



Rapid Communication

Amino acid substitutions that specifically impair the transcriptional activity of papillomavirus E2 affect binding to the long isoform of Brd4

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Abstract

The E2 protein of papillomaviruses binds to specific sites in the viral genome to regulate its transcription, replication and segregation in mitosis. Amino acid substitutions in the transactivation domain (TAD) of E2, of Arg37 and Ile73, have been shown previously to impair the transcriptional activity of the protein but not its ability to support viral DNA replication. To understand the biochemical basis of this defect, we have used the TADs of a low-risk (HPV11) and a high-risk (HPV31) human papillomavirus (HPV) as affinity ligands to capture proteins from whole cell extracts that can associate with these domains. The major TAD-binding protein was identified by mass spectrometry and western blotting as the long isoform of Brd4. Binding to Brd4 was also demonstrated for the E2 TADs of other papillomaviruses including cutaneous and animal types. For HPV11, HPV31 and CRPV E2, we found that binding to Brd4 is significantly reduced by substitutions of Arg37 and Ile73. Since these amino acids are located near each other in the 3-dimensional structure of the TAD, we suggest that they define a conserved surface involved in binding Brd4 to regulate viral gene transcription.

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Introduction

Papillomaviruses are a family of small double-stranded DNA viruses that induce benign and malignant hyperproliferative lesions of the differentiating epithelium (reviewed in Zur Hausen and de Villiers, 1994; Lowy and Howley, 2001; Hebner and Laimins, 2005). These viruses infect the basal cell layer where they maintain their double stranded DNA genome as a circular episome in the nucleus of infected cells. Maintenance of the HPV episome in primary keratinocyte cultures requires four viral proteins: the two viral oncogenes, E6 and E7, the E1 replicative helicase and the E2 protein (Howley and Lowy, 2001; Longworth and Laimins, 2004). E2

is a multifunctional protein that binds to specific sites in the regulatory region of the viral genome to promote its replication, regulate its transcription and ensure its proper segregation to daughter cells at mitosis (reviewed in Blachon and Demeret, 2003). E2 is comprised of two functional domains, a N-terminal transactivation domain (TAD) and a C-terminal DNA-binding/dimerization domain separated by a hinge region (Figs. 1A and B). The TAD has been shown to be a protein interaction module that binds to the viral E1 helicase to promote replication of the genome and to cellular transcription factors to regulate viral gene transcription (reviewed in Blachon and Demeret, 2003; Hebner and Laimins, 2005). As a transcription factor, E2 has been shown to act either as an activator or a repressor, depending on the promoter context. Reporter gene assays have shown that E2 can activate transcription from a minimal promoter

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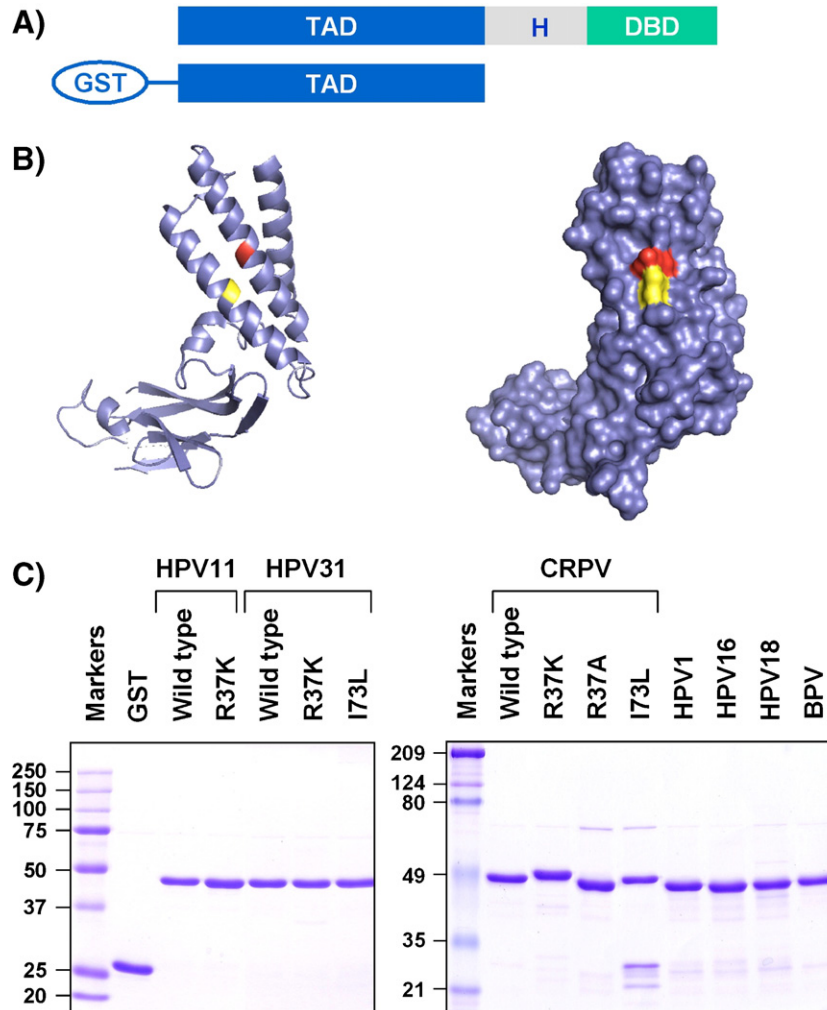


Fig. 1. Purified GST-E2 TAD proteins used in this study. (A) Domain structure of the complete E2 protein. The transactivation domain (TAD) is linked by a hinge (H) to the C-terminal dimerization and DNA binding domain (DBD). (B) Ribbon (left) and surface (right) representations of the HPV11 E2 TAD (Wang et al., 2004). Arg37 and Ile73 have been colored in red and yellow, respectively. (C) Purified GST-E2 TAD proteins used in this study. 3 μ g of each protein was separated on a 10% SDS-PAGE and stained with Coomassie blue. The sizes (kDa) of the molecular weight markers are shown on the left.

under the control of multimerized E2 binding sites (Kovelman et al., 1996). In contrast, in the context of the viral genome, E2 is primarily a repressor of viral transcription initiated in the LCR (Thierry and Yaniv, 1987; Bernard et al., 1989; Demeret et al., 1997; Soeda et al., 2006). As a segregation factor, E2 tethers the viral episome to mitotic chromatin (Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998; Bastien and McBride, 2000; Ilves et al., 1999). Studies using the E2 protein from bovine papillomavirus (BPV) have shown that the long isoform of the bromodomain-containing protein 4, Brd4(L), is the receptor to which E2 binds on mitotic chromosomes (You et al., 2004, 2005; McPhillips et al., 2005; reviewed in McBride et al., 2004). However, a recent study by Oliveira et al. (2006) has suggested that there are important differences in the way that alpha-papillomavirus E2, like those of HPV11 and HPV31, bind to mitotic chromosomes. One group also suggested that HPV11 E2 associates primarily with the mitotic spindle and with centrosomes, rather than chromatin in mitosis (Van Tine et al., 2004).

Mutagenesis of the E2 TAD led to the identification of amino acid substitutions that genetically separate the transcription from the replication function of the protein. In particular, substitutions of arginine 37 for alanine and of isoleucine 73 for leucine or alanine have been shown to impair the ability of E2 to activate and repress transcription while having little or no effect on its ability to interact with E1 and support viral DNA replication (Abroi et al., 1996; Breiding et al., 1996; Brokaw et al., 1996; Ferguson and Botchan, 1996; Sakai et al., 1996; Grossel et al., 1996; Cooper et al., 1998; Nishimura et al., 2000). Interestingly the conserved R37 and I73 are located close to each other at the surface of the TAD, in a region opposite to that involved in binding the E1 helicase (Fig. 1B). To understand the biochemical basis of this defect, we have used the TADs of a low-risk (HPV11) and a high-risk (HPV31) HPV type as affinity ligands to capture proteins from whole cell extracts that can associate with wild type but not transactivation-defective versions of these domains.

Results

Identification of cellular proteins that bind to the transactivation domain of E2

We used protein affinity chromatography (Formosa et al., 1991) as a method to identify, in a systematic fashion, cellular proteins that can bind to the E2 TAD of low- and high-risk HPV types. As affinity ligands, we used the TADs of HPV11 E2 (amino acids 1–209) and HPV31 E2 (amino acids 1–208), which we expressed and purified from bacteria as fusion proteins with glutathione-S-transferase (GST). These purified proteins are shown in Fig. 1C. First, we prepared a 40 μ l affinity column by immobilizing the GST-E2 TAD of HPV11 on glutathione-sepharose at a concentration of 4 mg/ml. A similar column was prepared using GST, as a control. These columns were loaded with 30 mg of whole cell extract prepared from 293 or HeLa cells, washed sequentially with buffer containing 0.1 M NaCl, and the remaining bound proteins eluted with 20 mM reduced glutathione. Analysis of the column eluates by SDS-PAGE and silver staining revealed one cellular protein that consistently and specifically associated with the GST-E2 TAD of HPV11 and not with GST or the glutathione-sepharose resin (Figs. 2A and B, arrow). Identification of this protein by tandem mass spectrometry (MS) revealed that it corresponds to the long isoform of the bromodomain-containing protein 4, Brd4(L) (tryptic peptides identified by MS are shown in Fig. 2E). To conclusively ascertain the identity of this protein, the column eluates were probed by western blotting with antibodies against Brd4(L). As can be seen in Figs. 2A and B, Brd4(L) was indeed detected in the eluates from the GST-E2 TAD column but not those from control columns. Similar experiments were then performed with the E2 TAD from HPV31. For this type also, the eluate from the column containing the GST-E2 TAD contained one major and specific band, which was identified as Brd4(L) by MS and western blotting (Figs. 2C and D). From these studies, we concluded that Brd4(L) is the major E2 TAD-binding proteins present in 293 and HeLa whole cell extracts. In additional experiments, we confirmed that the interaction of Brd4(L) with the E2 TAD of HPV11 and HPV31 is resistant to high salt concentrations (1M NaCl, data not shown), suggesting that it is primarily hydrophobic in nature.

Brd4(L) binds to the E2 TAD of different papillomaviruses

To investigate if the E2 proteins of other papillomaviruses also associate with Brd4(L), we performed affinity chromatography experiments with the TADs of several other viral types: one cutaneous type, HPV1, two other high-risk anogenital types, HPV16 and HPV18, and two prototypical animal types, BPV and cottontail rabbit papillomavirus (CRPV). The TADs of HPV11 and HPV31 were also used as positive controls. These TADs, purified as fusions with GST (Fig. 1C), were immobilized on glutathione-sepharose resin at two separate concentrations of 0.5 and 2.0 mg/ml. Affinity chromatography with 293 or HeLa cell extract was

then performed essentially as described above, and the bound proteins eluted with buffer containing reduced glutathione. Aliquots of the input whole cell extract and of the glutathione eluates were analyzed by western blotting with the anti-Brd4(L) antibody. As can be seen in Fig. 3A, all E2 TAD tested were capable of binding Brd4(L), indicating that this interaction is highly conserved among papillomaviruses. Recently, the transcription elongation factor P-TEFb, a kinase that phosphorylates the C-terminal domain (CTD) of RNA polymerase II, was shown to associate with Brd4 (Jang et al., 2005; Yang et al., 2005). We were unable to detect Cdk9, a component of P-TEFb, in our E2 TAD column eluates, perhaps because this interaction is too weak to be detected by affinity chromatography (data not shown).

To determine if binding to Brd4(L) was a characteristic specific to the E2 TAD or one shared with other transactivation domains, we performed affinity chromatography with the acidic activation domains of herpes simplex (HSV) VP16 and of the tumor suppressor protein p53. These proteins were purified from bacteria as fusions with GST (Fig. 3B), and immobilized on columns at concentrations of 2 and 4 mg/ml. As can be seen in Fig. 3C, the amount of Brd4(L) that was retained by the VP16 and p53 TAD was much lower than that retained by the HPV E2 TAD. These results argue that binding to Brd4(L) is not a general property of activation domains. They also provide further evidence that the interaction of E2 with Brd4(L) is specific.

Substitutions of R37 and I73 impair the binding of Brd4(L) to the E2 TAD of HPV11, HPV31 and CRPV

To examine if the association of Brd4(L) with the HPV11 and HPV31 E2 TAD is sensitive to amino acid substitutions affecting the transcriptional activity of the proteins, we performed affinity chromatography experiments with wild type and mutant TAD carrying substitutions at R37 and I73. For each TAD, a series of columns containing increasing amounts of immobilized protein was used. Eluates were then probed by western blotting for the presence of Brd4(L). As can be seen in Fig. 4A, binding of Brd4(L) to wild type HPV11 and HPV31 E2 was dose responsive, as anticipated for a genuine binary interaction. Similar analysis performed with R37 and I73 mutant proteins clearly indicated that these proteins are defective in binding Brd4(L). Thus R37 and I73 are essential for the specific binding of Brd4(L) to the E2 TAD. Interestingly, both amino acids are located close to each other in the 3-dimensional structure of the E2 TAD (Fig. 1B), on a surface of the TAD opposite to that involved in binding to the E1 helicase (Abbate et al., 2004). The simplest interpretation of these findings is that R37 and I73 are part of a surface involved in binding Brd4(L). It is unlikely that these two substitutions affect the folding of the TAD since they do not affect viral DNA replication (see Introduction), which is also dependent on the integrity of the TAD.

We then carried out a similar series of affinity chromatography experiments with the E2 TAD of CRPV, albeit at lower concentrations of immobilized proteins because we repeatedly observed

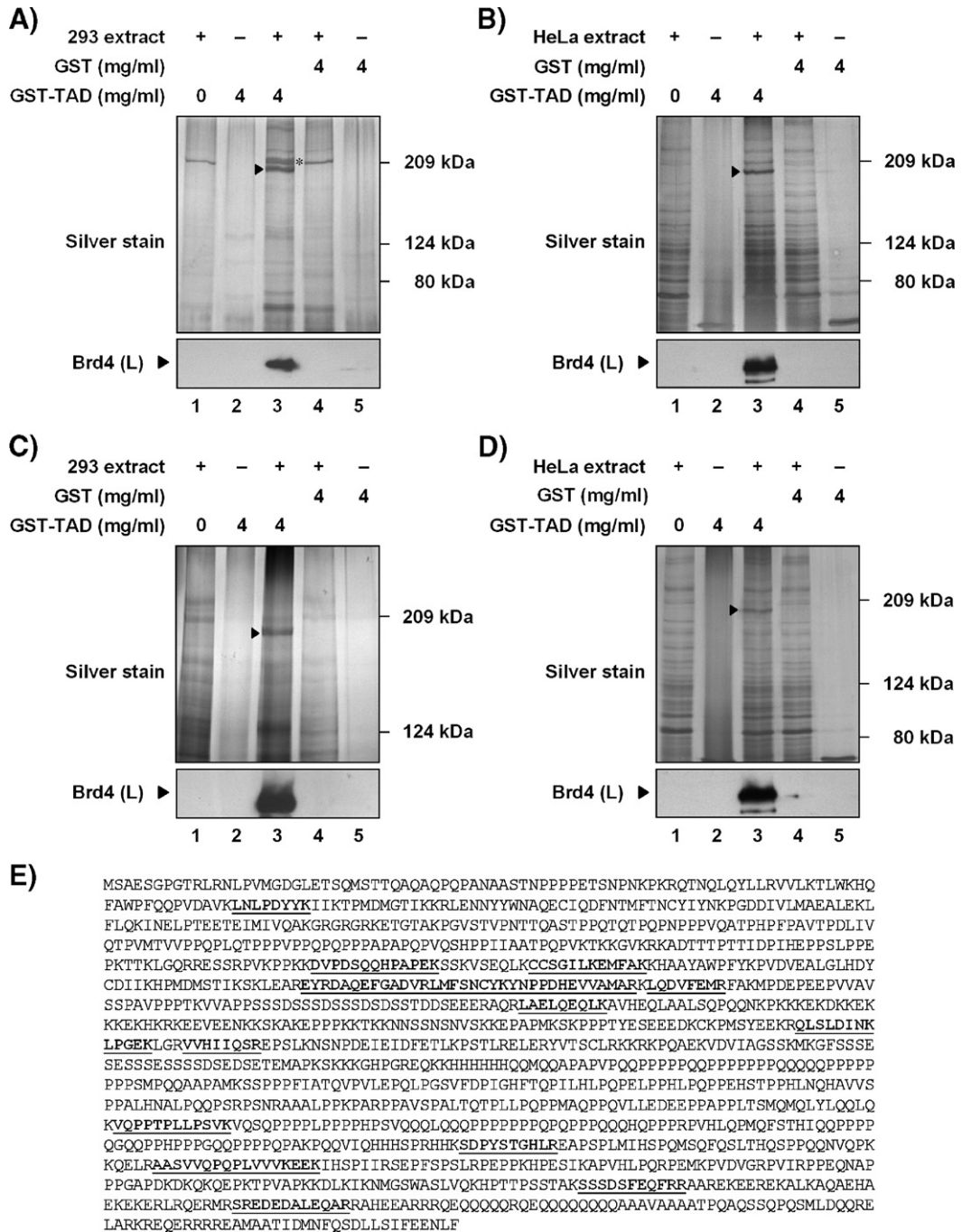


Fig. 2. Purification of Brd4(L) by affinity chromatography with the HPV11 and HPV31 E2 TAD. (A to D) Affinity chromatography using the E2 TADs from HPV11 (A and B) and HPV31 (C and D). For each TAD, a silver stained, 6% SDS-PAGE is shown that reveals the presence of the long isoform of Brd4, Brd4(L) (black arrow), in the glutathione eluates of the GST-E2 TAD column (lane 3), but not in that of a GST control column (lane 4). Also shown are the eluates from additional control columns, either not loaded with whole cell extract (lanes 2 and 5) or containing no protein (glutathione-sepharose resin only, lane 1). GST-E2 TAD and GST were immobilized at a concentration of 4 mg/ml and loaded (+) or not (-) with whole cell extract prepared from 293 cells (A and C) or HeLa cells (B and D), as indicated. Note that small proteins have been purposely run out of the gel to better separate proteins of larger molecular weight. In other experiments, we determined that there was no obvious E2-binding protein of molecular weight smaller than 75 kDa (data not shown). The positions of molecular weight markers are shown on the left. The major non-specific band in lanes 1, 3 and 4 was identified by MS as myosin heavy polypeptide 9 (shown by an asterisk in lane 4). Shown below each gel is the result of a western blot using a rabbit polyclonal antiserum directed against amino acids 1300–1400 of Brd4(L). (E) Brd4(L) peptides identified by mass spectrometry. Tryptic peptides identified by tandem MS are underlined in the amino acid sequence of Brd4(L).

that it binds with increased affinity to Brd4(L) as compared to the other TADs tested. For CRPV E2, substitutions R37A and I73L, but not R37K, were previously shown to affect its ability to trans-

activate a reporter gene under the control of multiple E2 binding sites (Jeckel et al., 2002). Accordingly, we found that the E2 TAD containing R37A and I73L showed reduced binding to Brd4(L),

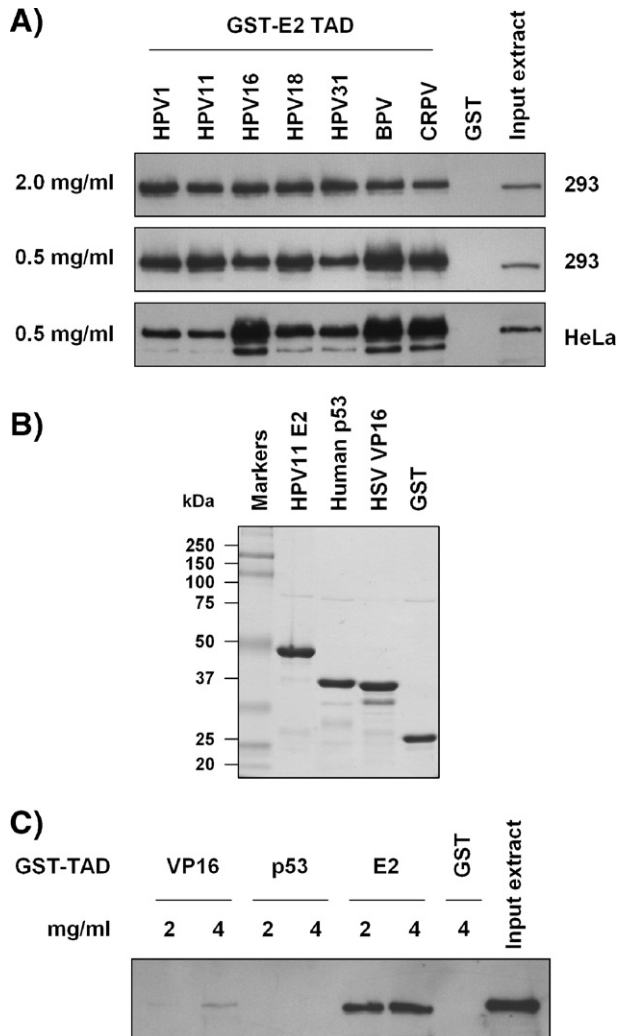


Fig. 3. Binding of Brd4(L) to other viral and cellular transactivation domains. (A) Binding of Brd4(L) to the E2 TADs of other papillomaviruses. Affinity chromatography was performed with GST-E2 TAD proteins of HPV1, 11, 16, 18, 31, bovine papillomavirus (BPV) and cottontail rabbit papillomavirus (CRPV), or with GST as a control. Two series of affinity columns were prepared at concentrations of immobilized proteins of 0.5 mg/ml and 2.0 mg/ml, as indicated, and loaded with 293 or HeLa whole cell extract, as indicated. Each column was then washed with buffer containing 0.1 M NaCl and the bound proteins eluted with reduced glutathione. Shown in the figure are the results of western blots with anti-Brd4(L) antibodies to reveal the presence of this protein in the different column eluates. 150 μ g of input of whole cell extract (input extract) was used as a positive control. (B) Purified TAD from HSV VP16 and human p53. 3 μ g of each purified GST-fusion protein was separated on a 10% SDS-PAGE and stained with Coomassie blue. (C) Brd4(L) does not bind to the TADs of VP16 and p53. Affinity chromatography was performed with GST-VP16, GST-p53, GST-E2 TAD (HPV11) or with GST as a control. Proteins were immobilized at a concentration of 2 and 4 mg/ml, as indicated. Each column was loaded with 293 cell extract, washed with buffer containing 0.1 M NaCl and the bound proteins eluted with reduced glutathione. Aliquots of each column eluates were subject to western blotting with antibodies against Brd4(L). 100 μ g of input of whole cell extract (input extract) was used as a positive control.

Discussion

Amino acid substitutions that specifically impair the transcriptional activity of E2 affect binding to Brd4(L)

In this study, we have used protein affinity chromatography to identify, in a systematic fashion, proteins that bind to the E2 TAD of HPV11, a low-risk type, and HPV31, a high-risk, oncogenic type. In both cases, we identified the long isoform of Brd4, Brd4(L), as the major TAD-binding protein present in 293 and HeLa whole cell extracts. Although 293 cells are not transformed by HPV, they were used because they support all of the known activities of HPV11 E2, namely its capacity to support viral DNA replication (Chiang et al., 1992; Kuo et al., 1994), regulate viral gene expression (Zou et al., 2000) and associate with the mitotic spindle and centrosomes (Van Tine et al., 2004; Dao et al., 2006). Interestingly, we found that the interaction of Brd4(L) with E2 is sensitive to

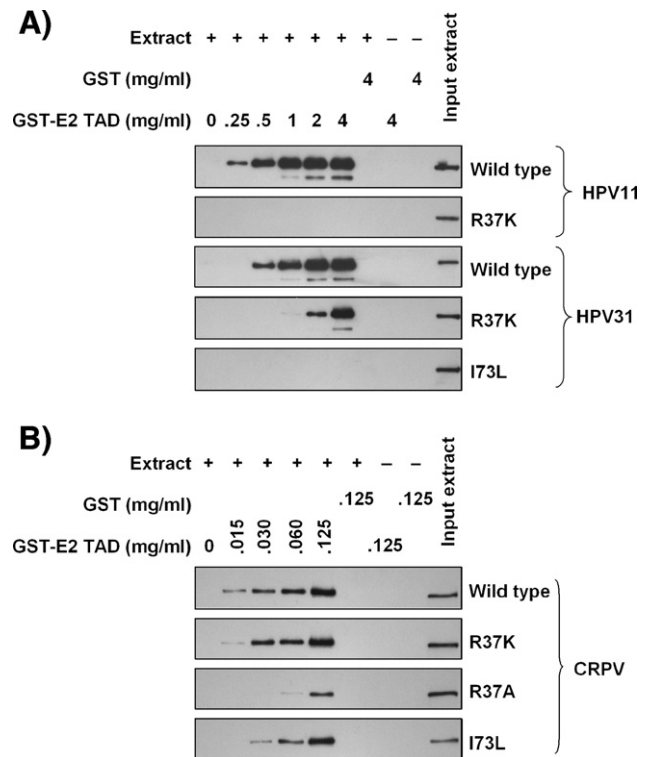


Fig. 4. Amino acid substitutions that specifically impair the transcriptional activity of E2 reduce binding to Brd4(L). (A) Affinity chromatography was performed with GST-E2 TAD proteins of HPV11 (wild type and R37K mutants) and HPV31 (wild type, R37K and I73L mutants), or with GST as a control. Affinity columns were prepared with increasing concentrations of immobilized protein, ranging from 0.25 to 4 mg/ml, and loaded (+) or not (-) with 293 whole cell extract, as indicated. Each column was then washed with buffer containing 0.1 M NaCl and the bound proteins eluted with reduced glutathione. Shown in the figure are the results of western blots with anti-Brd4(L) antibodies to reveal the presence of this protein in the different column eluates. 100 μ g of input of whole cell extract (input extract) was used as a positive control. (B) Affinity chromatography with GST-E2 TAD proteins of CRPV (wild type, R37K, R37A and I73L mutants), or with GST as a control. Affinity chromatography was performed as described in (A) but at lower concentrations of immobilized protein, ranging from 0.015 to 0.125 mg/ml.

whereas the transactivation competent R37K protein behaved like wild type. This correlation between transcriptional activity and Brd4(L)-binding further supports the notion that Brd4(L) is a mediator of E2's transcriptional function.

substitutions in the TAD of residues R37 and I73, which specifically impair its transcriptional activation and repression activities. Our results are in agreement with recent studies which also indicated that the binding of Brd4(L) to the E2 proteins of BPV and HPV16 is affected by substitutions of R37 and I73 (Baxter et al., 2005, Schweiger et al., 2006) and that Brd4(L) is involved in E2-mediated transcriptional activity (Ilves et al., 2006; Schweiger et al., 2006). Our results with other papillomavirus types thus provide further evidence that Brd4(L) is involved in the transcriptional activity of E2. The fact that R37 and I73 are adjacent to each other in the three dimensional structure of the TAD suggests that they define a surface of the TAD involved in binding Brd4(L). We note that this surface contains several hydrophobic residues, consistent with our finding that the E2–Brd4(L) interaction is primarily hydrophobic in nature. This surface lies opposite to that involved in interaction with the E1 helicase and involved in viral DNA replication (Abbate et al., 2004). It remains a formal possibility that Brd4(L) and E1 can form a ternary complex with E2, although the interaction with Brd4(L) does not appear to be required for viral DNA replication *per se*, based on the fact that substitutions such as I73L have little or no effect on viral DNA replication (see Introduction).

Based on the structure of the HPV16 E2 TAD, Antson et al. (2000) proposed the existence of a dimerization interface within the E2 TAD, comprised of the second and third α -helices, and involving, among others, residues Arg37 and Ile73. However, it is noteworthy that we observed quite a distinct monomer–monomer interaction interface in both of our published crystal structures of the HPV11 E2 TAD (free and in complex with a small-molecule inhibitor of the E1–E2 protein interaction) (Wang et al., 2004). In addition, our previous characterization of the quaternary structure of the HPV11 E2 TAD by size-exclusion chromatography and ultracentrifugation revealed that this domain is monomeric at concentrations up to approximately 20 μ M (Wang et al., 2004). Based on these findings and those reported in this study, we believe that the major role of Arg37 and Ile73, at least for HPV11 E2, is in forming a binding surface for Brd4(L) rather than dimerization of the TAD.

Role of the E2–Brd4(L) interaction in the viral life cycle and pathogenesis

An important reason why we chose to study the R37K and I73L substitutions in the context of HPV31 E2 is because these substitutions have been characterized previously in one of our laboratories (L.A.L) for their effect on maintenance of the HPV31 episome in primary keratinocyte cultures as well as on expression of the viral early and late genes upon differentiation in methylcellulose or in organotypic raft cultures (Stubenrauch et al., 1998). It is therefore informative to re-examine these results in light of the fact that R37K and I73L impair binding to Brd4(L). While both substitutions had little effect on transient viral DNA replication, they differed in their effect on maintenance of the episome in keratinocytes. Specifically, the copy number of the R37K mutant episome was greatly reduced while that of the I73L genome was closer to wild type

(Stubenrauch et al., 1998). Consistent with its more severe phenotype, little amplification of the R37K episome was observed upon keratinocyte differentiation (Stubenrauch et al., 1998). In contrast, the level of amplification of the I73L mutant episome was only slightly reduced and expression of the late genes was still detected, albeit at a reduced level compared to wild type (Stubenrauch et al., 1998). Considering our finding that I73L greatly reduces binding to Brd4(L) (Fig. 4A), these results would suggest that the E2–Brd4(L) interaction is neither essential for the replication and maintenance of the viral episome in keratinocytes nor for its amplification upon differentiation, although it may contribute to the overall efficiency of these events. An equally important question is whether the interaction of E2 with Brd4(L) contributes to pathogenesis. The effects of R37A, R37K and I73L substitutions in E2 have been characterized in the CRPV infection model and both were found to drastically reduce pathogenesis, as measured by induction of tumors (papillomas) following gene-gun inoculation of the viral genome into the skin of domestic rabbits (Jeckel et al., 2002). In this study, we have shown that CRPV E2 interacts with Brd4(L) and that this interaction is affected by R37A and I73L, but not R37K. These findings suggest that the interaction of E2 with Brd4(L) is essential for pathogenesis and thus warrants the search for small molecule inhibitors of the E2–Brd4(L) interaction as potential antiviral agents. However, the fact that R37K also reduces pathogenesis without affecting transcriptional activity (Jeckel et al., 2002) and binding to Brd4(L) (this study) suggests that this surface of the TAD may also be involved in additional yet undefined function of E2 essential for pathogenesis.

Materials and methods

Plasmid constructions and mutagenesis

Plasmids to express the E2 TADs as fusions with GST were constructed by inserting PCR fragments encoding these TADs into plasmid pGEX-2T (GE Healthcare, formerly Amersham Biosciences). The E2 TAD was defined by sequence alignment as residues 1–200 for HPV1, 1–208 for HPV11, 1–208 for HPV16, 1–213 for HPV18, 1–208 for HPV31, 1–211 for BPV1 and 1–211 for CRPV. Details on construction of these plasmids will be made available upon request. Mutagenesis was performed with the QuickChange Site-Directed Mutagenesis kit (Stratagene). Plasmids used to express the activation domains of HSV VP16 (amino acids 412 to 490) and p53 (amino acids 1 to 73) as GST fusion proteins were a kind gift from Dr. Jim Omichinski (University of Montreal).

Protein expression and purification

Expression vectors for the various GST-E2 TADs were introduced into *E. coli* BL21 (Novagen). Bacterial cultures were grown in LB media supplemented with 2% ethanol at 25 °C to an optical density of 0.5–0.8 (at 595 nm) and then induced for 4 h by the addition of 0.5 mM IPTG. GST-fusion proteins were purified essentially as described previously (Titolo et al., 1999).

Protein concentrations were determined by the method of Bradford (Bio-Rad protein assay) using BSA as a standard.

Protein affinity chromatography

40- μ l columns containing the various GST-E2 TAD, or GST, immobilized on glutathione–sepharose beads at a concentration of 4 mg/ml (or as otherwise indicated), were prepared in 200 μ l pipette tips and chromatography performed by gravity, essentially as described previously (Formosa et al., 1991). Columns were first equilibrated with 200 μ l of ACB buffer (10 mM Tris pH 8.0, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol) containing 0.1 M NaCl and then loaded with 30 mg of 293 or HeLa whole cell extract that had been previously depleted on glutathione–sepharose. Whole cell extracts at a final protein concentration of 6–8 mg/ml were prepared as described previously (Sopta et al., 1985) and dialyzed against buffer comprised of 10 mM HEPES pH 7.9, 0.1 M NaCl, 0.1 M K₂Ac, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol. After loading, the columns were washed with a 4 \times 100 μ l of ACB buffer containing 0.1 M NaCl. The bound proteins were eluted with 120 μ l of ACB buffer containing 0.1 M NaCl and 20 mM reduced glutathione.

Western blotting

Western blotting was performed with the enhanced chemiluminescence procedure (GE Healthcare). Purified rabbit polyclonal antibodies directed against amino acids 1300–1400 of the long isoform of human Brd4 were purchased from Abgent (AP8051b).

Protein identification by tandem mass spectrometry

The GST-E2 TAD column eluates were separated on 6% SDS-PAGE, stained with silver or Sypro Ruby (Bio-Rad) and gel slices were excised and digested with trypsin as previously described (Jeronimo et al., 2004). The resulting tryptic peptides were purified and identified by tandem mass spectrometry (LC-MS/MS) with microcapillary reversed-phase high-pressure liquid chromatography coupled to an LCQ DecaXP (Thermo-Finnigan) or an LTQ (ThermoElectron) quadrupole ion trap mass spectrometer with a nanospray interface. Resulting peptide MS/MS spectra were interpreted using the MASCOT (Matrix Science) software and searched against proteins in the National Center for Biotechnology Information (NCBI) non-redundant protein database or Uniref protein database (Bairoch et al., 2005). Many peptide sequences were confirmed by manual inspection of the spectrum.

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