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An Interaction between Human Papillomavirus 16 E2 and TopBP1 Is Required for Optimum Viral DNA Replication and Episomal Genome Establishment

Mary M. Donaldson, a Lorna J. Mackintosh, a Jason M. Bodily, b Edward S. Dornan, a Laimonis A. Laimins, b and Iain M. Morgan a
Medical Research Council–University of Glasgow Centre for Virus Research, Glasgow, United Kingdom, a and Department of Microbiology-Immunology, Northwestern University, Chicago, Illinois, USA b

In human papillomavirus DNA replication, the viral protein E2 forms homodimers and binds to 12-bp palindromic DNA sequences surrounding the origin of DNA replication. Via a protein–protein interaction, it then recruits the viral helicase E1 to an A/T-rich origin of replication, whereupon a dihexamer forms, resulting in DNA replication initiation. In order to carry out DNA replication, the viral proteins must interact with host factors that are currently not all known. An attractive cellular candidate for regulating viral replication is TopBP1, a known interactor of the E2 protein. In mammalian DNA replication, TopBP1 loads DNA polymerases onto the replicative helicase after the G1-to-S transition, and this process is tightly cell cycle controlled. The direct interaction between E2 and TopBP1 would allow E2 to bypass this cell cycle control, resulting in DNA replication more than once per cell cycle, which is a requirement for the viral life cycle. We report here the generation of an HPV16 E2 mutant compromised in TopBP1 interaction in vivo and demonstrate that this mutant retains transcriptional activation and repression functions but has suboptimal DNA replication potential. Introduction of this mutant into a viral life cycle model results in the failure to establish viral episomes. The results present a potential new antiviral target, the E2-TopBP1 interaction, and increase our understanding of the viral life cycle, suggesting that the E2-TopBP1 interaction is essential.

There are more than 100 types of human papillomavirus (HPV) involved in a host of epithelial lesions, ranging from hand warts and genital warts to cervical cancer (69). So-called high-risk HPV are those associated with cancer, and type 16 is the most commonly detected, being present in ca. 50% of cervical carcinomas and increasingly detected in head and neck cancers (30). All HPV encode two proteins, E1 and E2, required for replication of their double-stranded DNA genome in association with cellular partner proteins. The E2 protein forms homodimers and binds to 12-bp palindromic sequences surrounding the origin of replication and via a protein–protein interaction recruits the E1 protein to the A/T-rich origin (9, 40, 61). E1 then forms a dihexameric helicase that interacts with the cellular DNA polymerase machinery, resulting in DNA replication initiation (36, 38, 46, 55). The origin of replication is located in the long control region (LCR), a noncoding part of the genome that controls the initial transcription from the viral genome by cellular factors (50). The E2 protein can also regulate viral genome transcription; it can act as either an activator or a repressor of viral oncogene expression depending upon E2 levels and the cell type under study (10, 15, 60). The carboxyl terminus domain of E2 is required for homodimerization and DNA binding, while the amino terminus interacts with E1 and a number of cellular transcription factors (16, 47, 54, 56, 63). E2 can also associate with mitotic chromatin and is proposed as a viral genome segregation factor by binding the viral genome to the cellular DNA during mitosis ensuring recruitment of the viral genome into the nuclei of the resulting daughter cells (3, 6, 45, 67). For some E2 proteins, but not HPV16, the cellular protein Brd4 is the mitotic chromatin receptor (37, 43, 67); Brd4 is also an essential transcriptional coactivator for all E2 proteins (54).

The essential role that E2 plays in transcription, replication, and genome segregation makes it an antiviral target. In order to increase understanding of HPV16 E2 (from now on E2 will mean HPV16 unless stated otherwise), we carried out a yeast two-hybrid screen and identified the cellular protein TopBP1 as a binding partner (13, 14). TopBP1 is an excellent candidate protein for mediating E2 properties since it is involved in DNA replication initiation and transcriptional control and it associates with mitotic bodies (5, 23, 27, 28, 32, 35, 49). It has eight BRCA1 carboxyl-terminal (BRCT) domains, which are hydrophobic pockets first identified in BRCA1 that act as interacting domains for other proteins, damaged DNA, and phosphor-proteins (29). In yeast and Xenopus model systems, TopBP1 (and its homologues) is required for interacting with origin recognition complex proteins and loading Cdc45 and the GINS (Go, Ichi, Nii, San) complex onto MCM2-7 in an S-phase kinase-specific manner at the G1-S transition to form the replication helicase (28, 48, 68). TopBP1 also acts as a transcription cofactor. A direct interaction regulates transcription and apoptotic properties of E2F1 (32, 34). TopBP1 also acts as a transcriptional repressor for the Miz1 protein, thus regulating the function of c-Myc (20). TopBP1 can regulate p53 target genes via complexing directly with the p53 protein (31), as well as regulating properties of mutant p53 proteins that contribute toward transformation (33). In addition to regulating transcription and replication, TopBP1 is also a key component of the DNA damage response pathway. It binds directly to ATR to activate

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Address correspondence to Iain M. Morgan, iain.morgan@glasgow.ac.uk.
* Present address: Louisiana State University Health Sciences Center, Shreveport, Louisiana, USA.
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kinase activity (26) and also regulates ATM activation of ATR activity by acting as an ATM substrate, resulting in enhanced ATR signaling (65). It is also proposed that TopBP1 plays a role in homologous recombination by directly interacting with NBS1, resulting in DNA damage signaling (42, 66). The DNA damage and replication functions of TopBP1 can be dissected, demonstrating that this multi-BRCT domain containing scaffold protein can play key roles in multiple nucleic acid metabolism processes within the cell (19).

Our previous studies have demonstrated the regulation of E2 function and chromatin interaction by TopBP1 (14, 17). We report here the in vivo identification and characterization of an E2 mutant severely compromised in TopBP1 binding. This mutant is functional since it retains transcriptional activation and repression functions but is defective in DNA replication. Introduction of this mutation into a viral life cycle model results in failure to establish episomal viral genomes in primary epithelial cells, as would be predicted for a mutant that did not induce optimal DNA replication. This is the first report, to our knowledge, describing an HPV16 E2 mutant that fails to support the viral life cycle. It is also the first report to describe a mutant E2 protein, from any species, that has altered DNA replication potential with a candidate cellular cofactor explaining the phenotype identified. Overall, the results strongly suggest that the E2-TopBP1 interaction is essential for the viral life cycle and that disruption of this interaction may be a novel antiviral target.

**MATERIALS AND METHODS**

**Cell culture and transfection.** HEK293T and C33a cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) and 1× penicillin-streptomycin (pen-strep; Invitrogen Life Technologies) in a 5% CO₂ atmosphere at 37°C. Cultures were split 1 in 10 every 3 days. The cells used for transfection were plated in 60-mm plates for the transcription assays and the cycloheximide experiments (2×10⁵ cells/plate) or in 100-mm plates for the replication assays and for protein preparation for immunoprecipitation (6×10⁵ cells/plate). The following day, the cells were transfected using calcium phosphate, and 16 h later the cells were washed twice with phosphate-buffered saline (PBS) and re-fed with DMEM containing 10% FCS with pen-strep. The cells were harvested 48 h posttransfection for all experiments, with the exception of the replication assays (harvested 72 h posttransfection). Human foreskin keratinocytes (HKFs) and HKF-derived cell lines were cultured in E-medium with NIH 3T3 J2 fibroblast feeders as described previously (39). Cell lines containing HPV16 genomes were created by cotransfection of pEGFP Ni HPV16 with an expression vector for Cre recombinase and a neoymycin resistance plasmid, followed by G418 selection as described previously (11). Differentiation was induced by suspending cells in 1.5% methylcellulose for 24 h, followed by washes in PBS (51).

**Protein preparation for Western blotting and immunoprecipitation.** Cells were harvested by trypsinization, and the pellet was washed with PBS. The cells were then resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-Cl [pH 8], 0.5% NP-40, 1× Roche protease inhibitor cocktail) and lysed on ice for 30 min. Lysates were cleared by centrifugation (20,800 relative centrifugal force, 4°C, 30 min), and the supernatant was probed for 10 min at room temperature. Proteins were transferred onto nitrocellulose membranes using the Invitrogen iBlot system. The membranes were blocked in blocking solution (PBS, 0.1% Tween, 5% nonfat milk powder) for 16 h at 4°C or 1 h at room temperature. After both primary and secondary antibody incubations, membranes were washed in PBS–0.1% Tween. Bands were revealed by chemiluminescence (ECL+; Amersham).

**Antibodies.** R1180 rabbit polyclonal anti-TopBP1 antibody (14) was used for Western blotting and immunoprecipitation (Fig. 1B). TVG261 mouse monoclonal anti-HPV16 E2 antibody (21) was used for Western blotting (see Fig. 1B, 3A, 4A, and 4C). Mouse monoclonal anti-TopBP1 antibody (BD Biosciences) was used for Western blot analyses (see Fig. 1B). Mouse monoclonal anti-hemaggulutinin (HA) antibody HA.11 (Covance) was used for immunoprecipitation (see Fig. 3A) and Western blot analyses (see Fig. 3A and C). GTU/88 mouse monoclonal anti-gamma tubulin (Sigma) was used as a Western blot loading control (see Fig. 4A and C). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse antibody (GE Healthcare) was used as a secondary antibody to mouse primary antibodies in Western blot analyses. HRP-conjugated goat anti-rabbit antibody (Sigma) was used as a secondary antibody to rabbit primary antibodies in Western blot analyses.

**Site-directed mutagenesis.** Mutagenic PCRs were set up as follows. We used 5 µl of 10× KOD buffer with 5 µl of 2 mM deoxynucleoside triphosphates, 4 µl of 25 mM MgSO₄, 1 µl of dimethyl sulfoxide, forward primer, reverse primer, 100 ng of template DNA, 1 U of KOD Hot-Start DNA polymerase (Novagen), and distilled H₂O to 50 µl. Reactions were cycled as follows: 94°C for 4 min, followed by 18 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final 72°C for 4 min. After completion of the mutagenic PCR, 10 U of DpnI was added to the reaction, followed by incubation for 90 min at 37°C. Then, 5 µl of the reaction was transformed into Escherichia coli DH5α, and transformed cells were selected on Luria-Bertani/ampicillin plates. Positive colonies were picked, and plasmids were recovered by using a miniprep procedure and sequenced to determine the mutation. The primers used for the generation of the N89YE90V mutant were as follows: forward, 5’-CAATATAACTCACAATATAGTTATGTAAAG; reverse, 5’-CTGTATATGGTCACCTITACAAACATATTATGTGATATATTAG-3’. Immunoprecipitation. A total of 200 µg of protein lysate was pre-cooled with 10 µl of a protein A-Sepharose bead slurry equilibrated in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8], 0.5% NP-40). The volume was made up to 100 µl with immunoprecipitation buffer, followed by incubation for 1 h with rotation at 4°C. The beads were pelleted, and the cleared lysate was moved to a clean tube. Then, 1 µl of antiserum was added to lysate, followed by incubation for 1 to 2 h with rotation at 4°C. Next, 20 µl of bovine serum albumin (BSA)-blocked bead slurry (the washed beads were incubated in 1% BSA fraction V in PBS overnight at 4°C with rotation; the beads were then washed four times with 25 bed volumes immunoprecipitation buffer and resuspended in 1 volume of immunoprecipitation buffer) was added to the cell lysate, followed by incubation for 1 h with rotation at 4°C. The beads were then washed four times with 25 bed volumes immunoprecipitation buffer. The beads were then prepared for Western blotting.

**Transcription assays.** At 48 h posttransfection, the medium was discarded, and the plates were washed twice with PBS. A total of 300 µl of 1× reporter lysis buffer (Promega) was added to each plate, and the cells were lysed in situ for 10 min at room temperature. The lysates were scraped, transferred into Eppendorf tubes, and cleared by centrifugation. Then, 80 µl of each lysate was assayed for luciferase activity (Promega). The protein concentrations of the lysates were also determined by BCA assay (Sigma), and the results are expressed as the luciferase activity relative to the protein concentration.

**Replication assays.** At 72 h posttransfection, the medium was discarded, and the plates were washed twice with PBS. The samples were processed for reverse transcriptase quantative PCR (RT-qPCR)-detected transient replication assay, as described previously (39).

**Cycloheximide time course and densitometry.** At 36 h posttransfection, cycloheximide (Sigma) was added to the plates to a final concentration of 100 µg/ml. The plates were then incubated for the figures in being harvested by trypsinization. The cell pellets
were then processed for BCA assay (Sigma) and Western blotting. Exposed films from Western blots were scanned at high resolution in grayscale, and the gel analysis software in ImageJ was used to determine the intensity of each band. The intensity of each band of interest was calculated relative to the loading control band for that sample. Densitometry experiments were repeated in triplicate, and the average relative intensities were graphed.

**Southern blotting and RT-qPCR.** Total DNAs were isolated and digested with XhoI (which does not cut the HPV16 genome) or BamH1 (which cuts the HPV16 genome once) and analyzed by Southern blotting as described previously (11). Total RNAs were harvested using RNASTAT 60 (TelTest, Inc.) and converted to cDNAs using the qScript cDNA synthesis kit (Quanta). qPCR was performed using a StepOnePlus real-time PCR instrument. E7-containing transcripts were detected with PerfeCTa SYBR green Supermix (Quanta) using 16E7 SYBR 5’ (ATGAG CAATTTAAGTACAGCTT) and 16E7 SYBR 3’ (GACACTTTTACAAT) as the primers. Cyclophilin (57) was assayed as an internal control using qCyc SYBR 5’ (CTTGGGCCGCTTCC) and qCyc SYBR 3’ (GCGAGACCTTTATACCAATC) as primers. E1‘E4-containing transcripts were detected using the TaqMan Fast advanced master mix containing a TaqMan probe (FAM) spanning the splice junction masterplexed with a cyclophilin (VIC) internal control probe.

**RESULTS**

The role of TopBP1 in mammalian DNA replication led us to hypothesize that this protein contributed to E1-E2 mediated DNA replication via binding to E2. To test this hypothesis, an E2 protein compromised in TopBP1 binding is required; depletion of TopBP1 from cells is toxic due to the role of TopBP1 in DNA replication and also in regulating the DNA damage response, making siRNA/shRNA experiments with TopBP1 difficult to interpret, particularly in relation to replication (23, 24).

**Identification of an E2 mutant compromised in TopBP1 interaction.** The crystal structure of the E2 amino-terminal domain is known (4), and this was used to predict which amino acids may be contact residues for TopBP1. The amino-terminal domain was broken down into 50-amino-acid subdomains based on the crystal structure. In Fig. 1A, amino acids 50 to 100 are shown. In order to direct our mutagenesis strategy, we eliminated E2 residues with prior defined functions (Fig. 1A). Our previous results demonstrated that knocking down TopBP1 protein levels with siRNA did not affect E2 transcription properties (17); therefore, we eliminated the residues required for E2 transcription function, as detailed in the E2-Brd4 crystal structure (2). We also predicted that an E2 interaction with both TopBP1 and E1 would occur simultaneously to allow DNA polymerase loading onto the E1 helicase; therefore, the contact points on E2 for E1 could also be eliminated (1). Many previous studies had also identified residues whose mutation resulted in multiple defective phenotypes; therefore, these were also eliminated. Finally, the residues proposed to be involved in the homodimerization of the E2 amino terminus were also eliminated, leaving 17 amino acids as candidate residues for TopBP1 (4). Several of these amino acids had previously been mutated to alanine residues with no discernible phenotype; therefore, the residues were mutated in pairs and/or clusters and not necessarily to alanines in order to identify a mutant compromised in TopBP1 interaction. We prepared several E2 mutants with changes in the 17 amino acids and assayed their replication potential; we hypothesized that a compromised replication would relate to a compromised TopBP1 interaction. The results of these replication assays are shown in Fig. 1B, and the mutants are described in Fig. 1C. Mutant 8 failed to induce replication, and the ability of this mutant to interact with TopBP1 in vivo was tested by coimmunoprecipitation (Fig. 1D). In lane 3, it is clear that wild-type E2 coimmunoprecipitates with TopBP1, whereas mutant 8 fails to interact efficiently (lane 4). This result supported the hypothesis that an interaction between E2 and TopBP1 is involved in viral replication.

Our initial goal was to test whether the E2-TopBP1 interaction was essential for the HPV16 life cycle by generating an E2 mutant compromised in TopBP1 interaction. It is clear that mutant 8 is compromised in TopBP1 interaction, but the eight amino acid changes in this mutant (Fig. 1B) make it unsuitable for testing in a life cycle model. We therefore set out to reduce the number of residues required to be mutated to compromise the interaction with TopBP1. Figure 2A describes subsequent mutants that were prepared and assayed in DNA replication assays (Fig. 2B). All of the mutants studied remained significantly compromised in DNA replication except mutant 28, where there was no statistically significant difference with wild-type E2. The ability of the mutants to interact efficiently with TopBP1 was then tested (Fig. 2C). Lanes 1 to 7 demonstrate efficient expression of the mutant E2 proteins, and lanes 8 to 23 show the coimmunoprecipitation with TopBP1 (even-numbered lanes, anti-TopBP1 antisera; odd-numbered lanes, preimmune sera). Wild-type E2 interacts with TopBP1 (lane 10), while mutant 28 also interacts efficiently with TopBP1 (lane 20). All other mutants fail to interact efficiently with TopBP1 in this coimmunoprecipitation assay. Since mutant 28 was the only mutant not to have significantly reduced replication potential, we conclude that there is a correlation between compromised TopBP1 binding and a significant reduction in E2 replication properties. Mutant 25 has only two amino acid changes (N89YE90V) and retains compromised DNA replication activity; we designate this mutant E2TopBP1 for further study. The results suggest there are other contact points for TopBP1 on E2 but that the area around 89/90 is essential for an efficient interaction.

**Further characterization of E2TopBP1 (mutant 25).** The compromised TopBP1 interaction of E2TopBP1 was reproducible, and an additional result from a coimmunoprecipitation with E2TopBP1 and the wild-type protein (E2WT) is shown in Fig. 3A. The shadow band visible with E2TopBP1 is perhaps due to a residual interaction between E2TopBP1 and TopBP1; it could also be due to the natural stickiness of E2 to beads used in these coimmunoprecipitation experiments (14, 18). What is clear from this experiment is that E2TopBP1 is reproducibly compromised in the TopBP1 interaction. Figure 2B demonstrated a significant reduction in E2TopBP1 replication properties, and it is possible that this was due to a compromised interaction with the E1 protein, even though our mutational strategy did not mutate any predicted E1-interacting residues (Fig. 1A). An HA-tagged version of E1 was coexpressed with E2WT, E2TopBP1, and E2E39A, which is a known non-E1-interacting mutant (52). Lysates from these cells were used in coimmunoprecipitation assays with an HA antibody (Fig. 3B). The input levels of HA-E1 (upper panel) and the E2 proteins (lower panels) are shown in lanes 1 to 4. In lanes 5 to 8, it is clear that the HA immunoprecipitation has pulled down HA-E1 (upper panel) and also E2WT and E2TopBP1 in a coimmunoprecipitation (lanes 6 and 7 in the lower panel), whereas E2E39A does not interact with HA-E1 as predicted (lane 8, lower panel). The E39A mutant shows no residual “stickiness” to the beads (lane 8) since a short exposure time is required to detect the E1-E2 interaction due to E1 overex-
pression. These results confirm that E2TopBP1 interacts with E1 similarly to E2WT. The interaction between E3WT and E2TopBP1 with HAE1 was also no different when the coimmunoprecipitation experiments were carried out at 37°C (as opposed to the 4°C shown in Fig. 3B), demonstrating that the failure to replicate properly at 37°C by E2TopBP1 is not due to failure to interact with E1 (data not shown). Finally, replication assays were carried out with reduced levels of E2TopBP1 and E2WT plasmids to confirm the compromised replication phenotype of E2TopBP1. At 10 ng of E2 input plasmid, E2TopBP1 replicates at 18% compared to E2WT, demonstrating again that it is compromised in replication function (Fig. 3C); this difference is statistically significant.

**FIG 1** Identification of a non-TopBP1 interacting E2 mutant. (A) Residues between E2 amino acids 50 to 100 that interact with Brd4 and E1, amino acids shown previously to be multiply defective, indicating improper folding, and amino acids involved in the homodimerization of the E2 protein are highlighted by row as indicated. The remaining amino acids were TopBP1 interaction candidates. (B) A panel of mutants was prepared, and DNA replication assays were carried out in 293T cells with 100 ng of input E2 plasmid. Please note the logarithmic scale, demonstrating poor replication by mutants 7 and 17, and no replication by mutant 8. (C) A table describing the mutants used in panel B. (D) Mutant 8 does not interact efficiently with TopBP1 *in vivo*. Lanes 1 and 2 show the levels of input E2 proteins for the coimmunoprecipitation experiments shown in lanes 3 and 4 with a loading control; TopBP1 is often difficult to detect in the input lanes and is not shown here. Lanes 3 and 4 are Western blots of TopBP1 immunoprecipitations. In lane 3, it is clear in the lower panel that E2WT interacts well with endogenous TopBP1, whereas mutant 8 is severely compromised, as shown in lane 5. The shadow band observed may indicate some residual binding, but there may also be a contribution to this from the “stickiness” of E2 for the beads (14, 18).
onstrate that E2TopBP1 activates transcription. The pattern of activation is distinct from E2WT with no squelching at higher levels, and there is a significant difference between E2TopBP1 and E2WT activity at 10 ng of input plasmid. These results demonstrate that E2TopBP1 is folded correctly and can bind to target DNA sequences and that there are differences between the transcriptional activation properties of this mutant and E2WT. The fold activation in transcription over that obtained when no E2 was coexpressed is shown in the boxes below the graph. E2 can also act as a transcriptional repressor of papillomavirus control regions, and the ability of E2WT and E2TopBP1 to repress the HPV18 long control region (LCR) was tested. The results from these experiments are shown in Fig. 4B. In lanes 1 to 4, it is shown that E2WT represses transcription from the HPV18 LCR as predicted, and in lanes 5 to 8 it is clear that the E2TopBP1 mutant retains this repressive function. This again demonstrates that the E2TopBP1 protein is correctly folded, binds to DNA, and is functional. There were no statistical differences between E2WT and E2TopBP1 in the transcriptional repression assays.

The stability of E2TopBP1 is similar to E2WT. One explanation for the compromised replication potential of E2TopBP1 could be that the protein is relatively unstable. To check for this, a cycloheximide chase experiment was carried out with E2WT and E2TopBP1, and the results of this experiment are shown in Fig. 5A. Lanes 1 to 5 represent the cycloheximide chase of E2WT cells, whereas lanes 6 to 10 show the results for E2TopBP1. It is clear that

<table>
<thead>
<tr>
<th>Mutant</th>
<th>F51A</th>
<th>K52A</th>
<th>Q71A</th>
<th>E74A</th>
<th>Q86A</th>
<th>Y87A</th>
<th>N98Y</th>
<th>E90V</th>
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<tr>
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<td>K52A</td>
<td>Q71A</td>
<td>E74A</td>
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<td>Y87A</td>
<td>N98Y</td>
<td>E90V</td>
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<tr>
<td>Mutant 10</td>
<td>F51A</td>
<td>K52A</td>
<td>Q71A</td>
<td>E74A</td>
<td>Q86A</td>
<td>Y87A</td>
<td>N98Y</td>
<td>E90V</td>
</tr>
<tr>
<td>Mutant 12</td>
<td>F51A</td>
<td>K52A</td>
<td>Q71A</td>
<td>E74A</td>
<td>Q86A</td>
<td>Y87A</td>
<td>N98Y</td>
<td>E90V</td>
</tr>
<tr>
<td>Mutant 14</td>
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<td>Q71A</td>
<td>E74A</td>
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<td>Y87A</td>
<td>N98Y</td>
<td>E90V</td>
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<tr>
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<td>Q71A</td>
<td>E74A</td>
<td>Q86A</td>
<td>Y87A</td>
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there are no significant differences in the rate of protein level reduction between the two samples. This analysis was carried out three times and quantitated with the results shown in Fig. 5B, which confirm that there is no significant difference in the stability of the E2WT and E2TopBP1 proteins. We recently demonstrated that the E2 protein is stabilized in the presence of E1 (25), and we therefore checked the ability of E2TopBP1 to be stabilized by E1. The results of this experiment are shown in Fig. 5C, where it is clear that the E2TopBP1 binds HA-E1 like E2WT, while E2E39A fails to interact as expected. (C) DNA replication assays were carried out in 293T cells with input levels of E2WT and E2TopBP1 plasmids of 10 ng. E1 was held steady at 5 μg in these assays since replication does not work well below this level (59). The results are expressed as picograms of replicated DNA detected. Below the bar chart is a summary of the actual numbers from the experiments. The results represent the average of three independent experiments. Bars represent ± the standard errors of the mean. E2TopBP1 has a significantly reduced replication potential, indicated by an asterisk (*), as determined by a Student t test. These results confirm that E2TopBP1 is compromised in TopBP1 binding and DNA replication potential, while retaining wild-type levels of interaction with E1.

An HPV16 genome containing the E2TopBP1 mutation fails to establish episomes in primary HFKs. In order to test the effect of the E2TopBP1 mutations in the context of the complete HPV16 genome, primary HFKs were transfected with either wild-type or E2TopBP1 mutant HPV16 DNA and selected with G418. Immortal cell lines grew out from both wild-type and mutant transfections, demonstrating that the E2 mutations did not abrogate the ability of HPV to immortalize primary keratinocytes. As shown in Fig.
6A, the growth rates of cell lines containing mutant genomes were very similar to the wild type. To determine whether viral genomes harboring E2TopBP1 mutations were capable of maintaining themselves as episomes, total DNA was isolated from pooled cell lines and subjected to Southern blotting. In three independent experiments, the wild-type HPV16 genomes were all episomal, but none of the E2TopBP1 cell lines retained episomal genomes (a representative Southern blot is shown in Fig. 6B). Indeed, in one of the three experiments, the E2TopBP1 mutant genome failed to yield stable cell lines. It is notable that after differentiation the levels of wild-type HPV16 genome are not increased, as would be predicted. This has been observed previously, but viral particles are produced using this system (11); perhaps cervical keratinocytes are required to observe amplification of the HPV16 genome following differentiation in this system. The expression of viral RNAs was then investigated (Fig. 7A). Cells were grown in monolayers or suspended in methylcellulose to induce differentiation. Total RNA was isolated and subjected to RT-qPCR using primers specific for a portion of the E7 gene, which is transcribed from the early promoter p97 (Fig. 7A). This transcript was detected in E2WT and E2TopBP1 cells (Fig. 7B). Consistent with previous data, transcripts from p97 were not increased upon differentiation in cells containing wild-type genomes (12, 44; J. Bodily, unpublished data), nor were they increased in the E2TopBP1 cells (Fig. 7B). The fact that transcript levels were not elevated in the in the E2TopBP1 samples relative to the wild type indicates that genome integration was not sufficient by itself to increase transcript levels in this system. Using a qPCR probe spanning the E1/E4 splice junction, which is immediately downstream of the late promoter p670 (Fig.
replication is not subject to the tight “once and once only” regulation. HPV DNA is replicated more than once per cell cycle; therefore, HPV DNA replication is instrumental in initiating host DNA replication following the G$_1$-to-S transition. The *Saccharomyces cerevisiae* homologue of TopBP1, Dpb11, interacts with two cellular proteins Sld2 and Sld3 after they are phosphorylated by Cdk at the G$_1$-S transition. This is critical for replication initiation since it recruits Dpb11 to the prereplication minichromosome maintenance (MCM) complex loading on Cdc45 and GINS, resulting in polymerase loading and replication initiation (58, 68). The human functional counterpart of Sld3 is likely Treslin (27, 28), while RecQ4 is a candidate for fulfilling Sld2 function (22). In neither yeast nor humans does Dpb11/TopBP1 directly contact the origin recognition complex or the MCM helicase without recruitment by the phosphorylated Sld2/Sld3 and RecQ4/Treslin proteins, ensuring that the initiation of DNA replication occurs in a cell cycle-regulated manner. In this report we demonstrate that a failure of E2 and TopBP1 to interact optimally results in reduction in replication potential and failure to establish episomes in primary epithelial cells. This direct recruitment of TopBP1 to the HPV replication complex would allow polymerase loading onto the E1 replication helicase in a cell cycle-independent manner, giving the virus the potential to replicate more than once per cell cycle. As described above, this is a necessary function for the HPV life cycle.

The mutational strategy described in Fig. 1 and 2 identified several E2 mutants compromised in DNA replication function and TopBP1 binding. The correlation between these phenotypes strongly implicates TopBP1 in regulating E2 DNA replication properties in association with E1. E2$^{TopBP1}$ mutant genomes are unable to establish episomes in primary HFKs (Fig. 6, and 7). Figure 6B shows the results of a typical Southern blot carried out on the cells generated after transfection with E2$^{WT}$ and E2$^{TopBP1}$ genomes. The HPV16 genome is clearly seen in this figure as episomal in the E2$^{WT}$ cells but is not detected in the E2$^{TopBP1}$ cells. This lack of detection is due to multiple integration events in the pooled cells preventing detection of one band, as would be expected in a clonal line. Longer exposure of the Southern blot detects a smear in the E2$^{TopBP1}$ cells, demonstrating the presence of multiple integrated genomes in these pools (data not shown). The E2$^{TopBP1}$ cells clearly maintain expression of E6 and E7 sufficient for immortalization, and their inability to maintain episomal genomes is consistent with a defect in E1/E2-mediated DNA replication, as shown for E2$^{TopBP1}$ in Fig. 2 and 3. However, E2$^{TopBP1}$ also has altered transcription function (Fig. 4A), although it can activate transcription to E2$^{WT}$ levels when overexpressed, unlike in DNA replication, where it cannot. This transcription activation function and the ability to repress transcription as efficiently as E2$^{WT}$ demonstrates that E2$^{TopBP1}$ retains binding to Brd4. The alterations in transcription and defects in DNA replication are not due to the altered stability of the E2$^{TopBP1}$ protein (Fig. 5) compared to E2$^{WT}$, demonstrating that these are functional differences rather than due to the levels of protein expressed. Our previous studies with siRNA targeting TopBP1 demonstrated that in the absence of TopBP1 E2$^{WT}$ had an altered affinity for chromatin. However, the E2$^{TopBP1}$ mutant does not show this alteration for chromatin affinity (data not shown), suggesting that the studies with siRNA against TopBP1 may alter the cellular environment that influences E2$^{WT}$ affinity for chromatin. We favor a model where E2$^{TopBP1}$ deficiency in DNA replication is responsible for the lack of episomal establishment by E2$^{TopBP1}$ genomes. This view is supported by the observation that E2 cannot regulate tran-
scription from HPV16 episomal genomes (8). However, this will require further investigation of the E2\textsuperscript{NT} and E2\textsuperscript{TopBP1} replication and transcription complexes in vivo. Studies with BPV1 E2 mutants support the role of the E90 residue in DNA replication since a D89A E90A mutant had wild-type transcriptional activation properties but compromised DNA replication potential (7). We have previously shown that BPV1 E2 can interact with TopBP1 (17), and our preliminary results suggest that mutation of amino acids 89 and 90 in BPV1 E2 compromise interaction with TopBP1 (data not shown). Future studies on E2-TopBP1 will extend to a variety of PV types to confirm the essential nature of this interaction in PV life cycles.

Although effective prophylactic vaccines against HPV16/18/6/11 have been successfully introduced (53), it remains a priority to develop novel antiviral therapies for several reasons. First, individuals currently infected will not be helped by these vaccines, and there are currently no therapeutic vaccines. Second, the introduced vaccines target only a subset of HPV that induce disease. Third, other high-risk oncogenic HPVs, or 16/18 subtypes partially resistant to the vaccine, may become more prevalent following the reduction of the 16/18 load; therefore, cervical cancer rates may not fall as predicted. Fourth, in most developing nations, where ca. 80% of cervical cancer deaths occur, it will be very difficult to successfully introduce the current vaccines to a sufficient percentage of the population. The role of the E2-TopBP1 interaction in mediating E2 function and the potential requirement for this interaction in episomal establishment suggests that disruption of this interaction represents a therapeutic target for the treatment of HPV disease. Inhibition of HPV DNA replication is a therapeutic target, and disruptors of E1-E2 interactions exist that reduce DNA replication (64); however, these inhibitors are restricted in their usefulness since they do not operate efficiently across a variety of HPV types due to the subtle differences in the E1-E2 interactions. It is more likely that interaction between viral and host proteins are closely conserved across HPV types. Therefore, it is possible that inhibition of the E2-TopBP1 interaction could reduce viral replication and load in all HPV-related diseases. We have already confirmed interaction between BPV1 E2 and TopBP1 (17), suggesting that this interaction will be widely conserved across different PV types from different species. We have already demonstrated a direct interaction between E2 and TopBP1 bacterial recombinant proteins (data not shown), and future studies will focus on determining the crystal structure of the E2-TopBP1 interaction and confirming the essential nature of this interaction for the viral life cycle.

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