Genetic analysis of the E2 transactivation domain dimerization interface from bovine papillomavirus type 1

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Abstract

The bovine papillomavirus type 1 (BPV1) E2 protein binds as a dimer to the viral genome to promote its transcription, replication and maintenance in keratinocytes. Although BPV1 E2 dimerizes primarily through its DNA-binding domain, it was shown previously that its transactivation domain (TAD) can also dimerize in vitro through formation of a disulfide bond between cysteine 57 (C57) of adjacent monomers and of an ion pair between arginine 172 (R172) and aspartic acid 175 (D175). The function of this TAD dimerization interface in vivo remains unknown. Here, we report the effects of substituting C57, R172 and D175 by alanine on the transactivation activity of BPV E2 as well as on its ability to support viral DNA replication using a novel luciferase-based assay. Results for this mutational analysis suggest that the TAD dimerization interface is not essential for either process but may contribute to the DNA replication activity of BPV1 E2.

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Introduction

Papillomaviruses are double-stranded DNA viruses that induce benign and malignant hyperproliferative lesions of the differentiating epithelium (Hebner and Laimins, 2006; Howley and Lowy, 2001; zur Hausen and de Villier, 1994). The life cycle of papillomaviruses is coupled to the cellular differentiation program that keratinocytes undergo within the epithelium (Doorbar, 2005; Howley and Lowy, 2001; Longworth and Laimins, 2004). 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 episome in these cells requires the activities of the viral E2 protein, a regulatory factor that binds to specific sites in the long-control-region (LCR) of the viral genome to promote its replication, regulate its transcription and ensure its segregation to daughter cells in mitosis (Blachon and Demeret, 2003). As a transcription factor, E2 can either activate or repress transcription, depending on the promoter context (Bernard, 2002). E2 is comprised of two functional domains, an N-terminal transactivation domain (TAD) and a C-terminal DNA-binding domain (DBD) separated by a hinge region. The TAD is a protein interaction module that binds to the viral E1 helicase and to host proteins such as TopBP1 (Donaldson et al., 2012), and possibly also to RPA (Li and Botchan, 1993) and topoisomerase I (Clower et al., 2006) which were reported to interact with full-length E2, to promote replication of the viral genome. The E2 TAD also associates with cellular transcription factors, including the bromodomain containing protein 4 (Brd4), to regulate viral gene transcription (Abbate et al., 2004, 2006; Benson and Howley, 1995; Sakai et al., 1996; Wu and Chiang, 2007). Brd4 was first identified as the cellular chromatin component that is bound by the bovine papillomavirus type 1 (BPV1) E2 to tether the viral episome to host chromosomes in order to prevent its loss during mitosis (You et al., 2004, 2005). Subsequent studies revealed that Brd4 is also needed for E2 to activate or repress transcription (Baxter et al., 2005; Gagnon et al., 2009; McPhillips et al., 2006; Schweiger et al., 2006; Senechal et al., 2007; Wu et al., 2006). Several crystal structures of the E2 TAD, either alone (free form) or in complex with the C-terminal helicase domain of E1, or with a Brd4 peptide, have been reported (Abbate et al., 2004, 2006; Antson et al., 2000; Harris and Botchan, 1999; Sanders et al., 2007; Wang et al.,
These structures revealed that the E2 TAD has an L-shape comprised of three anti-parallel \( \alpha \)-helices followed by a \( \beta \)-sheet region (Fig. 1) (Sanders et al., 2007). They also revealed that E1 and Brd4 bind on opposite faces of the TAD.

E2 binds to DNA as a dimer and is also dimeric in solution. Its main dimerization interface is located within the C-terminal DBD (Hegde, 2002). However, the arrangement of the N-terminal TAD within the lattices of some of the crystal structures mentioned above suggested that this domain can also homodimerize, but through different surfaces depending on the papillomavirus type. For E2 from human papillomavirus type 16 (HPV16), dimerization of the TAD was found to involve two of the three \( \alpha \)-helices that form part of the Brd4-binding surface (Antson et al., 2000). This TAD–TAD interface was shown to occur between different E2 dimers as it can promote DNA-looping between E2 dimers bound at distant sites in vitro (Antson et al., 2000; Hernandez-Ramon et al., 2008). A very different TAD dimerization interface was identified for BPV1 E2. Sanders et al. (Sanders et al., 2007) reported that the BPV1 E2 TAD can dimerize, in a redox-dependent manner, in part through the formation of a disulfide bond between cysteine 57 (C57) of two adjacent monomers. They also identified other interactions mediating dimer formation; namely a pair of hydrogen bonds between glutamine 12 (Q12) and arginine 68 (R68) and an ion pair between arginine 172 (R172) and aspartic acid 175 (D175) (Sanders et al., 2007). Biochemical studies provided evidence that this mode of dimerization occurs between TADs within the same E2 dimer, rather than between separate dimers. In vitro, the purified BPV1 E2 TAD was found to dimerize under non-reducing conditions, a phenomenon that could be abolished by substitution to alanine of either C57 or R172 and, to a lesser extent, of D175. The locations of C57, R172, and D175 within the crystal structure of the BPV1 E2 TAD are shown in Fig. 1. Despite the clear demonstration that dimerization of the TAD occurs in vitro under oxidizing conditions, it is unclear if it also occurs in vivo. Unfortunately, demonstrating the specific dimerization of the TAD in vivo is likely to be technically challenging given that full-length E2 dimerizes primarily via its C-terminal DBD. In this study, we have opted to investigate the function of the putative TAD dimerization interface in vivo by characterizing the effect of the C57A, R172A and D175A substitutions, either alone or in combination, on the transactivation and DNA replication activities of BPV1 E2 in transfected cells. Our results suggest that the TAD dimerization interface is not essential for either function of BPV1 E2 but may play a role in its DNA replication activity.

**Results**

Expression of BPV1 E2 mutant proteins with amino acid substitutions in the TAD dimerization interface

As a first step towards determining the function of the TAD dimerization interface in vivo, we constructed plasmids expressing untagged BPV1 E2 from the CMV promoter, either the wild type protein or mutant derivatives bearing the C57A, R172A and D175A substitutions alone or in combination. In the remainder of this paper, we will refer to these three substitutions as CA, RA and DA, respectively. These vectors were transfected into C33A human cervical carcinoma cells and the expression of E2 assessed 24 h later by Western blotting using the anti-BPV1 E2 antibody 1E4 (Kurg et al., 1999). Five different experiments were performed using separate DNA preparations for transfection. All five Western blots yielded similar results and were analyzed by densitometry to determine the average expression level of each mutant protein relative to that of wild type E2, which was set at 100% (Table 1). One of the blots is shown in Fig. 2A. All seven E2 mutant proteins were expressed at the anticipated molecular weight albeit some at lower levels than wild type E2 (Fig. 2A). The CA single and CA/RA/DA triple mutant proteins were the least expressed, at levels approximately 50–55% that of wild type E2 \( (p<0.0001) \). The two double mutant proteins containing the CA substitution (CA/RA and CA/DA) also consistently displayed reduced expression, accumulating at levels approximately 80–85% that of wild type E2, although these differences did not reach statistical significance. The RA single mutant protein was the only one that was consistently expressed at slightly higher levels than wild type E2.

**Table 1**

Summary of the expression and activities of BPV1 E2 mutant proteins.

<table>
<thead>
<tr>
<th>BPV1 E2</th>
<th>Expression</th>
<th>Transactivation</th>
<th>DNA replication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% ( + )</td>
<td>AUC (%) ( + )</td>
<td>AUC (%) ( + )</td>
</tr>
<tr>
<td>WT</td>
<td>100</td>
<td>100 ± 12</td>
<td>100 ± 32</td>
</tr>
<tr>
<td>CA</td>
<td>52 ± 10</td>
<td>&lt;0.0001</td>
<td>54 ± 4</td>
</tr>
<tr>
<td>RA</td>
<td>126 ± 18</td>
<td>0.03</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>DA</td>
<td>104 ± 21</td>
<td>ns</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>CA/RA</td>
<td>80 ± 17</td>
<td>ns</td>
<td>84 ± 8</td>
</tr>
<tr>
<td>CA/DA</td>
<td>86 ± 15</td>
<td>ns</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>CA/RA/DA</td>
<td>102 ± 11</td>
<td>ns</td>
<td>64 ± 6</td>
</tr>
</tbody>
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\( ^{a} \) From Fig. 2A.

\( ^{b} \) Variance relative to WT E2 assessed by one-way ANOVA followed by Dunnett’s post-hoc analysis.

\( ^{c} \) From Fig. 3A.

\( ^{d} \) Variance relative to WT E2 assessed by two-way ANOVA followed by Dunnett’s post-hoc analysis.

\( ^{e} \) From Fig. 6A.

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**Fig. 1. Structure of the BPV1 E2 TAD dimer showing the location of amino acids mutated in this study.** (A) Surface and (B) ribbon diagram of the E2 TAD dimer (PDB accession code 2jeu). The two TAD monomers are colored in red and blue, respectively. (C) Location of C57, R172 and D175. C57 is visible only in the blue monomer and is colored in red. R172 and D175 are visible only in the red monomer and are colored in blue. Filled arrows point to residues on the visible face of each monomer while dashed arrows point to the analogous residues located on the hidden face of the opposite monomer. Images were generated with PyMol.
that substitutions in the TAD dimerization interface do not dramatically alter the expression of BPV1 E2, the levels of the protein being reduced at the most by half in the case of the CA and CA/RA/DA triple mutant E2. As an approximation of the transcriptional activities of the different E2 proteins, we measured the area under the curve (AUC) generated for each protein. These AUC values, presented in Table 1 relative to the AUC determined for wild type E2 (set at 100%), allowed for a comparison of the transcriptional activity of wild type BPV1 E2 transactivation activities of the BPV1 E2 mutant proteins

Next, we tested the ability of the different E2 proteins to transactivate a firefly luciferase (Fluc) gene under the control of four E2-binding sites. C33A cells were transfected with increasing amounts of E2 expression vector and constant amounts of the Fluc reporter gene and of an internal control vector for normalization (pRL, expressing Renilla luciferase from the CMV promoter). The levels of luciferase activities were measured 24 h post transfection and the normalized values used to calculate the fold-transactivation of the reporter gene presented in Fig. 3A. Under our assay conditions, wild type BPV1 E2 could transactivate the reporter gene nearly 10-fold at the highest concentration of expression vector tested. All of the mutant proteins showed reduced level of transactivation but none were completely defective for this activity. As an approximation of the transcriptional activities of the different E2 proteins, we measured the area under the curve (AUC) generated for each protein. These AUC values, presented in Table 1 relative to the AUC determined for wild type E2 (set at 100%), allowed for the different E2 proteins to be compared. The transcriptional activities of the RA and DA single mutant proteins were not statistically different from that of wild type E2. The activity of the CA/RA protein was only slightly lower than wild type (p = 0.03). In contrast, the remaining four mutant proteins, CA, CA/DA, RA/DA and CA/RA/DA, were all significantly less potent at transactivating the reporter gene than wild type E2 (p < 0.001). The CA single and CA/RA/DA triple mutant proteins were the least active, displaying 50–55% of the activity of wild type E2 (Fig. 3A and Table 1), a result suggesting that C57 is important, albeit not essential, for the transcriptional activity of E2. Because these two proteins were the least expressed (Fig. 2A), we wondered if their reduced transactivation activity was a consequence of their lower steady-state levels. To test this hypothesis, we repeated the transactivation assay with the CA/RA/DA triple mutant protein but this time using higher amounts of E2-expression vector to saturate the reporter gene. As can be seen in Fig. 3B, titration of increasing amounts of wild type E2 resulted in high levels of transactivation that reached a plateau at approximately 23-fold. As for the CA/RA/DA triple mutant protein, it could transactivate the reporter gene essentially to the same level as wild type E2, indicating that it is in not inherently defective for this activity. However, the amount of E2 expression vector required to achieve half-maximal transactivation of the reporter gene (EC50) was approximately 2.5-fold higher for the triple mutant protein (EC50 = 12 ng) than for wild type E2 (EC50 = 5 ng). These results

Wild type BPV1 E2 accumulates primarily in the nucleus of transfected cells. To test if the TAD dimerization interface is required for nuclear accumulation of E2, we compared the intracellular localization of the CA/RA/DA triple mutant protein to that of wild type E2 by immunofluorescence in transfected C33A cells, using the 1E4 antibody. As expected, the signal intensity of the CA/RA/DA protein was weaker than that of wild type E2, confirming its lower expression. More importantly, the intracellular distribution of the mutant protein was similar to that of wild type E2; the protein being exclusively nuclear in approximately two-third of the cells and present in both the nucleus and cytoplasm in the remaining third (Fig. 2B). Both the wild type and mutant E2 accumulated within discrete foci when in the nucleus, as previously reported (Baxter et al., 2005; Oliveira et al., 2006; Penrose and McBride, 2000). The more punctate appearance of cells expressing the triple mutant E2 protein simply reflects its lower expression; indeed cells in which wild type E2 was expressed showed lower levels also appeared more punctate (data not shown). From these results, we conclude that residues C57, R172 and D175 are not required for the nuclear accumulation of BPV1 E2 and, by extension, that the integrity of the TAD dimerization interface is also not needed for this process.

Transcriptional transactivation activities of the BPV1 E2 mutant proteins

The integrity of the TAD dimerization interface is not required for the nuclear localization of BPV1 E2

Wild type BPV1 E2 accumulates primarily in the nucleus of transfected cells. To test if the TAD dimerization interface is required for nuclear accumulation of E2, we compared the intracellular accumulation of wild type E2 (~125%, p = 0.03). The remaining mutant proteins accumulated at wild type levels. Collectively, these results indicate that substitutions in the TAD dimerization interface do not dramatically alter the expression of BPV1 E2, the levels of the protein being reduced at the most by half in the case of the CA and CA/RA/DA substitutions.

The integrity of the TAD dimerization interface is not required for the nuclear localization of BPV1 E2

Wild type BPV1 E2 accumulates primarily in the nucleus of transfected cells. To test if the TAD dimerization interface is required for nuclear accumulation of E2, we compared the intracellular

Fig. 2. Expression and intracellular localization of BPV1 E2 mutant proteins. (A) Representative Western blot analysis of C33A cells transiently expressing the indicated wild type (WT) or mutant E2. Untagged E2 proteins were detected with the 1E4 antibody. Half (1/2) and one quarter (1/4) of the amount of WT E2-containing extract were used as standards. β-tubulin was used as a loading control. The bar graph shows the expression levels of WT and mutant E2 proteins quantified from five independent Western blots, including the one presented in panel A. Standard deviations are indicated. (B) The intracellular localization of untagged wild type or CA/RA/DA triple mutant E2 was determined in transiently expressing C33A cells by immunofluorescence confocal microscopy using the 1E4 antibody. Nuclei were stained with DAPI. The percentages of transfected cells showing accumulation of E2 either exclusively in the nucleus (N), or in both the nucleus and cytoplasm (NC), were derived from the analysis of 300 cells and are indicated on the left.
sigmoidal dose–response curve using the following equation:

\[ Y = \frac{T_{\text{Top}}}{1 + \left(\frac{X}{T_{\text{max}}}\right)^n} \]

\( Y \) is the fold-transactivation value, \( T_{\text{Top}} \) is the maximal level of transactivation achieved at saturating amount of E2 expression vector, \( n \) is the hill coefficient. "Goodness-of-fit" values were \( R^2 = 0.98 \) for WT E2 and \( R^2 = 0.97 \) for the CA/RA/DA triple mutant protein.

Development of a luciferase-based BPV1 DNA replication assay

To compare the DNA replication activities of the different BPV1 E2 mutant proteins, we first established a quantitative, transient BPV1 DNA replication assay based on a luciferase readout, essentially as we reported previously for HPV31 (Fradet-Turcotte et al., 2010) (Fig. 4). The assay is based on a plasmid containing both the BPV1 origin of DNA replication and a CMV-Fluc reporter gene (pFLORI-BPV1, Fig. 4A), whose replication by E1 and E2 in C33A cells results in increased Fluc expression. A second plasmid expressing Fluc from the CMV promoter (pRL, lacking an origin, Fig. 4A) is used as an internal control, allowing the DNA replication results to be quantified as Fluc/RLuc ratios. To validate this assay, we confirmed that replication of the CMV-Fluc plasmid was dependent on the presence of the BPV1 origin and on expression of E1 and E2 (Fig. 4B). Two different origin fragments were tested, either a short one (nt 7891-31 in the BPV1 genome sequence, Genbank accession X02346.1) or a longer one (nt 7476-57) (Plisook et al., 1996), with comparable results (Fig. 4B). Similarly, two different E1-expression vectors were compared, either pCG E1Eag 1235 , which contains a mutation of the major splice site located within the E1 ORF, or a precursor plasmid lacking this mutation. It was previously shown that mutation of this splice site results in higher levels of DNA replication, presumably because of increased E1 expression (Ustav and Stenlund, 1991). Our results confirmed this finding, as shown by the observation that the splice site mutation increased DNA replication by approximately 5-fold (Fig. 4B). For the remainder of this study, the assay will be performed with the E1 expression vector containing the splice site mutation in combination with the firefly luciferase vector carrying the long origin. To further validate this novel assay, we verified that replication of the ori-plasmid could be inhibited by the known DNA replication inhibitors hydroxyurea (EC50 = 470 μM), gemcitabine (EC50 = 6 nM) and aphidicolin (EC50 = 164 nM) (Fig. 5). From these results, we conclude that the levels of firefly luciferase measured in our assay increase as a function of ori-plasmid replication by E1 and E2.

DNA replication activities of the BPV1 E2 mutant proteins

The luciferase assay presented above was then used to measure the DNA replication activities of the different E2 mutant proteins, at increasing amounts of expression vector, 72 h following transfection in C33A cells. These studies revealed that all 7 mutant proteins were defective but not completely inactive in supporting transient DNA replication (Fig. 6A). To estimate the DNA replication activity of each mutant protein relative to that of wild type, we measured the area under the curve (AUC) generated for each E2 protein (Table 1), essentially as we did in Fig. 3 to assess their transcriptional activity. All E2 mutant proteins were found to have a statistically significant lower DNA replication activity than wild type E2, except for the RA protein. The CA/RA double and CA/RA/DA triple mutant proteins were the least active, displaying approximately 30–40% the activity of wild type E2. To determine if the lower activity of the CA/RA/DA triple mutant E2 was due to its lower expression level or to an intrinsic DNA replication defect, we repeated the DNA replication assay using higher and saturating amounts of E2 expression vector. Under these conditions, the maximal level of replication
with the triple mutant E2 remained at 45% that obtained with the wild type protein (Fig. 6B). These results suggest that the CA/RA/DA mutant E2 is intrinsically deficient for DNA replication. Collectively, the results presented above point to the TAD dimerization interface as being important, albeit not essential, for viral DNA replication. As in any mutational analysis, however, we cannot rule out that the CA, RA and DA substitutions also affect DNA replication by another yet-undefined mechanism distinct from interfering with dimerization of the TAD.

Discussion

The BPV1 E2 TAD dimerization interface was discovered and extensively characterized in vitro by Sanders et al. (Sanders et al., 2007). This study is the first to investigate the role of this TAD dimerization interface in vivo. To do so, we took advantage of the three amino acid substitutions, CA, RA and DA, previously shown by Sanders et al. to significantly reduce dimerization of the TAD in vitro. Our results, summarized in Table 1, indicate that the CA...
Fig. 6. DNA replication activities of wild type and mutant BPV1 E2. (A) The DNA replication activities of wild type (WT) and mutant E2 proteins obtained at increasing amounts of E2-expression vector. The dose–response curve obtained for WT E2 is repeated in each graph for comparison. DNA replication activities were determined from C33A cells co-transfected with a plasmid that encodes the BPV1 origin and a firefly luciferase (Fluc) reporter gene (pFLORI-BPV1), together with an E1-expression plasmid (pCG E1Eag 1235) and increasing amounts of E2-expression vector (from 0.016 to 150 ng in 2.5-fold increments). A Renilla luciferase (Rluc) expression plasmid was used for normalization.

(A) Same as in panel A but using higher amounts of E2-expression vector (from 0.03 to 0.50 ng in two-fold increments). The partially replication-defective BPV1 E2 R47Q/K48Q/K49Q/R58Q/H61N mutant protein (D2 mutant in Baxter et al., 2005)) was used as a control (CH). For each of the indicated E2 protein, the dose–response curve that is presented was obtained from two independent experiments, each done in duplicates using a 2.5-fold increased amount of E2-expression vector. The dose–response curve obtained for WT E2 was fitted to a sigmoidal dose–response curve using the following equation: $Y = \text{Bottom} + \left(\text{Top} - \text{Bottom}\right) \left(1 - 10^{\left(-\frac{\text{LogEC}_{50} - X}{n}\right)}\right)$, in which $Y$ is the DNA replication value, Bottom is the DNA replication value measured in absence of E2, Top is the maximal level of DNA replication obtained at saturating amounts of E2 expression plasmid, $X$ is the amount of E2 expression plasmid (in grams), and $n$ is the Hill coefficient. “Goodness-of–fit” values were $R^2 = 0.98$ for WT E2, $R^2 = 0.96$ for the CA/RA/DA triple mutant protein and $R^2 = 0.98$ for the control protein.

(B) Same as in panel A but using higher amounts of E2-expression vector (from 0.016 to 150 ng in 2.5-fold increments). The data was fitted to a sigmoidal dose–response curve using the following equation:

Although not strictly essential, the TAD dimerization interface may contribute to some extent to the functions of E2, as suggested by the phenotypes of the double (CA/RA and CA/DA) and triple (CA/RA/DA) mutant proteins in which formation of the C57 disulfide bond and R172–D175 salt bridges between adjacent E2 monomers is essential for the expression as well as transcription and DNA replication activities of E2. As such, they suggest that dimerization of the TAD is not crucial for these activities, a notion that is in agreement with previous studies indicating that BPV1 E2 heterodimers containing a single TAD are functional, albeit at reduced levels compared to full-length E2 homodimers (Kurg et al., 2006, 2009, 2010).

Although the single, double or triple amino acid substitutions that we introduced in the TAD did not completely abolished the transactivation and DNA replication activities of E2, some could substitution, alone or in combination with the other substitutions, had generally the greatest effect on the expression, transcriptional activity and DNA replication activity of E2. This is in contrast to the RA and DA substitutions which, alone or together, were better tolerated; the largest effect being a 30% reduction in DNA replication activity imposed by the RA single and RA/DA double substitutions. The CA substitution should abolish the possibility of disulfide bond formation between adjacent TADs and thus prevent any form of redox-dependent regulation of this domain in vivo. Our finding that the CA single mutant E2 retains 50–60% of transactivation and DNA-replication activities suggests that formation of a disulfide bond between the two C57 of adjacent TADs is not essential for these functions of E2. These results are also consistent with those of Sanders et al. indicating that the CA substitution does not significantly impair the interaction of E2 with E1 in vitro (Sanders et al., 2007). The formation of the two salt bridges between R172 and D175 of adjacent TADs also appears to be non-essential for the transactivation and DNA replication activities of E2, as both the single (RA and DA) and double (RA/DA) mutant proteins were well expressed and retained significant levels of transactivation and DNA replication activity. Collectively, these results indicate that neither the C57–C57 disulfide bond nor the two R172–D175 salt bridges between adjacent E2 monomers are essential for the expression as well as transcription and DNA replication activities of E2 in vivo. As such, they suggest that dimerization of the TAD is not crucial for these activities, a notion that is in agreement with previous studies indicating that BPV1 E2 heterodimers containing a single TAD are functional, albeit at reduced levels compared to full-length E2 homodimers (Kurg et al., 2006, 2009, 2010).

Although not strictly essential, the TAD dimerization interface may contribute to some extent to the functions of E2, as suggested by the phenotypes of the double (CA/RA and CA/DA) and triple (CA/RA/DA) mutant proteins in which formation of the C57 disulfide bond and R172–D175 salt bridges are prevented simultaneously. These E2 mutant proteins should in theory be the most impaired for TAD dimerization and, accordingly, displayed the most severe phenotypes in the DNA replication assay. They were also significantly compromised for transactivation, with the exception of the CA/RA double mutant which retained approximately 85% of the activity of wild type E2. Not surprisingly, the CA/RA/DA triple mutant E2 was the least active showing a 50–60% reduction in transactivation and DNA replication activity. Because it was the most defective, we chose to further characterize this triple mutant protein, relative to wild type E2, using full dose–response curves. The transactivation and DNA replication data could be fitted by non-linear regression to a classical sigmoid dose–response curve, with excellent goodness-of–fit values, thus validating the robustness of our assays for characterizing and comparing mutant proteins (Fig. 3B and Fig. 6B). These studies revealed that the 50% weaker transcriptional activity of the triple mutant E2 could be rescued by overexpression of the protein. This finding suggests that the triple mutant E2 is transcriptionally compromised primarily because of its reduced expression and not because it is intrinsically deficient for this activity. In stark contrast, overexpression of the triple mutant E2 could not rescue its DNA replication defect, the activity of the protein remaining approximately 45% that of wild type E2. This key result suggests that the triple mutant E2 is intrinsically deficient for some aspect of viral DNA replication. As such, it raises the possibility that the TAD dimerization interface, although not essential for BPV1 DNA replication, may be required structurally and/or functionally for this process.

Although the single, double or triple amino acid substitutions that we introduced in the TAD did not completely abolished the transactivation and DNA replication activities of E2, some could
nevertheless have dramatic effects on the BPV1 life cycle. Indeed, it has been observed previously that many amino acid substitutions in E2 that only partially reduce its transactivation and/or DNA replication activity often impose a more severe phenotype in longer-term assays involving the maintenance and/or segregation of the viral episome in immortalized proliferating cells (Abroi et al., 2004; Brokaw et al., 1996). As an example relevant to this study, Kurg et al. (2006) observed that an E2 heterodimer carrying a single TAD is only partially defective in a transient transactivation assay but severely compromised in a BPV plasmid segregation assay performed over several cell divisions; the phenotype in each assay likely being caused by the weaker binding of the single TAD E2 to the cellular protein Brd4, a key mediator of E2’s transcription and segregation activities. By analogy, it is possible that the CA single and CA/RA/DA triple mutant proteins characterized in this study, which display ~50% transactivation activity, would also be defective in a segregation assay. Additional studies will be required to test this possibility and, more generally, to determine the capacity of these mutant proteins to interact with factors such as Brd4 and the viral E1 helicase to promote transcription, replication and segregation of the BPV1 episome. In summary, the results from our mutational analysis indicate that the TAD dimerization interface is not strictly required for the transactivation and DNA replication activities of BPV1 E2 in transient assays, but also support the notion that the two TADs within an E2 dimer are not completely independent and must productively dimerize for full activity, in particular to facilitate replication of the viral episome in infected cells.

Materials and methods

Plasmid constructions and mutagenesis

The plasmid used to express untagged BPV1 E2 was constructed in two steps. First, a BamHI-XbaI digested PCR fragment encoding the E2 ORF was inserted between the BamHI and XbaI sites of pRLuc-3xFLAG (Gagnon et al., 2009). The following pairs of primers were used for amplification of E2: 5′-CCATTGGATCCGAGACAGCATGC-GAAGCCTTACATGT-3′ and 5′-CCCTCTAGATCAGAAGTCCAAGCTGGC-CCATTGGATCCGAGACAGCATGC-GAAGCCTTACATGT-3′ and 5′-CCCGAATTCGGTGCTC-GCGGATACCC-3′. In the second step, most of the pRLuc-3xFLAG-E2 plasmid, except for the region encoding the RLuc-3xFlag tag, was amplified with the following pair of primers, 5′-CCCGAATTCATGGAATCCAGTTCTACATGT-3′ and 5′-CCCGAATTCGGTGCTC-GCGGATACCC-3′, digested with EcoRI and re-circularized. The resulting plasmid, pBPV1E2, expresses untagged E2 from the CMV promoter. Mutations in the BPV E2 ORF were constructed using the Quick Change Site-Directed Mutagenesis kit (Agilent). All DNA constructs were verified by sequencing. Further details about their construction will be made available upon request.

Antibodies and Western blotting

For Western blot analysis, proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. BPV1 E2 was detected with the mouse monoclonal antibody 1E4 obtained from Santa Cruz Biotechnology (catalogue no. sc-57644) and used at a dilution of 1:500. β-tubulin, used as a loading control, was detected using a monoclonal antibody from Sigma-Aldrich (catalogue no.T4026) at a dilution of 1:2000. Proteins were visualized using a horseradish peroxidase-linked sheep anti-mouse secondary antibody purchased from GE Healthcare (catalogue no. NA931, used at a 1:5000 dilution) and the chemiluminescent PLUS Western blotting detection reagents procedure from GE Healthcare. Blots were quantified using a ChemiDoc MP Imaging System and the Quantity One 1-D Analysis Software (Bio-Rad).

Cell culture and transfections

Human cervical carcinoma C33A cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 I.U. of penicillin/ml, 50 μg of streptomycin/ml and 2 mM of l-glutamine. Transfections were performed using the Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen).

Confocal fluorescence microscopy

C33A cells were grown on coverslips. For immunofluorescence, cells were fixed 24 h transfection with 4% formaldehyde, permeabilized with 0.2% Triton X-100, and incubated with the 1E4 antibody diluted 1:200 in PBS-2% milk for 1.5 h, followed by 1 h of incubation with a 1:1000 dilution of Alexa Fluor 633-conjugated secondary antibody. Cells were washed with PBS between each step and then mounted using VectaShield mounting medium (Vector Laboratories). Images were acquired using a LSM510 confocal laser coupled to an Axiovert 100M inverted scanning microscope (Zeiss, Toronto, Ontario, Canada) and analyzed by using LSM Image Browser version 3.2.0.70 (Zeiss).

Transient transactivation assay

Transactivation assays were performed in 96-wells plates in which C33A cells were plated 24 h before transfection at a density of 25,000 cells. Transfection reactions contained increasing quantities of E2-expression plasmid, as indicated in the text, completed with the appropriate amount of pCI plasmid (Promega) in order to maintain constant the total amount of DNA in each well. Transfections reactions also contained 160 ng of pK4xE2BS reporter plasmid expressing firefly luciferase under the control of four E2 binding sites (kind gift from Laimons A. Laimins, Northwestern University) and 0.5 ng of the pRL control vector expressing Renilla luciferase. Dual-luciferase measurements were performed 24 h post transfection as described above.

Transient BPV1 DNA replication assay

This assay was developed and performed essentially as described previously for HPV31 (Fradet-Turcotte et al., 2010) but using E1, E2 and the origin of DNA replication from BPV1. Briefly, ~20 × 10^6 C33A cells were transfected with four plasmids encoding, respectively, E1 (pCG E1Eag 1235−) (Ustav and Stenlund, 1991), E2 (pBPV1E2), the origin of DNA replication in cis of a firefly luciferase reporter gene (pFLORI-BPV1), and Renilla luciferase (pRL). Under standard conditions, 0.03 to 0.50 ng of E2-expression vector, in two-fold increments, were used along with constant amounts of the other plasmids (20 ng of pCG E1Eag 1235−, 2.5 ng of pFLORI-BPV1, and 0.5 ng of pRL). For all conditions, replication of the origin-containing plasmid was quantified 72 h post transfection by measuring the levels of firefly and Renilla luciferase activities using the Dual-Glo luciferase assay system (Promega). EC_{50} values for hydroxyurea, gemcitabine and aphidicolin were calculated with the GraphPad Prism 5 software by fitting the data to the following equation: % DNA replication = Low + (100 – Low)/[1 + 10^((LogEC_{50} – [I]) ÷ n)], in which Low represents the lowest percentage of DNA replication reached at saturating amounts of inhibitor, [I] is the concentration of the inhibitor (M), and n is the Hill coefficient.
Statistical analysis

ANOVA with Dunnnett’s post-hoc analysis, as well as calculation of AUC values, were performed using GraphPad Prism version 6.00.

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