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Interaction of the Bovine Papillomavirus E2 Protein with Brd4 Tethers the Viral DNA to Host Mitotic Chromosomes

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Summary

The papillomavirus E2 protein tethers viral genomes to host mitotic chromosomes to ensure genome maintenance. We have identified the bromodomain protein Brd4 as a major cellular interacting partner of the bovine papillomavirus E2. Brd4 associates with mitotic chromosomes and colocalizes with E2 on mitotic chromosomes. The site of E2 binding maps to the C-terminal domain of Brd4. Expression of this C-terminal Brd4 domain functions in a dominant-negative manner to abrogate the colocalization of E2 with Brd4 on mitotic chromosomes, to block association of the viral episomes with Brd4, and to inhibit BPV-1 DNA-mediated cellular transformation. Brd4 also associates with HPV16 E2, indicating that Brd4 binding may be a shared property of all papillomavirus E2 proteins. The interaction of E2 with Brd4 is required to ensure the tethering of viral genomes to the host mitotic chromosomes for persistence of viral episomes in PV-infected cells.

Introduction

The papillomaviruses (PVs) comprise a group of small DNA viruses that induce benign lesions in a variety of higher vertebrates, including humans. Certain human papillomavirus (HPVs) such as HPV16 and 18 are also closely associated with the pathogenesis of cervical cancer and other human tumors (zur Hausen, 2002). The PVs have a specific tropism for squamous epithelial cells and must infect cells within the dividing basal epithelial cell layer to establish an infection. The life cycle of the virus is intimately associated with the differentiation program of the squamous epithelial cell. Late gene expression, lytic DNA amplification, and viral production are restricted to the more differentiated keratinocytes near the surface of the epithelium (Howley and Lowy, 2001).

During latent infection period, the viral genomes are maintained as autonomous replicating extrachromosomal elements (episomes) at a low copy number in the nuclei of proliferating host cells (Gilbert and Cohen, 1987). In BPV-1-transformed mouse cells, the viral genomes also replicate as multicopy nuclear plasmids that can persist over long periods of time to maintain transformation status of the cells (Law et al., 1981). To ensure persistence in both infected and transformed cells, the replicated viral episomes need to faithfully partition to the nuclei of daughter cells during mitosis. Without a mechanism to ensure the localization of the viral genome within the nuclear envelope following nuclear reassembly, the viral genome could be left behind in the cytoplasm and lost either through degradation or dilution after cell division.

Faithful partitioning of genetic materials between the daughter cells during cell proliferation is vital for genomic stability of all organisms. In eukaryotes, the centromere/kinetochor complex is dedicated to faithfully separate replicated chromosomes into daughter cells. However, the relatively small genomes of episomal DNA viruses, such as PV, prevent them from sparing millions of base pairs of DNA for centromere function during the host mitosis. Nonetheless, an adequate strategy was developed by these viruses to colocalize with the chromosomes through a noncovalent interaction.

Several groups have shown that the bovine papillomavirus (BPV) E2 protein is a key player for long-term episomal maintenance of viral genomes within replicating cells (Bastien and McBride, 2000; Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). The first insight into this E2 function came from the observations that E2 as well as BPV-1 episomes are closely associated with mitotic chromatin in dividing cells (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). E2 plays a critical role in viral DNA replication by binding cooperatively with PV E1 protein to the viral origin (Howley and Lowy, 2001). Although this interaction and the minimal origin of replication are sufficient to support the transient replication of viral plasmids, long-term maintenance of the viral episomes requires, in addition, a *cis* minichromosome maintenance element (MME), which consists of multiple E2 binding sites (Piirsoo et al., 1996). In the presence of E2 protein, the MME is attached to the mitotic chromosomes. Furthermore, the chromatin attachment function correlates perfectly with the stable episomal maintenance of the viral plasmids (Ilves et al., 1999). Taken together, these studies strongly suggest that E2 facilitates viral genome segregation by interacting simultaneously with condensed mitotic chromatin and viral genomes.

However, very little is known about the cellular factors involved in the long-term maintenance of the PV genomes. It was shown that the DNA binding function of E2 is not necessary for its association with mitotic chromosomes (Skiadopoulos and McBride, 1998), therefore, the interaction of E2 with mitotic chromosomes cannot be mediated by E2 binding directly to cellular DNA. A cellular protein has therefore been postulated to more likely link E2 to the cellular mitotic chromosomes. However, this cellular mitotic receptor has not yet been identified.

Utilizing a proteomic approach to systematically characterize cellular proteins that associate with E2 in vivo, we identified the human protein Brd4 (bromodomain-

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containing protein 4) as an E2-interacting protein. Brd4 contains two bromodomains, which are conserved sequence motifs that have been implicated in chromatin targeting. Little is known about the human Brd4 protein or its mouse homolog, MCAP. Brd4 is a member of the BET family proteins that carry two bromodomains, and it associates with mitotic chromosomes (Dey et al., 2000). It has recently been shown to bind to acetylated chromatin with preferential binding for acetylated histone H4 (Dey et al., 2003). The human *Brd4* gene is located on chromosome 19 and is the target of translocation t(15;19)(q13;p13.1), which defines a lethal upper respiratory tract carcinoma in young people (French et al., 2001). In addition, Brd4 has been shown to play an important role in cellular growth control and cell cycle progression (Houzelstein et al., 2002; Maruyama et al., 2002).

In this study, we show that Brd4 functions as the major cellular receptor for E2 and viral genome on mitotic chromosomes. Blocking the E2/Brd4 interaction prevented the mitotic chromosomal targeting of both E2 protein and the viral genome. In addition, blocking the binding of E2 to Brd4 inhibited transformation of mouse C127 cells by cloned viral BPV-1 DNA. We demonstrated that Brd4 also associates with the HPV16 E2 protein, indicating that interaction with Brd4 is not specific for BPV-1 E2 and may be a general property of all PV E2 proteins.

Results

Identification of Brd4 as a BPV-1 E2-Interacting Protein

The PV E2 protein plays critical roles in the viral life cycle, regulating viral transcription, as an accessory factor for DNA replication, and in ensuring plasmid maintenance. These diverse functions suggested that E2 might interact with a variety of different cellular partners. Although some cellular partners have already been identified in the literature using the yeast two-hybrid system and other biochemical approaches (Howley and Lowy, 2001), we decided to use a proteomic approach applying the tandem affinity purification (TAP) technology (Ikura et al., 2000; Ogawa et al., 2002) to identify additional E2 in vivo interacting proteins. The PV E2 proteins are well conserved among different PV types and across species. The biological functions of E2 have been best studied for the genital tract HPVs and for BPV-1(Howley and Lowy, 2001). In our study, we chose BPV-1 E2 as a bait protein trying to identify cellular E2-interacting proteins. The full-length BPV-1 E2 protein (E2TA) consists of well-conserved N-terminal and C-terminal domains connected by a flexible hinge region (Figure 1A). The N terminus features a transcriptional activation domain that functions in transcriptional activation, transcriptional repression, ensuring plasmid partitioning, and interacting with the viral E1 protein. The E2 C terminus contains the DNA binding and dimerization domain (Howley and Lowy, 2001). BPV-1 also encodes two shorter E2 proteins, E2TR that initiates at an internal initiation codon within the E2 open reading frame (orf) and E8/E2 that is expressed from a spliced mRNA fusing the E8 and E2 orfs. Both of these shorter forms lack the transactivation domain of the protein and consist primarily of the C-terminal DNA binding/dimerization domain. Both E2TR and E8/E2 can inhibit the N-terminal-dependent functions of E2TA through heterodimerization with the full-length protein and competitive binding to its cognate DNA binding sites (Howley and Lowy, 2001). Since we were particularly interested in further understanding the biological functions of E2 that have been assigned to its N-terminal transactivation domain, we performed a comparative TAP analysis of E2TA and the shorter E2TR.

For stable expression of the E2 proteins, the HPV-negative human cervical carcinoma C33A cell line, which carries a defective p53 and does not undergo cell cycle arrest and apoptosis upon E2 expression (Desaintes et al., 1997), was transduced with a recombinant retrovirus expressing a bicistronic mRNA encoding FLAG-HA-tagged E2 and an ILR2 α subunit surface marker (Ikura et al., 2000). The E2-expressing subpopulation of cells was purified by repeated cycles of affinity sorting using anti-ILR2 antibodies. E2s and their cellular interacting proteins were purified though two steps of immunoaffinity chromatography using immobilized FLAG and HA antibodies, respectively. Proteins copurified with E2TA, but not E2TR, were identified by mass spectrometry analysis.

As demonstrated by previous studies (Ikura et al., 2000; Ogawa et al., 2002), one advantage of the TAP system that we employed here using retroviruses is that the bait protein is expressed at a low to moderate level, more comparable to the levels of endogenous cellular proteins. Since it has been reported that the use of a strong promoter to overexpress E2 protein can result in misfolding and disruption of E2 activity (Bastien and McBride, 2000), we believe that this TAP system is particularly suited for expression of E2 and affinity purification of its natural interacting partners. Prior to the TAP protein purification, we have demonstrated that the FLAG-HA-tagged E2TA proteins stably expressed in cells were functional in an E2 transactivation assay (data not shown).

As shown in Figure 1B, comparison of proteins copurified with E2TA and E2TR on SDS-PAGE gel identified major and minor high molecular weight protein bands uniquely present in the E2TA immunoprecipitation (IP) sample. Mass spec analysis identifies both of these bands as the human Brd4 bromodomain protein. To confirm the mass spec identification result, an antibody, C-MCAP, which recognizes the C-terminal 14 amino acids of both human and mouse Brd4, was used to blot the protein samples coimmunoprecipitated with E2TA and E2TR after tandem FLAG and HA affinity purification (Figure 1C). The Western blot detected a major band of $\sim$200 kDa and two shorter fragments present in sample coimmunoprecipitated with E2TA but not with E2TR. This 200 kDa band is the expected full-length product of Brd4. We believed that the two shorter fragments may be proteolytic-cleavage products of Brd4 that contain the last 14 amino acid of the protein because similar bands were also detected by mass spec analysis in the E2TA TAP sample (Figure 1B). This experiment confirmed the specific interaction of human Brd4 protein with E2TA and not with E2TR, thus demonstrating a requirement for the E2 N-terminal transactivation do-

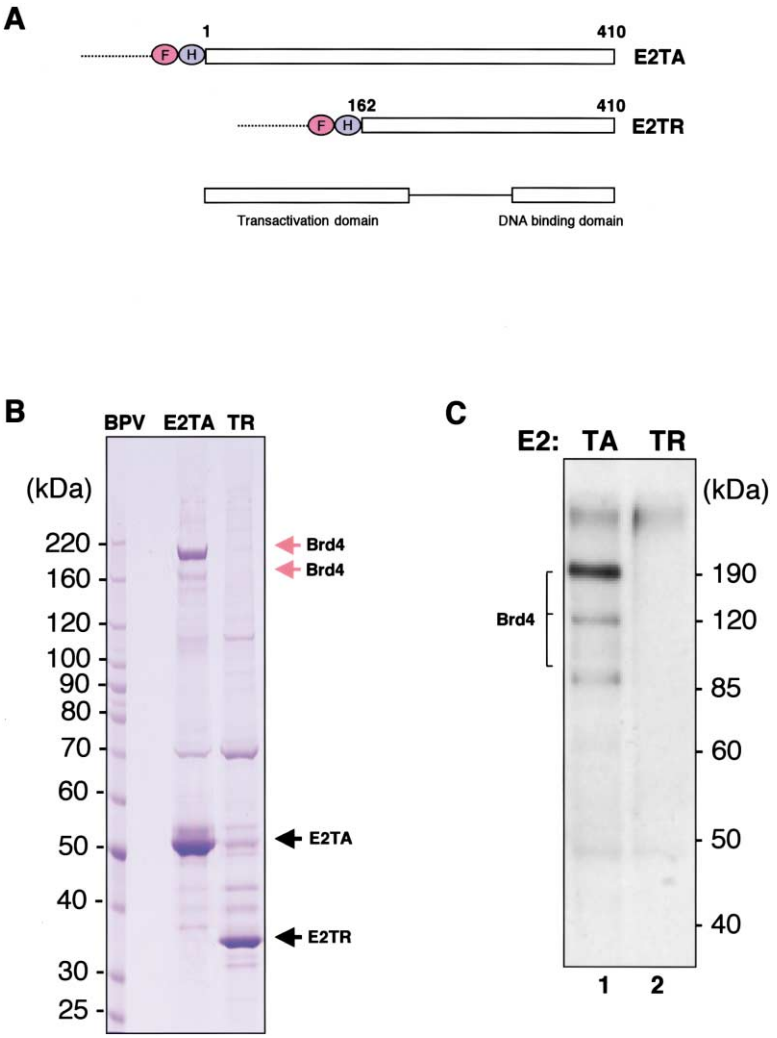


Figure 1. Identification of Brd4 as a Major Cellular Interacting Partner of the BPV-1 E2 Protein

(A) FLAG-HA-tagged E2 constructs for stable expression in C33A cells. Shown are the domain structures of BPV-1 E2TA and E2TR proteins. (F = FLAG tag; H = HA tag).

(B) Proteomic analysis of cellular proteins that associate with E2 in vivo. Nuclear lysates from C33A/E2TA and C33A/E2TR stable cells were immunoprecipitated with anti-FLAG M2 beads followed by anti-HA antibody-conjugated sepharose. Proteins copurified with E2TA and E2TR were analyzed on SDS-PAGE gel and stained with Coomassie blue. Molecular weights of the markers are indicated on the left. The identities of the marked bands were obtained by MALDI-TOF mass spectrometry.

(C) Anti-Brd4 immunoblot of cellular proteins coimmunoprecipitated with E2. An anti-Brd4 rabbit polyclonal antibody C-MCAP (recognizes both human and mouse Brd4 protein) was used to show that human Brd4 protein is uniquely present in the sample coimmunoprecipitated with E2TA after FLAG and HA affinity purification.

main for this interaction. In a separate experiment in which C33A/E2TA and C33A/E2TR stable cells were transiently transfected with FLAG-tagged mouse *Brd4* gene, we also showed that ectopically expressed mouse Brd4 also specifically interacted with E2TA and not with E2TR (data not shown).

E2 and Brd4 Protein Colocalize on Human Mitotic Chromosomes

Previous work has shown that the transactivation domain of E2TA is necessary and sufficient for the association of E2 with mitotic chromosomes (Bastien and McBride, 2000). Since the transactivation domain was also critical for binding to Brd4, which binds to mitotic chromosomes, we next analyzed the cellular localization of Brd4 and E2 protein in cells.

As shown in Figure 2A, Brd4 protein was detected in the nucleus mainly as highly condensed dots. This same dot localization was observed in C33A cells expressing E2TR or no E2 protein (data not shown). In contrast, E2TA protein displayed a speckled pattern of staining in some of the cells and remained diffuse in others. However, in mitotic cells, similar patterns of punctate dots were observed for Brd4 and for E2 over condensed mitotic chromosomes (Figure 2). The overlay of the E2 and Brd4 immunofluorescent images demonstrated that these two proteins colocalized specifically on mitotic chromosomes, where the green E2 staining (Figure 2B) and the red Brd4 staining (Figure 2A) appeared as merged bright yellow dots (Figure 2D). Therefore, E2TA and Brd4 proteins colocalize on mitotic chromosomes. The colocalization of E2 and Brd4 on metaphase chromosomes was also observed in BPV-1-transformed C127 cells (data not shown).

In contrast, staining of E2TR in stable C33A/E2TR cells revealed diffuse localization throughout the nucleus in all cells. Furthermore, E2TR was excluded from mitotic chromosomes in metaphase cells while Brd4 remained associated with the mitotic chromosomes (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/117/3/349/DC1). This result indicated that the colocalization of E2TA with Brd4 in punctate dots on mitotic chromosomes required the E2 transactivation domain. In previous work, it has been demonstrated that E2TR and E8/E2, another E2 protein also lacking the transactivation domain, do not associate with mitotic chromatin (Skiadopoulos and McBride, 1998). Thus the E2 transactivation domain is required for both the association

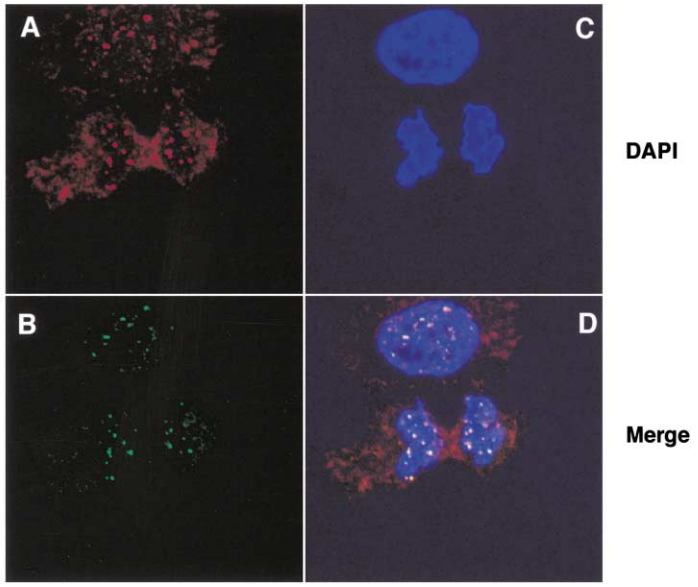


Figure 2. Colocalization of Brd4 and E2TA on Mitotic Chromosomes

C33A/E2TA cells were double-stained with an anti-Brd4 rabbit polyclonal antibody N-MCAP and an anti-E2TA mouse monoclonal antibody IE4. The staining was detected by incubation with a Alexa Fluor 594 goat anti-rabbit IgG (A) and a Alexa Fluor 488 goat anti-mouse IgG (B), respectively. Cells were also counterstained with DAPI to help identify nuclei and mitotic chromosomes (C). Cells were examined under a Zeiss LSM 510 UV upright confocal microscope and the images from (A)–(C) were merged using a Zeiss LSM 510 software as shown in (D). Colocalization of hBrd4 and E2 protein was distinctly observed on mitotic chromosomes as bright yellow dots (D).

of E2 with mitotic chromosomes and the interaction with the Brd4 protein. These data indicated that Brd4 could represent the docking site for E2 on mitotic chromosomes.

Cloning of Human *Brd4* cDNA and Mapping of Its E2 Binding Domain

The two bromodomains on the predicted human Brd4 protein serve as the potential mitotic chromosome binding sites. To identify the domain(s) of human Brd4 that tether E2 protein onto mitotic chromosomes, we proceeded next to clone the previously unavailable human *Brd4* cDNA. All the human Brd4 EST clones available from ATCC I.M.A.G.E. bank only partially covered the predicted *Brd4* cDNA (see Experimental Procedures).

To map the E2 binding domain on Brd4 protein, fragments from human Brd4 covering different regions of the protein (Figure 3A) were translated and labeled by S^{35}Met using in vitro transcription and translation (TNT). Each Brd4 TNT fragment translated from identical amount of DNA template was incubated with either GST-E2TA or GST-E2TR immobilized on glutathione resin. After extensive washing, bound proteins were eluted from GST-E2TA beads (Figure 3B, lane A) and GST-E2TR beads (lane R) and compared with 30% of the input (Figure 3B, lane I). Since our previous in vivo IP experiment had shown that the endogenous Brd4 bound E2TA but not E2TR, the GST-E2TR fusion protein served as a negative control for background binding. Only fragments that showed significantly increased signal in GST-E2TA elution than that observed in either the 30% input or the E2TR elution were identified as E2 binding positive. As shown in Figure 3B and summarized in Figure 3A, all fragments containing the C-terminal aa1047–1362 region bound specifically to E2TA, but not E2TR, demonstrating that the E2 binding domain of Brd4 resided in this 315 C-terminal amino acid segment. Using a reciprocal binding assay, we confirmed that the C-terminal 315 aa of Brd4 fused to GST could pull down E2TA synthesized in vitro, but not E2TR (data not shown).

To further define the E2 binding region within the aa1047–1362 segment of Brd4, we subcloned the *Brd4* cDNA encoding the C-terminal 315 amino acids and expressed them as ~100 aa fragments by TNT. The GST-E2TA binding analysis of these smaller fragments mapped the E2 binding region to the C-terminal 139 amino acids (aa1224–1362) of Brd4 (Figure 3C). Structural prediction analysis indicated that this C-terminal 139 aa region might have a coil-coil structure. In these binding experiments, we have shown that the interaction of Brd4 with E2TA in vitro is mediated by the C-terminal 139 amino acids of Brd4 and the N-terminal transactivation domain of E2TA. The mapping of the E2 binding domain to the Brd4 C-terminal region suggested a strategy to disrupt the binding between E2 and Brd4 in cells in order to investigate the importance of this interaction to the biology of the virus.

Transient Expression of the Brd4 C-Terminal Domain Blocks E2/Brd4 Interaction In Vivo

Based on our in vitro binding results, we next examined whether the Brd4 C-terminal 1047–1362 domain (CTD) could exert a dominant-negative effect on the E2/Brd4 interaction. We expressed the CTD of Brd4 fused to a SV40 nuclear localization signal in cells to test whether it would disrupt Brd4 and E2 binding.

C33A/E2TA cells were transfected with either a pcDNA4C plasmid encoding His-Xpress-SV40NLS-hBrd4-1047-1362 (CTD) or empty vector (V). Forty-eight hours after transfection, nuclear extracts were prepared and immunoprecipitated with anti-FLAG antibody and Western blots performed to detect E2, endogenous Brd4, and the transfected Brd4-CTD. Similar levels of E2TA were observed in cells transfected with vector or the CTD expression plasmid (Figure 4, lanes 5 and 6). Full-length endogenous Brd4 protein (and its proteolytic-cleavage products) was complexed with E2 in cells transfected with empty vector (Figure 4, lane 1), but not in cells transfected with the Brd4-CTD. Instead, the tagged Brd4-CTD protein was detected in the immunoprecipi-

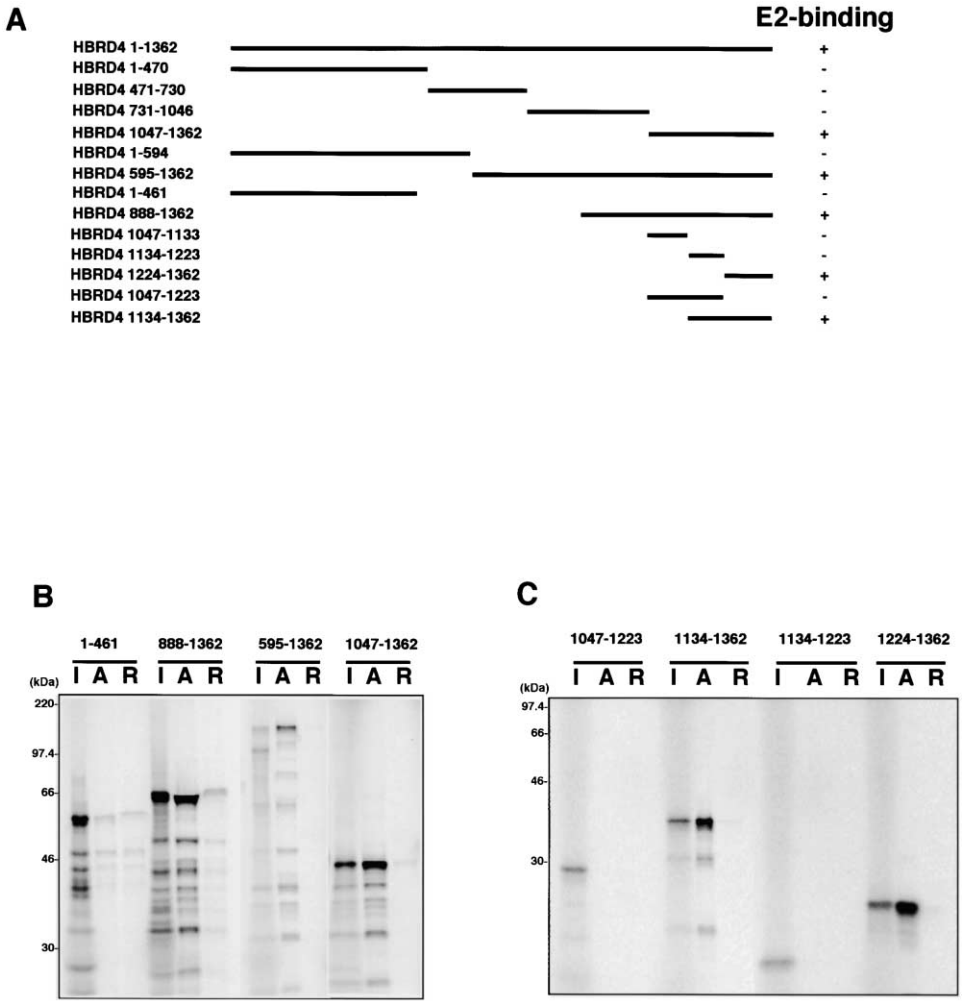


Figure 3. Mapping of E2 Binding Domain on the Human Brd4 Protein

(A) Diagrams of human Brd4 full-length protein and fragments used to map the E2 binding domain. The full-length Brd4 and each indicated subfragment were translated and labeled by S^{35}Met using in vitro transcription and translation (TNT). Their abilities to bind E2TA protein are summarized on the right.

(B) In vitro binding of Brd4 to E2 protein. Each Brd4 TNT product was tested for E2 binding on GST-E2TA or GST-E2TR protein immobilized on glutathione resin. Aliquots (10 μl) of sample eluted from GST-E2TA (lanes A) and GST-E2TR (lanes R) were resolved by SDS-PAGE along with 30% of the input sample (lanes I) and detected by autoradiography. Only the fragments that showed significantly increased signal in lane A over lane I were identified as positive E2 binding samples, as summarized in (A). Shown are the representative gels for both E2 binding positive and negative fragments.

(C) Finer mapping of the E2 binding region on Brd4. Smaller fragments spanning the C-terminal 315 amino acids of Brd4 were generated by in vitro transcription and translation. Their binding to GST-E2 fusion was tested as in (B).

tated sample by the Brd4 antibody, which recognizes the last 14 amino acids of Brd4 protein. In addition, an anti-Xpress antibody also detected the same CTD protein coimmunoprecipitated with E2, confirming the identity of the faster migrating band as the expressed His-Xpress-SV40NLS-hBrd4-CTD product, and not a proteolytic cleavage fragment of endogenous Brd4 protein.

These data confirmed the above in vitro mapping data by showing that the aa1047–1362 fragment of Brd4, when expressed in human cells, interacts with E2 protein. Furthermore, these data established that Brd4 CTD could function in a dominant-negative manner to compete in the binding of E2 to endogenous Brd4 in vivo.

Stable Expression of the Brd4 C-Terminal Domain Prevents E2 Localization to Mitotic Chromosomes

To further address the biological significance of E2/Brd4 binding, we next tested whether disruption of the interaction of E2 with Brd4 would affect E2 mitotic chromosome association. C33A/E2TA cells stably expressing Brd4-CTD or carrying an empty vector were established, and the cells were double-stained with Brd4 and E2TA antibodies and counterstained with DAPI. In the E2 cells transduced by the vector alone, the red Brd4 staining (Figure 4D) and the green E2 staining (Figure 4E) on mitotic chromosomes overlapped completely in a merged image (data not shown), consistent with the data presented in Figure 2 demonstrating the colocaliza-

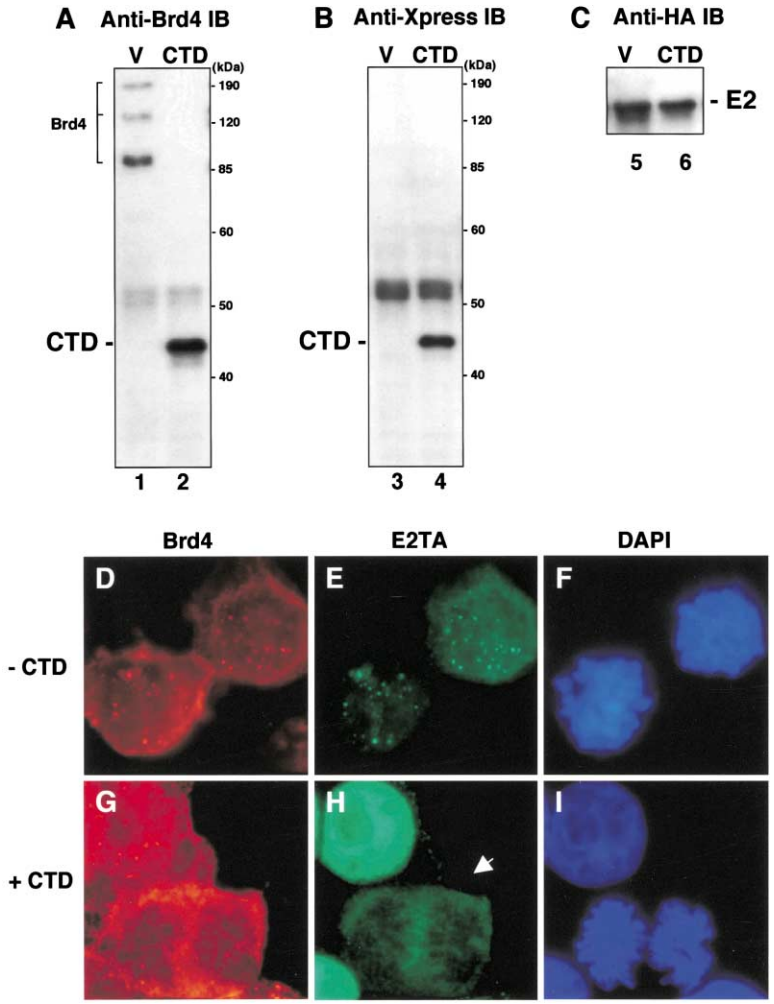


Figure 4. Expression of the C-Terminal Domain of Brd4 in Cells Disrupts the Brd4 and E2 Interaction and Prevents the Localization of E2 Protein on Mitotic Chromosomes

(A) Anti-Brd4 immunoblot detection of the competitive inhibition of Brd4 binding to E2 protein by a dominant-negative inhibitor. C33A cells stably expressing the FLAG-HA-E2TA protein were transiently transfected with either a pcDNA4C plasmid expressing His-Xpress-SV40NLS-hBrd4-CTD or an empty vector (V). Forty-eight hours after transfection, nuclear extracts (NE) were prepared from these cells and immunoprecipitated with anti-FLAG M2 beads.

(B) Anti-Xpress immunoblot detection of the interaction between E2 and the Brd4-CTD. In cells expressing His-Xpress-SV40NLS-hBrd4-CTD, the Brd4-CTD fragment was coimmunoprecipitated with E2 by anti-FLAG beads.

(C) Anti-HA immunoblot detection of the immunoprecipitated E2 protein. The membrane for immunoblot (A) was stripped and re-blotted with an anti-HA peroxidase to demonstrate that anti-FLAG immunoprecipitation of E2 was not affected by the Brd4-CTD.

(D–I) C33A/E2TA cells stably expressing Brd4-CTD (G–I) or carrying an empty vector (D–F) were double-stained with an anti-Brd4 antibody (D and G) and an anti-BPV-1 E2 antibody (E and H). The anti-Brd4 antibody is directed to the N terminus of the protein and therefore does not recognize the Brd4-CTD. Cells were also counterstained with DAPI to label the nucleus and mitotic chromosomes (F and I).

tion of E2 and Brd4 as punctate dots on the mitotic chromosomes. However, in cells stably expressing Brd4-CTD, E2 staining was completely excluded from mitotic chromosomes (panel H), whereas Brd4 remained in the dots on mitotic chromosomes (panels G–I). These patterns of staining were observed in 16 of 16 sets of mitotic chromosomes in C33A/E2TA + vector cells and in 17 of 18 sets of mitotic chromosomes from C33A/E2TA +CTD cells. The one C33A/E2TA +CTD cell line in which no CTD effect was noted is likely due to the lack of CTD expression since we were able to demonstrate CTD expression in only 95% of the C33A/E2TA +CTD cells. This result demonstrated that disruption of the E2/Brd4 interaction by Brd4-CTD prevented the tethering of E2 to host mitotic chromosomes. The direct correlation between E2/Brd4 binding and E2 mitotic chromosome localization demonstrated in this experiment provides further support for the identification of Brd4 as the E2 mitotic receptor.

Disruption of E2/Brd4 Interaction Abrogates Tethering of BPV-1 Genome onto Brd4

As the E2 mitotic chromosome receptor, Brd4 would provide the cellular docking site for BPV-1 episomes to tether onto mitotic chromosomes through E2 (Figure 5A). To test this model, we examined whether Brd4 was associated with BPV-1 DNA and if the interaction depended upon the presence of E2 by chromatin immunoprecipitation (ChIP) analysis.

C127ClH2 cells are BPV-1-transformed C127 cells that carry BPV-1 DNA exclusively as episomes (C. Baker, NCI, personal communication). These cells were transduced with retroviruses expressing the dominant-negative inhibitor His-Xpress-SV40NLS-hBrd4-CTD or vector alone to generate stable cell lines H2-CTD or H2-V. Chromatin prepared from C127, C127ClH2, H2-V, and H2-CTD cells was assayed by ChIP using a Brd4 antibody to an N-terminal segment of Brd4 that would recognize endogenous Brd4 but not the C-terminal domain. As controls for background binding, the Brd4 antibody was either omitted in the binding (−) or replaced by a control nonimmune normal rabbit IgG. The presence of BPV-1 episomes in the DNA/protein complexes recovered was analyzed by PCR using primers that specifically amplify a region of BPV-1 genome. As shown in Figure 5B, BPV-1 DNA could be detected in complex with Brd4 in H2-V cells with the Brd4 antibody at levels significantly above the background signals seen with no antibody (−) or with normal rabbit IgG. Similar ChIP results were also obtained using the nontransduced C127Cl H2 cells (data

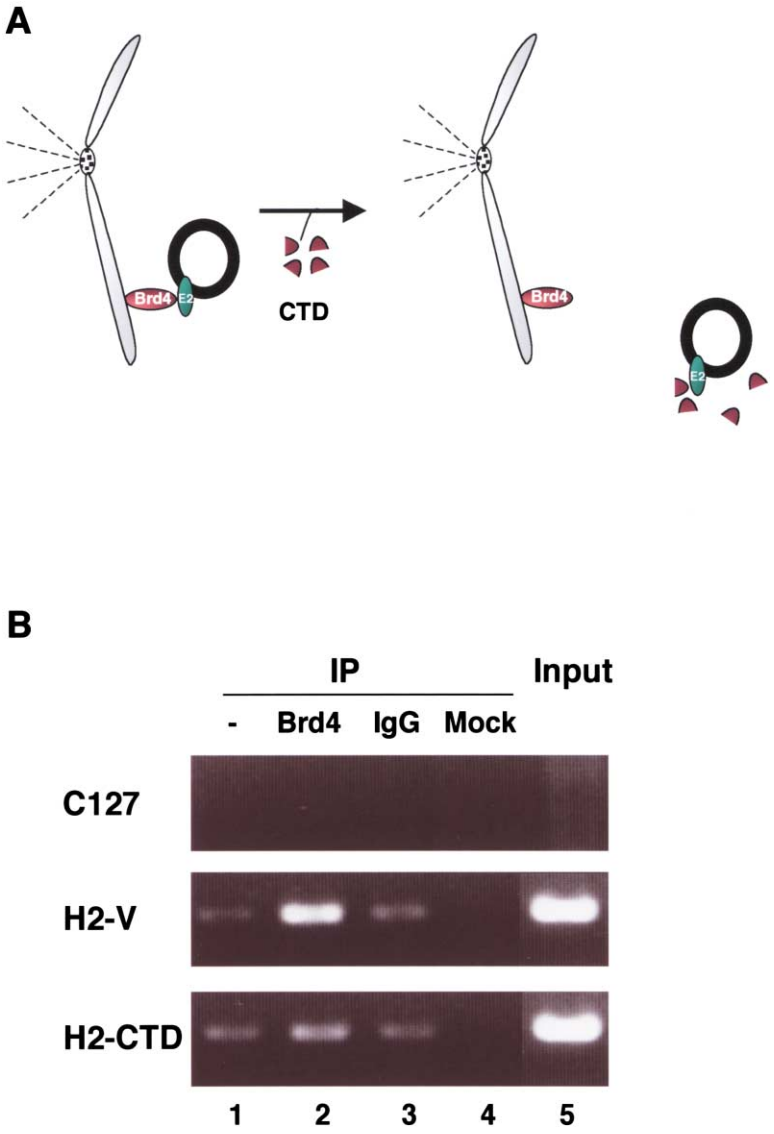


Figure 5. Stable Expression of the Brd4 C-Terminal Domain Abrogates Association of BPV-1 Genome with Brd4

(A) Proposed model for Brd4 as the receptor for papillomavirus E2/DNA complex on mitotic chromosomes. The Brd4 CTD prevents the association of Brd4 with E2, abrogating the docking of E2 protein onto mitotic chromosomes and disrupting the tethering of the viral DNA to the chromosome by the Brd4 protein.

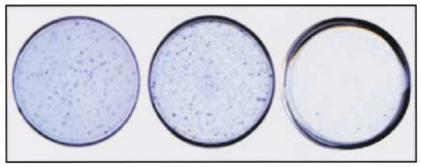
(B) Association of Brd4 with BPV-1 DNA in C127ClH2 cells. C127ClH2 cells were transduced with a CTD retrovirus or an empty vector retrovirus. Transduced cells were then selected for 4 days in puromycin. Crosslinked chromatin from C127, H2-V, and H2-CTD cells was precipitated using antibody specific to an N-terminal domain of Brd4 (Brd4), normal rabbit IgG (IgG), or without any antibody (−) as a negative control. For the "mock" chromatin IP, IP buffer was used instead of chromatin. The recovered DNA was tested by PCR using primers that specifically amplify a region of BPV-1 genome. For lane 5, the input chromatin from each cell line was tested in parallel with the immunoprecipitated DNA. Shown is the image of an agarose gel stained with ethidium bromide.

not shown). The PCR product was not seen in the "mock" ChIP, where the chromatin input was replaced with ChIP buffer, nor was it detected in nontransformed C127 cells, which do not harbor BPV-1 episomes, indicating that it is specific for the BPV-1 genome. Significantly, in the H2-CTD cells, the amount of Brd4 chromatin-associated BPV DNA, as detected by the PCR, was reduced to nearly background level. Using a standard curve to quantitate the relative amounts of DNA in the samples, we estimate a 95% reduction in BPV-1DNA levels in H2-CTD compared to HT-V cells. Furthermore, using fluorescence in situ hybridization (FISH), we were able to show that the BPV-1 episomes that were readily detected associated with mitotic chromosomes in the H2-V cells were undetectable in the H2-CTD cells (data not shown). These results provide direct evidence that Brd4 is associated with BPV-1 DNA in cells that stably maintain viral episomes. By blocking the E2/Brd4 interaction using the Brd4-CTD, we could specifically disrupt the association of BPV-1 genome to Brd4, confirming that the Brd4-episome interaction is mediated by E2. Furthermore, quantitative analysis of viral episome levels in CTD-expressing H2 cells revealed a progressive loss of BPV-1 DNA with cell passage (data not shown).

Blocking E2/Brd4 Interaction Inhibits BPV-1 Transformation of C127 Cells

In BPV-1-transformed mouse C127 cells, the viral genome is maintained stably in an extrachromosomal state (Law et al., 1981). Since BPV-1 ably transforms C127 cells, we reasoned that blocking the tethering of the viral DNA to the mitotic chromosomes should result in a loss of the viral DNA and a decrease in the efficiency of transformation. We therefore generated C127 cells that stably expressed the Brd4-CTD using retrovirus transduction as well as vector control cells and tested their respective susceptibilities to BPV-1 transformation. Stable expression of the Brd4-CTD was verified in the C127 cells by immunofluorescent staining; furthermore, no differences were noted in the appearance or growth parameters between Brd4-CTD-expressing cells and vector control cells or nontransduced C127 cells

A



C127 +BPV-1 C127+V+BPV-1 C127+CTD+BPV-1

B

Cell	Foci/1ug BPV1	Average	Stdev.
C127	150 146 155	150.3	4.5
C127+V	141 140 127	136.0	7.8
C127+CTD	8 10 10	9.3	1.2

Figure 6. Stable Expression of the Brd4 C-Terminal Domain Inhibited BPV-1 Viral Genome-Induced Transformation of Mouse C127 Cells

Mouse C127 cells were infected with retrovirus to generate cells stably expressing His-Xpress-SV40NLS-HBrd4-CTD (C127+CTD) or carrying an empty vector as a control (C127+V). C127 and the stable cells were each transfected with 1 μg of BPV-1 DNA that has been purified after BamHI digestion of the p142-6 plasmid in which the full-length BPV-1 genome is cloned into pML2 at the BamH1 site. After 21 days, the cells were fixed, stained with methylene blue, and the number of transformed foci determined, as shown in (A). The results from three independent transfections into each cell line are summarized in (B).

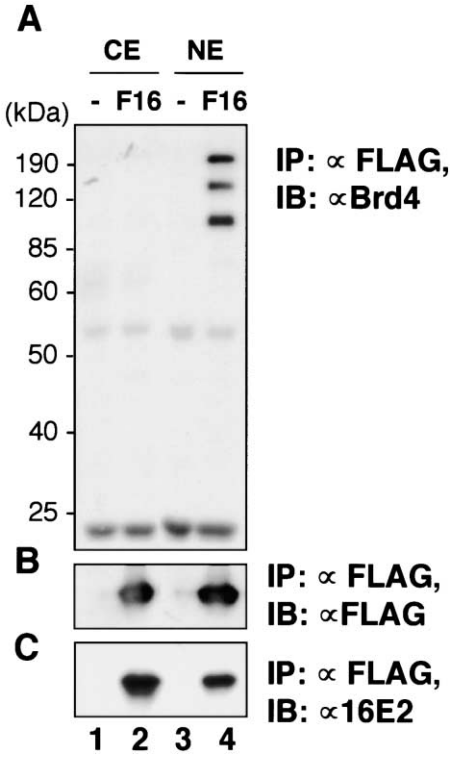


Figure 7. Brd4 Interacts with HPV16 E2

(A) Anti-Brd4 immunoblot analysis of anti-FLAG-16E2 immunoprecipitation. C33A cells were transfected with FLAG-16E2 plasmid (F16) or an empty vector (−). Cytoplasmic extracts (CE) and nuclear extracts (NE) immunoprecipitated with anti-FLAG M2 beads were immunoblotted with the Brd4 antibody C-MCAP.

(B) Anti-FLAG immunoblot of M2 immunoprecipitation. Anti-FLAG immunoprecipitates from (A) were also immunoblotted with an anti-FLAG antibody to detect the FLAG-tagged HPV16E2.

(C) Anti-16E2 immunoblot of M2 immunoprecipitation. Membrane for immunoblot (B) was striped and reblotted with an anti-16E2 antibody to detect the presence of 16E2 protein.

(data not shown). As shown in Figure 6, a comparable level of transformation assayed by focus formation was seen in the vector control cells and normal C127 cells. In contrast, the numbers of colonies observed in the Brd4-CTD-expressing cells were decreased more than 90%. This decrease in transformation efficiency suggests that the E2/Brd4 interaction could represent an excellent target for developing antiviral inhibitors.

Brd4 Interacts with HPV16 E2 Protein

To determine whether Brd4 might serve as the tether for other PV E2 proteins, we tested if the HPV16 E2 also interacted with Brd4. C33A cells were transfected with pCMV4-FLAG-16E2 or vector. Cytoplasmic and nuclear extracts prepared from these cells were subjected to anti-FLAG IP to pull down the HPV16 E2 protein and associated factors. A Brd4 immunoblot showed that the Brd4 protein and its proteolytic-cleavage products coimmunoprecipitated with E2 using anti-FLAG beads only in the nuclear extract of cells that were transfected with FLAG-16E2 plasmid (Figure 7A, lane 4). The set of bands corresponding to Brd4 protein and its proteolytic-cleavage products were not detected in C33A cells transfected with the empty vector plasmid (lane 3), nor were they present in the cytoplasmic IP. Both anti-FLAG and anti-16E2 immunoblots showed the IP of the HPV16 E2 protein only in cells transfected with FLAG-16E2 plasmid (Figures 7B and 7C, respectively). This result showed that Brd4 protein was coimmunoprecipitated from the nuclear extract with 16E2, demonstrating that the interaction between Brd4 and BPV-1 E2 also applies to other PV E2 proteins.

Discussion

Some DNA viruses, like the PVs and the lymphotropic herpesviruses, which establish persistent or latent infections, must be able to maintain their genomes as stable episomes in the nuclei of infected cells. Although elaborate mechanisms have been demonstrated for the effective segregation of low copy number plasmids in prokaryotes (Niki and Hiraga, 1997; Nordstrom and Austin, 1989), the mechanisms by which eukaryotic episomal viruses ensure genome maintenance have not been yet fully elaborated.

A major obstacle for maintaining plasmids in eukaryotes is presented by the breakdown and reassembly of

the nuclear membrane during cell division. Even high-copy-number extrachromosomal viral genomes could be excluded from the nucleus and subject to degradation in the cytoplasm if not tethered to the chromosomes during mitosis. For the PVs, the consequence is the very rapid and catastrophic loss of the viral genomes (Lehman and Botchan, 1998). Noncovalent association with cellular chromosomes appears to be the principle strategy employed by episomal DNA viruses to ensure that their genomes are enclosed within the new nuclear envelopes and thus maintained in progeny cells (Ballestas et al., 1999; Hung et al., 2001; Ilves et al., 1999; Skiadopoulos and McBride, 1998).

During the life cycle of the PVs, the viral genome is maintained as an episome at a low copy level in the infected basal squamous epithelial cells (Howley and Lowy, 2001). Therefore, a specific mechanism is necessary to ensure that the viral DNA is not lost from the dividing basal cells of a papilloma. The E1 and E2 proteins are sufficient for transient replication of viral genome containing the viral origin of replication; however, stable maintenance of origin-containing plasmids requires E2 and regions from the viral long control region (LCR) that contain E2-specific DNA binding sites (Piirsoo et al., 1996). Previous work from several laboratories has suggested that E2 binds to these sites and tethers the viral genomes to mitotic chromosomes (Bastien and McBride, 2000; Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998).

Our study identifies the bromodomain protein Brd4 as the mitotic chromosomal receptor for E2. The interaction between BPV-1 E2 and Brd4 was confirmed both in vivo and in vitro. We mapped the E2 binding region of Brd4 to a 139 aa C-terminal domain. Brd4 interacts with the N-terminal transactivation domain of E2, which has previously been shown to be responsible for the colocalization of E2 with Brd4 on mitotic chromosomes (Skiadopoulos and McBride, 1998). By ChIP and FISH analyses, we show that Brd4 associates with extrachromosomal PV DNA in an E2-dependent manner. Our data thus provide a molecular mechanism for E2-mediated plasmid maintenance: mitotic chromosome-associated Brd4 interacts with the transactivation domain of E2, and the C-terminal E2 DNA binding domain binds the viral DNA through cognate E2 binding sites (Figure 5A). Through these molecular interactions, the PV DNA becomes associated with host mitotic chromosomes before division of the sister chromatids.

To fulfill its viral genome maintenance function, E2 must tether the viral DNA to the condensed chromosomes early in mitosis and remain bound until after the nuclear envelope has been reformed. To this end, E2 has been observed associated with condensed chromosome at all stages of mitosis, at times when many other transcription factors have been displaced from mitotic chromatin (Bastien and McBride, 2000). Brd4 association with chromosomes through mitosis reveals a temporal pattern that coincides with the E2-chromosome association (Dey et al., 2000).

The colocalization of E2 with Brd4 was most distinctly observed in mitotic cells, in which both proteins appeared in high-density punctate dots on mitotic chromosomes. However, at what stage of cell cycle E2 becomes associated with Brd4 remains an open question. For instance, E2 was observed to localize diffusely in some of the cells in which it was stably expressed and to be present in nuclear dots in others, raising the possibility that E2 may be associated with Brd4 in some fraction of cells that are not yet in mitosis. Additional insight into the mechanisms involved in the regulation of the E2/Brd4 interaction awaits further investigation.

Association of viral genomes with host mitotic chromosomes via a viral encoded protein is a strategy employed by a number of different latent DNA viruses. The EBNA1 protein of Epstein-Barr virus and latency-associated nuclear antigen (LANA) of human herpesvirus-8 are each required for stable viral genome maintenance (Ballestas et al., 1999; Hung et al., 2001). Like E2, each of these herpesvirus proteins is a sequence-specific DNA binding protein that associates with mitotic chromosomes (Ballestas et al., 1999; Hung et al., 2001). Although several candidate chromosomal binding partners have been identified for both EBNA1 and LANA (Kapoor and Frappier, 2003; Shire et al., 1999; Cotter and Robertson, 1999; Krithivas et al., 2002; Platt et al., 1999), none has been shown unequivocally to function as their mitotic receptors.

We have demonstrated the direct interaction of Brd4 and the viral E2 protein and the colocalization of E2 with endogenous Brd4 on host mitotic chromosomes. In addition, we developed the E2 binding domain of Brd4, CTD, as a dominant-negative inhibitor to prevent E2 association with endogenous Brd4. Using the Brd4 CTD, we abolished both the mitotic chromosome association of E2 protein and the tethering of BPV-1 DNA to Brd4 (see Figure 5A). The direct mitotic chromosome association feature of Brd4 is further strengthened by the demonstration that Brd4 selectively associates with acetylated histone H4 (Dey et al., 2000, 2003). These in vivo data demonstrate that Brd4 represents the previously unidentified cellular factor that serves as the receptor of E2/viral DNA complex on mitotic chromosomes. By preincubation of recombinant E2TA protein with the Brd4 CTD, we could prevent the association of E2 with human mitotic chromosomes (data not shown). Thus the region of the E2 transactivation domain to which Brd4-CTD binds is necessary for E2 association with mitotic chromosomes and suggests that Brd4 may be the sole E2 mitotic receptor. Brd4 is thus the first mitotic chromosome receptor to be identified for a DNA virus.

Although our studies have focused on the BPV-1, a requirement for mitotic association of the viral DNA in dividing cells is no doubt a general feature of all PVs, including the HPVs (Howley and Lowy, 2001). Indeed several cell lines derived from HPV16 and HPV31 preneoplastic lesions have been shown to harbor predominantly extrachromosomal viral DNA (Bedell et al., 1991; Stanley et al., 1989). Furthermore, replication assays using HPV31 have shown roles for E2 and E2 binding sites in viral genome maintenance in cultured cells (Hummel et al., 1992; Stubenrauch et al., 1998, 2000). Although detailed studies on genome maintenance functions for the HPVs have not yet been carried out, it seems likely that this E2 genome maintenance function will be conserved among the PVs and that Brd4 may serve as the mitotic receptor for E2 from different PVs. In our studies, we have shown that HPV16 E2 protein

interacts with endogenous Brd4, supporting the hypothesis that different PVs share a common pathway for tethering to mitotic chromosomes through Brd4.

Using the Brd4 CTD, we have shown that disruption of E2/Brd4 binding can inhibit viral transformation, presumably by inhibiting the maintenance of the viral DNA in the infected cell. This observation suggests that E2/Brd4 binding could be a new target for the development of PV antivirals. Studies are underway to test if the Brd4 CTD can similarly disrupt the association between HPV episome and host mitotic chromosomes. A minimum peptide region responsible for mediating the E2/Brd4 interaction could be used to design small molecules and screen for compounds that could be useful in the treatment or prevention of HPV infections and associated diseases.

Experimental Procedures

Recombinant Plasmid Construction

To construct retrovirus expression vectors for FLAG and HA epitope-tagged E2 proteins, E2TA and E2TR coding sequences were PCR amplified and subcloned into pOZ-N vector using XhoI and NotI sites (Ogawa et al., 2002). The resulting plasmids are pOZN-E2TA and pOZN-E2TR. Three I.M.A.G.E. Consortium clones spanning different regions of the human *Brd4* cDNA were obtained from ATCC: clone 5214463 (spanning nt. 1–1626), clone 4138081 (spanning nt. 1382–2161), and clone 380968 (spanning nt. 2601–4090). A 558 bp fragment spanning nt. 2133–2691 of human *Brd4* cDNA missing in all of the available cDNA clones was isolated from a human cDNA library (Panomics) by PCR. The cDNA fragments were appropriately amplified by PCR and cloned into pGEX-6P-1 to construct a full-length cDNA, which was then subcloned into pcDNA4C plasmid using BamHI and NotI digestion. Fragments of human *Brd4* cDNA were cloned into pcDNA3.1/V5-His using PCR and TOPO TA Expression Kit (Invitrogen) for expression controlled by both a CMV promoter and a T7 promoter. These plasmids were used for in vitro transcription and translation. To construct an expression plasmid for the Brd4-CTD, the SV40 nuclear localization signal (NLS) was inserted in frame in front of the Brd4-CTD coding region by PCR and the product was subcloned into pcDNA4C vector using BamHI and NotI sites. The resulting plasmid is pcDNA4C-His-Xpress-SV40NLS-hBrd4-CTD. The His-Xpress-NLS-hBrd4-CTD cassette was reamplified by PCR and subcloned into pLPCX plasmid (BD Biosciences) to generate the pLPCX-His-Xpress-NLS-hBrd4-CTD plasmid for stable cell line construction. pGEX-E2TA, pGEX-E2TR, and pCMV-FLAG16E2 were from our laboratory plasmid bank. All plasmid constructs were verified by DNA sequencing.

Cells Culture and Cell Lines

C33A and murine C127 cells were maintained as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The BPV-1-transformed C127ClH2 cell line carrying extrachromosomal BPV-1 DNA was generously provided by Dr. Carl Baker (NCI).

Stable Cell Line Construction

Using standard retrovirus production and transfection procedures, pOZN-E2TA and pOZN-E2TR plasmids were used to generate C33A cells stably expressing FLAG-HA-tagged E2TA and E2TR, designated C33A/E2TA and C33A/E2TR. To block the Brd4 and E2 interaction in C33A/E2TA and C127ClH2 cells, the pLPCX-His-Xpress-NLS-hBrd4-CTD plasmid was used to establish stable expression of the Brd4 CTD. The resulting cell lines are C33A/E2TA-CTD and H2-CTD, respectively. As a control, retrovirus produced from pLPCX empty vector was used to transduce the same parental cells to prepare stable cell lines carrying the vector backbone, C33A/E2TA-V and H2-V, respectively.

Tandem Affinity Purification of E2-Interacting Protein

C33A cells stably expressing FLAG-HA-E2TA and E2TR were prepared as described previously (Ikura et al., 2000; Ogawa et al., 2002). Nuclear extracts of C33A/E2TA and C33A/E2TR stable cells were prepared as described (Groisman et al., 2003). The E2s and associated components were isolated from nuclear extracts by IP with anti-FLAG antibody followed by anti-HA antibody according to established procedure (Ikura et al., 2000; Ogawa et al., 2002). E2-associated protein bands were excised from SDS-PAGE gels, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry for peptide sequence at the DFCI Molecular Biology Core Facility.

Immunoprecipitation and Western Blot Analysis

For transient protein expression, 40%–80% confluent cells growing in 10 cm dishes were transfected with total 16 μg of plasmid DNA using FuGENE 6 Transfection Reagent (Roche). Cells were harvested at 48∼72 hr after transfection. Cytoplasmic and nuclear extracts were prepared as described previously (Schreiber et al., 1989).

For IP, soluble extract proteins were mixed with 10 μl of anti-FLAG M2 agarose (Sigma) and rotated at 4°C for 4 hr. The beads were washed three times with 1 ml 0.1 M KCl base buffer (20 mM Tris-HCl [pH 8.0], 10% glycerol, 5 mM $MgCl_2$, 0.1% Tween 20, 0.1 M KCl, 0.2 mM PMSF, and 0.5 mM DTT), and bound proteins were eluted with 30 μl of sample buffer. Aliquots (10 μl) were resolved on a 10% gel. Proteins were transferred to Immobilon-P (Millipore) and blotted with specific antibody to detect the protein of interest (ECL detection). Antibodies employed in the Western blot analysis were as follows: the rabbit polyclonal antibody against Brd4, C-MCAP, has been described previously (Dey et al., 2000); the anti-FLAG M2 monoclonal antibody was obtained from Sigma; the anti-Xpress mouse monoclonal antibody was from Invitrogen; the rabbit polyclonal antibody against HPV16E2 has been described previously (Sakai et al., 1996); and the anti-HA-Peroxidase was purchased from Roche.

Immunofluorescent Staining

C33A/E2TA stable cells were cultured on coverslips in DMEM containing 10% fetal calf serum. Cells fixed with 3% paraformaldehyde were incubated for 10 min at room temperature in blocking/permeabilization buffer (0.5% Triton X-100 and 3% BSA in PBS) and stained with anti-Brd4 rabbit polyclonal antibody, N-MCAP ([Dey et al., 2000], 1/500 dilution), and an anti-BPVE2 mouse monoclonal antibody, 1E4 ([Kurg et al., 1999], 1/50 dilution) at room temperature for 60 min. Cells were then washed three times with blocking/permeabilization buffer and incubated for an additional 60 min using a Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, 1/500 dilution) and a Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes, 1/1000 dilution). Cells were counterstained with 0.3 μM DAPI and examined using either a Zeiss LSM 510 Meta UV upright confocal microscope and associated Zeiss LSM 510 software or a Leica DMLB epifluorescence microscope equipped with a Leica DC 500 digital camera.

In Vitro Binding of Brd4 to GST-E2

S^{35}Met-labeled Brd4 full-length protein or fragments were produced by in vitro transcription/translation in reticulocyte lysate (Promega) using plasmids pcDNA3.1/V5-His carrying the human Brd4 full-length or partial cDNA, respectively. GST-E2TA and -E2TR fusion proteins were produced in *E. coli* using the respective pGEX plasmids. A 20 μl aliquot of each translation mixture was mixed with 5 μl of immobilized GST-E2TA or GST-E2TR (1 μg of protein/μl beads) and 0.18 ml PBS prior to 4 hr incubation at 4°C. The beads were washed three times with 0.5 ml of 0.1 M KCl base buffer and eluted with 30 μl of SDS-PAGE sample buffer. An aliquot of each eluate (10 μl) was analyzed by SDS-PAGE and autoradiography.

Chromatin Immunoprecipitation (ChIP)

Chromatin of C127, C127ClH2-V, or C127ClH2-CTD cells was prepared as described (Weinmann et al., 2001). Chromatin from 3 × 10^7 cells was incubated with 5 μg of either rabbit polyclonal anti-Brd4 antibody, N-MCAP (Dey et al., 2000), a control normal rabbit IgG (Upstate), or no antibody at 4°C for 12 hr. Recovery of immune complexes and de-crosslinking of chromatin were carried out as

described (Weinmann et al., 2001). After phenol/chloroform extraction and ethanol precipitation, DNA was resuspended in 30 μl of H_2O and analyzed by PCR using primers specifically amplifying a region of BPV-1 genome.

PCR was performed using 2 μl of IP or 2 μl of a 1:300 dilution of the total input chromatin in a 40 μl reaction containing 0.4 μM of each primer; 0.2 mM of dNTP; 1X Pfu polymerase buffer; and 2 U of Pfu DNA polymerase (Stratagene). Following 30 cycles of amplification, the PCR products were separated on a 1.2% agarose gel and detected with ethidium bromide. The PCR primers were designed to amplify 432 bp spanning nt. 2601–3032 of BPV-1 plasmid. Primer sequences are available on request.

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