53 protein in cells. The p53 protein level was therefore analyzed by Western blotting to monitor the repression of E6 transcription. Both BPV1 E2TA and HPV18 E2 were used as positive controls for the E6/E7 repression assay. We consistently observed that when cotransfected with control siRNA, both E2s are expressed at very low levels and the HPV18 E2 protein is below the detection level of the Western blotting. However, the Northern and Western blotting data show that expression of either BPV1 E2TA or HPV18 E2 leads to efficient downregulation of HPV18 E6/E7 expression and significantly increased p53 protein levels (Fig. 5A). P-TEFb siRNA is able to efficiently knockdown both Cdk9 and cyclin T1 (Fig. 5A). Remarkably, knocking down P-TEFb also results in the repression of HPV18 E6/E7 transcription and an elevated p53 protein level compared to the case for the control siRNA-treated cells (Fig. 5A, compare lane 4 with lane 1). To further validate P-TEFb’s role in E6/E7 oncogene transcription, we performed the RNA interference experiment with CaSki cells, which harbor about 500 intrachromosomal copies of the HPV16 genome. In these cells, P-TEFb knockdown also leads to significant repression of HPV16 E6/E7 expression and p53 accumulation (Fig. 5B). Hence, using cell lines containing integrated HPV16 and HPV18 genomes, we demonstrate that P-TEFb is essential for stimulating E6/E7 transcription. These data predict that E2 may repress E6/E7 expression through blocking Brd4-mediated P-TEFb recruitment to the viral chromatin template in vivo.

E2 dissociates P-TEFb from Brd4 on the integrated HPV18 viral chromatin template. Since E2 binding to Brd4 disrupts the Brd4-P-TEFb interaction in vivo, we hypothesized that E2 may abrogate the recruitment of P-TEFb by Brd4 to the viral chromatin template, leading to active repression of the HPV E6/E7 promoter (Fig. 6A). HPV oncogene transcription from the integrated viral genome thus provides a biological model to further investigate the functional impact of the competitive binding of E2 and P-TEFb to Brd4. To test our model, we first examined the effect of E2 expression on P-TEFb binding to Brd4 on the viral chromatin template using ChIP. HeLa cells were either mock transfected or transfected with an E2TA expression construct. E2 protein expression was confirmed by Western blotting (Fig. 6B). Cellular chromatin from these cells was immunoprecipitated by either Brd4 antibody or Cdk9 antibody. Normal rabbit IgG was used as a negative control. Recovery of the viral chromatin template was detected by real-time PCR using specific primers amplifying a promoter-proximal region of the HPV18 E6/E7 gene and the E6/E7 coding sequence. As shown in Fig. 6C, in the anti-Brd4 ChIP, similar amounts of viral chromatin were immunoprecipitated with Brd4 antibody from cells either with or without E2 expression, indicating that the E2 protein does not affect the Brd4-chromatin interaction (Fig. 6C). Normal rabbit IgG did not isolate any viral chromatin template, confirming the specific association of Brd4 with the viral chromatin template (data not shown). In the ChIP using Cdk9 antibody, viral DNA template was recovered in the absence of E2, indicating that P-TEFb and Brd4 coexist on the HPV18 E6/E7 promoter and coding sequence in HeLa cells (Fig. 6C). However, P-TEFb bound to the viral chromatin template was reduced by nearly
In cells, P-TEFb is kept in a functional equilibrium through alternately interacting with its positive regulator Brd4 and negative regulator, the HEXIM1 protein and 7SKsnRNA complex. When bound to Brd4, the activated P-TEFb stimulates transcriptional elongation by phosphorylating the Ser 2 residue in the CTD of RNAP II (Fig. 6A). To determine how E2 competitive binding on Brd4 affects the functional equilibrium of P-TEFb, we examined the kinase activity of P-TEFb in stable C33A and E2TA cells. RNAP II CTD Ser 2 phosphorylation was reduced by ~31% in stable E2TA cells compared to C33A cells (Fig. 6D). This observation indicates that, by competing the P-TEFb interaction with Brd4, E2TA converts some of the Brd4-associated active P-TEFb molecules into the inactive form associated with the 7SKsnRNA/HEXIM1 complex (Fig. 6A). Based on these data, we predict that E2 binding to the viral chromatin would inhibit the RNAP II CTD Ser 2 phosphorylation on the viral chromatin template. Indeed, the RNAP II CTD Ser 2 phosphorylation associated with the viral chromatin template was significantly reduced in the presence of E2TA (Fig. 6C). To further examine this notion in the presence of HPV18 E2, we transfected the pOZN-HPV18 E2 construct into HeLa cells and performed ChIP analysis to examine the amount of RNAP II CTD Ser 2 phosphorylation as well as Cdk9 associated with the viral chromatin template. Expression of the HPV18 E2 protein is shown in Fig. 6E. ChIP analysis show that, compared to the vector control, HPV18 E2 expression also prevents the recruitment of Cdk9 to the viral chromatin template and inhibits RNAP II CTD Ser 2 phosphorylation on both the HPV18 E6/E7 promoter and E6/E7 coding sequence (Fig. 6F). Our result supports that E2 competitive inhibition of the P-TEFb and Brd4 interaction impairs the recruitment of P-TEFb to the viral E6/E7 promoter and coding sequence, leading to the repression of viral oncogene transcription.

**Dissociation of Brd4 from chromatin template inhibits HPV18 E6/E7 transcription.** Brd4 recruitment of E2 to the endogenous HPV18 E6/E7 promoter in HeLa cells is necessary for E2-mediated repression (64). On the other hand, deletion of the Brd4 bromodomains abrogates the transcriptional activity of the Brd4–P-TEFb complex (33, 66), highlighting the importance of bromodomain-dependent association of the Brd4–P-TEFb complex with chromatin in this process. To further determine if Brd4-mediated P-TEFb association with chromatin contributes to E6/E7 transcription from the integrated viral promoter, we developed a dominant-negative in-control. Ser2P signal intensity was normalized with RNAP II intensity and is indicated. (E) HeLa cells were transfected with either the empty vector (V) or pOZN-HPV18E2 (18E2). At 48 hours posttransfection, the cross-linked chromatin was harvested and analyzed as for panel B to detect the HA-tagged E2 protein. (F) Cross-linked chromatin from cells transfected for panel E was precipitated with either Cdk9 antibody, Ser2P, or normal rabbit IgG. The recovered DNA was quantitated using the Bio-Rad iQ 5 multicolor real-time PCR detection system (Bio-Rad) with primers that specifically amplify the HPV18 E6/E7 promoter-proximal region and the E6/E7 coding sequence (CS). Each assay was performed in triplicate, and the average signal for each ChIP sample was normalized by the input chromatin signal. ChIP signals were expressed as percentages of chromatin DNA immunoprecipitated from vector control cells. (D) C33A cells and stable C33A/E2TA cells (1 × 10^6) were lysed directly in SDS sample buffer. The lysates were separated on a 5.5% SDS-polyacrylamide gel and immunoblotted with Ser2P to detect Cdk9-mediated phosphorylation. The RNAP II signal is shown as a control. Ser2P signal intensity was normalized with RNAP II intensity and is indicated. (E) HeLa cells were transfected with either the empty vector (V) or pOZN-HPV18E2 (18E2). At 48 hours posttransfection, the cross-linked chromatin was harvested and analyzed as for panel B to detect the HA-tagged E2 protein. (F) Cross-linked chromatin from cells transfected for panel E was precipitated with either Cdk9 antibody, Ser2P, or normal rabbit IgG. The recovered DNA was analyzed as for panel C. Shown are representative results from two independent experiments.

FIG. 6. E2 dissociates the P-TEFb from the viral chromatin template and inhibits P-TEFb phosphorylation of RNAP II CTD. (A) Proposed model for E2 inhibition of Brd4 and P-TEFb interaction during viral oncogene expression. Brd4 stimulates transcription by recruiting P-TEFb to phosphorylate the RNAP II CTD along the integrated viral chromatin template. E2 binding to Brd4 disrupts the Brd4–P-TEFb interaction. E2 association with the integrated HPV promoter abrogates the recruitment of P-TEFb by Brd4 and converts the Brd4-associated active P-TEFb molecules into the inactive form associated with the 7SKsnRNA/HEXIM1 complex. This competitive E2 binding inhibits RNAP II CTD Ser 2 phosphorylation and leads to active repression of the HPV E6/E7 promoter. (B) HeLa cells were transfected with either an empty vector (V) or pOZN-E2TA (E2). At 48 hours posttransfection, cross-linked chromatin was harvested. Fifty micrograms of chromatin samples was resolved on an SDS-polyacrylamide gel and immunoblotted with anti-HA–HRP to detect the HA-tagged E2 protein. (C) The cross-linked chromatin harvested for panel (B) was precipitated using either Brd4 antibody, RNAP II CTD phospho-Ser 2 antibody(Ser2P), Cdk9 antibody, or normal rabbit IgG. The recovered DNA was quantitated using the Bio-Rad iQ 5 multicolor real-time PCR detection system (Bio-Rad) with primers that specifically amplify the HPV18 E6/E7 promoter-proximal region and the E6/E7 coding sequence (CS). Each assay was performed in triplicate, and the average signal for each ChIP sample was normalized by the input chromatin signal. ChIP signals were expressed as percentages of chromatin DNA immunoprecipitated from vector control cells. (D) C33A cells and stable C33A/E2TA cells (1 × 10^6) were lysed directly in SDS sample buffer. The lysates were separated on a 5.5% SDS-polyacrylamide gel and immunoblotted with Ser2P to detect Cdk9-mediated phosphorylation. The RNAP II signal is shown as a 80% in the presence of E2 protein (Fig. 6C). This study demonstrates that E2 abrogates recruitment of P-TEFb to the E6/E7 promoter and coding sequence by blocking its association with Brd4. The RNAP II CTD Ser 2 phosphorylation was reduced by ~31% in stable E2TA cells compared to C33A cells (Fig. 6D). This observation indicates that, by competing the P-TEFb interaction with Brd4, E2TA converts some of the Brd4-associated active P-TEFb molecules into the inactive form associated with the 7SKsnRNA/HEXIM1 complex (Fig. 6A). Based on these data, we predict that E2 binding to the viral chromatin would inhibit the RNAP II CTD Ser 2 phosphorylation on the viral chromatin template. Indeed, the RNAP II CTD Ser 2 phosphorylation associated with the viral chromatin template was significantly reduced in the presence of E2TA (Fig. 6C). To further examine this notion in the presence of HPV18 E2, we transfected the pOZN-HPV18 E2 construct into HeLa cells and performed ChIP analysis to examine the amount of RNAP II CTD Ser 2 phosphorylation as well as Cdk9 associated with the viral chromatin template. Expression of the HPV18 E2 protein is shown in Fig. 6E. ChIP analysis show that, compared to the vector control, HPV18 E2 expression also prevents the recruitment of Cdk9 to the viral chromatin template and inhibits RNAP II CTD Ser 2 phosphorylation on both the HPV18 E6/E7 promoter and E6/E7 coding sequence (Fig. 6F). Our result supports that E2 competitive inhibition of the P-TEFb and Brd4 interaction impairs the recruitment of P-TEFb to the viral E6/E7 promoter and coding sequence, leading to the repression of viral oncogene transcription.
hbinator to block Brd4 and associated proteins from localizing to cellular chromatin. The cDNA fragment encoding the double bromodomains of Brd4 (aa 1 to 470, BDI/II) was subcloned downstream of an Xpress epitope tag and an NLS in the pcDNA4C vector (see Materials and Methods). In C33A cells, Brd4 normally localizes to punctate dots on cellular chromatin (Fig. 7A). However, when the Xpress-BDI/II protein was expressed in C33A cells, it localized to these punctuate chromatin dots instead (Fig. 7A). In these cells, the amount of Brd4 protein associated with punctate chromatin dots was dramatically reduced (Fig. 7A). The Xpress-BDI/II thus demonstrates a dominant-negative effect in preventing endogenous Brd4 from binding to its native sites in cells. In addition, BDI/II expression in HeLa cells also inhibits the recruitment of Cdk9 to the HPV18 E6/E7 promoter and E6/E7 coding sequence (Fig. 7B). To test how dissociation of Brd4 and P-TEFb from the chromatin template affects HPV E6/E7 transcription, HeLa cells were transfected with the BDI/II expression construct either alone or in combination with an E2 construct (pcDNA4C-E2TA or pcDNA4C-HPV18E2). Both BPV1 E2TA and HPV18 E2 were used in this study as positive controls for E6/E7 repression function. As indicated above, HPV18 E2 is consistently expressed at a much lower level than BPV1 E2TA. However, expression of either E2 protein alone can efficiently repress E6/E7 transcription (Fig. 7C, compare lanes 3 and 5 to lane 1). Importantly, expression of BDI/II in HeLa cells also causes significant repression of E6/E7 and accumulation of p53 protein compared to those in mock-transfected cells (Fig. 7C, compare lane 2 to lane 1). Coexpression of the BDI/II protein together with an E2 construct does not further repress the E6/E7 transcription (Fig. 7C, compare lanes 4 to 6 to lanes 3 and lane 5, respectively). These data are consistent with the notion that the BDI/II protein is likely to act upstream of E2 due to its ability to dissociate Brd4 and associated proteins from the cellular chromatin. Notably, coexpression of the BDI/II has repeatedly led to BPV1 E2 protein stabilization, the mechanism of which remains to be further investigated. Since dissociation of Brd4 and associated proteins from chromatin by the BDI/II protein can significantly repress viral E6/E7 oncogene transcription, it is suggested that the binding of Brd4 and associated P-TEFb with viral chromatin is essential for integrated viral oncogene E6/E7 transcription. This experiment therefore provides further evidence to support that E2 may repress E6/E7 transcription in carcinoma cells carrying integrated viral genome by competing P-TEFb away from Brd4 on the viral chromatin template.

**DISCUSSION**

E2 plays important roles in viral genome replication and episome maintenance in host cells. E2 also contributes to a tight regulation of viral oncogene transcription. The loss of E2 during the integration of the viral DNA into the host genome in HPV-positive lesions causes dysregulated viral oncogene expression and has been linked to malignant progression of HPV-associated cancers (29). Recent studies of E2 and Brd4 have established Brd4 as a key host receptor that contributes to multiple E2 functions, including viral episome maintenance, viral transcriptional activation, and repression of the viral oncogene transcription from the integrated viral genome in can-
cer cells (32, 38, 40, 53, 63). However, the molecular mechanisms underlying these multiple functions of E2-Brd4 interaction remained poorly understood.

Brd4 functions as a molecular adapter, with its bromodomain targeting acetylated chromatin and CTD binding to E2. The multiple functions of E2 have been linked to its ability to interact with Brd4 and become associated with cellular chromatin. Interestingly, an important cellular transcription regulator, P-TEFb, also binds to the C-terminal region of Brd4 that overlaps with the E2 binding site. In this study, we investigated how E2 binding to Brd4 affects the recruitment of P-TEFb by Brd4 and the functional impact on E2-mediated repression of the viral oncogene from the integrated HPV genome. We demonstrate that E2 and P-TEFb bind to Brd4 in a mutually exclusive manner in vivo. ChIP data establish that E2 prevents P-TEFb association with Brd4 on the integrated viral chromatin template. In cancer cells carrying integrated high-risk HPV genomes, either knockdown of P-TEFb or dissociation of the Brd4-P-TEFb complex from the integrated viral chromatin template causes repression of the E6/E7 oncogene and accumulation of p53 protein. Our data establish that P-TEFb interacts with Brd4 to contribute to HPV oncogene transcription and that E2 actively represses viral E6/E7 transcription by blocking the formation of the active Brd4-P-TEFb complex on the viral chromatin template. Taken together, these studies provide a molecular mechanism underlying the function of E2-Brd4 interaction in transcriptional repression of HPV oncogenes in cancer cells carrying integrated viral genomes. Our current study establishes that E2 inhibition of P-TEFb and Brd4 interaction contributes to viral oncogene repression in the integrated viral genome. How these molecular interactions regulate viral oncogene expression during the productive viral life cycle in HPV-infected cells carrying episomal viral DNA remains an important question for future studies.

It has been suggested that the E2 transcriptional repression function cannot simply be achieved by binding to the DNA sequence within the E6/E7 promoter and displacing other cellular transcription factors. It appears that inhibition of HPV oncogene transcription additionally requires the E2 N-terminal transactivation domain (12, 18, 25, 46). Brd4 was identified as a cellular factor that binds to this region of E2 to mediate E6/E7 repression (64). Recent findings indicate that Brd4 accomplishes the corepressor function by recruiting E2 to block the assembly of a functional preinitiation complex on the viral promoter chromatin template (35, 63). Since P-TEFb is an essential component of the preinitiation complex that stimulates RNA Pol II transcription (49), our data provide additional evidence to support a model in which E2 inhibits HPV E6/E7 transcription by binding to chromatin-associated Brd4 and preventing preinitiation complex assembly on the viral promoter chromatin.

This study also provides new clues to understand a number of previous observations. First, mapping of P-TEFb and E2 binding sites to the CTD of Brd4 explains why the Brd4 CTD does not repress, but rather further augments, the E2-mediated E6/E7 transcriptional repression in vivo (52). It is conceivable that the Brd4 CTD not only disrupts the Brd4-E2 interaction but also displaces P-TEFb from endogenous Brd4, leading to added E6/E7 repression. Second, because E2 abrogation of P-TEFb-Brd4 interaction on the viral chromatin template relies on its abilities to bind cognate DNA sites and to associate with Brd4, it explains the requirement for both the N-terminal region and the C-terminal DNA binding domain of E2 as well as the extreme C terminus and the bromodomain region of Brd4 for E2 repression function (46, 64).

The E2-Brd4 complex plays a dual role in E2-mediated viral gene activation and repression. It has been demonstrated that E2 regulates viral promoter activity in a dose-dependent manner that results in either activation (at low levels of E2) or repression (at high concentrations of E2) of E6/E7 expression from the viral early promoter (7, 57). Notably, under the conditions used in our experiment, E2 binding to Brd4 does not completely abolish P-TEFb association. Some P-TEFb molecules remain associated with Brd4 in the presence of E2 (Fig. 4). Since the E2-Brd4 complex also contributes to the E2-mediated transactivation (53), it remains to be investigated whether E2 inhibits the P-TEFb and Brd4 interaction in a dose-dependent manner and whether, at low concentration, E2 may recruit the Brd4-P-TEFb complex to stimulate viral transcription.

Brd4 interacts with E2s from many different types of human and animal PVs (1, 3, 8, 32, 40, 41, 54, 67). The involvement of the E2-Brd4 complex in the multiple aspects of the PV life cycle establishes it as an important target for the development of antiviral therapeutics (1, 67). The viral oncogenes E6 and E7 are the main arbitrators of HPV-induced oncogenesis. Understanding the mechanism of E2-Brd4-mediated E6/E7 repression will offer new strategies to prevent PV-induced human cancer. Brd4 by itself plays an important role in host cellular growth control and cancer development (11, 22, 42). The E2-Brd4 interaction therefore offers an excellent molecular model to investigate how the oncogenic HPV, while hitchhiking in host cells, hijacks Brd4 functions to achieve viral persistence and malignant progression.

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