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Inhibition of Human Papillomavirus DNA Replication by an E1-Derived p80/UAF1-Binding Peptide

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The papillomavirus E1 helicase is recruited by E2 to the viral origin, where it assembles into a double hexamer that orchestrates replication of the viral genome. We previously identified the cellular WD40 repeat-containing protein p80/UAF1 as a novel interaction partner of E1 from anogenital human papillomavirus (HPV) types. p80 was found to interact with the first 40 residues of HPV type 31 (HPV31) E1, and amino acid substitutions within this domain abrogated the maintenance of the viral episome in keratinocytes. In this study, we report that these p80-binding substitutions reduce by 70% the ability of E1 to support transient viral DNA replication without affecting its interaction with E2 and assembly at the origin *in vivo*. Microscopy studies revealed that p80 is relocalized from the cytoplasm to discrete subnuclear foci by E1 and E2. Chromatin immunoprecipitation assays further revealed that p80 is recruited to the viral origin in an E1- and E2-dependent manner. Interestingly, overexpression of a 40-amino-acid-long p80-binding peptide, derived from HPV31 E1, was found to inhibit viral DNA replication by preventing the recruitment of endogenous p80 to the origin. Mutant peptides defective for p80 interaction were not inhibitory, demonstrating the specificity of this effect. Characterization of this E1 peptide by nuclear magnetic resonance (NMR) showed that it is intrinsically disordered in solution, while mapping studies indicated that the WD repeats of p80 are required for E1 interaction. These results provide additional evidence for the requirement for p80 in anogenital HPV DNA replication and highlight the potential of E1-p80 interaction as a novel antiviral target.

Human papillomaviruses (HPVs) are small double-stranded DNA viruses that replicate and cause hyperproliferative lesions within stratified epithelia (reviewed in references 26 and 34). Among the 100 described HPV genotypes, more than 30 infect the anogenital mucosa (3, 15). These mucosal types are classified as low- or high-risk viruses according to their capacity to cause benign or cancerous lesions, respectively. High-risk HPVs are the etiological agents of cervical cancer, the second leading cause of cancer in women worldwide (47). These viral types have also been linked with other malignancies of the genital tract and with the majority of anal cancers in both men and women (2, 13, 24, 48). Specific high-risk types, and in particular HPV type 16 (HPV16), are also increasingly being associated with a subset of head and neck cancers (32).

HPVs infect keratinocytes of the basal cell layer. Upon infection, the viral genome is established as a multicopy episome in the nuclei of these cells through several rounds of viral DNA replication. Maintenance of the viral episome at 50 to 100 copies in these undifferentiated cells is then achieved through low levels of DNA replication and is essential for the persistence of the infection, itself a risk factor for virus-induced carcinogenesis. As the infected cells differentiate and reach the upper layers of the epithelium, the virus switches from the maintenance to the productive phase of its life cycle, characterized by the amplification of the viral episome to greater than 1,000 copies per cell and the expression of the late genes encoding the capsid proteins (reviewed in reference 26). HPV DNA replication, which is required for establishment and maintenance of the viral episome in undifferentiated cells, as well as for its amplification in response to differentiation, is achieved by the viral E1 and E2 proteins in concert with the host DNA replication machinery. E1, the viral helicase, is recruited by E2 to the viral origin (ori), where it assembles into a double-hexameric complex capable of unwinding DNA ahead of the bidirectional replication fork (1, 22, 23, 39, 51, 53, 54, 64). E1 also recruits several cellular DNA replication factors required for viral DNA synthesis, such as the single-stranded DNA-binding protein RPA, topoisomerase I, and the polymerase α-primase complex (8, 11, 25, 35, 36, 46).

E1 can be subdivided into three functional regions: a C-terminal helicase/ATPase domain, a central origin-binding domain (OBD) that mediates interaction with specific sequences within the ori, and an N-terminal regulatory region. The last encodes several functional elements, such as a nuclear localization sequence (NLS), a nuclear export sequence (NES), a cyclin-binding motif, and Cdk2 phosphorylation sites. Altogether, these sequences mediate the nucleocytoplasmic shuttling of E1 and its nuclear accumulation during S phase, when viral DNA replication occurs (14, 19). We previously reported that the N-terminal regulatory region of E1 from anogenital HPV types also interacts with the cellular protein p80 (12), alternatively known as UAF1 or WDR48. Deletion analysis revealed that amino acids (aa) 10 to 40 of E1, a region that is well conserved among low- and high-risk HPV genotypes, constitutes the minimal p80-binding domain (Fig. 1A). Three independent double amino acid substitutions to alanine within this domain of E1 (highlighted in black in Fig. 1A) that prevent p80 binding were identified. These substitutions were found to abrogate the maintenance of the viral genome in immor-

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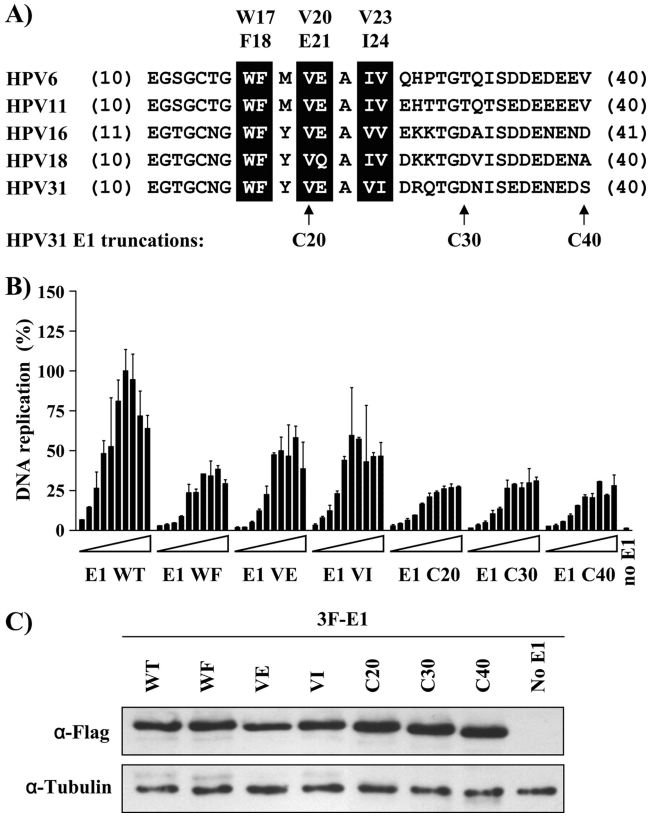

FIG 1 The p80-binding domain of E1 is required for efficient HPV DNA replication. (A) Amino acid sequence alignment of the p80-binding domains of E1 proteins from different anogenital HPV types. The residues highlighted in black are those mutated to alanines in the p80-binding mutants (WF, VE, and VI) of HPV31 E1. The boundaries of the different N-terminal truncations generated in HPV31 E1 (C20, C30, and C40) are indicated below the alignment by arrows. (B) DNA replication activities of 3F-E1 WT compared with the indicated E1 mutant proteins or truncated derivatives using a broad gradient of E1 expression vectors (0.625, 1.25, 2.5, 5, 10, 20, 25, 50, 75, and 90 ng). Cells transfected without an E1 expression plasmid were used as a negative control (no E1). Replication activity is reported as a percentage of the maximal signal obtained with WT E1. The error bars represent the standard deviations of duplicate values. The results are representative of at least three independent experiments. (C) Anti-Flag Western blot showing the expression of the different E1 proteins. β-Tubulin was used as a loading control.

talized keratinocytes and to reduce transient HPV DNA replication (12, 20). The substitutions appeared to specifically prevent the interaction of E1 with p80, as they affected neither the ability of E1 to bind to DNA or to Cdk2 *in vitro* nor its nuclear accumulation *in vivo* in transfected cells. Collectively, these findings suggested that p80 is essential for the maintenance of the HPV episome in keratinocytes.

Although p80 was first identified as an interaction partner of the Tip protein from herpes saimiri virus (6, 7, 44, 45), several recent studies have begun to address its role in uninfected cells. Various cellular proteins that bind p80 have been described, including the deubiquitinating enzymes USP1, USP12, and USP46 (9, 10). Interestingly, p80 was found to enhance the deubiquitinating activity of these enzymes, although the exact substrates of the different p80-USP complexes are only beginning to be unraveled. Among those that may be relevant to HPV DNA replication are monoubiquitinated FANCD2 and PCNA, which are deubiquitinated by USP1 as a means of downregulating, respectively, the Fanconi anemia (FA) DNA repair pathway (30, 43) and DNA translesion synthesis, a DNA damage tolerance process (27). As for USP12 and -46, they were recently shown to associate with p80 to deubiquitinate monoubiquitinated histones H2A and H2B, perhaps as a way to modulate chromatin structure during transcription (21, 29). Structure-function studies have indicated that p80 interacts with USP1, -12, and -46 through its N-terminal WD40 repeats, predicted to fold as a β-propeller. More recently, the C-terminal domain of p80 was found to contain a ubiquitin-like motif, either a UBX domain (50) or a SUMO-like domain (SLD), that may be involved in targeting specific p80-USP complexes to their appropriate substrates (66).

Despite the studies presented above, our understanding of the function of p80 in normal cell physiology and in HPV episomal maintenance remains limited. This prompted us to characterize in greater detail the role of p80 in HPV DNA replication with an emphasis on determining the potential of the E1-p80 interaction as a therapeutic target. In this study, we report that the DNA replication defect of E1 mutant proteins deficient for p80 binding is unrelated to their capacity to interact with E2 and to assemble into the E1-E2-ori preinitiation complex *in vivo*. We also demonstrate that in the presence of E1 and E2, p80 is relocalized into nuclear foci and recruited to the viral origin prior to the initiation of DNA synthesis. Importantly, we determined that overexpression of an E1-derived peptide (N40), encompassing the p80-binding motif of HPV31 E1, prevents the recruitment of p80 to the viral genome and, accordingly, inhibits transient HPV DNA replication. Nuclear magnetic resonance (NMR) and biochemical studies showed that this peptide is intrinsically disordered in solution and likely binds to the WD repeat region of p80. Collectively, these results provide further evidence that p80 is essential for efficient HPV DNA replication at a step following assembly of the E1-E2-ori complex. Furthermore, they suggest that the E1-p80 interaction may be a valid therapeutic target, as highlighted by the inhibitory effect of the E1 N40 peptide on HPV DNA replication.

MATERIALS AND METHODS

Plasmid construction and mutagenesis. The plasmids encoding green fluorescent protein (GFP)-E1 and yellow fluorescent protein (YFP)-E1 were previously described (18). The plasmid used to express p80 fused at its N terminus to a triple Flag epitope (3F) was constructed by inserting the PCR-amplified p80 open reading frame (ORF) between the EcoRV and XhoI sites of pCMV-3Tag-1a (Stratagene). Plasmids expressing codon-optimized 3F-E1 (p31E1) and 3F-E2 (p31E2) were described previously (20). The plasmid used to express untagged E1 was constructed by excising the 3× Flag coding region from pCMV-3Tag-1a by NotI/BamHI digestion and replacing it with the codon-optimized E1 ORF. The plasmids expressing the red fluorescent protein (RFP) and RFP-p80 have been described previously (12). The RFP-E2 expression plasmid was constructed by PCR amplification of the codon-optimized E2 ORF and ligation between the NotI and XbaI sites of the RFP vector. The N40-YFP expression plasmids were obtained by inserting the amplified p80-binding domain coding region of E1 (amino acids 1 to 40) between the BamHI and AgeI sites of pVenus-N1 (42), upstream of the Venus variant of the YFP coding region. Plasmids to express the N40 and N83 peptides fused to glutathione *S*-transferase (GST) were constructed by inserting the E1 peptide coding regions (amino acids 1 to 40 and 1 to 83, respectively) between the EcoRI and SalI restriction sites of pGEX-4T-1 (GE Healthcare). 3F-p80 truncations were generated by cloning PCR-amplified p80 fragments between the EcoRV and XhoI sites of pCMV-3Tag-1a for *in vivo* expression or between the NcoI and XhoI sites of

pTM1 (61) for *in vitro* translation. E1 mutants were generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene). All DNA constructs were verified by sequencing. The sequences of primers and additional details on the construction of these plasmids will be made available upon request.

Antibodies and Western blotting. 3× Flag-tagged proteins and β-tubulin were detected using mouse monoclonal antibodies from Sigma-Aldrich (M2-Flag antibody catalog no. F1804 and β-tubulin antibody catalog no. T0426). The mixture of two mouse monoclonal antibodies against GFP was purchased from Roche (catalog no. 11814460001), and the mouse monoclonal antibody against RFP was from Abcam (catalog no. AB65856). The hemagglutinin (HA) antibody was purchased from Covance (HA.11 catalog no. MMS-101P). Rabbit polyclonal antibodies against p80 or E1 were raised, respectively, by injecting rabbits (Open Biosystems) with a purified C-terminal fragment of p80 (amino acids 400 to 677) or an E1 fragment (amino acids 50 to 332) produced in bacteria as a GST-6×His fusion protein. Protein purification and cleavage of the GST moiety were performed as previously described (12). For Western blot analysis, proteins were transferred onto polyvinylidene difluoride membranes and were detected using horseradish peroxidase-conjugated secondary antibodies from GE Healthcare, either sheep anti-mouse IgG (catalog no. NA931) or donkey anti-rabbit IgG (NA934V), and an enhanced chemiluminescence detection kit (GE Healthcare).

Cell culture and transfections. The human cervical carcinoma cell line C33A was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. For the selection and the growth of stably transfected cell lines (YFP constructs), the culture medium was supplemented with 15 μg/ml bleomycin (Bleocin; EMD Millipore catalog no. 203408). Transfections of C33A were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Luciferase HPV DNA replication assay. The HPV DNA replication assay was performed as previously described (20). Briefly, C33A cells were plated at a density of 25,000 cells/well in white flat-bottom 96-well plates and were transfected 24 h later with a mixture of four plasmids: an origin-containing plasmid with a firefly luciferase (FLuc) reporter in *cis* (pFLORI31), a *Renilla* luciferase (RLuc) plasmid as an internal control (pRL), and the indicated quantities of codon-optimized HPV31 E1 (p31E1) and HPV31 E2 (p31E2) expression vectors. For all experiments, the total quantity of plasmid DNA transfected was adjusted to 100 ng (or to 150 ng when N40-YFP was added) with the pCMV-3Tag-1a vector as carrier DNA. Firefly and *Renilla* luciferase activities were measured using the Dual-Glo Luciferase assay system (Promega) 72 h posttransfection. When mentioned in the text, two-way analysis of variance (ANOVA) was performed to determine if differences in the replication signal were significant.

Coimmunoprecipitation assays. C33A cells were grown on 100-mm-diameter plates and transfected with the indicated DNA. Cells were harvested 48 h posttransfection in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and the following protease inhibitors: 10 μg/ml antipain, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). Cleared cellular extracts were then immunoprecipitated for 3 h with 40 μl protein G-Sepharose (GE Healthcare) conjugated to 1 μg of anti-Flag or anti-GFP antibodies. The resin was washed 3 times with Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), and the bound proteins were analyzed by Western blotting.

ChIP. The chromatin immunoprecipitation (ChIP) protocol was modified from a previously published procedure (65). C33A cells were plated at a density of 3.6×10^6 cells on 100-mm-diameter plates and transfected 24 h later with the following plasmids: 0.5 μg pFLORI31, 0.1 μg pRL, 1 μg p31E1, and 2 μg RFP-E2. In N40 inhibition studies, 4.2 μg of the plasmid N40-YFP was added. In all experiments, the total quantity of DNA was adjusted to 12 μg with pUC18 DNA as a carrier. Twenty-four hours posttransfection, cells were cross-linked with 1% formaldehyde, lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8, and protease inhibitors) and sonicated to shear the DNA. The lysate was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl, and protease inhibitors) and precleared using protein G-Sepharose (GE Healthcare catalog no. 16-0618) preblocked with 5 mg/ml bovine serum albumin (BSA) and 0.5 mg/ml salmon sperm DNA. The lysates (1 million cells per IP) were then incubated overnight with the indicated antibodies, and protein G-Sepharose was added and mixed for 3 h to capture the complexes. The resin was then successively washed with the following buffers: once with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8, 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8, 500 mM NaCl), and LiCl buffer (250 mM LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris, pH 8) and twice with TE buffer (10 mM Tris, pH 8, 1 mM EDTA). The immunoprecipitate was recovered by two successive elutions in elution buffer (1% SDS, 0.1 M $NaHCO_3$) at 65°C and treated with RNase A, and the cross-links were reversed by overnight incubation in 0.2 M NaCl at 65°C. The eluates were treated with proteinase K for 1 h at 45°C, and DNA was purified using QIAgen spin columns. The eluates and corresponding input DNA were then analyzed by quantitative real-time PCR (qPCR).

Quantitative real-time PCR. Quantification of DNA samples was done using TaqMan probes and the PerfeCTa PCR SuperMix, UNG, Low ROX kit (Quanta Biosciences) on a Viaa 7 real-time PCR system (Applied Biosystems). The primers (Invitrogen) were designed to amplify a fragment overlapping the HPV31 origin within the pFLORI31 plasmid or a portion of the *Renilla* luciferase gene present in the pRL internal control vector. The TaqMan probes were synthesized by Eurogentec and labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) at their 5′ and 3′ ends, respectively. Real-time qPCR was performed using 900 nM primers and 125 nM probe. Quantification was performed using a 7-point standard curve of pFLORI31 or pRL plasmid ranging from 10^4 to 10^{-2} pg (10-fold serial dilutions) with the following PCR amplification conditions: 95°C for 15 s and 60°C for 1 min for 40 cycles. The normalized enrichment levels of the ori plasmid are reported in pg of origin detected in the immunoprecipitate per pg of pRL vector found in the corresponding input DNA. The primers for the ori were 5′-CGAAAACGGTTGGTATATAAAGCA-3′ and 5′-GGGAGCCCCCG ATTTAGAG-3′, and the primers for RLuc were 5′-GCTACGAGCACCA GGACAAGA-3′ and 5′-TCGTCCCAGCTCTCGATCAC-3′. The sequences of the TaqMan probes are 5′-AACCTACGCCGGCTTTCCCC G-3′ for the ori and 5′-AAGGCCATCGTGCACGCCGA-3′ for RLuc.

Confocal fluorescence microscopy. C33A cells were plated at a density of 6×10^5 cells/well on coverslips and were transfected 24 h later with the indicated plasmids. Twenty-four hours posttransfection, the cells were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100, and their DNA was stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen catalog no. D1306). Slides were mounted using Vectashield mounting medium (Vector Laboratories). Images were collected with a Zeiss LSM710 laser scanning confocal microscope and analyzed using Zen 2009 LE software.

Flow cytometry analysis. (i) Cell cycle analysis. Cell cycle profiles were obtained by staining live cells 48 h posttransfection with 6.3 μg/ml Hoescht and 50 μM Verapamil.

(ii) BrdU incorporation. Forty-eight hours posttransfection, cells were pulsed for 1 h with 10 μM bromodeoxyuridine (BrdU) and were then fixed and stained using the APC BrdU Flow kit (BD Pharmingen catalog no. 522598). BrdU was detected using allophycocyanin (APC)-conjugated anti-BrdU antibodies, and DNA was stained with 7-amino-actinomycin D (7-AAD). The percentage of cells incorporating BrdU was determined using the FlowJo software program (v.8.1). All the flow cytometry acquisitions were done using a BD LSR flow cytometer gated on the YFP-positive population. When indicated, aphidicolin (1.7 μg/ml) was added to the transfected cells for 2 h before the addition of BrdU.

Colony formation assay. C33A cells (~1.2×10^6) were transfected with 1.5 μg of the indicated plasmids in a 6-well plate. Twenty-four hours posttransfection, the cells were trypsinized, and 2/5 of the cells were plated onto 100-mm-diameter plates in fresh medium. Twenty-four hours later, bleomycin-containing medium was added and changed every 3 to 4 days for a period of about 3 weeks or until fully resistant cells were selected. Colonies were then fixed in cold methanol for 10 min and stained for 2 min at room temperature with methylene blue (1% [wt/vol] in 60% methanol-H_2O).

NMR spectroscopy. Peptides comprising amino acids 1 to 40 (N40) or 1 to 83 (N83) of HPV31 E1, containing the C14A and C14A/C54S substitutions, respectively, were produced as ^{15}N-labeled GST fusion proteins in *Escherichia coli* BL21(DE3) (Novagen) as previously described (16). The GST moiety was cleaved with thrombin, and the peptides were purified to homogeneity using anion-exchange fast protein liquid chromatography (FPLC) (Q-Sepharose). The peptides were lyophilized and resuspended at 400 μM in 20 mM phosphate buffer, pH 6.5, containing 90% H_2O and 10% D_2O. NMR spectra were collected at 300 K on a Varian Unity Inova 600-MHz NMR spectrometer equipped with a *z* pulsed-field gradient unit and a triple resonance probe.

GST pulldown assays. GST fusion proteins were produced in *E. coli* BL21(DE3) (Novagen) as previously described (55). 3F-p80 truncations were expressed using the TnT T7 Quick Coupled Transcription/Translation System (Promega catalog no. L1170) with unlabeled methionine. Purified GST and GST-E1 were immobilized on glutathione beads (GE Healthcare) at a concentration of 4 mg/ml. GST pulldown assays were performed as described elsewhere (61), but with a modified buffer A (10 mM Tris, pH 8, 1 M NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol [DTT], and 10% glycerol) and binding buffer (10 mM Tris, pH 8, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol). Bound proteins were eluted with binding buffer containing 20 mM reduced glutathione and analyzed by Western blotting.

RESULTS

The p80-binding domain of E1 is required for efficient viral DNA replication. We previously reported three double amino acid substitutions in the p80-binding domain of HPV31 E1 (W17A/F18A, V20A/E21A, and V23A/I24A) (Fig. 1A) that abrogate its interaction with p80 and its ability to maintain the viral episome in immortalized keratinocytes (12). These three substitutions were also found to reduce transient HPV31 DNA replication, with little to no effect on the ability of E1 to bind to DNA *in vitro* and to localize to the nucleus and interact with Cdk2 *in vivo*, indicating that they do not grossly alter the structure of E1 (12, 20). Here, we used our recently described luciferase-based DNA replication assay to further characterize and quantify the DNA replication defect imposed by the WF, VE, and VI substitutions. First, we investigated if this defect could be rescued by overexpression of the mutant E1 proteins, as could be expected if the p80-binding substitutions reduce the expression and/or stability of E1, for example. To do so, increasing amounts of expression vector for wild-type (WT) or the p80-binding-defective E1 proteins, tagged with a triple Flag epitope (3F-E1), were transfected into C33A cells, along with a constant amount of E2 expression vector and of a third plasmid encoding the origin in *cis* of a firefly luciferase reporter gene. A fourth plasmid encoding *Renilla* luciferase was used as an internal control to normalize for differences in transfection efficiency and cell viability. As expected, the DNA replication signal obtained with WT E1 increased in a dose-dependent manner, eventually reaching a maximum (Fig. 1B). The three E1 mutant proteins also supported DNA replication in a dose-dependent manner, but their maximum levels were consistently lower than that achieved with WT E1. While WF E1 reached a maximum at approximately 25 to 35% of the WT, overexpression of the VE and VI mutant proteins led to higher values (~50 to 60% of WT levels). These results indicate that the replication defect of WF E1 is more pronounced than that observed with the other two mutant proteins. These findings are in complete agreement with results from coimmunoprecipitation experiments indicating that the VE and VI E1 proteins retain weak residual p80-binding activity while WF E1 is completely defective (data not shown).

In addition to these three amino acid substitutions, we previously reported three N-terminal deletions that partially or completely remove the p80-binding domain of E1 (C20, C30, and C40) (Fig. 1A) and reduce its capacity to support viral DNA replication (41). Similarly to what was described above, we tested if overexpression of these truncated E1 proteins could rescue their DNA replication defect but found that none could reach a plateau greater than 30% of WT levels (Fig. 1B). These defects are similar to that observed with WF E1. Western blotting was used to confirm that all E1 proteins tested were expressed at comparable levels and thus that the lower DNA replication activities of the p80-binding-defective E1 mutant proteins are not due to lower expression (Fig. 1C). Collectively, these results indicate that mutation or deletion of the p80-binding domain of E1 reduces transient DNA replication by more than 70% and cannot be compensated for by overexpression of the protein. As such, they provide further evidence that the p80-binding domain of E1 is required for efficient viral DNA replication.

The p80-binding domain of E1 is dispensable for the assembly of the E1-E2-ori complex. HPV DNA replication is initiated by the cooperative binding of E1 and E2 to the viral origin (ori). Assembly of the E1-E2-ori complex requires the DNA-binding activities of E1 and E2, as well as a critical protein-protein interaction between the two proteins. To test if the DNA replication defects of the p80-binding-defective E1 were due to impaired association with E2, these mutant proteins were tested for E2 binding in coimmunoprecipitation assays. C33A cells were cotransfected with an expression plasmid for RFP-E2, together with a vector encoding 3F-E1, either the WT, WF mutant, or C40-truncated protein. For later studies, a mutant E1 protein devoid of ATPase activity (K463A) was also included. The results presented in Fig. 2A show that all three mutant proteins are as competent as WT E1 for binding to E2. Thus, the p80-binding domain of E1 is not required for interaction with E2.

Next, we sought to determine if the p80-binding domain was needed for assembly of the E1-E2-ori complex *in vivo*. To do so, we made use of the previous observation that the ATPase-negative K463A E1 has a dominant-negative effect on HPV DNA replication (41). This mutant protein retains the ability to interact with E2, as shown by coimmunoprecipitation (Fig. 2A). As such, K463A E1 can compete with WT E1 for assembly into the E1-E2-ori complex; this competition is very efficient, as K463A E1 inhibits DNA replication by 50% when expressed in a 1:1 ratio relative to WT E1. Based on these findings, we rationalized that if the WF substitution and C40 truncation affect the formation of the E1-E2-ori complex, they should attenuate the dominant-negative effect of K463A E1. This possibility was addressed by transfecting cells with plasmids encoding WT E1, E2, and the origin (the plasmid mixture normally used in the HPV DNA replication) assay, together with increasing concentrations of expression vector for K463A E1 or for the same mutant E1 in combination with the

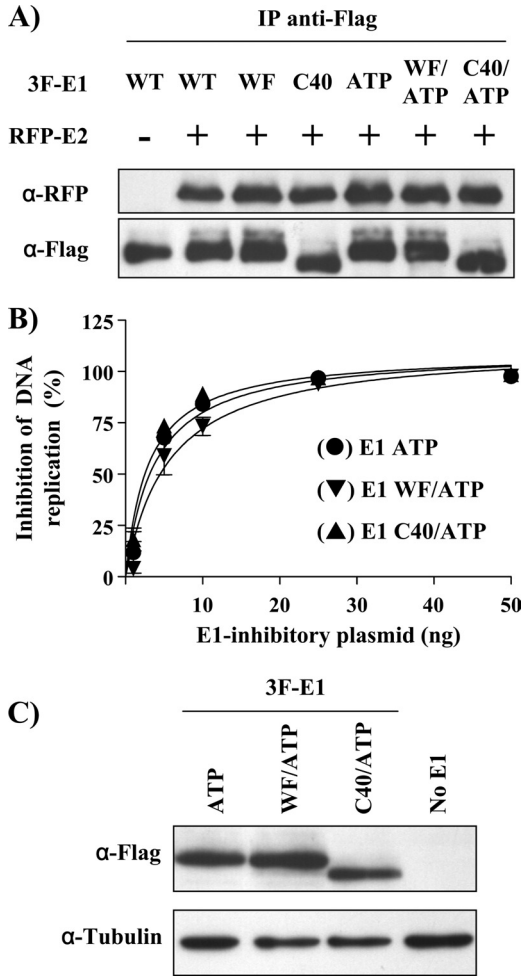

FIG 2 The p80-binding domain of E1 is dispensable for the assembly of the E1-E2-ori complex. (A) Interaction of HPV31 E1 and E2 *in vivo*. 3F-tagged E1 WT, the ATPase mutant K463A (ATP), or p80-binding mutants (WF and C40), alone or in combination, were cotransfected in C33A with RFP-tagged E2. Cells were harvested 48 h posttransfection, and whole-cell extracts were submitted to immunoprecipitation using an anti-Flag antibody. The immunoprecipitates were analyzed by Western blotting using anti-RFP and anti-Flag antibodies. (B) Inhibition of HPV DNA replication by the ATPase-negative K463A E1 and derivatives containing the WF or C40 mutations. DNA replication was performed using 5 ng of WT E1 expression plasmid and the indicated amounts of E1-inhibitory plasmid. The error bars represent the standard deviations of duplicate values. The results are representative of at least three independent experiments. (C) Western blot showing the expression of mutant E1 proteins. β-Tubulin was used as a loading control.

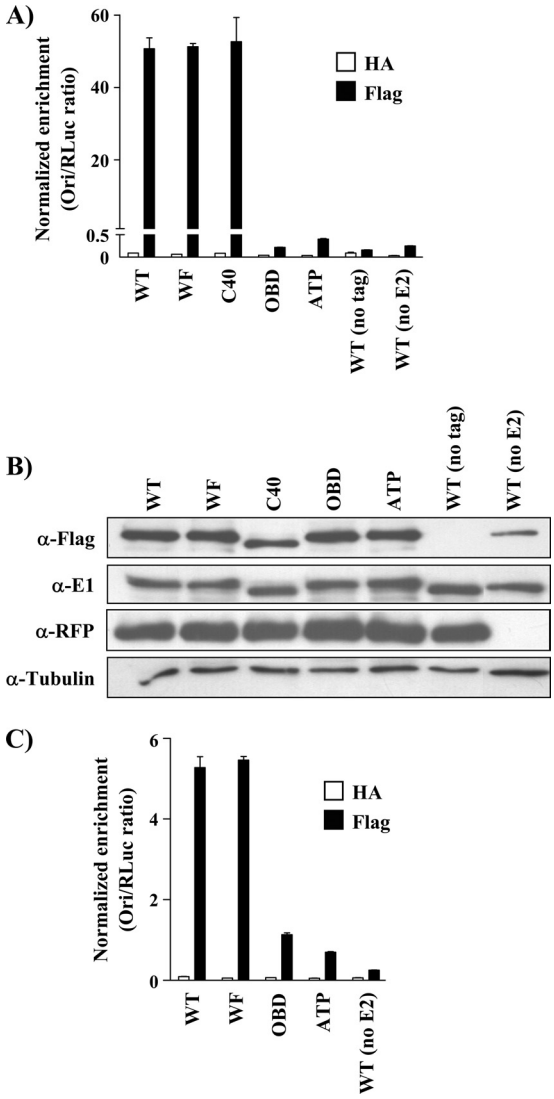

FIG 3 The p80-binding domain is not required for the stable assembly of E1 at the viral origin. (A) ChIP assays were performed in C33A cells transfected with 3F-E1 (WT or mutant), RFP-E2, and an origin plasmid (pFLORI31). Equal quantities of cell lysates were immunoprecipitated with anti-Flag (E1) or anti-HA (nonspecific) antibodies. The results of the ori enrichment levels determined by qPCR are shown after normalization to input DNA, using the internal control pRL (RLuc). Each value is the average of at least two replicates, with the standard deviations presented as error bars. (B) Western blot showing the expression of E1 mutant proteins detected by either the anti-Flag or anti-E1 antibody and of RFP-E2 using an anti-RFP antibody. β-Tubulin was used as a loading control. (C) C33A cells were transfected with plasmids expressing 3F-E1 (WT or mutant) and RFP-E2 and one containing the origin (pFLORI31). The cells were treated with 5 μg/ml of aphidicolin 4 h posttransfection, and ChIP assays were performed on the lysates using anti-Flag and anti-HA antibodies. The results of the ori enrichment levels determined by qPCR are shown after normalization to input DNA using the internal control pRL (RLuc). Each value is the average of at least two replicates, with the standard deviations presented as error bars.

p80-binding mutations WF and C40. The double-mutant proteins WF/K463A and C40/K463A were found to inhibit viral DNA replication as efficiently as K463A E1 (50% inhibition at a 1:1 ratio relative to WT E1) (Fig. 2B). All three mutant E1 proteins were expressed at comparable levels as determined by Western blotting (Fig. 2C). These results provide evidence that the p80-binding domain of E1 is not required for its assembly with E2 at the origin *in vivo*.

The p80-binding domain is not required for the stable assembly of E1 at the viral origin. To directly monitor the binding of WT and mutant E1 proteins to the viral origin of replication *in vivo*, we developed a ChIP assay using cells transiently expressing E1 and E2 and containing a plasmid encoding the origin, under conditions similar to those used in the DNA replication assay. E1 was immunoprecipitated using an anti-Flag antibody, and the amount of coprecipitated origin plasmid was measured by qPCR. As shown in Fig. 3A, immunoprecipitation of 3F-E1 with the anti-

Flag antibody led to significant enrichment of the ori plasmid, which was not detected when an isotype-matched irrelevant antibody (anti-HA) was used as a control. To further confirm the specificity of the immunoprecipitation, the experiment was repeated with an untagged E1 protein (WT [no tag]), and no enrichment of the viral origin was observed. As additional negative controls, we performed the ChIP assay with a mutant E1 protein defective for DNA binding (KR265–267AA [OBD]) and, separately, with WT E1 in the absence of E2. In both cases, little to no enrichment of the ori plasmid was observed, confirming that the presence of E1 at the origin requires its DNA-binding activity and interaction with E2. Interestingly, we also observed that the ATPase-negative mutant K463A E1 did not coprecipitate the origin. This result indicates that the ATP-binding site is required for the stable association of E1 with the origin *in vivo*, similar to what we previously observed *in vitro* (60). The requirement for a functional ATP-binding site raises the possibility that the ori-bound E1 that is immunoprecipitated in our ChIP assay is oligomeric and, by extension, that the binding of monomeric E1 to DNA may be too weak and/or transient to be detected. Interestingly, when using the p80-binding-defective mutant WF and C40 E1, enrichment levels similar to those of WT E1 were obtained, suggesting that the p80-binding domain is not required for the assembly of E1 into a stable complex at the origin *in vivo*. The expression levels of the different E1 proteins were found to be comparable, as shown in Fig. 3B.

A potential caveat in the above-mentioned experiments is the fact that the ori plasmid can be replicated in cells expressing WT E1 and E2 but not in control cells expressing either the OBD or ATPase mutant E1 or lacking E2. Because replication of the ori plasmid could potentially contribute to its levels of enrichment in the ChIP experiments, we were careful to perform these assays 24 h posttransfection, when viral DNA replication is minimal, as we previously reported (20) and confirmed here by quantification of the total amount of input and replicated DNA (data not shown). However, to definitively rule out an effect of ori plasmid replication, we repeated the ChIP experiments in cells treated with aphidicolin, a specific inhibitor of DNA polymerases α and δ. Aphidicolin treatment reduced by 10-fold the total amount of ori plasmid extracted from the cells prior to immunoprecipitation compared to untreated cells, indicating that it efficiently inhibited ori plasmid replication (data not shown). Aphidicolin also led to an approximately 10-fold decrease in the levels of ori plasmid coprecipitated with WT E1 and the WF mutant protein (Fig. 3C). Importantly, however, both E1 proteins were bound to the origin to similar extents and substantially above background levels. Altogether, these results provide further evidence that the p80-binding domain of E1 is not required for the E2-dependent binding of E1 at the origin. From these findings and those presented above, we surmise that the p80-binding domain of E1 is required for viral DNA replication at a step following assembly of the E1-E2-ori complex and for stable binding of E1 to the origin.

p80 is relocalized to nuclear foci in an E1- and E2-dependent manner. It is now well established that E1 is relocalized to discrete subnuclear domains when coexpressed with E2 in cells (19, 49, 59). Although the exact function of these nuclear foci remains uncertain, they were previously suggested to be the precursors of DNA replication centers (49, 59). As expected, our GFP-E1 fusion protein also accumulates in nuclear foci, in the presence of E2, where the two viral proteins colocalize (Fig. 4A). E1 mutant proteins defective for p80 binding also accumulate in similar foci (Fig. 4B and data not shown), suggesting that their DNA replication defect is not due to their mislocalization within the nucleus. Since our previous observations pointed to a role for the E1-p80 interaction during HPV DNA replication, we investigated if p80 is also present in these nuclear domains. When transiently expressed in C33A cells together with GFP-E1 and 3F-E2, RFP-p80 colocalized with E1 in nuclear foci (Fig. 4B). In contrast, RFP-p80 remained cytoplasmic in cells expressing any of the E1 mutants defective for p80 binding. These results indicate that p80 is recruited from the cytoplasm to nuclear foci through its association with E1, consistent with its role in viral DNA replication.

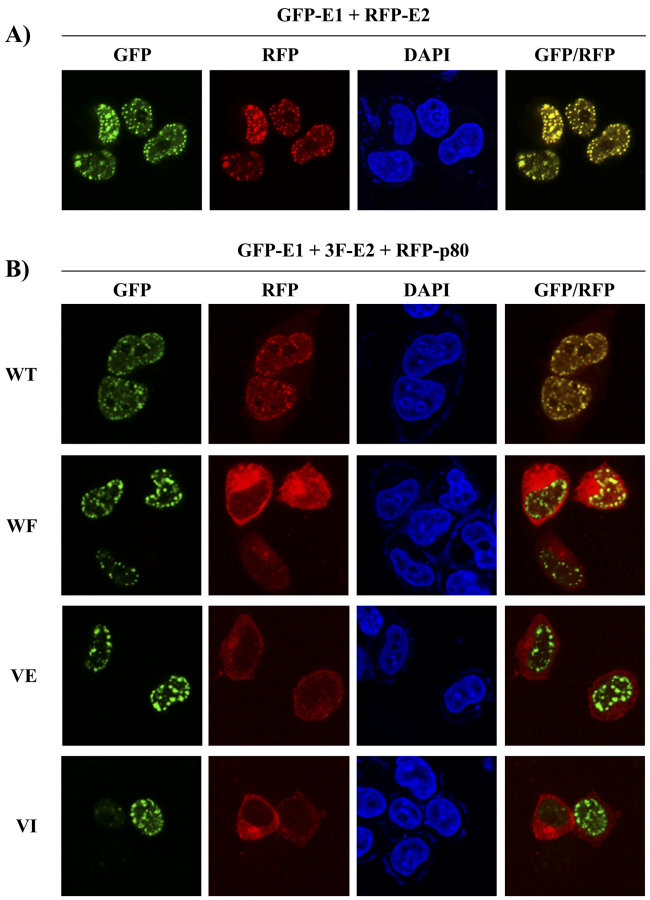

FIG 4 p80 is relocalized to nuclear foci in an E1- and E2-dependent manner. (A) Fluorescence confocal microscopy images showing the subcellular localization of GFP-E1 and RFP-E2. (B) Subcellular localization of RFP-p80 when coexpressed with 3F-E2 and WT or p80-binding-defective GFP-E1. Nuclei were stained with DAPI.

Endogenous p80 interacts with E1 and is recruited to the viral origin *in vivo*. All of our previous studies on p80 were performed with overexpressed and epitope-tagged versions of the protein due to the lack of a good antibody against endogenous p80. This prompted us to develop a rabbit polyclonal antibody against the C-terminal domain of p80 (see Materials and Methods) and to test by coimmunoprecipitation if E1 could interact with endogenous p80. To do so, 3F-E1, either WT or p80-binding mutant derivatives, were transiently expressed in C33A cells and immunoprecipitated 48 h posttransfection. Western blot analysis of the immunoprecipitate showed that WT E1 does interact with

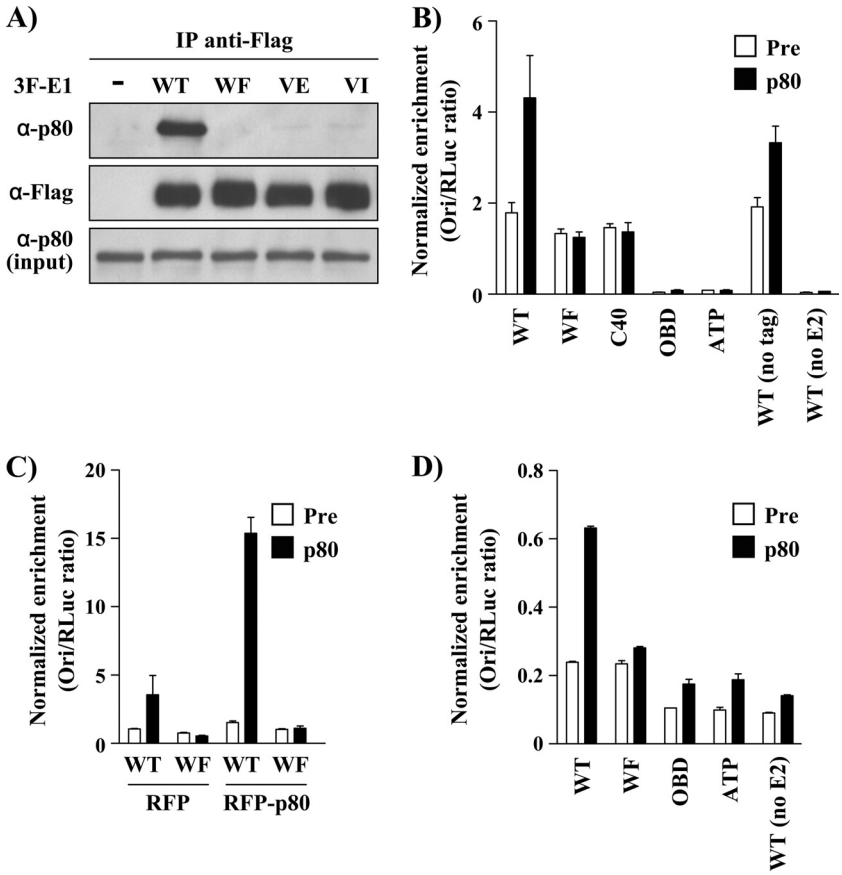

FIG 5 Endogenous p80 interacts with E1 and is recruited to the viral origin *in vivo*. (A) Coimmunoprecipitation of endogenous p80 with WT or p80-binding-defective 3F-E1 (WF, VE, and VI). C33A cells were transfected with the indicated E1 expression vectors and harvested 48 h posttransfection, and whole-cell extracts were subjected to immunoprecipitation using an anti-Flag antibody. The immunoprecipitates were analyzed using an anti-Flag antibody or a p80 antiserum. (B) ChIP assays were performed in C33A cells cotransfected with plasmids encoding 3F-E1 (WT or mutant) and RFP-E2 and one containing the origin (pFLORI31). Equal quantities of cell lysates were immunoprecipitated with a rabbit antiserum raised against p80 or the preimmune serum (Pre). (C) ChIP assays were repeated with WT and WF E1 under conditions similar to those described for panel B but in the presence of overexpressed RFP-p80. (D) C33A cells were transfected as described for panel B but were treated with 5 μg/ml of aphidicolin 4 h posttransfection. ChIP assays were performed on the lysates using a p80 antiserum or preimmune serum. For all ChIP experiments, the results of the ori enrichment levels determined by qPCR are shown after normalization to input DNA, using the internal control pRL (RLuc). Each value is the average of at least two replicates, with the standard deviations presented as error bars.

endogenous p80, in contrast to the three previously described E1 mutant proteins (WF, VE, and VI), which were used as controls (Fig. 5A). In addition to confirming the association of E1 with endogenous p80, these results also demonstrate the specificity of the anti-p80 antiserum.

To determine if p80 could be found in association with viral DNA, we next repeated the ChIP experiments using the p80-specific rabbit antiserum or the corresponding nonspecific preimmune serum as a control. As hypothesized, the ori plasmid was enriched in p80 immunoprecipitates from cells expressing WT E1 and E2 (Fig. 5B). This enrichment was specific and dependent upon the interaction of E1 with p80, as it was not observed with the preimmune serum or when using the E1 WF or C40 mutant protein. The use of E1 mutant proteins defective for DNA binding or ATPase activity, as well as conditions without E2, suggested that this enrichment was also dependent on the stable assembly of E1 at the origin. Not surprisingly, pulldown of p80 also led to a significant enrichment of the origin in cells transfected with vectors encoding E2 and untagged E1. We consistently observed throughout these studies that the levels of ori DNA coprecipitated by the anti-p80 antibody were much lower than those obtained by immunoprecipitation of E1, perhaps because the endogenous levels of p80 are limiting compared to those of overexpressed E1. In support of this suggestion, we found that overexpression of p80 (achieved by transfection of a p80 expression plasmid) greatly increased its presence at the origin (Fig. 5C). This greater association with the origin was specific, as it was not observed when the WF mutant E1 was used.

To rule out any artifactual bias due to ori plasmid replication, the ChIP assays were repeated in the presence of aphidicolin. Under these conditions, immunoprecipitation of p80 also led to enrichment of the ori plasmid when the latter was cotransfected with WT E1 but not with the WF mutant protein (Fig. 5D). These results demonstrate that the recruitment of p80 to the viral origin is specific and does not require viral DNA replication. From these findings, we conclude that endogenous p80 is recruited to the viral origin by E1 and E2 during the initiation of DNA replication.

Inhibition of the E1-p80 interaction by N40, a short peptide spanning the p80-binding domain of HPV31 E1. The results presented above strongly suggest a role for the E1-p80 interaction in

HPV DNA replication. To more directly assess the requirement for p80 in viral DNA replication, we sought to downregulate p80 using RNA interference. However, in both cell lines tested, C33A and HeLa, depletion of p80 with short hairpin RNA (shRNA) was found to significantly reduce cellular proliferation and viability, as well as cell cycle progression (data not shown). The need for p80 for the growth of noninfected cells is intriguing and is the subject of ongoing experiments, but unfortunately, it limits the study of its function in viral DNA replication, as inhibition of cell cycle progression almost inevitably results in abrogation of viral and host DNA synthesis. Accordingly, we confirmed that shRNA-mediated depletion of p80 reduces HPV DNA replication, but this effect is likely indirect (data not shown). To overcome this limitation, we aimed to specifically inhibit the E1-p80 interaction by overexpressing a short E1-derived peptide spanning the p80-binding domain. To do so, we constructed a plasmid encoding the first 40 amino acids of E1 fused at their C terminus to YFP (referred to as N40) (Fig. 6A). Similar N40 mutant peptides containing the p80-binding substitutions, WF, VE, and VI, were also constructed as specificity controls. First, coimmunoprecipitation experiments were used to confirm that the WT N40 peptide is sufficient for interaction with RFP-p80, in contrast to the three mutant peptides (Fig. 6B). We repeatedly observed that the VE N40 peptide migrates faster in SDS-PAGE than the WT and other mutant peptides, perhaps because removal of the negatively charged aspartate increases SDS binding on the VE peptide. We then investigated if overexpression of N40 could prevent the interaction between full-length E1 and p80 *in vivo*. Coimmunoprecipitation experiments showed that the expression of increasing amounts of N40 could efficiently inhibit the binding of full-length 3F-E1 to RFP-p80 in a dose-dependent manner (Fig. 6C). In contrast, expression of any of the three N40 mutant peptides, at the highest concentration tested, had no effect on formation of the full-length E1-p80 complex. These results indicate that N40 can efficiently compete with full-length E1 for binding to p80 and, furthermore, that it can be used as a tool to specifically antagonize the E1-p80 interaction *in vivo*.

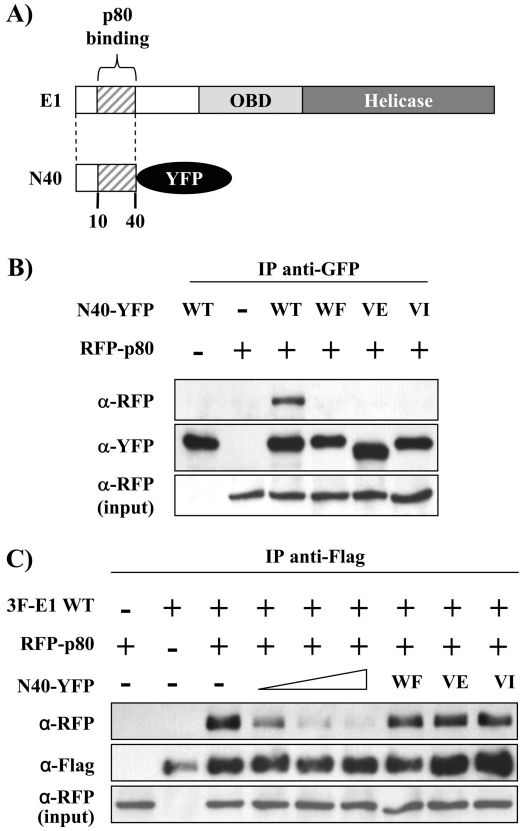

FIG 6 Inhibition of the E1-p80 interaction by N40. (A) Schematic representation of HPV31 E1 and of the N40 peptide. E1 is subdivided into its three functional domains: the N-terminal regulatory domain (white), the OBD (light gray), and the helicase domain (Helicase; dark gray). N40 encompasses amino acids 1 to 40 of HPV31 E1, which includes the p80-binding motif (hatched box), and is fused C terminally to the Venus YFP. (B) Coimmunoprecipitation of RFP-p80 with WT or p80-binding-defective N40 (WF, VE, and VI) fused to YFP (N40-YFP). C33A cells were cotransfected with the indicated N40-YFP and RFP-p80 expression vectors and harvested 48 h posttransfection, and whole-cell extracts were subjected to immunoprecipitation using anti-GFP antibodies that also recognize YFP. (C) Inhibitory effect of N40-YFP on the coimmunoprecipitation of RFP-p80 with full-length WT 3F-E1. C33A cells were cotransfected with the indicated E1 and p80 expression vectors, as well as with increasing amounts of WT N40 expression plasmid, and harvested 48 h posttransfection. The WF, VE, and VI mutant peptides were also tested as controls at the highest level of expression vector. Whole-cell extracts were subjected to immunoprecipitation using an anti-Flag antibody.

N40 prevents the E1- and E2-dependent recruitment of p80 to the origin and inhibits viral DNA replication. Next, we used the N40 peptide to further probe the requirement for the E1-p80 interaction in viral DNA replication. First, we performed ChIP assays to determine if N40 was able to prevent the recruitment of p80, by E1 and E2, to the viral origin. We observed that N40 had no effect on the ability of E1 to associate with the origin (Fig. 7A). In contrast, the results shown in Fig. 7B indicated that N40, but none of the three N40 mutant peptides or YFP alone (ctl), could efficiently inhibit the recruitment of p80 to the origin. Thus, N40 can be used to exclude p80 from the viral DNA replication complex.

We next tested the effect of N40 on viral DNA replication *per se* by transfecting increasing amounts of N40 expression vector in the luciferase-based DNA replication assay. Satisfyingly, N40 could efficiently inhibit viral DNA replication in a dose-dependent manner (Fig. 7C). This effect was specific, as neither YFP alone (ctl) nor any of the three N40 mutant peptides was inhibitory. In several experiments, we noted that WT N40 could not reduce viral DNA replication by more than 75%, consistent with the previous observation that p80-binding-defective E1 mutant proteins still support viral DNA replication at approximately 30% of WT levels. From these results, we could also determine that N40 inhibits 50% of the DNA replication signal when its expression plasmid is transfected in an ~2:1 ratio relative to that of full-length E1, thus providing a measure of its potency. As an important control for the specificity of N40, we verified that it has no effect on the expression of the luciferase reporter gene in the absence of the viral proteins (i.e., in the absence of ori plasmid replication) (Fig. 7D). Coimmunoprecipitation experiments also indicated that N40 does not prevent the interaction between E1 and E2 (data not shown). Altogether, these results indicate that overexpression of the N40 peptide inhibits HPV DNA replication in a manner that recapitulates the effect of the p80-binding substitutions in E1 and, as such, provides additional evidence that p80 is required for efficient HPV DNA replication.

N40 expression has little to no effect on cell cycle progression and DNA synthesis. HPV DNA replication takes place during the

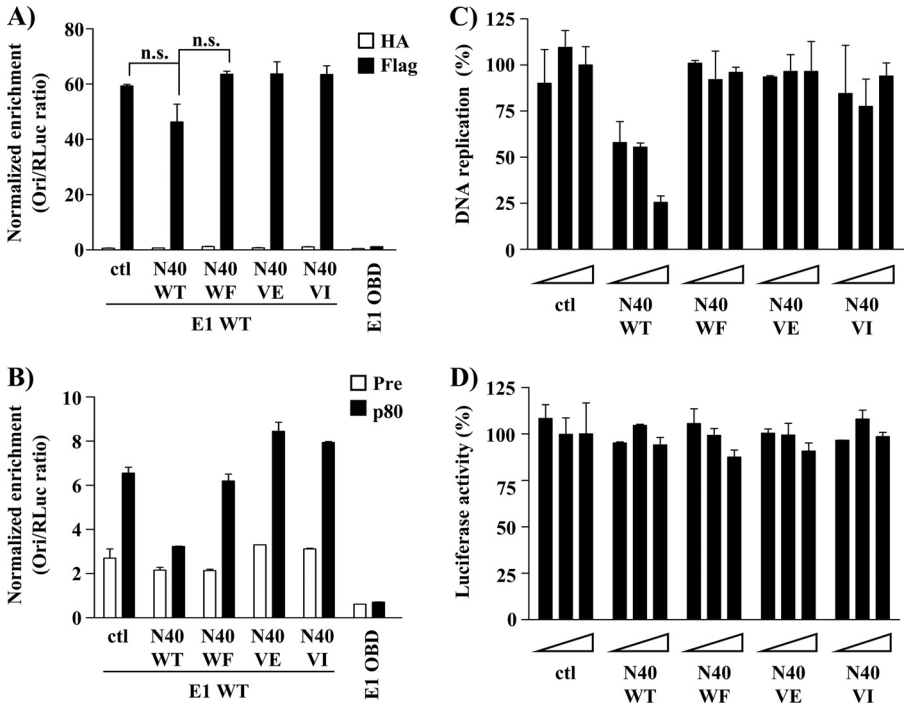

FIG 7 N40 prevents the recruitment of p80 to the origin and inhibits viral DNA replication. (A and B) C33A cells were transfected with 3F-E1, RFP-E2, and N40 (WT or mutant) expression vectors, together with an origin plasmid. ChIP assays were performed on the lysates using anti-Flag and anti-HA antibodies (A) or with a p80 antiserum or preimmune serum (B). The results of the ori enrichment levels determined by qPCR are shown after normalization to input DNA, using the internal control pRL (RLuc). Each value is the average of at least two replicates, with the standard deviations presented as error bars. An unpaired two-tailed Student's *t* test was used to determine if differences in enrichment levels were significant. Differences with a *P* value greater than 0.05 were deemed to be nonsignificant (n.s.) and are indicated. (C) DNA replication levels in cells expressing a gradient of WT or mutant N40 peptide (1, 10, and 50 ng of N40-YFP expression vector). Cells cotransfected with the empty YFP vector were used as a negative control (ctl). DNA replication activity is reported as a percentage of the signal obtained at the largest amount of YFP control vector. The error bars represent the standard deviations of duplicate values. The results are representative of at least three independent experiments. (D) Effect of N40-YFP on expression of cytomegalovirus (CMV)-FLuc in the absence of the viral proteins. The luciferase signal is reported as a percentage of the signal obtained with the largest amount of empty YFP control vector. The error bars represent the standard deviations of duplicate values. The results are representative of at least three independent experiments.

S phase of the cell cycle, and we have previously shown that interfering with cellular proliferation, with the use of chemical inhibitors or shRNA against p80, affects viral DNA replication indirectly (reference 20 and data not shown). This prompted us to investigate if the inhibitory effect of N40 on viral DNA replication was due to an indirect effect on cell cycle progression. We observed by fluorescence-activated cell sorter (FACS) analysis that transient overexpression of N40 had no major effect on the cell cycle distribution of an asynchronously growing cell population, except for a slight accumulation of cells in G_2/M that was not noticed in cells expressing the N40 WF mutant peptide or YFP alone (Fig. 8A). Note that only YFP-positive cells were analyzed (i.e., gated) in these experiments. To determine if N40-expressing cells could progress normally through S phase, they were synchronized in G_1/S with the chemical inhibitor mimosine and released in fresh medium lacking mimosine but containing nocodazole to prevent them from progressing past G_2/M. The results shown in Fig. 8A demonstrate that N40-expressing cells are able to resume cell cycle progression and accumulate in G_2/M as efficiently as control cells.

Since host DNA replication factors are absolutely essential for HPV DNA replication, we next tested if the N40 peptide had any effect on cellular DNA synthesis. This was done by measuring BrdU incorporation as a function of cell cycle progression, the latter being determined by staining cellular DNA and measuring its content with 7-AAD. Once again, no major effect on cell cycle distribution was observed in N40-expressing cells, as determined by their DNA-staining profile (Fig. 8B). Furthermore, N40-expressing cells incorporated BrdU to the same extent as cells expressing the N40 WF mutant peptide or YFP alone. Treatment with aphidicolin was used as a control to ensure that BrdU incorporation was indeed the result of DNA synthesis. For each condition, the percentage of cells incorporating BrdU was determined from three independent experiments and is reported in Fig. 8B. This quantification confirmed that cells expressing the WT or WF N40 mutant peptide incorporate BrdU as efficiently as the control YFP-expressing cells. Collectively, these results indicate that N40 expression has little to no effect on cell cycle progression and cellular DNA synthesis. As such, they suggest that the inhibitory effect of N40 on viral DNA replication is not indirect.

HPV DNA replication is attenuated in N40-expressing stable cell lines. The results presented above suggested that N40 does not affect cellular proliferation. To extend these findings, we performed colony formation assays and confirmed that cells stably expressing the N40 peptide, either the WT or mutant versions, are viable (Fig. 9A). These cells, as well as YFP-expressing control cells, were successfully established as stable lines that remained proliferative for several passages without any detectable defect while maintaining continuous expression of N40 (Fig. 9B). These

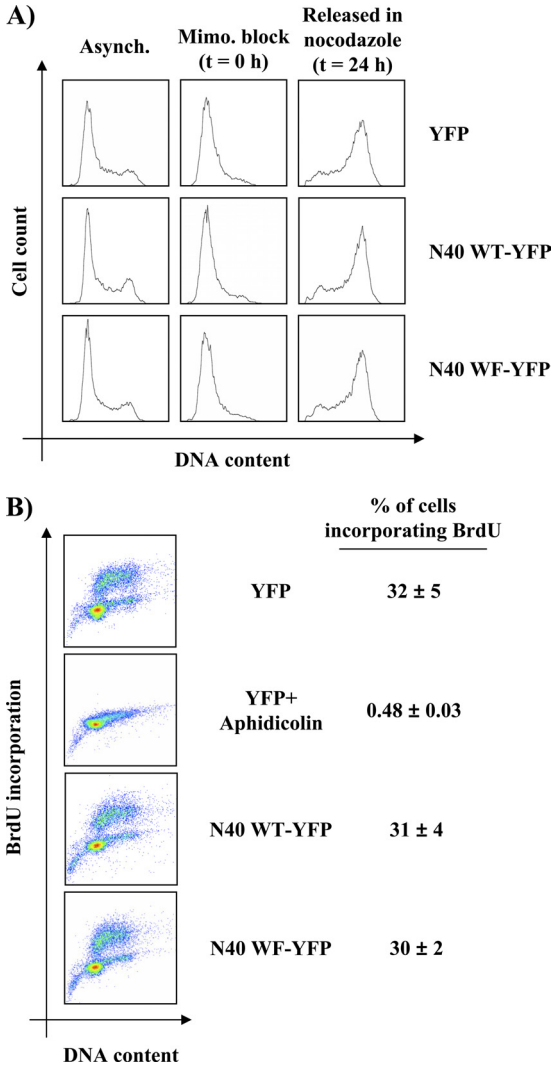

FIG 8 N40 expression has little to no effect on cell cycle progression and DNA synthesis. (A) Cell cycle analysis of C33A cells transiently expressing YFP alone or YFP-tagged WT or WF N40. The DNA content was determined by Hoechst staining and flow cytometry. For each transfection, the cells were either grown asynchronously (Asynch.) or synchronized with mimosine for 24 h (Mimo. block) and then released in nocodazole for another 24 h (Released in nocodazole). (B) BrdU incorporation of C33A cells transiently expressing YFP alone or YFP-tagged WT or WF N40. Cells were pulsed for 1 h with BrdU 48 h posttransfection and were then dually labeled for BrdU and DNA using an APC-coupled antibody and 7-AAD staining, respectively. For each sample, BrdU incorporation is graphed as a function of the DNA content. BrdU incorporation of cells treated with aphidicolin is shown as a negative control. Quantification of the percentage of cells incorporating BrdU, from three independent experiments, is shown on the right (average ± standard deviation).

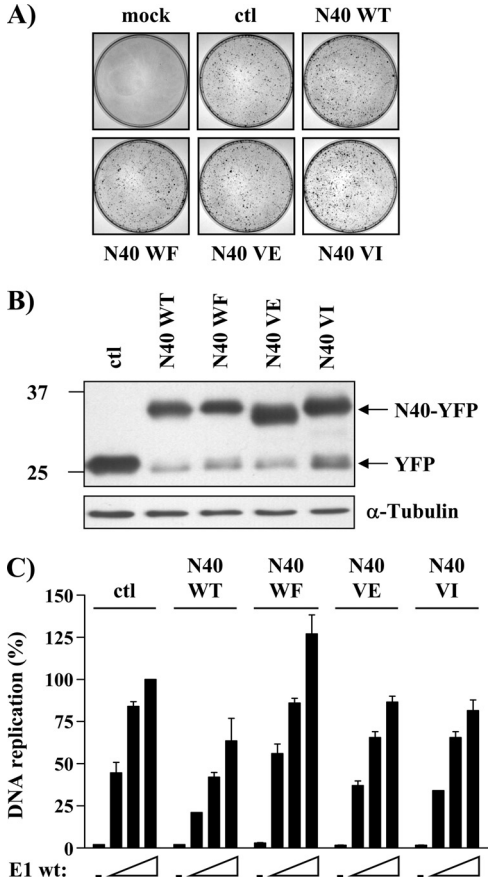

FIG 9 HPV DNA replication is attenuated in N40-expressing stable cell lines. (A) Colony formation assay. C33A cells were transfected with the indicated expression vectors and then selected for approximately 3 weeks in bleomycin-containing media. Drug-resistant colonies were fixed with methanol and stained with methylene blue. (B) Western blot showing the expression of the N40, WT, or p80-binding mutant protein (WF, VE, and VI), in the established stable cell lines. β-Tubulin was used as a loading control. (C) The DNA replication activities of WT E1 were tested by transfecting three different quantities of E1 expression vector (2.5, 5, and 10 ng) in the indicated N40 stable cell lines. DNA replication was also measured in a control cell line expressing YFP alone (ctl). DNA replication activity is reported as a percentage of the signal obtained with 10 ng of WT E1 expression vector in the control cell line. The error bars represent the standard deviations of duplicate values. The results are representative of at least three independent experiments.

stable cell lines were then tested for their capacity to support transient HPV DNA replication. As anticipated, the N40 WT stable cell line was able to support lower levels of viral DNA replication than the three N40 mutant lines or the control YFP-expressing cell line (Fig. 9C). In three independent experiments, each performed with three different amounts of E1 expression vector, the levels of viral DNA replication were reduced by 30 to 50% in N40-YFP-expressing cells compared to YFP-expressing control cells (two-way ANOVA; $P < 0.005$). In contrast, no statistically significant reduction in viral DNA replication was observed in cell lines expressing the three N40-YFP mutant peptides. These results provide further evidence that the inhibition of HPV DNA replication by N40 is not caused by an indirect effect on cellular proliferation and indicate that long-term expression of N40 is not deleterious to cell viability. Together, the specificity of the N40 peptide for p80 and its lack of cytotoxicity suggest that the E1-p80 interaction may be a valuable antiviral target (see Discussion).

The N40 p80-binding peptide is intrinsically disordered. We previously reported that the first 83 amino acids of HPV31 E1 (N83), which include the p80-binding domain, are predicted to constitute an intrinsically disordered domain (IDD) (41). To test this prediction, we set out to analyze the structures of the E1-derived N40 and N83 peptides by NMR. However, this region of HPV31 E1 is not ideal for NMR analysis, as it contains two cysteine residues, at positions 14 and 54, that could possibly become oxidized and complicate the interpretation of the data. To circum-

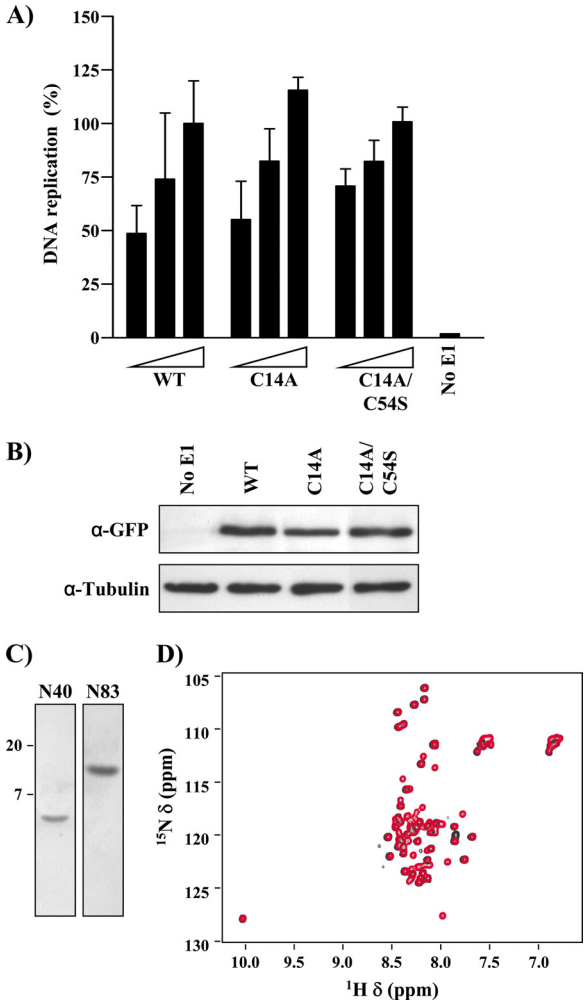

FIG 10 The N40 p80-binding peptide is intrinsically disordered. (A) The DNA replication activities of WT E1 were compared to those of the C14A or C14A/C54S E1 mutant by transfecting three different quantities of E1 expression vector (1.25, 2.5, and 5 ng). C33A cells transfected without an E1 plasmid were used as a negative control (No E1). Replication activity is reported as a percentage of the signal obtained with 5 ng of WT E1. The error bars represent the standard deviations of duplicate values. The results are representative of at least three independent experiments. (B) Anti-GFP Western blot showing the expression of the E1 mutant proteins in C33A cells. β-Tubulin was used as a loading control. (C) Coomassie gel showing the purified ^{15}N-labeled peptides. (D) Overlay of the 2D ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled E1 N-terminal peptides N40 (aa 1 to 40; black) and N83 (aa 1 to 83; red).

vent this problem, the cysteines were changed to alanine and serine, respectively (C14A and C54S). To test if these substitutions had any effect on the function of E1, they were introduced in the context of full-length YFP-E1, and the resulting mutant proteins were tested for their ability to support transient DNA replication. Satisfyingly, both substitutions, either alone or in combination, had little to no effect on viral DNA replication (Fig. 10A) and E1 levels (Fig. 10B). We then produced ^{15}N-labeled versions of the N40 and N83 peptides (containing the cysteine substitutions) as GST fusions in bacteria, purified the proteins, and removed the GST moiety by thrombin cleavage. The purified untagged peptides are shown in Fig. 10C. Two-dimensional (2D) ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) spectra were then acquired for both peptides (Fig. 10D). The limited chemical shift dispersion of the signals strongly suggests the absence of either secondary or tertiary structural elements. Moreover, the nearly perfect superposition of the two spectra shows that amino acids 40 to 83 do not influence the folding of N40. The absence of secondary structural elements was further verified by the absence of medium or long-range nuclear Overhauser effects (NOEs) in a three-dimensional ^{15}N nuclear Overhauser effect spectroscopy (NOESY) experiment (data not shown). Collectively, these results provide direct evidence that N40 and N83 are disordered in solution. They also support our previous suggestion that the N-terminal region of E1 constitutes an IDD, which could fold upon interaction with binding partners, such as p80.

E1 interacts with amino acids 1 to 573 of p80. To complete the characterization of the E1-p80 interaction, we mapped the domain of p80 required for E1 binding *in vitro* and *in vivo* using the series of p80 truncations shown in Fig. 11A. For the *in vitro* studies, each p80 truncation was expressed as fusions to the triple Flag epitope (3F-p80) in a rabbit reticulocyte lysate and tested for binding to the N-terminal domain of E1 (aa 1 to 170), fused to GST, in pulldown assays. The results shown in Fig. 11B revealed that the first 573 amino acids of p80 are required for robust binding to E1. This interaction was specific, as it was not observed with GST alone and, importantly, was abolished by the VE substitution in E1. Shorter fragments of p80 spanning the first 400 amino acids showed weaker binding to E1, indicating that the WD40 repeat region may constitute the minimal E1 interaction motif. Accordingly, little to no binding of the C-terminal domain of p80 (aa 389 to 677) to E1 was detected.

The same p80 truncations were then tested for interaction with full-length E1 *in vivo* by coimmunoprecipitation. Specifically, the various 3F-p80 fragments were expressed in C33A cells, together with YFP-E1, and immunoprecipitated using an anti-Flag antibody, and the precipitates were probed for the presence of YFP-E1 by Western blotting with an anti-GFP antibody. These studies confirmed that the first 573 amino acids of p80 (aa 1 to 573) interact with E1 as efficiently as the full-length p80 (Fig. 11C). However, and in contrast to what was observed *in vitro*, none of the shorter N-terminal fragments of p80 were able to interact with E1, possibly because of the lower concentration of these fragments and of E1 *in vivo* compared to the *in vitro* assay. Finally, no interaction was detected between E1 and the C-terminal fragment of p80, which contains the putative UBX/SLD. Altogether, these results indicate that the N-terminal 573 amino acids of p80, including the WD40 repeat region, are necessary for interaction with E1.

DISCUSSION

We previously reported that E1 interacts with p80 to promote the maintenance of the viral genome in undifferentiated keratinocytes and mapped the p80-binding domain on E1 to a short N-terminal region of the protein located between amino acids 10 and 40. This sequence is highly conserved among E1 proteins from mucosal HPV types but less so between cutaneous types. Accordingly, we previously found that only the E1 proteins from anogenital HPV types interact with p80. In this study, we determined that the p80-binding domain of E1 is essential for efficient transient HPV DNA replication, a finding that likely accounts for the failure of viral episomes expressing a p80-binding-defective E1 to be properly maintained in keratinocytes. Furthermore, we have shown that overexpression of these E1 mutant proteins cannot suppress their

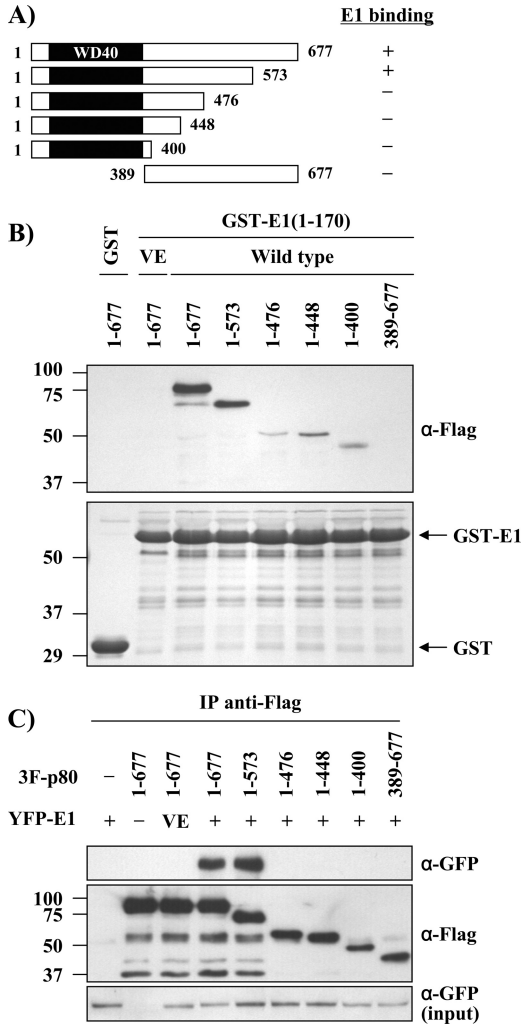

FIG 11 E1 interacts with amino acids 1 to 573 of p80. (A) Schematic representation of the p80 truncations used for mapping the E1 binding domain on p80. The WD40 repeats of p80 are represented by black boxes. (B) *In vitro* pulldown assays. 3F-p80 truncations were expressed in rabbit reticulocyte lysates and tested for binding to the N-terminal domain of E1 (aa 1 to 170) fused to GST. GST alone or GST-E1 VE was used as a negative control. Bound proteins were detected by Western blotting using an anti-Flag antibody. At the bottom is shown a Coomassie blue-stained SDS-PAGE gel of the purified GST fusion proteins used in these pulldown experiments. The sizes of molecular weight markers (in thousands) are indicated on the left. (C) *In vivo co*immunoprecipitation assays. The indicated 3F-p80 truncations were expressed in C33A cells together with YFP-E1 and immunoprecipitated using anti-Flag antibodies. The presence of YFP-E1 in the immunoprecipitates was determined by Western blotting using an anti-GFP antibody. Cells expressing the YFP-E1 VE mutant protein and full-length 3F-p80 were used as a negative control.

transient DNA replication defect, suggesting it is not due to lower expression and/or the stability of these proteins. Importantly, we have also gathered evidence in functional assays and by ChIP that these E1 mutant proteins retain the capacity to interact with E2 at the viral origin *in vivo*. Collectively, these findings suggest that p80-binding-deficient E1 proteins are intrinsically impaired for viral DNA replication at a step following the assembly of the E1-E2-ori initiation complex.

The results summarized above suggest that the interaction of anogenital HPV E1 with p80 is essential for efficient viral DNA replication. In support of this suggestion, we observed for the different E1 mutant proteins that the magnitude of their DNA replication defects correlate with their capacity to interact with p80. Namely, we found that the WF and C40 E1 proteins, which are completely defective for p80 interaction, are more severely compromised for DNA replication than the VI or VE mutant proteins, which both retain weak binding to p80. Additional evidence that the E1-p80 interaction is required for viral DNA replication came from the observation that p80 is recruited from the cytoplasm to discrete nuclear foci and to the viral origin by E1 and E2 in a manner dependent on the integrity of the E1 p80-binding domain. Of particular significance was the demonstration that the HPV31 E1-derived peptide N40, which efficiently binds to p80 and antagonizes the E1-p80 interaction *in vivo*, can prevent the recruitment of p80 onto the viral origin and inhibit viral DNA replication. The antagonistic effect of this peptide was specific to viral DNA replication, as it did not affect host DNA synthesis and cell cycle progression or any other host functions required for cellular proliferation and viability. Further evidence of specificity came from the observation that N40 mutant peptides defective for p80 binding have little to no effect on the recruitment of p80 to the viral origin and on transient HPV DNA replication. Together, these findings provide strong evidence that the interaction of E1 with p80 is required for efficient HPV DNA replication.

While the E1-p80 interaction is not strictly essential for transient DNA replication, it greatly enhances the overall efficiency of the process. Indeed, amino acid substitutions or truncations in E1 that abrogate p80 binding or overexpression of the N40 peptide reduce transient DNA replication by 70 to 75% but never completely abolish it. Although the E1-p80 interaction is not essential in transient DNA replication assays, it appears from our previous study to be indispensable for the proper maintenance of the viral episome in keratinocytes. Thus, the E1-p80 interaction seems to be more stringently required for episomal maintenance in immortalized keratinocytes than for viral DNA synthesis in transient assays, perhaps because the assays are performed under the less physiologically relevant condition of E1 and E2 overexpression. Although the exact molecular mechanism by which p80 promotes viral DNA replication remains elusive, we can discuss some possibilities based on our current understanding of the structure and functions of p80. The N-terminal half of p80 is comprised of WD40 repeats, which are known to fold into β-propeller structures that often act as protein-protein interaction modules (56). In this respect, our finding that the WD40 repeats of p80 are needed for interaction with E1 is not surprising. Thus, one possible role of p80 in viral DNA replication may be to act as a scaffold to facilitate either the assembly of a replication-competent E1 double hexamer, its licensing, its processivity during DNA synthesis, or its recycling for further rounds of DNA replication. As such, the p80-binding domain of E1 could play a role similar to that of the simian virus 40 (SV40) large T antigen J domain in polyomavirus DNA replication (57, 63). One reason for raising this possibility is the previous report that p80 copurifies with Hsp70, although this interaction was deemed to be nonspecific (45). Our finding that p80 is recruited to the viral origin during the initiation of viral DNA replication (i.e., in aphidicolin-treated cells) is consistent with p80 playing a role in the assembly of the viral DNA replication complex. However, the fact that the WF and C40 E1 proteins still assemble at the origin in our ChIP assays, an activity

that requires the ATPase function of E1, suggests that these mutant proteins are capable of oligomerizing at the origin. Thus, we surmise that the requirement for p80 arises after E1 oligomerization, such as for the licensing, processivity, or recycling of the helicase.

Interestingly, p80 was recently shown to associate with and to stimulate the activity of three deubiquitinating enzymes (DUBs), namely, USP1, USP12, and USP46 (9, 10). Similar to what we found for E1, the WD repeats of p80 were shown to be required for interaction with these enzymes. This prompted us to investigate if E1 competes with these DUBs for binding to p80. On the contrary, we found in coimmunoprecipitation assays that E1 can form a ternary complex with p80 and any of the three USPs (M. Lehoux and J. Archambault, unpublished data), indicating that their interactions are not mutually exclusive. USP1 has been implicated in two different aspects of the cellular DNA damage response (DDR), namely, the Fanconi anemia pathway, which coordinates the repair of DNA damage, such as DNA cross-links, and the translesion synthesis pathway, which allows DNA replication to proceed past certain DNA lesions. It has been found that USP1 can downregulate these two pathways by deubiquitinating monoubiquitinated FANCD2 and monoubiquitinated PCNA, respectively. Interestingly, FANCD2 activation and recruitment to nuclear foci was previously observed in cells expressing SV40 large T antigen (5). This activation was shown to correlate with DNA replication-induced stress and to be required for SV40 DNA replication. In the context of HPV, it is tempting to speculate that the presence of p80 at the replication fork may facilitate the recruitment of DDR proteins to promote viral DNA replication or, conversely, to alleviate problems linked with a stalled replication fork. However, we and others recently showed that DNA damage neither interferes with nor stimulates transient HPV DNA replication (18, 31), suggesting that the effect of p80 in this context is independent of DDR signaling. Furthermore, Moody et al. also observed, using the chemical inhibitor KU-55933, that the ATM pathway has no effect on the stable maintenance of the HPV episome in undifferentiated keratinocytes (40), in contrast to p80, whose interaction with E1 is essential for this process (12). Altogether, these results suggest a function for p80 in episomal replication and maintenance that is independent of DNA damage signaling events. Another possibility is that p80 could serve to modulate the monoubiquitination status of PCNA during viral DNA replication through its association with E1 and USP1, perhaps as a means to recruit translesion synthesis polymerases. In this context, it is intriguing that a previous study addressing the protein requirement for papillomavirus DNA replication *in vitro* indicated the need for an unknown cellular factor present in the same biochemical fraction as polymerases α and δ, but different from these two enzymes, that could potentially be a third polymerase, such as polymerase ε (37).

As for USP12 and USP46, they have been shown to deubiquitinate monoubiquitinated histones H2A and H2B (29). Thus, one possible role of p80 in viral DNA replication may be to modify chromatin to facilitate the assembly of the E1-containing DNA replication complex and/or its progression at the fork. Two other E1-associated proteins, histone H1 and hSNF5, were previously suggested to affect chromatin structure for the benefit of viral DNA replication (33, 58). Interestingly, the initiator protein EBNA1 of Epstein-Barr virus (EBV) has recently been shown to recruit USP7 to the latent viral origin in order to locally deubiquitinate H2B (52). However, the exact function of this process in the EBV life cycle remains to be determined. While a role for USP1, -12, and -46 in the HPV life cycle remains attractive, our current unpublished results obtained using dominant-negative versions of these enzymes suggest that their catalytic activity is not required for transient viral DNA replication, although these results should be interpreted with caution, as with any other negative findings. In summary, it appears that the role of p80 in transient HPV DNA replication does not rely on its association with catalytically active DUBs, a conclusion that does not preclude a role for these interactions in other aspects of the viral life cycle but one that suggests that p80 can also function independently of these enzymes.

As a first step toward understanding the mechanism of action of the N40-inhibitory peptide, we used NMR to analyze its structure and that of a longer E1 fragment spanning the first 83 amino acids. Both peptides were found to be intrinsically disordered in solution, a finding consistent with our previous *in silico* predictions that the N-terminal region of E1 is predominantly unstructured (41). In addition to the p80-binding domain, we previously showed that the first 83 residues of E1 contain a conserved $\Phi X \Phi \Phi$ motif (in which Φ indicates a hydrophobic amino acid) that is required for efficient DNA replication and that may fold as an amphipathic helix with structural and functional similarity to those found in the p53 TAD2 and VP16C transactivation domains (41). These protein domains are known to be intrinsically disordered in solution and to fold into α-helices upon binding to their interaction partners, such as the Tfb1/p62 subunit of the transcription factor TFIIH (4, 16, 28, 62). By analogy, it is possible that the first 83 amino acids of E1 undergo folding upon interaction with binding partners, such as p80. Similarly, the E1 N40 peptide may interact with the WD40 repeat region of p80 through an induced-fit mechanism. From a thermodynamic point of view, this type of peptide-protein interaction is disfavored entropically but is often compensated for by highly specific and extensive interaction surfaces comprised of several low-affinity contacts (17). By their very nature, these kinds of reversible interactions have been suggested to be ideal targets for therapeutic intervention (38). The finding that the E1-p80 interaction is essential for episomal maintenance and the discovery of an E1-derived, p80-binding peptide that inhibits viral DNA replication and is well tolerated in cells represent important steps in the validation of the E1-p80 interface as a novel antiviral target.

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REFERENCES

1. **Androphy EJ, Lowy DR, Schiller JT.** 1987. Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. Nature **325**:70–73.
2. **Baseman JG, Koutsky LA.** 2005. The epidemiology of human papillomavirus infections. J. Clin. Virol. **32**(Suppl. 1):S16–S24.
3. **Bernard HU, et al.** 2010. Classification of papillomaviruses (PVs) based

on 189 PV types and proposal of taxonomic amendments. Virology **401**: 70–79.
4. **Bochkareva E, et al.** 2005. Single-stranded DNA mimicry in the p53 transactivation domain interaction with replication protein A. Proc. Natl. Acad. Sci. U. S. A. **102**:15412–15417.
5. **Boichuk S, Hu L, Hein J, Gjoerup OV.** 2010. Multiple DNA damage signaling and repair pathways deregulated by simian virus 40 large T antigen. J. Virol. **84**:8007–8020.
6. **Cho NH, et al.** 2004. Inhibition of T cell receptor signal transduction by tyrosine kinase-interacting protein of Herpesvirus saimiri. J. Exp. Med. **200**:681–687.
7. **Cho NH, et al.** 2006. Association of herpesvirus saimiri tip with lipid raft is essential for downregulation of T-cell receptor and CD4 coreceptor. J. Virol. **80**:108–118.
8. **Clower RV, Fisk JC, Melendy T.** 2006. Papillomavirus E1 protein binds to and stimulates human topoisomerase I. J. Virol. **80**:1584–1587.
9. **Cohn MA, Kee Y, Haas W, Gygi SP, D'Andrea AD.** 2009. UAF1 is a subunit of multiple deubiquitinating enzyme complexes. J. Biol. Chem. **284**:5343–5351.
10. **Cohn MA, et al.** 2007. A UAF1-containing multisubunit protein complex regulates the Fanconi anemia pathway. Mol. Cell **28**:786–797.
11. **Conger KL, Liu JS, Kuo SR, Chow LT, Wang TS.** 1999. Human papillomavirus DNA replication. Interactions between the viral E1 protein and two subunits of human DNA polymerase alpha/primase. J. Biol. Chem. **274**:2696–2705.
12. **Cote-Martin A, et al.** 2008. Human papillomavirus E1 helicase interacts with the WD repeat protein p80 to promote maintenance of the viral genome in keratinocytes. J. Virol. **82**:1271–1283.
13. **Daling JR, et al.** 2004. Human papillomavirus, smoking, and sexual practices in the etiology of anal cancer. Cancer **101**:270–280.
14. **Deng W, et al.** 2004. Cyclin/CDK regulates the nucleocytoplasmic localization of the human papillomavirus E1 DNA helicase. J. Virol. **78**:13954–13965.
15. **de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H.** 2004. Classification of papillomaviruses. Virology **324**:17–27.
16. **Di Lello P, et al.** 2006. Structure of the Tfb1/p53 complex: insights into the interaction between the p62/Tfb1 subunit of TFIIH and the activation domain of p53. Mol. Cell **22**:731–740.
17. **Dyson HJ, Wright PE.** 2005. Intrinsically unstructured proteins and their functions. Nat. Rev. Mol. Cell Biol. **6**:197–208.
18. **Fradet-Turcotte A, et al.** 2011. Nuclear accumulation of the papillomavirus E1 helicase blocks S-phase progression and triggers an ATM-dependent DNA damage response. J. Virol. **85**:8996–9012.
19. **Fradet-Turcotte A, Moody C, Laimins LA, Archambault J.** 2010. Nuclear export of human papillomavirus type 31 E1 is regulated by Cdk2 phosphorylation and required for viral genome maintenance. J. Virol. **84**:11747–11760.
20. **Fradet-Turcotte A, Morin G, Lehoux M, Bullock PA, Archambault J.** 2010. Development of quantitative and high-throughput assays of polyomavirus and papillomavirus DNA replication. Virology **399**:65–76.
21. **Frappier L, Verrijzer CP.** 2011. Gene expression control by protein deubiquitinases. Curr. Opin. Genet. Dev. **21**:207–213.
22. **Frattini MG, Laimins LA.** 1994. Binding of the human papillomavirus E1 origin-recognition protein is regulated through complex formation with the E2 enhancer-binding protein. Proc. Natl. Acad. Sci. U. S. A. **91**:12398–12402.
23. **Frattini MG, Laimins LA.** 1994. The role of the E1 and E2 proteins in the replication of human papillomavirus type 31b. Virology **204**:799–804.
24. **Frisch M, et al.** 1999. Variants of squamous cell carcinoma of the anal canal and perianal skin and their relation to human papillomaviruses. Cancer Res. **59**:753–757.
25. **Han Y, Loo YM, Militello KT, Melendy T.** 1999. Interactions of the papovavirus DNA replication initiator proteins, bovine papillomavirus type 1 E1 and simian virus 40 large T antigen, with human replication protein A. J. Virol. **73**:4899–4907.
26. **Hebner CM, Laimins LA.** 2006. Human papillomaviruses: basic mechanisms of pathogenesis and oncogenicity. Rev. Med. Virol. **16**:83–97.
27. **Huang TT, et al.** 2006. Regulation of monoubiquitinated PCNA by DUB autocleavage. Nat. Cell Biol. **8**:339–347.
28. **Jonker HR, Wechselberger RW, Boelens R, Folkers GE, Kaptein R.** 2005. Structural properties of the promiscuous VP16 activation domain. Biochemistry **44**:827–839.
29. **Joo HY, et al.** 2011. Regulation of histone H2A and H2B deubiquitination and Xenopus development by USP12 and USP46. J. Biol. Chem. **286**: 7190–7201.
30. **Kim JM, et al.** 2009. Inactivation of murine Usp1 results in genomic instability and a Fanconi anemia phenotype. Dev. Cell **16**:314–320.
31. **King LE, et al.** 2010. Human papillomavirus E1 and E2 mediated DNA replication is not arrested by DNA damage signalling. Virology **406**:95–102.
32. **Kreimer AR, Clifford GM, Boyle P, Franceschi S.** 2005. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol. Biomarkers Prev. **14**:467–475.
33. **Lee D, Sohn H, Kalpana GV, Choe J.** 1999. Interaction of E1 and hSNF5 proteins stimulates replication of human papillomavirus DNA. Nature **399**:487–491.
34. **Lehoux M, D'Abramo CM, Archambault J.** 2009. Molecular mechanisms of human papillomavirus-induced carcinogenesis. Public Health Genomics **12**:268–280.
35. **Loo YM, Melendy T.** 2004. Recruitment of replication protein A by the papillomavirus E1 protein and modulation by single-stranded DNA. J. Virol. **78**:1605–1615.
36. **Masterson PJ, Stanley MA, Lewis AP, Romanos MA.** 1998. A C-terminal helicase domain of the human papillomavirus E1 protein binds E2 and the DNA polymerase alpha-primase p68 subunit. J. Virol. **72**:7407–7419.
37. **Melendy T, Sedman J, Stenlund A.** 1995. Cellular factors required for papillomavirus DNA replication. J. Virol. **69**:7857–7867.
38. **Metallo SJ.** 2010. Intrinsically disordered proteins are potential drug targets. Curr. Opin. Chem. Biol. **14**:481–488.
39. **Mohr IJ, et al.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. Science **250**:1694–1699.
40. **Moody CA, Laimins LA.** 2009. Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. PLoS Pathog. **5**:e1000605.
41. **Morin G, et al.** 2011. A conserved amphipathic helix in the N-terminal regulatory region of the papillomavirus E1 helicase is required for efficient viral DNA replication. J. Virol. **85**:5287–5300.
42. **Nagai T, et al.** 2002. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat. Biotechnol. **20**: 87–90.
43. **Nijman SM, et al.** 2005. The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. Mol. Cell **17**:331–339.
44. **Park J, et al.** 2003. Distinct roles of cellular Lck and p80 proteins in herpesvirus saimiri Tip function on lipid rafts. J. Virol. **77**:9041–9051.
45. **Park J, et al.** 2002. Herpesviral protein targets a cellular WD repeat endosomal protein to downregulate T lymphocyte receptor expression. Immunity **17**:221–233.
46. **Park P, et al.** 1994. The cellular DNA polymerase alpha-primase is required for papillomavirus DNA replication and associates with the viral E1 helicase. Proc. Natl. Acad. Sci. U. S. A. **91**:8700–8704.
47. **Parkin DM, Bray F, Ferlay J, Pisani P.** 2005. Global cancer statistics, 2002. CA Cancer J. Clin. **55**:74–108.
48. **Partridge JM, Koutsky LA.** 2006. Genital human papillomavirus infection in men. Lancet Infect. Dis. **6**:21–31.
49. **Sakakibara N, Mitra R, McBride AA.** 2011. The papillomavirus E1 helicase activates a cellular DNA damage response in viral replication foci. J. Virol. **85**:8981–8995.
50. **Sanchez-Pulido L, Devos D, Sung ZR, Calonje M.** 2008. RAWUL: a new ubiquitin-like domain in PRC1 ring finger proteins that unveils putative plant and worm PRC1 orthologs. BMC Genomics **9**:308.
51. **Sanders CM, Stenlund A.** 2001. Mechanism and requirements for bovine papillomavirus, type 1, E1 initiator complex assembly promoted by the E2 transcription factor bound to distal sites. J. Biol. Chem. **276**:23689–23699.
52. **Sarkari F, et al.** 2009. EBNA1-mediated recruitment of a histone H2B deubiquitylating complex to the Epstein-Barr virus latent origin of DNA replication. PLoS Pathog. **5**:e1000624.
53. **Schuck S, Stenlund A.** 2005. Assembly of a double hexameric helicase. Mol. Cell **20**:377–389.
54. **Schuck S, Stenlund A.** 2005. Role of papillomavirus E1 initiator dimerization in DNA replication. J. Virol. **79**:8661–8664.
55. **Senechal H, Poirier GG, Coulombe B, Laimins LA, Archambault J.** 2007. Amino acid substitutions that specifically impair the transcriptional activity of papillomavirus E2 affect binding to the long isoform of Brd4. Virology **358**:10–17.
56. **Smith TF, Gaitatzes C, Saxena K, Neer EJ.** 1999. The WD repeat: a

common architecture for diverse functions. Trends Biochem. Sci. **24**:181–185.
57. **Sullivan CS, Pipas JM.** 2002. T antigens of simian virus 40: molecular chaperones for viral replication and tumorigenesis. Microbiol. Mol. Biol. Rev. **66**:179–202.
58. **Swindle CS, Engler JA.** 1998. Association of the human papillomavirus type 11 E1 protein with histone H1. J. Virol. **72**:1994–2001.
59. **Swindle CS, et al.** 1999. Human papillomavirus DNA replication compartments in a transient DNA replication system. J. Virol. **73**:1001–1009.
60. **Titolo S, et al.** 2000. Identification of domains of the human papillomavirus type 11 E1 helicase involved in oligomerization and binding to the viral origin. J. Virol. **74**:7349–7361.
61. **Titolo S, et al.** 1999. Role of the ATP-binding domain of the human papillomavirus type 11 E1 helicase in E2-dependent binding to the origin. J. Virol. **73**:5282–5293.
62. **Uesugi M, Nyanguile O, Lu H, Levine AJ, Verdine GL.** 1997. Induced alpha helix in the VP16 activation domain upon binding to a human TAF. Science **277**:1310–1313.
63. **Weisshart K, et al.** 1999. Two regions of simian virus 40 T antigen determine cooperativity of double-hexamer assembly on the viral origin of DNA replication and promote hexamer interactions during bidirectional origin DNA unwinding. J. Virol. **73**:2201–2211.
64. **Wilson VG, West M, Woytek K, Rangasamy D.** 2002. Papillomavirus E1 proteins: form, function, and features. Virus Genes **24**:275–290.
65. **Wooldridge TR, Laimins LA.** 2008. Regulation of human papillomavirus type 31 gene expression during the differentiation-dependent life cycle through histone modifications and transcription factor binding. Virology **374**:371–380.
66. **Yang K, et al.** 2011. Regulation of the Fanconi anemia pathway by a SUMO-like delivery network. Genes Dev. **25**:1847–1858.