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The Human Papillomavirus Type 11 E1^E4 Protein Is Phosphorylated in Genital Epithelium

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The most abundant viral transcript in human papillomavirus (HPV) 11-infected xenograft tissue has been shown to encode the E1^E4 protein. The function of E1^E4 protein has not been determined. Several potential phosphorylation sequence motifs were identified in the HPV 11 E1^E4 protein, including potential sites of phosphorylation by mitogen-activated protein kinase (MAPK), cAMP-dependent protein kinase (PKA), casein kinase II, and protein kinase C. To test phosphorylation of the HPV 11 E1^E4 protein, a soluble maltose binding protein (MBP) fusion was produced in *Escherichia coli.* Only MAPK and PKA phosphorylated the E1^E4 protein. Phosphoamino acid analysis showed that one or more threonine residues were phosphorylated by MAPK, and both serine and threonine residues were phosphorylated by PKA. MBP–E1^E4 mutant proteins were designed to delineate the E1^E4 phosphoacceptor residues. MAPK was shown to phosphorylate E1^E4 on threonine 53 within a MAPK consensus phorphorylation sequence motif. PKA was shown to phosphorylate E1^E4 at two residues: threonine 36 within a consensus motif and serine 44 within a variant of the PKA consensus phosphorylation sequence motif. HPV 11-infected human genital tissue grown as a xenograft in an athymic mouse was labeled with [^{32}P]orthophosphate. Phosphoamino acid analysis of E1^E4 protein immunoprecipitated from ^{32}P-labeled tissue revealed that both serine and threonine residues were phosphorylated. Analysis by liquid chromatography–mass spectrophotometry was consistent with phosphorylation of residues within the PKA and MAPK phosphorylation sequence motifs. Expression of E1^E4 protein containing phosphorylation substitution mutations showed that the PKA mutant did not differ from wild-type E1^E4 protein in intracellular distribution. In contrast, the MAPK mutant did not localize exclusively to the cytoplasm nor did it colocalize with wild-type E1^E4 protein. We conclude that HPV 11 E1^E4 protein is phosphorylated *in vitro* and *in vivo.* Our data are consistent with phosphorylation of HPV 11 E1^E4 protein by MAPK and PKA in infected tissue.

INTRODUCTION

Human papillomaviruses (HPVs) are small DNA viruses that infect epithelial surfaces, causing a range of disease states (Shah and Howley, 1996). Approximately 30 HPV types are regularly detected in genital tract lesions. These lesions include condylomata acuminata (genital warts), characterized by epithelial proliferation and a low risk of dysplastic abnormalities. Genital warts are most often caused by HPV types 6a and 11 (Brown *et al.,* 1993; Gissmann *et al.,* 1983; Pfister, 1987). The most abundant viral mRNA detected in HPV 11-infected genital wart tissue potentially encodes a 10-kDa E1^E4 spliced gene product that joins a short segment of open reading frame (ORF) E1 to ORF E4 (Chow *et al.,* 1987; Nasseri *et al.,* 1987). The E1^E4 protein is encoded by two transcripts: the E1^E4,E5 transcript and the E1^E4^L1 transcript (Brown *et al.,* 1996, 1998; Rotenberg *et al.,* 1989). The E1^E4,E5 transcript contains the sequences of the spliced E1^E4 ORF and continues downstream through the E5a and E5b ORFs. This transcript is detected early in HPV 11 infection, throughout the epithelial layers (Stoler and Broker, 1986). The multiply spliced, polycistronic E1^E4^L1 transcript encodes both the E1^E4 protein and the L1 major capsid protein (Brown *et al.,* 1996; Chow *et al.,* 1987; Rotenberg *et al.,* 1989). Despite the wide distribution of E1^E4-encoding transcripts, the HPV 11 E1^E4 protein is detected only in the cytoplasm of differentiated cells that are also expressing L1 protein (Brown *et al.,* 1994, 1995). In immunoblots of HPV 11-infected tissue, the E1^E4 protein is detected as a 10/11-kDa doublet and higher molecular weight forms thought to be oligomers (Brown *et al.,* 1988).

The function of the E1^E4 protein has not been determined. There is evidence that the E1^E4 proteins of HPV types 1, 16, and 31b interact with the intermediate filament network when expressed in cells grown in culture (Doorbar, 1991; Doorbar *et al.,* 1991; Pray and Laimins, 1995; Roberts *et al.,* 1993). However, in an analysis of HPV 1-infected tissue, Doorbar *et al.* (1996) showed that greater than 95% of the E4 protein existed as complexes that did not include keratins. The proposed complexes were E4 multimers. Immunoblot analysis of HPV 11-infected human genital tissue grown as a xenograft in an athymic mouse revealed a 10/11-kDa doublet, believed to be the monomeric form of E1^E4, and additional immu-

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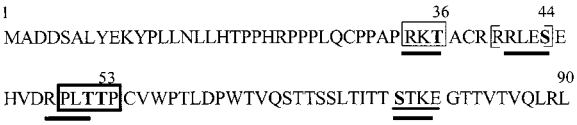

FIG. 1. Amino acid sequence of HPV 11 E1^E4 protein from the Hershey isolate (Kreider, 1987) in single-letter code. Numbers above the sequence indicate amino acid position. Putative phosphorylation sites are indicated as follows: one PKA phosphorylation recognition motif is boxed with thin black lines; one MAPK phosphorylation recognition motif is boxed with heavy black lines; four PKC phosphorylation recognition motifs are underlined with heavy black lines; one CKII phosphorylation recognition motif is underlined with a thin black line. A degenerate PKA site, experimentally determined, is contained within brackets. The putative phosphoacceptor amino acid residues are indicated in boldface type.

noreactive bands at 18 and 29 kDa (Brown *et al.,* 1988). HPV 11 E1^E4 protein oligomerization has been demonstrated with amino acid residues in the carboxyl-terminus shown to be required for self-association (Bryan *et al.l,* 1998). Oligomerization as well as posttranslational modifications may potentially influence E1^E4 protein function.

Protein phosphorylation is a controlling step in the regulation of many biochemical pathways, influencing cell signaling events, cell cycling, protein localization, and gene expression. Demonstration of potential phosphorylation sequence motifs may provide clues to protein function. We identified several potential consensus phosphorylation sequence motifs by computer analysis of the HPV 11 E1^E4 primary sequence. These included consensus sites for kinases known to be involved in cell signaling pathways. Based on these observations, we have undertaken an investigation of the HPV 11 E1^E4 protein and its ability to be phosphorylated by specific protein kinases.

RESULTS

The HPV 11 E1^E4 protein is phosphorylated *in vitro* by cAMP-dependent protein kinase and mitogen-activated protein kinase

The HPV 11 E1^E4 protein sequence was examined using ScanProsite to search for potential phosphorylation sites (Fig. 1). Sites were found within the protein corresponding to motifs for four kinases: mitogen-activated protein kinase (MAPK), cAMP-dependent protein kinase (PKA), casein kinase II (CKII), and protein kinase C (PKC). The phosphorylation consensus sequence motifs of these four kinases are PKC (S/TXR/K, K/RXXS/T, and K/RXS/T) (Russo *et al.,* 1992), MAPK (PXXS/TP and PXS/TP) (Davis, 1993), CKII (S/TXXD/E) (Russo *et al.,* 1992), and PKA (RXS/T and RR/KXS/T) (Pearson and Kemp, 1991). The HPV 11 E1^E4 protein was predicted to contain seven putative phosphorylation sites: four PKC sites, one MAPK site at amino acids 50–54, one CKII site at amino acids 77–80, and one PKA site at amino acids 34–36. The CKII and the last PKC consensus patterns overlap, sharing the same phosphoacceptor, as do the PKA and the first PKC consensus sequence.

Because the HPV 11 E1^E4 protein is insoluble in aqueous buffers (unpublished observation), a fusion of the maltose binding protein (MBP) and the HPV 11 E1^E4 protein was produced in *Escherichia coli.* This fusion was affinity-purified, found to be soluble in aqueous buffers, and used for *in vitro* phosphorylation reactions. In the *in vitro* experiments, both PKA and MAPK phosphorylated MBP–E1^E4 but not MBP (Fig. 2). This was demonstrated by the introduction and ^{32}P into the MBP–E1^E4 fusion protein with detection by autoradiography following SDS–PAGE. Upon MBP–E1^E4 cleavage with Factor Xa, only the E1^E4 peptide was detected as a ^{32}P-labeled protein (Fig. 2, Lanes 3 and 6). CKII phosphorylated phosvitin, a positive control protein. However, CKII failed to phosphorylate MBP or MBP–E1^E4 (data not shown). Similarly, PKC failed to phosphorylate MBP or MBP–E1^E4, but did phosphorylate Type III-S histone, a positive control protein (data not shown).

For the phosphoamino acid analysis, the MBP–E1^E4 fusion was phosphorylated by either PKA or MAPK in the presence of [γ-^{32}P]ATP (Fig. 3). The E1^E4 peptide was then cleaved from MBP with Factor Xa and subjected to acid hydrolysis and thin-layer electrophoresis (Fig. 3B, Lanes 1 and 4). E1^E4 was phosphorylated by PKA at both serine and threonine residues; MAPK phosphorylated E1^E4 only on a threonine residue(s). A short exposure time of PKA E1^E4 phosphorylation is presented in Fig. 3B, Lane 1, to show the distinction between the phosphoserine and the phosphothreonine residues.

Based on the consensus PKA recognition motifs (R-X-

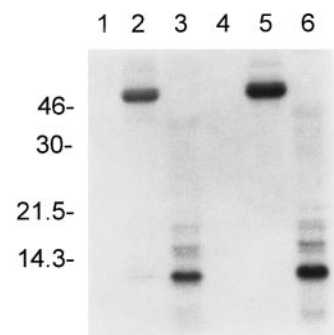

FIG. 2. PKA and MAPK phosphorylate the MBP–E1^E4 protein *in vitro.* Shown is an autoradiography of *in vitro* phosphorylated recombinant MBP and MBP–E1^E4 proteins separated by electrophoresis on 15% SDS–PAGE. Lanes 1, 2, and 3 contain phosphorylation reactions by PKA. Lane 1, nonfused MBP protein; Lane 2, MBP–E1^E4 protein; Lane 3, MBP–E1^E4 protein cleaved with Factor Xa generating MBP and E1^E4 peptides. Proteins in Lanes 4, 5, and 6 contain phosphorylation reactions by MAPK. Lane 4, nonfused MBP protein; Lane 5, MBP–E1^E4 protein; Lane 6, MBP–E1^E4 protein cleaved with Factor Xa generating MBP and E1^E4 peptides. Molecular mass markers are indicated on the left side of the figure in kilodaltons.

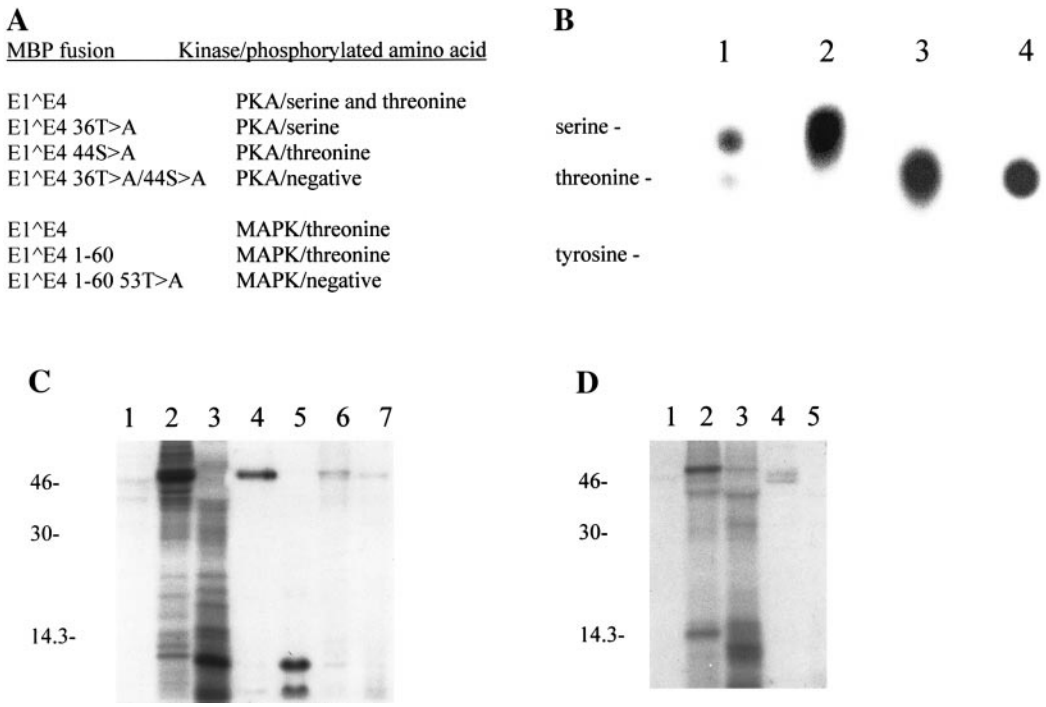

FIG. 3. PKA and MAPK phosphorylate specific amino acids in the E1^E4 protein. (A) MBP–E1^E4 mutant proteins (as described in text), followed by the kinase and the amino acid residues shown to phosphorylated. (B) Phosphoamino acid analysis of E1^E4 peptides cleaved from MBP fusions. Lane 1, native E1^E4 phosphorylated by PKA; Lane 2, E1^E4 36T > A phosphorylated by PKA; Lane 3, E1^E4 44S > A phosphorylated by PKA; Lane 4, native E1^E4 phosphorylated by MAPK. Phosphoserine, phosphothreonine, and phosphotyrosine markers are indicated on the left. (C) Autoradiograph of SDS–PAGE showing PKA-phosphorylated MBP fusions. Lane 1, nonfused MBP; Lane 2, MBP–E1^E4 44S > A; Lane 3, Factor Xa-cleaved MBP–E1^E4 44S > A; Lane 4, MBP–E1^E4 36T > A; Lane 5, Factor Xa-cleaved MBP–E1^E4 36T > A; Lane 6, MBP–E1^E4 36T > A/44S > A; Lane 7, Factor Xa-cleaved MBP–E1^E4 36T > A/44S > A. (D) Autoradiograph of SDS–PAGE showing MAPK-phosphorylated MBP fusions. Lane 1, nonfused MBP; Lane 2, MBP–E1^E4 1–60; Lane 3, Factor Xa-cleaved MBP–E1^E4 1–60; Lane 4, MBP–E1^E4 1–60 53T > A; Lane 5, Factor Xa-cleaved MBP–E1^E4 1–60 53T > A. Molecular mass markers in kilodaltons are indicated to the left of C and D.

S/T, R-R/K-X-S/T), the HPV 11 E1^E4 protein is predicted to be phosphorylated only at the threonine residue at position 36. However, the amino acid sequence "RRLES" (amino acids 40–44) in the E1^E4 protein is very similar to the PKA consensus recognition motif "R-R/K-X-S/T," which could explain the serine (amino acid 44) phosphorylation by PKA *in vitro.* To test these predictions, amino acids 36 and 44 were mutated to alanine residues both separately and together and were expressed as MBP fusions (Fig. 3A). Both MBP–E1^E4 36T > A and MBP–E1^E4 44S > A were phosphorylated by PKA (Fig. 3C, Lanes 2 and 4). Mutating both sites (MBP–E1^E4 36T > A/44S > A) resulted in no phosphorylation above background (Fig. 3C, Lane 6). Phosphoamino acid analysis of PKA phosphorylated E1^E4 36T > A revealed only a phosphoserine residue (Fig. 3B, Lane 2). Phosphoamino acid analysis of PKA phosphorylated E1^E4 44S > A revealed only a phosphothreonine residues (Fig. 3B, Lane 3). These data clearly demonstrate that PKA phosphorylates E1^E4 threonine 36 and serine 44 *in vitro.*

The predicted phosphoacceptor residue for E1^E4 phosphorylation by MAPK is threonine 53. To facilitate cloning and mutagenesis of this site, the E1^E4 protein was truncated to contain only the first 60 amino acids. Threonine 53 was mutated to an alanine and expressed as an MBP–E1^E4 1–60 53T > A fusion protein (Fig. 3A). MBP–E1^E4 1–60 was shown to be phosphorylated by MAPK (Fig. 3D, Lane 2). MAPK was not able to phosphorylate MBP–E1^E4 1–60 53T > A in which threonine 53 was mutated to alanine (Fig. 3D, Lane 4). Although a faint signal can be seen in Lane 4 below the predicted molecular weight of MBP–E1^E4 1–60 53T > A, no phosphorylation of the E1^E4 1–60 53T > A peptide was detected following Factor Xa cleavage (Fig. 3D, Lane 5). These data demonstrate that MAPK phosphorylates E1^E4 *in vitro* at threonine 53.

E1^E4 protein is phosphorylated in HPV 11-infected tissue

To determine whether the E1^E4 protein is phosphorylated by kinases present in human epithelium, HPV 11-infected tissue was grown as xenografts in athymic mice for 3 months. To identify E1^E4 protein in HPV 11-infected xenograft tissue labeled with [^{32}P]orthophosphate, an immunoprecipitation was performed (Fig. 4). The immunoprecipitated proteins were

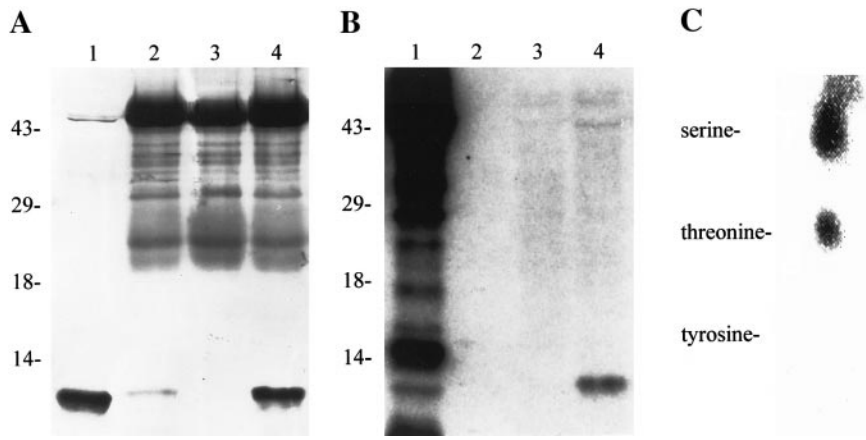

FIG. 4. E1^E4 protein is phosphorylated in tissue at serine and threonine residues. (A) Immunoblot using anti-E1^E4 serum. Lane 1, crude extract of ^{32}P-labeled HPV 11-infected xenograft tissue; Lane 2, immunoprecipitation of unlabeled HPV 11-infected xenograft tissue using anti-E1^E4 serum; Lane 3, immunoprecipitation of ^{32}P-labeled HPV 11-infected xenograft tissue using preimmune serum; Lane 4, immunoprecipitation of ^{32}P-labeled HPV 11-infected xenograft tissue using anti-E1^E4 serum. (B) Autoradiography of the immunoblot presented in A. Molecular mass markers in kilodaltons are indicated on the left of A and B. (C) Phosphoamino acid analysis of immunoprecipitated E1^E4 protein. Phosphoserine, phosphothreonine, and phosphotyrosine markers are indicated on the left.

separated on SDS–PAGE and transferred to nitrocellulose. An immunoassay was performed to detect E1^E4 protein (Fig. 4A). Both the 10- and 11-kDa E1^E4 proteins were specifically immunoprecipitated by anti-E1^E4 serum (Fig. 4A, Lanes 2 and 4) but not preimmune rabbit serum (Fig. 4, Lane 3). The immunoblot was then subjected to autoradiography (Fig. 4B). A large number of phosphorylated proteins in the HPV 11-infected xenograft tissue were present in the crude extract (Fig. 4B, Lane 1). A phosphorylated protein was detected at 11 kDa corresponding to E1^E4 protein (Fig. 4B, Lane 4). Additional phosphoproteins at approximately 50 to 55 kDa were immunoprecipitated by both preimmune rabbit serum (Fig. 4B, Lane 3) and anti-E1^E4 serum (Fig. 4B, Lane 4). The significance of these nonspecifically immunoprecipitated proteins is not known. Phosphoamino acid analysis of the 11-kDa immunoprecipitated E1^E4 protein was performed, revealing weak phosphoserine and phosphothreonine signals on autoradiography but no phosphotyrosine signal (Fig. 4C).

Lambda phosphatase treatment of immunoprecipitated E1^E4 protein

Lambda phosphatase (λ-PPase) (New England Biolabs, Beverly, MA) removes phosphate groups from phosphoserine and phosphothreonine residues. A [^{32}P]orthophosphate-labeled extract of HPV 11-infected xenograft tissue was immunoprecipitated with preimmune or anti-E1^E4 sera. A portion of immunoprecipitated E1^E4 protein was treated with λ-PPase. Immunoprecipitated proteins were separated by SDS–PAGE and transferred to nitrocellulose. Immunoblot analysis revealed immunoreactive bands corresponding to the 10/11-kDa E1^E4 doublet from the anti-E1^E4 immunoprecipitation (Fig. 5A, Lane 2). Following treatment of the immunoprecipitated E1^E4 protein with λ-PPase, a slightly faster migrating band was detected on the immunoblot (Fig. 5A, Lane 3). Autoradiography of the immunoblot was then performed (Fig. 5B). The phosphorylated immunoprecipitated E1^E4 protein was visualized at 11 kDa (Fig. 5B, Lane 2). Additional higher molecular weight proteins of approximately 50 to 55 kDa were nonspecifically immunoprecipitated by both preimmune rabbit serum (Fig. 5B, Lane 1) and anti-E1^E4 serum (Fig. 5B, Lane 2). The significance of these proteins is not known. Treatment of the immunoprecipitated E1^E4 protein with λ-PPase removed any detectable phosphorylation signal (Fig. 5B, Lane 3).

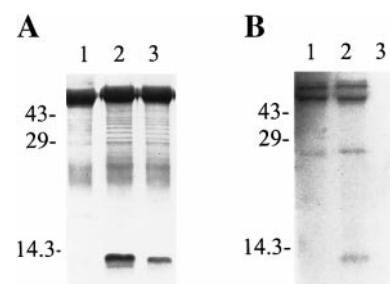

FIG. 5. Dephosphorylation of E1^E4 protein by λ-PPase. (A) Immunoblot of immunoprecipitated E1^E4 protein from ^{32}P-labeled HPV 11-infected xenograft tissue using anti-E1^E4 serum. Lane 1, immunoprecipitation with preimmune rabbit serum; Lane 2, immunoprecipitation with anti-E1^E4 serum; Lane 3, immunoprecipitation with anti-E1^E4 serum followed by λ-PPase treatment. (B) An autoradiograph of the immunoblot in A. Molecular mass markers in kilodaltons are indicated on the left of each panel.

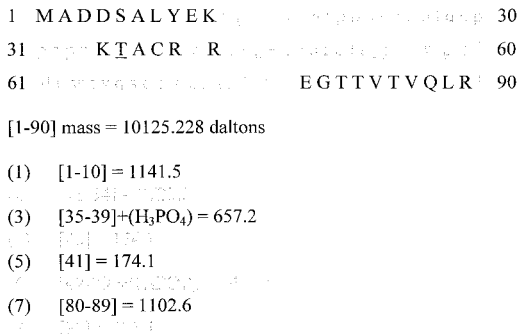

FIG. 6. Trypsin digestion of the HPV 11 E1^E4 protein. The amino acid sequence of the E1^E4 protein is shown using single-letter code. Individual trypsin fragments are indicated by alternating uppercase (black) and lowercase (gray) letters. Numbers on each side of the sequence represent the amino acid number. Shown below the E1^E4 sequence are the masses of the trypsin digestion fragments (determined using Protein Prospector), including the phosphate groups found to be present in peptides 3 and 6. The underlined amino acids in peptide fragments 3 and 6 were phosphorylated in the *in vitro* studies.

Analysis of E1^E4 protein using liquid chromatography–mass spectrophotometry (LC–MS)

Eight fragments, including five peptides and three single amino acid residues, were generated by in-gel trypsin digestion of E1^E4 protein (Fig. 6). LC–MS was performed, and peptides 3 and 6 were found to contain one and three phosphate groups, respectively. Phosphopeptide 3, corresponding to amino acids 35 through 39, contained a phosphothreonine at position 36, within the predicted PKA motif. Both the second potential PKA site at serine 44 and the MAPK site at threonine 53 found by our *in vitro* studies were contained in phosphopeptide 6 (amino acids 42 through 79). This peptide contains a total of 15 serine and threonine residues. It was identified by mass, 4467.1 Da, corresponding to a +4 charge state with three phosphate groups. Collision-induced dissociation of this large peptide resulted in too many fragments to assign ionized masses with confidence. The other E1^E4 peptides generated were shown to contain no phosphoamino acid residues. These included peptides containing a serine at position 5 (amino acids 1 through 10), a threonine at position 19 (amino acids 11 through 34), or three threonine residues at positions 81, 82, and 84 (amino acids 79 through 89).

Analysis of E1^E4 mutants expressed in human keratinocytes

To determine whether phosphorylation affects the intracellular location of the E1^E4 protein, wild-type and mutant proteins were expressed as fusions with either green fluorescent protein (GFP) or blue fluorescent proteins (BFP). Primary human keratinocytes (PHKs) were transfected and expressed GFP–E1^E4 containing wild-type sequences, GFP–E1^E4 PKA (mutant protein containing alanine substitutions for threonine 36 and serine 44), or GFP–E1^E4 MAPK (mutant protein containing an analine substitution for threonine 53). GFP–E1^E4 was detected exclusively in the cytoplasm, circling the nucleus and extending to the cell periphery in long filamentous strands (Fig. 7A). GFP–E1^E4-PKA was indistinguishable from GFP–E1^E4 (Fig. 7B). In contrast, GFP–E1^E4-MAPK was detected as diffuse fluorescence throughout the cell, with a minor degree of filamentous distribution (Fig. 7C).

The HPV 11 E1^E4 protein has been shown to oligomerize (Bryan *et al.,* 1998). As an indication of the potential to oligomerize, PHKs were cotransfected with the pEGFP–E1^E4 plasmid and the pEBFP–E1^E4 plasmid for expression of blue fluorescent protein fused to E1^E4 (BFP–E1^E4). As expected, the GFP–E1^E4 pattern of green fluorescence (Fig. 8A) was nearly identical to the BFP–E1^E4 blue fluorescence pattern (Fig. 8B), indicating colocalization. Together, double-image photography confirmed colocalization (Fig. 8C). GFP–E1^E4-PKA also colocalized with BFP–E1^E4 (data not shown). In contrast, GFP–E1^E4-MAPK did not colocalize with BFP–E1^E4. GFP–E1^E4-MAPK and BFP–E1^E4 were detected with two distinct expression patterns. GFP–E1^E4 MAPK was detected as diffuse green fluorescence throughout the cell (Fig. 8D). In contrast, BFP–E1^E4 was detected as blue fluorescence circling the nucleus and extending toward the cell periphery (Fig. 8E). Double-image photography was consistent with an absence of colocalization of the two fusions (Fig. 8F).

DISCUSSION

The studies reported here demonstrate that the HPV 11 E1^E4 protein is phosphorylated *in vivo* and that PKA and MAPK phosphorylate the protein *in vitro.* Determining the significance of posttranslational modifications and the association with cellular components will provide important information in evaluating potential roles for the E1^E4 protein. Phosphorylation represents a posttranslational modification that may profoundly influence protein function by altering the conformational structure and charge of the target protein. The E1^E4 protein is expressed only in differentiated keratinocytes, suggesting that its function is linked to events occurring specifically in these cells.

Immunoblot analysis of HPV 11-infected xenograft tissue shows an E1^E4 doublet of 10/11 kDa, as well as higher molecular weight proteins (Brown *et al.,* 1988). We have observed a variability in the relative abundance of the 10- and 11-kDa species in human biopsy samples of HPV 11-infected condylomata acuminata and in HPV 11-infected athymic mouse implants (Brown *et al.,* 1991, 1992). In HPV 11-infected xenograft tissue there may be several forms of the E1^E4 protein: phosphorylated forms at one or more amino acid residues and a nonphosphor-

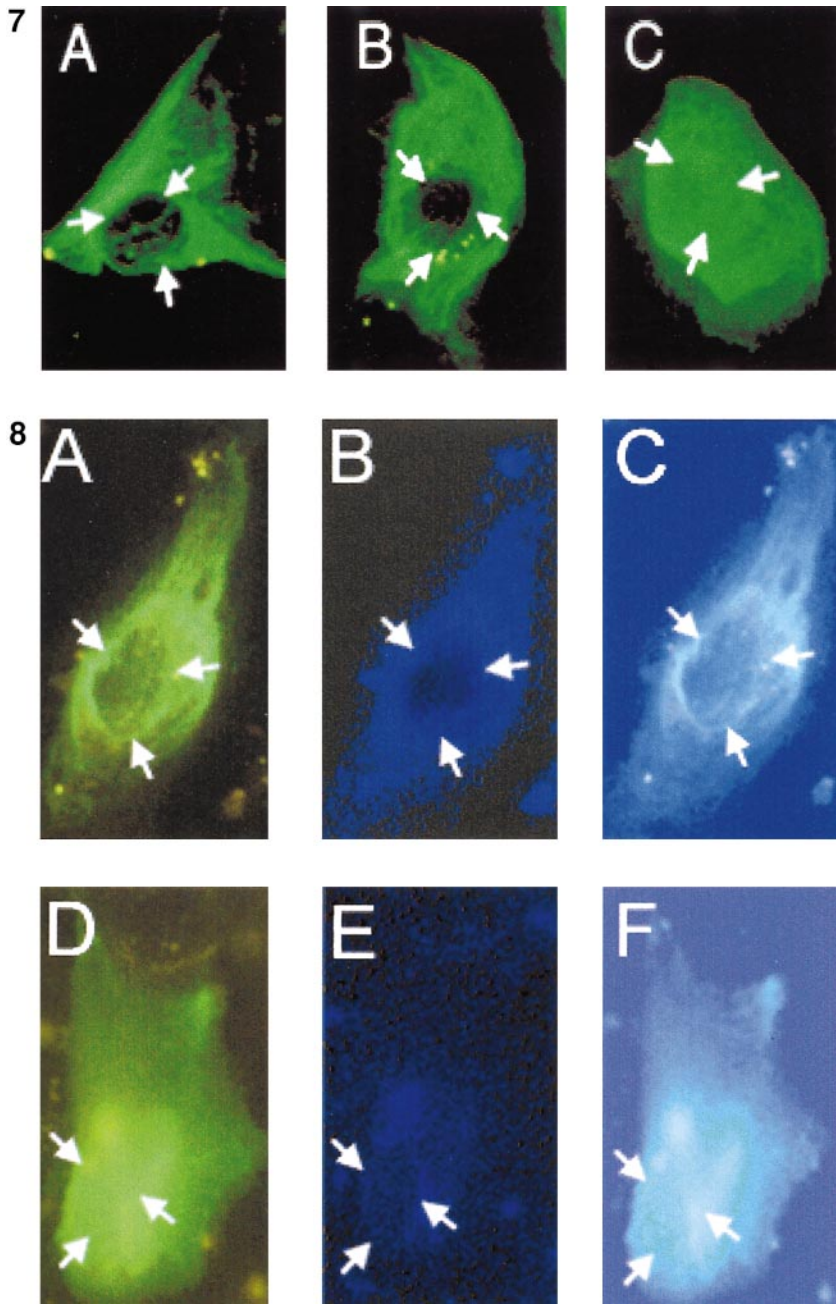

FIG. 7. Expression of GFP–E1^E4 phosphorylation substitution mutants in PHKs. (A) PHK expressing GFP–E1^E4 (wild-type E1^E4 protein fused to GFP); (B) PHK expressing GFP–E1^E4 PKA; (C) PHK expressing GFP-E1^E4 MAPK. Arrows indicate the periphery of the nucleus.

FIG. 8. Coexpression of wild-type E1^E4 protein fused to BFP (BFP–E1^E4) and GFP–E1^E4 phosphorylation substitution mutants. A PHK coexpressing GFP–E1^E4 and BFP–E1^E4 was examined using a FITC filter to detect the GFP–E1^E4 protein (A), a DAPI filter to detect the BFP–E1^E4 protein (B), or both filters to detect colocalization of the fusions (C). A PHK coexpressing GFP–E1^E4 MAPK substitution mutant and BFP–E1^E4 was examined using a FITC filter to detect GFP–E1^E4 MAPK (D), a DAPI filter to detect the BFP–E1^E4 protein (E), or both filters to determine the intracellular location of the fusions (F). In this case, the two fusions did not colocalize. Arrows indicate the periphery of the nucleus.

ylated form. Only one of these forms may be functional, or the various forms may have different functions.

Certain sequences within the E1^E4 protein appear to be highly conserved between genital HPV types of differing oncogenic potential, including several predicted phosphorylation sites. The HPV 11 E1^E4 sites shown to be phosphorylated by PKA are nearly identical to the sequences around serines 31 and 42 of the HPV 16 E1^E4 protein (Seedorf *et al.,* 1985). In addition, each of the HPV types 6 and 83 E1^E4 proteins contains one of the two PKA sites (Brown *et al.,* 1999; Schwarz *et al.,* 1983). Two overlapping MAPK consensus sequence motifs are present around threonine residues 51 and 54 in the HPV 16 E1^E4 protein. The latter threonine corre-

sponds to the HPV 11 E1^E4 threonine 53, which we have demonstrated is a MAPK phosphoacceptor. MAPK can also phosphorylate the HPV 16 E1^E4 protein *in vitro* (our unpublished data). MAPK motifs are also present in the E1^E4 sequences of HPV 6 (two overlapping motifs around serine 50 and threonine 54) and HPV 83 (around serine 61).

Our studies show that PKA is likely to be a kinase involved in HPV 11 E1^E4 phosphorylation. Phosphoamino acid analysis of E1^E4 protein purified from HPV 11-infected human tissue demonstrated serine and threonine phosphorylation, consistent with our observation that PKA phosphorylated E1^E4 *in vitro.* This result was further supported by studies using LC–MS. LC–MS followed by fractionation demonstrated a phosphopeptide corresponding to amino acids 35 through 39, containing a phosphothreonine at position 36, the location of a consensus PKA phosphorylation sequence motif. Three additional peptides were identified and shown to contain no phosphoamino acid residues. The second potential PKA phosphorylation sequence motif at serine 44 and the MAPK phosphorylation sequence motif at threonine 53 shown by our *in vitro* studies to be phosphorylated were both contained in a large trypsin digestion peptide (amino acids 42 through 79). This large peptide contained three phosphates, consistent with our two predicted sites plus one additional site that we could not identify.

PKA is activated by cyclic AMP and phosphorylates numerous cellular proteins (Soderling, 1990). PKA binds to A-kinase anchoring proteins (AKAPs) that help direct the kinase to discrete intracellular locations, including the cytoplasm (Colledge and Scott, 1999). Anchoring ensures that PKA is exposed to localized changes in cyclic AMP concentration and favors specific phosphorylation events by placing the kinase close to a particular subset of substrates. In addition, PKA can activate and can be activated by the MAPK signaling pathway (Colledge and Scott, 1999). HPV 11 E1^E4 protein has been localized to the cytoplasm, concentrated at the cell periphery of differentiated cells (Brown *et al.,* 1994). It is possible that PKA-bound AKAPs are responsible for directing E1^E4 phosphorylation.

In addition to PKA, the HPV 11 E1^E4 protein was phosphorylated by MAPK *in vitro.* The significance of this observation is not known, and the exact role of phosphorylation on E1^E4 function can be determined only through further study. The GFP–E1^E4 MAPK phosphorylation substitution mutant and BFP–E1^E4 colocalization studies demonstrated that the two fusion proteins did not colocalize to the same region of the cell. This observation suggests either that MAPK phosphorylation is necessary or that the threonine residue at position 53 is required for E1^E4 cytoplasmic localization and oligomerization. The E1^E4 may interact with components of a signaling cascade either by becoming modified by kinases such as PKA and MAPK or by interfering with normal signaling pathways. Previous studies suggest an interaction of E4 gene products with cytoskeletal proteins (Doorbar *et al.,* 1991; Roberts *et al.,* 1993). E1^E4 phosphorylation may have a role in such interactions, as was supported by the altered expression pattern of the GFP–E1^E4-MAPK substitution mutant in PHKs.

In summary, the HPV 11 E1^E4 protein was shown to be phosphorylated *in vitro* by PKA and MAPK. In HPV 11-infected human genital epithelium, E1^E4 protein was shown to be phosphorylated at serine and threonine residues, consistent with activity by these kinases. Further studies are needed to determine how phosphorylation influences E1^E4 protein function in terminally differentiated keratinocytes.

MATERIALS AND METHODS

Identification of kinase phosphorylation sites

Potential protein kinase phosphorylation sites were identified in the E1^E4 sequence using the computer program ScanProsite (http://www.expasy.ch/tools/scnpsite.html). The program scans the primary structure of a protein for the occurrence of patterns matching known consensus pattern sequences including many kinase phosphorylation sequence motifs.

Production of MBP–E1^E4 and mutant fusion proteins

The pMAL–E1^E4 construct was made by amplifying the complete HPV 11 E1^E4 sequence using reverse transcriptase-PCR and ligating in-frame into pMAL-c2 (New England Biolabs). The resulting construct was used to transform DH5-α cells (Life Technologies, Gibco BRL, Gaithersburg, MD). Expression of the MBP–E1^E4 fusion protein was induced by addition of IPTG. The MBP–E1^E4 fusion was purified using an amylose resin column as directed by the manufacturer (New England Biolabs).

PKA phosphorylation of E1^E4 amino acids serine 44 and threonine 36 was examined by mutating these residues to alanines. For the mutant with alanine substituted for serine at position 44 (MBP–E1^E4 44S > A), the sense strand oligonucleotide primer 5′ CGG CGC CG**C** CTA GGA **GC**C GAG CAC 3′ was used, which contained the E1^E4 internal *Nar*I site (underlined) and the substituted nucleotides (nt) indicated in boldface type. This primer was used to amplify E1^E4 115–278 nt (44S > A). Digestion at the *Nar*I site allowed the substitution of the 115–278 nt (44S > A) sequence for the wild-type 115–278 nt sequence in the pMAL–E1^E4 plasmid generating pMAL–E1^E4 44S > A. For the mutant with alanine substituted for threonine at position 36 (MBP–E1^E4 36T > A), the sense strand oligonucleotide primer 5′ CCA CAC AGA CCA CCG CCC CTA CAG TGT CCG CCT GCA CCA CGG AAG **G**CG GCG 3′ was used to introduce the

substitution, indicated in boldface type, by PCR. This amplified product encodes amino acids 21–90 of E1^E4 with the alanine substitution of amino acid 36. A second sense-strand oligonucleotide primer was then employed to extend the sequence 5′ to the E1^E4 ATG by a second PCR. The amplified product was cloned to generate pMAL–E1^E4 36T > A. The double-mutant was generated by using pMAL–E1^E4 44S > A as the template for the initial PCR with the oligonucleotide E1^E4 36T > A. This amplified product was cloned into pMAL-c2 and expressed to generate an MBP fusion protein, MBP–E1^E4 36T > A/44S > A, with a 20-amino-acid truncation at the amino-terminus and alanine substitutions of threonine 36 and serine 44.

MAPK phosphorylation of E1^E4 amino acid threonine 53 was examined in the context of the first 60 amino acids of the E1^E4 protein. MBP–E1^E4(1–60) was generated by PCR using the antisense oligonucleotide primer 5′ GTC GAC CTA TGA TGT TGG CCA CA 3′, which contains an in-frame stop codon (underlined) and a *Sal*I site extension. The amplified product was digested with *Sal*I and cloned into the pMAL-c2 vector. For MBP–E1^E4(1–60) 53T > A, the antisense oligonucleotide primer 5′ GTC GAC CTA TGA TGT TGG CCA CAC ACA GGG **C**G**C** 3′ which contains an in-frame stop codon (underlined), the substituted nucleotide (in boldface type), and a *Sal*I site extension. The amplified product was digested with *Sal*I and cloned into the pMAL-c2 vector. Sequence analysis was performed on all pMAL–E1^E4 plasmids to confirm in-frame cloning and nucleotide substitutions.

In vitro phosphorylation of the MBP–E1^E4 and mutant fusion proteins

MBP–E1^E4 fusion protein or, as a control, nonfused MBP was used for *in vitro* phosphorylation reactions with MAPK, CKII, PKC, and PKA. For MAPK, CKII, or PKA (New England Biolabs), MBP or MBP–E1^E4 (5 μg each) was combined with 1.7 mM ATP, 10 μCi [γ-^{32}P]ATP, and 50 units MAPK, CKII, or PKA in the appropriate reaction buffer. Phosvitin (Sigma, St. Louis, MO) was used as a control protein in CKII reactions. Reactions were conducted at 30°C for 90 min. Factor Xa was added to a portion of each reaction to cleave the E1^E4 peptide from MBP. Proteins were separated on 15% SDS–PAGE, the gel was dried, and autoradiography was performed. To confirm that the phosphorylated peptide observed on the autoradiography was E1^E4 protein, immunoblot analysis was performed using an antiserum raised against a trpE–E1^E4 fusion protein (Brown *et al.,* 1994). Autoradiography of the immunoblot was then performed.

For PKC (Promega), MBP (5 μg), MBP–E1^E4 (5 μg), MBP–E1^E4 (5 μg) plus Type III-S histone at 1 mg/ml (Sigma), or histone alone was used as substrate and combined with 20 mM HEPES (pH 7.4), 1.0 mM DTT, 10 mM ATP, 10 mM $MgCl_2$, 10 μCi [γ-^{32}P]ATP, and 12.5 ng PKC in the presence or in the absence of 600 μg/ml phosphatidyl serine and 1.7 mM $CaCl_2$. The reaction was conducted at 30°C for 90 min. MBP–E1^E4 was digested with Factor Xa, the resulting peptides were separated by SDS–PAGE, the gel was dried, and autoradiography was performed.

In vitro phosphorylation reactions using MBP–E1^E4 mutants were performed to determine the specific amino acid residues phosphorylated by PKA and MAPK. Reactions and analysis of phosphorylated proteins were conducted as described above.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed to determine the amino acid phosphoacceptors involved in phosphorylation reactions of the E1^E4 protein. MBP–E1^E4 protein was phosphorylated *in vitro* as described above, and E1^E4 sequences were separated from MBP by Factor Xa digestion. Proteins were then separated by SDS–PAGE and transferred to PVDF membrane (Bio-Rad, Hercules, CA). To identify the E1^E4 protein, immunoblot analysis was performed on a portion of the PVDF membrane using the anti-E1^E4 serum. A thin strip of PVDF membrane containing the ^{32}P-labeled E1^E4 protein from each experiment was hydrolyzed in HCl (5.7 N) at 110°C and lyophilized. Residues were suspended in water and applied to thin-layer cellulose plates (Eastman Kodak Co., Rochester, NY). After separation by thin-layer electrophoresis, plates were air-dried and standards (serine, threonine, and tyrosine) were visualized by ninhydrin (0.2% in acetone) application. ^{32}P-labeled phosphoamino acids were detected by autoradiography and compared to standards.

Phosphorylation of E1^E4 protein expressed in HPV 11-infected human epithelium

HPV 11-infected xenograft tissue was incubated with [^{32}P]orthophosphate in DMEM without phosphate (Life Technologies, Gibco BRL) for 18 h in 5% CO_2 at 37°C. The labeled tissue was washed with PBS and ground in a sterile disposable tissue grinder. Some material was reserved for analysis, and the remainder was extracted in immunoprecipitation (IP) buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 1% Nonidet-P40; 0.5% deoxycholic acid; 0.1% SDS; 4 M urea).

The HPV 11 E1^E4 protein was immunoprecipitated from the infected tissue extract as follows. Twenty microliters of Protein A–Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) beads was added to 500 μl of IP buffer. Ten microliters of anti-E1^E4 serum was added and incubated at 4°C for 16 h with agitation. As a control, preimmune rabbit serum was incubated with Protein A–Sepharose CL-4B beads in an identical manner. Sepharose beads were washed three times with 5 vol of

cold IP buffer. A 100-mg fragment of an HPV 11-infected human foreskin implant grown in an athymic mouse (Kreider *et al.,* 1987) was ground in 5 ml of IP buffer, heated to 100°C for 15 min, and clarified by centrifugation at 10,000*g* for 10 min. Five hundred microliters of clarified supernatant was added to the washed Sepharose beads bound to either preimmune antibodies or anti-E-E1^E4 antibodies. After incubation at 4°C for 16 h with agitation, the Sepharose beads were washed five times with IP buffer. The Sepharose beads were then suspended in Laemmli (1970) buffer and heated to 100°C and proteins were separated on 15% SDS–PAGE. To identify the E1^E4 protein, immunoblot analysis was performed using anti-E1^E4 serum.

Phosphoamino acid analysis was performed to determine the amino acid phosphoacceptor residues involved in phosphorylation of E1^E4 protein expressed in HPV 11-infected xenograft tissue. Immunoprecipitation of *in vivo* labeled tissue was performed as described above. SDS–PAGE and immunoblot analyses were performed as described for the *in vitro* phosphoamino acid reactions. The strip of PVDF membrane containing the ^{32}P-labeled E1^E4 protein was acid hydrolyzed and applied to thin-layer cellulose plates. After separation by thin-layer electrophoresis, ^{32}P-labeled phosphoamino acids were detected by autoradiography and compared to standards.

Phosphatase treatment of immunoprecipitated E1^E4 protein

To further verify E1^E4 phosphorylation in HPV 11-infected human genital epithelium, ^{32}P-labeled E1^E4 immunoprecipitated protein was treated with lambda phosphatase. λ-PPase is a Mn^{2+}-dependent protein phosphatase that dephosphorylates serine, threonine, and tyrosine residues. The Protein A–Sepharose CL-4B beads with bound E1^E4 were resuspended in 100 μl of λ-PPase reaction buffer (50 mM Tris–HCl, pH 7.8; 5 mM dithiothreitol; 2 mM $MnCl_2$; and 100 μg of bovine serum albumin per milliliter) with 2 μl of λ-PPase (400,000 U/ml) and incubated for 1 h at 37°C. The Sepharose beads were washed five times with cold IP buffer and resuspended in Laemmli buffer. After being heated to 100°C, samples were centrifuged and supernates analyzed by 15% SDS–PAGE. Immunoblot analysis was performed using anti-E1^E4 serum. Autoradiography of the immunoblot was then performed.

Liquid chromatography–mass spectrophotometry

To verify results of the *in vitro* phosphorylation studies, E1^E4 protein was derived from two sources and analyzed by LC–MS and collision-induced dissociation (LC–MS/MS) using a Finnigan LCQ Mass Spectrophotometer (ThermoQuest, San Jose, CA). First, as a relatively pure source, E1^E4 protein was immunoprecipitated from HPV 11-infected xenograft tissue. Second, to obtain a large sample of E1^E4 protein, a crude extract of a large condyloma acuminata lesion (containing HPV 11) was prepared. The E4 ORF was sequenced and shown to be identical to that from the HPV 11-infected xenograft tissue isolate. The E1^E4 protein was isolated from each preparation by 20% SDS–PAGE followed by identification using anti-E1^E4 serum in an immunoblot of a side portion of the gel. The E1^E4 protein was excised from the gel, and in-gel trypsin digestion was performed. Peptides were analyzed using LC–MS/MS and ionized fragments were identified using Protein Prospector (MS-Product) (http://prospector.ucsf.edu/ucsfhtml3.2/msprod.htm).

Analysis of E1^E4 mutants expressed in human keratinocytes

Three GFP fusions were constructed to analyze the characteristics of E1^E4 phosphorylation substitution mutants. These included full-length E1^E4 wild-type sequences, a mutant containing alanine substitutions for threonine 36 and serine 44 (PKA phosphoacceptors), and a mutant containing an alanine substitution for threonine 53 (MAPK phosphoacceptor). To produce these fusions, wild-type or mutant E1^E4 sequences were amplified by PCR and subcloned in-frame into pEGFP-C1 (Clontech, Palo Alto, CA). Sequencing was performed to confirm introduction of mutations and in-frame cloning. The GFP–E1^E4 fusion proteins were expressed in PHKs (Clonetics, San Diego, CA) by transient transfection with Effectene as directed by the manufacturer (Qiagen). PHKs were grown to 60% confluence on glass coverslips in keratinocyte growth medium (KGM, Clonetics) with 0.10 mM calcium. The transfection solution was applied for 16 h and replaced with fresh medium. Twenty-four hours after transfection, the cells were washed three times in PBS and mounted with GEL-MOUNT (Biomeda Corp., Foster City, CA) onto glass slides. Fluorescence microscopy using a FITC filter was performed to examine the intracellular localization of the GFP–E1^E4 PKA and MAPK phosphorylation substitution mutants compared to the wild-type E1^E4 protein.

PHKs were cotransfected with the pEGFP–E1^E4 plasmids and either pEBFP–C1 (Clontech) to express blue fluorescent protein or pEBFP–E1^E4 to express a BFP–E1^E4 fusion protein. Fluorescence microscopy using DAPI and FITC filters was performed to determine whether wild-type E1^E4 protein would colocalize with the phosphorylation substitution mutants.

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