

High-Risk Human Papillomavirus E6 Oncoproteins Interact with 14-3-3 ζ in a PDZ Binding Motif-Dependent Manner

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Cervical cancer develops through the combined activities of the human papillomavirus (HPV) E6 and E7 oncoproteins. A defining characteristic of E6 oncoproteins derived from cancer-causing HPV types is the presence of a PDZ binding motif (PBM) at the extreme carboxy terminus of the protein which is absent from E6 proteins derived from the so-called low-risk HPV types. Within this PBM is also a protein kinase A (PKA) phospho-acceptor site, which is thought to negatively regulate the association of E6 with its PDZ domain-containing substrates. We can now show that phosphorylation of E6 by PKA and/or AKT confers the ability to interact with 14-3-3 ζ . The interaction is direct and specific for the high-risk HPV E6 oncoproteins, although there are significant differences in the efficiencies with which HPV-16, HPV-18, and HPV-31 E6 oncoproteins can associate with 14-3-3 ζ ; this correlates directly with their respective susceptibilities to phosphorylation by PKA and/or AKT. We demonstrate here that the interaction between E6 and 14-3-3 ζ also requires integrity of the E6 PBM, and downregulation of 14-3-3 ζ results in a marked reduction in the levels of HPV-18 E6 expression in HeLa cells. Using phospho-specific anti-E6 antibodies, we also demonstrate significant levels of E6 phosphorylation *in vivo*. These studies redefine the potential relevance of the E6 PBM in the development of cervical cancer, suggesting that interaction with 14-3-3 ζ , as well as the more well-established interactions with PDZ domain-containing substrates, is likely to be responsible for the biological activities attributed to this region of the high-risk HPV E6 oncoproteins.

Human papillomaviruses (HPVs) are the causative agents of a large number of different human cancers, including cervical cancer, other anogenital cancers, and a growing number of head and neck cancers (reviewed in reference 1). Of these, cervical cancer is by far the most prevalent, being the second major cause of cancer-related death for women worldwide. Recently, the WHO classified 12 different cervical cancer-causing HPV types, with HPV-16 and HPV-18 being predominant (2). A defining feature of cervical cancers and derived cell lines is the continued expression of two viral oncoproteins, E6 and E7, many years after the initial immortalizing events (3–5). Indeed, abrogation of their respective functions or expression results in a cessation of cell growth and induction of senescence and/or apoptosis (6–8). Therefore, these two proteins represent ideal targets for potential therapeutic intervention in HPV-induced malignancies.

The HPV E6 and E7 proteins contribute to tumor development by directly interfering with critical growth-regulatory pathways (9, 10). The so-called high-risk cancer-causing HPV E7 oncoproteins interact with a very large number of cellular proteins, many of which are involved in the regulation of cell proliferation and control of the cell cycle, as well as gene expression and DNA damage response pathways. These include the pRb family of tumor suppressor proteins (reviewed in reference 10), which, with the aid of the ubiquitin proteasome pathway, are targeted for degradation by the HPV-16 E7 oncoprotein (11, 12). The net result of this E7 activity is the induction of unscheduled DNA replication (13) and DNA damage (14), with subsequent induction of a p53 response, signaling growth arrest, and apoptosis (15, 16). This activity of p53 is overcome largely through the activity of the E6 oncoprotein, which, together with the ubiquitin ligase E6AP, efficiently directs p53 degradation at the 26S proteasome (17).

While inactivation of the pocket proteins and of the p53 signaling networks is a central facet of the ability of high-risk HPV types to cause cancer, a number of other activities are likely to be

involved. Particularly intriguing are the PDZ domain-containing substrates of the high-risk HPV E6 oncoproteins. All HPV types that are defined as cancer causing produce E6 oncoproteins that have a class I PDZ binding motif (PBM) at their extreme carboxy termini (18, 19). This sequence is absent in all of the so-called low-risk non-cancer-causing HPV E6 oncoproteins. E6 has been reported to interact, through this PBM, with at least 10 different cellular PDZ domain-containing substrates, many of which are involved in regulating processes as diverse as cell polarity regulation, cell signaling, cell attachment, and cell proliferation (reviewed in reference 20). Mutational analysis of the E6 PBM has implicated this activity of E6 in a wide range of biological functions. In some cases, it has a role in keratinocyte immortalization and transformation (21, 22); in others, it has been linked to the induction of epithelial-mesenchymal transition (21, 23). In animal models, the integrity of the E6 PBM appears to play a role in the capacity of E6 to induce malignancy in both the skin and the cervix (24, 25). Furthermore, viral life cycle studies have shown a critical role for this function of E6 in contributing to the normal viral life cycle, with loss of the PBM binding capacity of E6, in the context of the whole viral genome, reducing the capacity of the virus to maintain itself episomally (26). Finally, recent studies have suggested a potential role for this domain of E6 in contributing to the stability of the E6 protein (27).

However, the ability of E6 to recognize PDZ domains is not constitutive. Embedded within the PBM is a protein kinase A

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(PKA) phospho-acceptor site, and numerous studies have shown that phosphorylation of the key serine/threonine residue within this site inhibits PDZ domain binding activity (28, 29). Intriguingly, like the PBM itself, the PKA consensus recognition sequence is very highly conserved in all HPV types that are defined as cancer causing and is absent from all the low-risk HPV E6 oncoproteins (28). This raises the question of whether the phosphorylation of E6 by PKA serves to only regulate PDZ binding activity or can confer interaction with other potential cellular target proteins. Obvious candidates are members of the 14-3-3 family of proteins. These are phospho-threonine/serine binding proteins (30) that have been implicated in the regulation of a large number of different cellular processes, with many of the 14-3-3 family members being implicated in processes directly relevant for cancer progression and malignancy (31, 32). Indeed, a recent proteomic analysis reported 14-3-3ζ to be a candidate E6 interaction partner (33). This was particularly intriguing since 14-3-3ζ has been implicated as a potential oncogene in a number of different human cancers, with overexpression frequently observed (32, 34–36).

Therefore, we have investigated whether 14-3-3ζ is an interaction partner of the high-risk HPV E6 oncoproteins. We show that high-risk HPV E6 can recognize 14-3-3ζ in a PKA or AKT phosphorylation-dependent manner, and we provide evidence to suggest that this interaction helps to maintain steady-state levels of the HPV-18 E6 oncoprotein.

MATERIALS AND METHODS

Cell culture and transfections. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/ml), and glutamine (300 μg/ml). Small interfering RNA (siRNA) transfections were performed using Lipofectamine 2000 (Invitrogen) for 72 h with approximately 1.2×10^5 cells seeded in 60-mm dishes. siRNA against luciferase was used as a negative control, and an ON-TARGETplus SMARTpool against 14-3-3ζ (human YWHAZ) was purchased from Thermo Scientific Dharmacon.

Plasmids. To generate glutathione S-transferase (GST) fusion proteins, HPV-11, HPV-16, and HPV-18 E6 proteins and Scribble were cloned and expressed in pGEX2T as described previously (37–39). The HPV-31 E6-GST fusion protein was generated by subcloning PCR-amplified HPV-31 E6 into compatible BamHI and EcoRI restriction sites in pGEX2T. The HPV-18 E6 mutants R153A, T156E, T156D, S82A, and S82A/T156E were produced by using the GeneTailor mutagenesis kit (Invitrogen). The primer sequences used were 5'-GAACGACTCCAACGAGCGAGAGAAACACAAGTA-3' (forward) and 5'-TCGTTGGAGTAGTTCCTGTCGTGC-3' (reverse) for R153A, 5'-CTCCAACGACGACGAGAGAGACCAAGTATAAG-3' (forward) and 5'-TTCTCTGCGTCGTTGGA GTCTGTTCC-3' (reverse) for T156D, and 5'-CAACGACGACGAGAAAG AACAAGTATAAGAATTC-3' (forward) and 5'-TTCTCTGCGTCGTTG GAGTCGTTCC-3' (reverse) for T156E. The resulting plasmids were all verified by DNA sequencing. These HPV-18 E6 mutants are depicted schematically in Fig. 1. The Dlg and MAGI-1 expression plasmids have been described previously (27), and the 14-3-3ζ expression plasmid was kindly provided by Haian Fu.

In vitro phosphorylation. GST fusion proteins were washed with $1 \times$ phosphate-buffered saline (PBS) containing 0.1% Tween 20 and then washed twice with the respective kinase buffers. The buffers used were PKA buffer (25 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, and 70 mM NaCl), p21-activated kinase (PAK) buffer (50 mM HEPES [pH 7.4], 12.5 mM NaCl, 1.5 mM MgCl₂, 0.5% Tween 20, and 1.5 mM MnCl₂), and AKT buffer (25 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, and 2 mM dithiothreitol [DTT]). Ca²⁺/calmodulin kinase II (CamKII) was diluted in $1 \times$ NEBuffer for protein kinases (New England BioLabs) supplemented with 200 μM ATP, 1.2 μM calmodulin, and 2 mM CaCl₂.

AKT consensus site

RXRXXS/T

PKA consensus site

RXXS/T

14-3-3 consensus

RRXS/T

PDZ binding motif

XS/TXV

	18 E6	82	156
RHYSDS.....	RRRETQV	
18 E6 T156ERHYSDS.....	RRRE ^Q QV	
18 E6 T156DRHYSDS.....	RRRE ^D QV	
18 E6 R153ARHYSDS.....	RARE ^T QV	
18 E6 S82ARHYADS.....	RRRETQV	
18 E6 S82A/T156ERHYADS.....	RRRE ^Q QV	

FIG 1 Amino acid sequence of HPV-18 E6 and its consensus recognition sites. The wild-type HPV-18 E6 sequence around residues S82 and T156 is shown. Upper section, consensus recognition sequences for phosphorylation with PKA (47) and AKT (49). Also shown is the consensus recognition motif for 14-3-3 (30) and the PDZ binding motif (48). Lower section, E6 mutants used in the study. Potential phospho-acceptor residues are shown in red, phosphomimic mutations are shown in green, and Ala substitutions are shown in blue.

In vitro phosphorylation of the fusion proteins was carried out at 20°C for 30 min in 20 μl kinase buffer containing 2.5 μCi [γ -³²P]ATP and 25 U of cAMP-dependent protein kinase, catalytic subunit (Promega), 250 U of activated CamKII (New England BioLabs), 10 pg of AKT-I (GenWay Biotech), or 37 U of PAK (Calbiochem).

In vivo phosphorylation and immunoprecipitation. HEK293 cells (7×10^5) were seeded onto 10-cm dishes and transfected with 10 μg of hemagglutinin (HA)-tagged HPV-18 or HPV-16 E6. Five hours posttransfection, cells were treated with 10 μM forskolin (Calbiochem) for 24 h. Cells were collected and lysed by using E1A buffer (250 mM NaCl, 0.1% NP-40, and 50 mM HEPES [pH 7.0]), with gentle syringing, and then placed on ice for 20 min. The cell lysate was then centrifuged at 14,000 rpm for 10 min, and the supernatant was incubated with 30 μl of monoclonal anti-HA agarose beads (Sigma) at 4°C for 3 h. Samples were washed thrice with E1A buffer and then subjected to Western blot analysis.

Fusion protein purification and in vitro binding assays. Purified GST fusion proteins (pre- and postphosphorylation with nonradiolabeled ATP) were incubated for 1 h at room temperature with *in vitro*-translated and -radiolabeled 14-3-3ζ, Dlg, and MAGI-1. After being washed with PBS containing 0.1% Tween 20, the bound proteins were subjected to SDS-PAGE and autoradiographic analysis.

For direct binding assays with purified His-tagged 14-3-3ζ recombinant protein (Abcam), the purified GST fusion proteins (pre- and postphosphorylation with nonradiolabeled ATP) were incubated with 10 ng of purified recombinant 14-3-3ζ at 4°C for 1 h. Complexed proteins were then washed thrice with PBS containing 0.1% Tween 20. Bound proteins were subjected to SDS-PAGE and Western blotting for the detection of His-tagged 14-3-3ζ.

Antibodies. Mouse monoclonal antibodies against HPV-18 E6 (1:1,000; N terminus no. 399) were generated and generously provided by the Arbor Vita Corporation. Mouse anti-6×His (BD Pharmingen), rabbit anti-α-actinin (Santa Cruz Biotechnology), and rabbit anti-14-3-3ζ (Santa Cruz Biotechnology) antibodies and appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) (Dako) were also used. The anti-phospho-E6-specific antibody (custom made by Eurogentec) was raised against the peptide H₂N-RQERLQRRRET(PO₃H₂)QV-

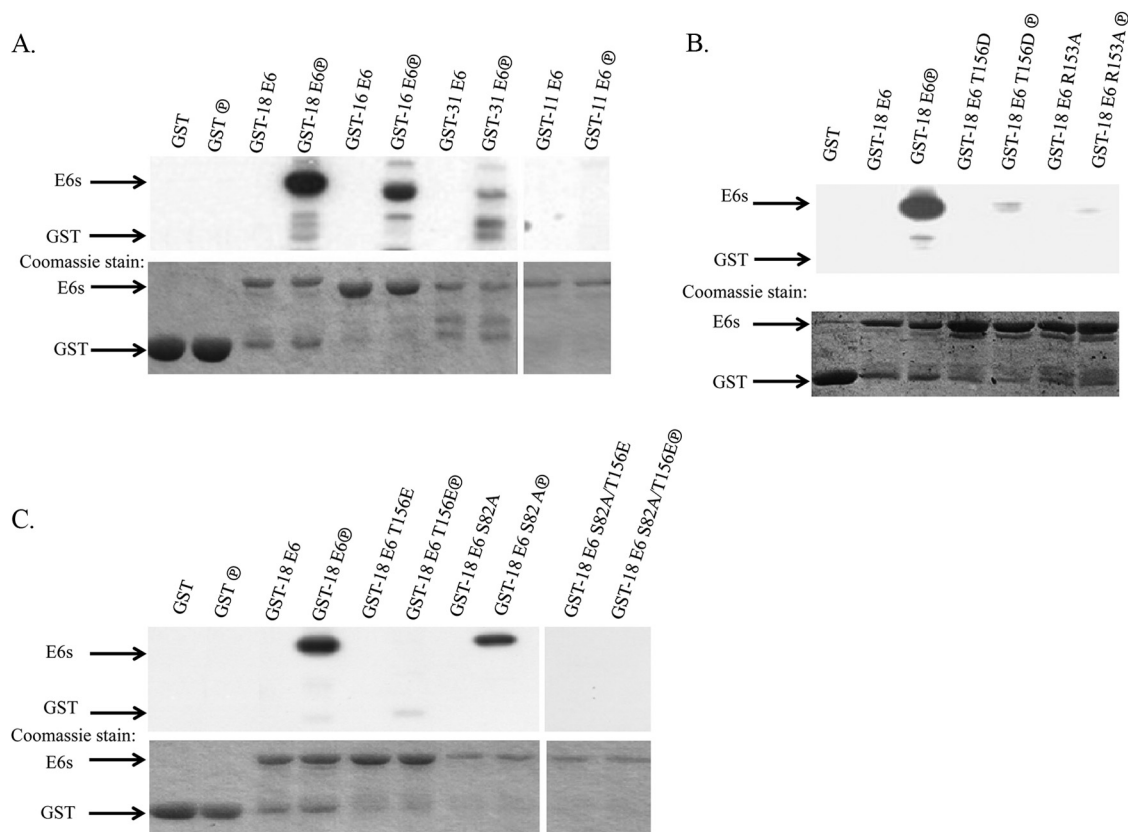


FIG 2 High-risk HPV E6 oncoproteins are phosphorylated to differing degrees by PKA. (A) The purified GST fusion proteins were either untreated or incubated with PKA and [γ - 32 P]ATP (circled P) as indicated. Proteins were then subjected to SDS-PAGE and autoradiographic analysis. Upper panel, autoradiogram from a series of parallel reactions; lower panel, the Coomassie blue-stained gel. (B and C) PKA phosphorylation of different HPV-18 E6 mutant-GST fusion proteins. Upper panels, autoradiograms; lower panels, the Coomassie blue-stained gels. Arrows indicate the relevant GST proteins. The residual levels of phosphorylation for the T156D and R153A mutants are approximately 1% of the level obtained with the wild-type E6 fusion protein.

COOH in rabbits. For the detection of phospho-E6 with this antibody in a Western blot, blocking was done using 5% bovine serum albumin (BSA) in 1× Tris-buffered saline containing 0.1% Tween 20 (1× TBST), with gentle rocking, at 4°C. The blot was then incubated overnight with anti-phospho-E6-specific antibody diluted with 5% BSA in 1× TBST (1:500), with gentle rocking. The blot was washed thrice with 1× TBST, incubated with anti-rabbit HRP (Dako) antibody, and developed by using the Amersham ECL detection system (GE Healthcare).

Western blotting. Immunoprecipitation samples or total cell extracts were obtained by lysing the cells directly in 2× SDS-PAGE sample buffer. Western blotting and processing were then performed as described previously (40). Protein band intensities were quantitated when possible by using the ImageJ quantification program.

RESULTS

High-risk HPV E6 oncoproteins are common substrates of PKA. Previous studies have shown that HPV-18 E6 and carboxy-terminal peptides derived from several other high-risk HPV E6 oncoproteins are substrates of PKA (28). In order to verify these results, we first investigated the susceptibility of different full-length E6 oncoproteins to phosphorylation by PKA. The high-risk HPV-16, HPV-18, and HPV-31 E6 and the low-risk HPV-11 E6 proteins were expressed as GST fusion proteins and purified. They were then incubated with purified PKA and radiolabeled ATP; after 20 min, the levels of phosphorylation were ascertained by SDS-PAGE and autoradiography. The results presented in Fig. 2A

demonstrate that HPV-16, HPV-18, and HPV-31 E6s are all substrates of PKA, while HPV-11 E6 is not, which is in agreement with previous studies (28). However, these results also demonstrate that HPV-18 E6 is the strongest substrate, followed by HPV-16 E6, with HPV-31 E6 being a much weaker PKA substrate.

It was shown previously that the major phospho-acceptor site on HPV-18 E6 is Thr156 (28); however, phospho-site predictions suggest that S82 might also be recognized by PKA. To determine whether Thr156 is indeed the major site of PKA phosphorylation of E6, we repeated the phosphorylation assays using wild-type HPV-18 E6 and the mutants (summarized in Fig. 1) T156D (Fig. 2B), T156E, and S82A, the double mutant S82A/T156E (Fig. 2C), and R153A (Fig. 2B); the latter mutant was predicted to destroy the PKA consensus recognition site. The results demonstrate that T156 is the major PKA phospho-acceptor site on HPV-18 E6, with mutations at this residue and at R153 largely destroying PKA phosphorylation of the E6 protein, while mutation of S82A had no major impact on the susceptibility of E6 to phosphorylation by PKA.

The above results demonstrate quite surprising differences in the susceptibilities of HPV-18, HPV-16, and HPV-31 E6 proteins to phosphorylation by PKA, despite their having very similar carboxy-terminal residues. This raised the possibility that other kinases might phosphorylate the different E6 oncoproteins. To in-

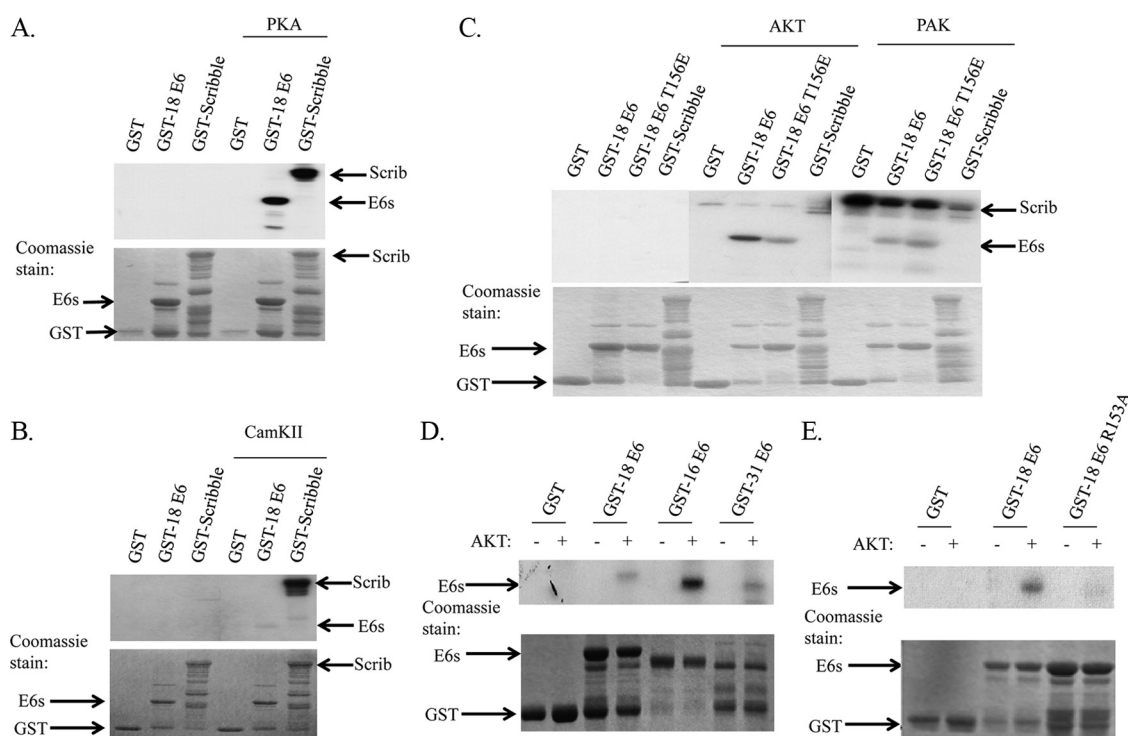


FIG 3 Comparative analysis of E6 phosphorylation by different kinases. (A through C) Phosphorylation of GST fusion proteins of HPV-18 E6, the HPV-18 E6 T156E mutant, and Scribble (Scrib) with PKA, CamKII, AKT, and PAK, as indicated. Upper panels, autoradiograms; lower panels, Coomassie blue-stained gels. (D and E) Phosphorylation of GST fusion proteins of HPV-18, HPV-16, and HPV-31 E6s and the HPV-18 E6 R153A mutant by AKT. Upper panels, autoradiograms; lower panels, the Coomassie blue-stained gels. Arrows indicate the relevant GST fusion proteins.

investigate this possibility, we extended the analysis to three kinases that all scored highly in the phospho-site prediction software analyses, i.e., p21-activated kinase (PAK), Ca^{2+} /calmodulin kinase II (CamKII), and protein kinase B (PKB) or AKT. We first analyzed the susceptibility of HPV-18 E6 to phosphorylation and compared the results with those for Scribble, a protein known to be phosphorylated by multiple kinases (39, 41). The results again confirm high levels of phosphorylation by PKA (Fig. 3A), with little or no phosphorylation by CamKII (Fig. 3B). There was very weak phosphorylation of E6 by PAK that was not T156 dependent (Fig. 3C). However, there was a significant level of phosphorylation by AKT (Fig. 3C) which occurred primarily at T156, although a second weaker site in E6 most likely also exists. To determine whether this was mediated by recognition of the carboxy-terminal consensus site (Fig. 1), the assay was repeated using the R153A mutant; as can be seen in Fig. 3E, this also abolished phosphorylation by AKT. Taken together, these results demonstrate that HPV-18 E6 can be phosphorylated by either PKA or AKT at residue T156, and the two kinases recognize the same consensus recognition site, as defined by the R153 residue. Having found that AKT was the kinase most likely to phosphorylate E6, in addition to PKA, we then analyzed HPV-16 and HPV-31 E6s. The results shown in Fig. 3D demonstrate that HPV-16 E6 is the better substrate for AKT, with HPV-31 E6 being phosphorylated more weakly (at a level similar to that seen for HPV-18 E6).

HPV-16 E6 and HPV-18 E6 are phosphorylated *in vivo*. The above results demonstrate that the high-risk HPV E6 proteins are potential substrates for PKA, although there is little evidence directly demonstrating E6 phosphorylation within its carboxy-ter-

минаl region *in vivo*. In order to address this, we generated phospho-specific antibodies directed against the HPV-18 E6 C terminus. As a means of assessing their specificities, we first performed Western blot analysis of HPV-18 and HPV-16 E6-GST fusion proteins that were either untreated or subjected to phosphorylation by PKA in the absence of radiolabeled ATP. The results shown in Fig. 4A show that only the phosphorylated E6 proteins were recognized by the anti-phospho-E6 antibody. Interestingly, the antibody appeared to recognize HPV-16 and HPV-18 E6s with similar efficiencies, which is consistent with the high degree of sequence homology in the immediate vicinity of the phospho-acceptor site between the two E6 oncoproteins. As a further control, we included the HPV-18 E6 T156E mutant. As can be seen, this was recognized by the anti-phospho antibody in the absence of phosphorylation, but the levels of detection did not change significantly following incubation with PKA. This suggests that the phospho-mimic charge mutation on HPV-18 E6 does behave as such, at least with respect to recognition by the anti-phospho-specific antibody.

We then investigated whether phosphorylation of E6 could be similarly detected *in vivo*. To do this, HEK293 cells were transfected with HA-tagged HPV-16 E6, HPV-18 E6, and HPV-18 E6 T156E expression constructs and incubated in the presence or absence of forskolin in order to stimulate endogenous PKA activity. After 24 h, the cells were harvested, and immunoprecipitations were performed using anti-HA antibody-conjugated agarose beads. The immunoprecipitates were then analyzed by Western blotting using the anti-HA antibody for total E6 and using the anti-E6 phospho-specific antibody for phospho-E6. The results

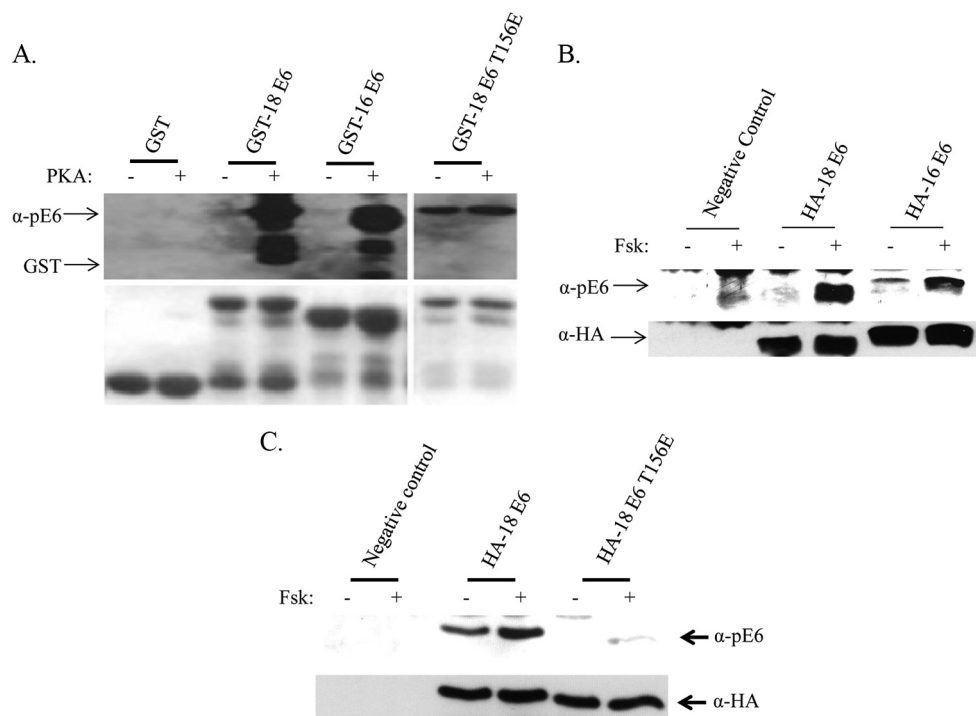


FIG 4 Detection of phospho-E6 *in vivo*. (A) The indicated GST fusion proteins were either untreated or subjected to phosphorylation with PKA (+) in the presence of nonradiolabeled ATP. Proteins were then detected by Western blotting with anti-E6 phospho-specific antibody. The lower panel, shows Ponceau staining of the nitrocellulose membrane, confirming equal levels of protein loading in each assay. (B and C) HEK293 cells were transfected with empty vector or the indicated HA-tagged E6 expression plasmids in the presence (+) or absence (–) of forskolin (Fsk). After 24 h, the cells were harvested and subjected to immunoprecipitation using anti-HA-conjugated agarose beads. The presence of E6 was then detected by Western blotting using either the anti-E6 phospho-specific antibody to detect phosphorylated E6 (upper panels) or the anti-HA antibody to detect total levels of E6 protein (lower panels).

shown in Fig. 4B and C demonstrate a number of interesting features. First, in the absence of forskolin, HPV-18 E6 appeared to be only weakly phosphorylated, while there was a slightly higher level of phosphorylation of HPV-16 E6 (Fig. 4B). However, incubation of the cells with forskolin resulted in dramatic increases in the levels of phosphorylation of both HPV-16 E6 and HPV-18 E6. In contrast, the HPV-18 E6 T156E mutant showed weak reactivity with the anti-phospho antibody, consistent with the mutation acting as a partial phospho-mimic (Fig. 4C). These results demonstrate that transiently transfected HPV-16 E6 and HPV-18 E6 are highly phosphorylated *in vivo* following stimulation of PKA. In the absence of PKA stimulation, the levels of E6 phosphorylation are low, although minor differences in the levels of phosphorylation of the two E6 proteins exist.

Phospho-E6 interacts with 14-3-3ζ. Previous studies have indicated that 14-3-3ζ is a potential interaction partner of HPV E6 (33). Therefore, we investigated whether phosphorylation of E6 could impart binding to 14-3-3ζ *in vitro*. To do this, the purified HPV-18 E6-GST fusion protein was subjected to phosphorylation by PKA with nonradiolabeled ATP. Binding assays were then performed with *in vitro*-translated radiolabeled 14-3-3ζ. For comparison, parallel assays were performed with *in vitro*-translated Dlg and MAGI-1, two PDZ domain-containing substrates of HPV-18 E6 whose binding to E6 would be predicted to be inhibited by PKA phosphorylation of the T156 residue. The results obtained are shown in Fig. 5A and demonstrate a number of interesting features. In the absence of phosphorylation, 14-3-3ζ did not interact with HPV-18 E6, whereas there was a strong association of

HPV-18 E6 with both Dlg and MAGI-1. However, following phosphorylation of HPV-18 E6, there was a dramatic decrease in the capacity of E6 to recognize both Dlg and MAGI-1, while there was a corresponding increase in the capacity of E6 to interact with 14-3-3ζ. These results demonstrate that the PBM/PKA module indeed has dual functions, depending on the phospho status of the T156 residue, and this confers either recognition of PDZ proteins or recognition of 14-3-3ζ.

Having found that HPV-18 E6 could interact with 14-3-3ζ, we proceeded to investigate whether HPV-16 E6 and HPV-31 E6 could also recognize 14-3-3ζ in a phosphorylation-dependent manner. Purified GST fusion proteins were subjected to phosphorylation by PKA using nonradiolabeled ATP, and binding assays were performed with *in vitro*-translated radiolabeled 14-3-3ζ. The results in Fig. 5B again show a strong interaction between HPV-18 phospho-E6 and 14-3-3ζ. A similar phospho-dependent interaction between HPV-16 E6 and 14-3-3ζ was also apparent; however, HPV-31 E6, which is only a poor substrate for PKA, largely failed to interact with 14-3-3ζ. As expected, no interaction between HPV-11 E6 and 14-3-3ζ was observed, consistent with the absence of the PBM/PKA module on the carboxy terminus of HPV-11 E6. These results demonstrate that the association between E6 and 14-3-3ζ is, in part, a reflection of the degree to which the particular E6 oncoprotein can be phosphorylated by PKA.

We next wanted to investigate the molecular basis for HPV-18 E6 recognition of 14-3-3ζ. To do this, we made use of the panel of HPV-18 E6 mutants (Fig. 1). These included the phospho-mimics T156D and T156E, the potential PKA phospho-acceptor site mu-

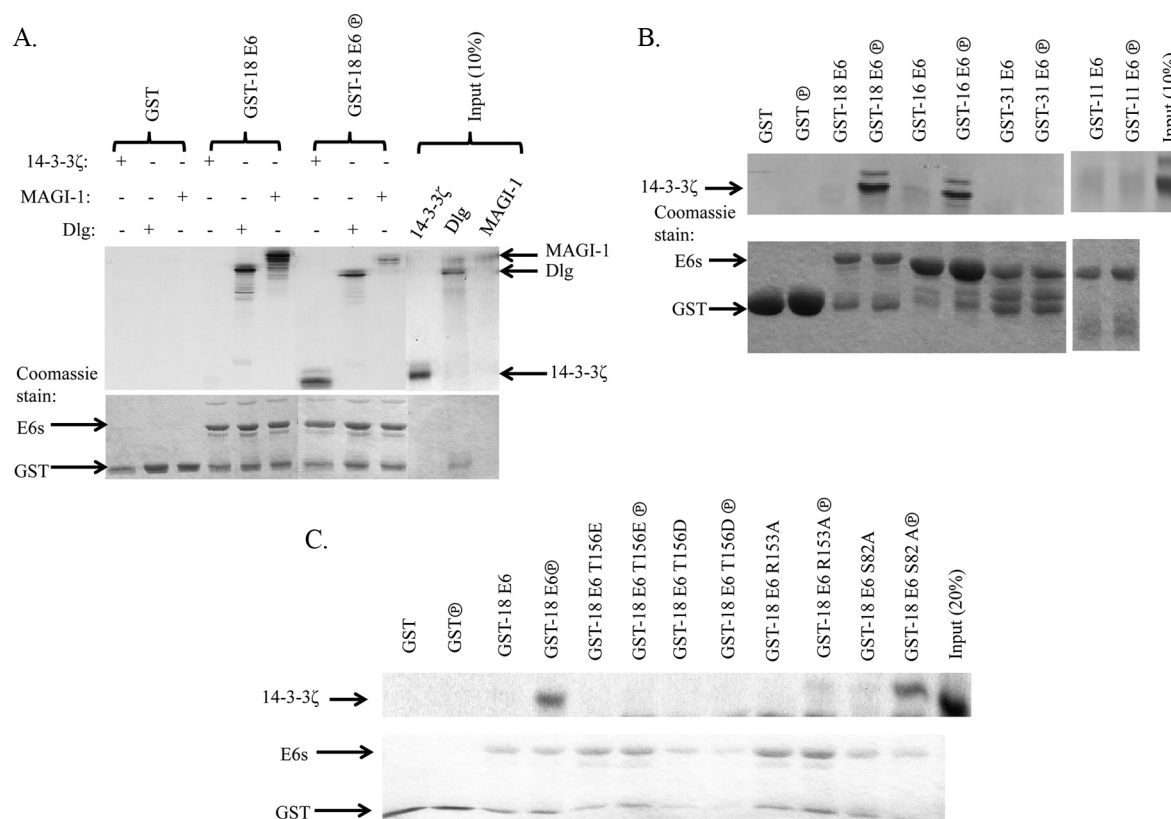


FIG 5 HPV E6 interacts with 14-3-3ζ in a phosphorylation-dependent manner. (A) The indicated GST fusion proteins were either untreated or subjected to phosphorylation with PKA (circled P) in the presence of nonradiolabeled ATP. They were then incubated with *in vitro*-translated radiolabeled 14-3-3ζ, MAGI-1, or Dlg as indicated. Following extensive washing, bound proteins were detected by using SDS-PAGE and autoradiography. Upper panel, autoradiogram; lower panel, the Coomassie blue-stained gel. The arrows indicate the relevant fusion proteins and translated products. (B) The indicated GST fusion proteins were either untreated or subjected to phosphorylation with PKA (circled P) in the presence of nonradiolabeled ATP. They were then incubated with *in vitro*-translated radiolabeled 14-3-3ζ. Following extensive washing, the bound 14-3-3ζ was detected using SDS-PAGE and autoradiography. Upper panel, autoradiogram; lower panel, the Coomassie blue-stained gel. Arrows indicate the relevant proteins. (C) Assays to monitor 14-3-3ζ interactions with the different HPV-18 E6 mutant-GST fusion proteins were performed as described for panel B.

tant S82A, and the R153A mutant, in which the PKA consensus site is abolished but the PBM remains intact. The purified fusion proteins were phosphorylated with PKA, and *in vitro* interaction assays with 14-3-3ζ were performed as described above. The results shown in Fig. 5C demonstrate that phosphorylation of HPV-18 E6 is essential for the interaction with 14-3-3ζ, since no association with the R153A mutant was seen. Furthermore, these results demonstrate little or no influence of the S82 residue on the ability of E6 to recognize 14-3-3ζ. Surprisingly, the two phosphomimics also failed to interact with 14-3-3ζ. This result demonstrates that recognition of 14-3-3ζ by HPV-18 E6 is strictly dependent on the phosphorylation of T156, and simple replacement with an acidic residue is not sufficient to confer interaction.

The interaction between phospho-E6 and 14-3-3ζ is direct. To confirm that the interaction between E6 and 14-3-3ζ is direct and not mediated through an intermediary protein, we repeated the interaction assays using commercially available purified 14-3-3ζ. Following PKA phosphorylation of the purified E6-GST fusion proteins, they were incubated with purified His-tagged 14-3-3ζ; after extensive washing, the bound 14-3-3ζ was detected by Western blotting using an anti-His antibody. The results obtained are shown in Fig. 6A (the results of quantitation are shown in Fig. 6B) and confirm the phospho-specific associations between HPV-16

and HPV-18 E6 and 14-3-3ζ, with little or no interaction between HPV-31 and HPV-11 E6s and 14-3-3ζ, again corresponding to their respective susceptibilities to phosphorylation by PKA.

Since the above results indicate that HPV-16 E6 and HPV-31 E6 can also be phosphorylated by AKT, we also analyzed whether AKT phosphorylation of these E6 proteins could stimulate interactions with 14-3-3ζ. The results of this analysis are shown in Fig. 6C and D and demonstrate that AKT phosphorylation of HPV-16 E6 by AKT does indeed promote interaction with purified 14-3-3ζ. Surprisingly, phosphorylation of HPV-31 E6 by AKT also conferred interaction with 14-3-3ζ. This result demonstrates that the capacity of high-risk E6 oncoproteins to interact with 14-3-3ζ is highly conserved across different HPV types and that differences are determined more by the identity of the kinase that recognizes the E6 phospho-acceptor site rather than any intrinsic difference in the PBM. This is borne out by the results obtained with HPV-18 E6; while HPV-18 E6 can be very weakly phosphorylated by AKT, the very low level of phosphorylation (approximately 1% of that seen with PKA) did not seem to be sufficient to confer any significant degree of association with 14-3-3ζ in this *in vitro* assay.

14-3-3ζ contributes to maintenance of the steady-state levels of HPV-18 E6 protein. Previous studies have shown that an intact

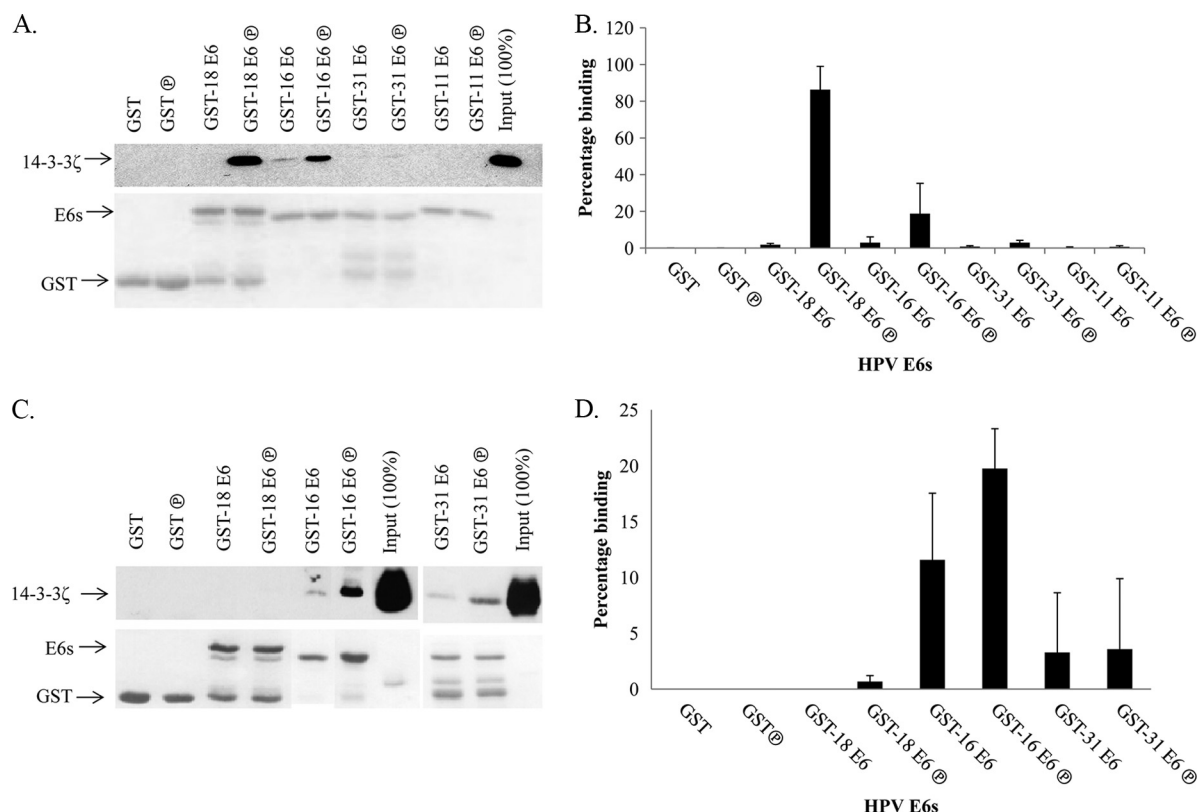


FIG 6 Direct interaction between HPV E6 and 14-3-3 ζ . (A) Interaction assay with purified 14-3-3 ζ . Purified GST fusion proteins were either untreated or subjected to phosphorylation with PKA (circled P) in the presence of nonradiolabeled ATP. They were then incubated with purified 14-3-3 ζ . After extensive washing, the bound protein was detected by Western blotting using anti-His antibody (upper panel). Ponceau staining of the nitrocellulose membrane was also performed (lower panel). (B) Results of quantitation from at least three independent assays. (C) As described for panel A, except phosphorylation was performed with AKT. (D) Results of quantitation from at least three independent assays.

PBM in E6 can contribute to maintaining the levels of E6, which appears to be related, in part, to the ability of HPV-18 E6 to recognize certain PDZ domain-containing substrates (27). However, based on the above analyses, we cannot exclude the possibility that loss of the PBM might result in lower levels of E6 through its inability to associate with 14-3-3 ζ . To investigate this, HPV-18-containing HeLa cells were transfected with an siRNA against 14-3-3 ζ , the cells were harvested after 72 h, and HPV-18 E6 expression levels were ascertained by Western blotting. The results obtained are shown in Fig. 7A, with the results of quantitation shown in Fig. 7B. As can be seen, knockdown of 14-3-3 ζ expression resulted in significant decreases in the levels of HPV-18 E6 in the cells. These results demonstrate that the interaction between HPV-18 E6 and 14-3-3 ζ can indeed contribute to maintaining the steady-state levels of HPV-18 E6 in a cervical tumor-derived cell line.

DISCUSSION

The 14-3-3 family of proteins is intimately associated with the development of many human malignancies (32). In the case of 14-3-3 ζ , the protein has been reported to be overexpressed in a variety of human cancers (35, 36, 42, 43), including cervical cancer (44), and the ablation of 14-3-3 ζ expression in such cells has been proposed as a potential therapeutic option for some tumor types (32, 43). While the modes of action of 14-3-3 ζ are diverse and affect multiple signaling pathways, a common theme is the ability

to recognize phosphorylated substrates and thereby directly modulate their respective modes of action. In the case of the high-risk HPV E6 oncoproteins, an important aspect of their ability to contribute to the development of cervical cancer is thought to be their capacity to target cellular proteins that harbor PDZ domains, through their highly conserved carboxy-terminal PBM (18, 19). It was long thought that loss of function of this region would result simply in a loss of the capacity for interactions with PDZ domain-containing substrates. Based on the studies described here, we now know that the situation is rather more complex and that loss of function within the PBM region of E6 can also be expected to perturb the capacity of E6 oncoproteins to interact with 14-3-3 ζ .

Previous studies have shown that phosphorylation of E6 by PKA is a feature of high-risk HPV E6 oncoproteins, and certainly the C-terminal peptides derived from HPV-16, HPV-18, HPV-31, and HPV-33 E6s were all subject to phosphorylation by PKA, with very similar kinetics (28). In the current study, we found that, in the context of the full-length E6 oncoproteins, patterns of phosphorylation by PKA are quite dissimilar among the different E6 oncoproteins. Thus, while HPV-18 E6 and, to a lesser extent, HPV-16 E6 are excellent substrates for phosphorylation by PKA, HPV-31 E6 is only a very weak substrate. It is intriguing to speculate that this might reflect a situation in which the carboxy-terminal Thr/Ser is phosphorylated by kinases other than PKA, and we provide evidence to suggest that AKT is potentially one such kinase. Thus, while all of the cancer-causing HPV E6 oncoproteins

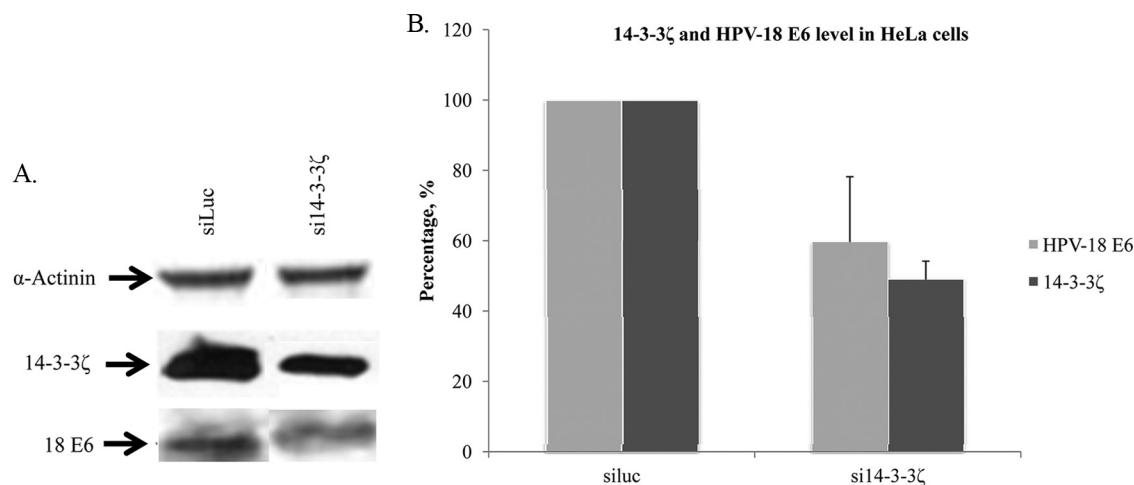


FIG 7 14-3-3 ζ contributes to maintaining HPV-18 E6 steady-state levels. (A) HeLa cells were transfected with control siRNA (siLuc) or siRNA against 14-3-3 ζ . After 72 h, the cells were harvested, and the levels of E6 were ascertained by Western blotting using anti-HPV-18 E6 monoclonal antibody. Also shown are the levels of 14-3-3 ζ and the loading control α -actinin. (B) Results of quantitation from at least three independent experiments showing the levels of 14-3-3 ζ knockdown and the effects on E6 levels.

possess PBMs and potential phospho-acceptor sites within them, it is quite clear that PKA is unlikely to be the common kinase that phosphorylates them all; further studies are required to identify the kinases responsible for phosphorylating the PBMs of the different high-risk HPV E6 oncoproteins, with AKT being high on the list of the other candidates.

Using phospho-specific antibodies directed against HPV-18 E6, we also were able to confirm that HPV-18 E6 is phosphorylated *in vivo*. Interestingly, these antibodies appear to cross-react with HPV-16 E6, which allows us to conclude that it is similarly phosphorylated *in vivo*. However, in transient-transfection assays, the levels of phosphorylation of the two E6 proteins are somewhat different, with low levels of phosphorylation occurring with HPV-18 E6 and readily detectable levels of phosphorylation being seen with HPV-16 E6. Stimulation of the cells with forskolin to induce endogenous PKA activity results in dramatic increases in the levels of phosphorylation of both proteins, confirming them as being PKA substrates *in vivo*, but the increase in HPV-18 E6 phosphorylation is more pronounced than that in HPV-16 E6 phosphorylation, consistent with the *in vitro* phosphorylation assay results. It is intriguing to speculate that, under conditions in which PKA is not stimulated, HPV-16 E6 might be phosphorylated by another kinase, and AKT is a compelling candidate for this. Finally, these results indicate that, in simple transfection experiments, most of the E6 protein is not phosphorylated, which allows interactions with PDZ domain-containing substrates. Whether this reflects the phosphorylation status of E6 in cells derived from cervical tumors is an important question that is under investigation.

It was shown previously that phosphorylation of HPV-18 E6 by PKA inhibits association with Dlg, and the molecular basis for this is well established (28, 29). We have extended this to demonstrate that association with MAGI-1 is likewise inhibited, confirming the general applicability of this negative regulation of the E6 PBM by phosphorylation. However, an outstanding question is whether such phosphorylated E6 proteins, when not bound to a PDZ domain, gain the capacity to associate with other as-yet-unidentified targets. The 14-3-3 proteins are prime candidates for such sub-

strates of E6, being defined as phospho-Ser/Thr binding proteins (30). Indeed, previous studies identified 14-3-3 ζ in a proteomic screen of HPV E6 interaction partners (33). We found that 14-3-3 ζ is indeed an interaction partner of HPV-16 E6, HPV-18 E6, and HPV-31 E6 but not of the low-risk HPV-11 E6. Furthermore, the interaction is direct and strictly dependent upon the capacity of the E6 oncoprotein to be phosphorylated within the PBM, which is absent in HPV-11 E6. In the case of HPV-18 E6, this is strictly dependent upon PKA. It is dependent upon either PKA or AKT in the case of HPV-16 E6, while it is dependent upon AKT for HPV-31 E6. All of this regulation reflects the degree to which E6 is phosphorylated by the different kinases. This suggests that different high-risk HPV E6 oncoproteins will also be regulated differently with respect to their capacities to recognize PDZ domains and 14-3-3 ζ . Interestingly, unlike for many phospho-driven interactions, replacement of Thr156 with an acidic residue does not yield a phospho-mimic with respect to interaction with 14-3-3 ζ (even though it can function as such with the anti-phospho-E6-specific antibody), which indicates that 14-3-3 ζ recognition is phosphate dependent and not amenable to simple charge substitution. This result has another very important consequence. Simple deletion, or even single-point mutational ablation of the Ser/Thr residue within the PBMs of high-risk HPV E6 oncoproteins, abolishes interactions with 14-3-3 ζ in addition to inhibiting PDZ association. It is therefore extremely important that this be taken into consideration when assigning biological functions to this particular region of the E6 oncoprotein.

Biologically, the relevance of the E6 interaction with 14-3-3 ζ remains to be elucidated. Certainly, 14-3-3 ζ levels do not seem to be unduly affected by the E6 oncoprotein (data not shown), suggesting that 14-3-3 ζ is not a standard degradation target of E6. Furthermore, we have been unable to demonstrate that 14-3-3 ζ has any significant effect on the cellular distribution of HPV-18 E6. However, previous studies have shown that the loss of the PBM binding capacity of E6 had a detrimental effect upon the levels of E6 expression (27). Therefore, we investigated whether this might be partly due to a loss of association with 14-3-3 ζ . In HPV-18-positive HeLa cells, knockdown of 14-3-3 ζ expression

leads to a significant reduction in the steady-state levels of HPV-18 E6, suggesting that the association between E6 and 14-3-3 ζ can contribute to maintaining normal levels of E6. Obviously, whether HPV-18 E6 can also affect the capacity of 14-3-3 ζ to recognize its normal cellular substrates is an open question that is under investigation.

Finally, it is worth emphasizing that, in addition to 14-3-3 ζ , there are six other members of the 14-3-3 family, many of which are differently associated with the development of human malignancies. While the studies presented here suggest that 14-3-3 ζ is an important partner of the high-risk HPV E6 oncoproteins, it remains to be determined whether other 14-3-3 family members are similarly bound by the high-risk HPV E6 oncoproteins. Studies to investigate these aspects further are currently in progress.

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