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# HPV16 E1^E4 protein is phosphorylated by Cdk2/cyclin A and relocalizes this complex to the cytoplasm

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**Abstract**

The human papillomavirus type 16 E1^E4 protein is expressed abundantly in cells supporting viral DNA amplification, but its expression is lost during malignant progression. In cell culture, 16E1^E4 causes G2 cell cycle arrest by associating with and preventing the nuclear entry of Cdk1/cyclin B1 complexes. Here, we show that 16E1^E4 is also able to associate with cyclin A and Cdk2 during the G2 phase of the cell cycle. Only a weak association was apparent during S-phase, and progression through S-phase appeared unaffected. As with cyclin B1, the interaction of 16E1^E4 with cyclin A is dependent on residues T22/T23 and results in the accumulation of cyclin A in the cytoplasm where it colocalizes with 16E1^E4. 16E1^E4 serine 32 was found to be phosphorylated by Cdk2/cyclin A. We hypothesize that the interaction of 16E1^E4 with cyclin A may serve to increase the efficiency with which 16E1^E4 is able to prevent mitotic entry.


*Keywords:* Papillomavirus; HPV; Cyclin A; Cdk; Cell cycle; E1^E4; E4; Phosphorylation

## Introduction

Papillomaviruses (PV) are small DNA viruses that show a high degree of specificity in the species they infect (Lowy and Howley, 2001). A further level of specificity is seen in the varied tropisms PV have for different types of epithelial tissue. Human papillomaviruses (HPV) are classed as being either low risk, e.g., HPV 1 and 11, or high risk, e.g., HPV16. The former are only associated with benign lesions (often called warts), while the latter cause lesions that may become malignant. The presence of HPV16 DNA is the major risk factor associated with cervical cancer (Walboomers et al., 1999).

Initial infection of the epithelium by the PV occurs in the basal cells and completion of the virus life-cycle is dependent on these cells dividing, differentiating, and migrating towards the surface of the epithelium (Howley and Lowy, 2001). The PV genome is normally maintained at low copy number in the basal cells and in the lower layers of the epithelium. Differentiation of the cells in the upper layers triggers a significant increase in levels of viral DNA replication (viral DNA amplification), and the new viral genomes are then packaged into infectious virions (Lowy and Howley, 2001).

During these productive infections, the E1^E4 protein, derived from a spliced transcript of the E1 and E4 open reading frames (Doorbar et al., 1990), is found abundantly in the cytoplasm of cells in the upper layers of the epithelium (Doorbar et al., 1997). Expression of 16E1^E4 results from activation of the differentiation dependent promoter, and its expression is lost if the lesion progresses towards malignancy (Middleton et al., 2003). Viral DNA amplification is detected in cells that are 16E1^E4-positive (Crum et al., 1990; Doorbar et al., 1997), and the current model suggests that genome amplification occurs in cells that contain E1^E4 and S phase proteins such as PCNA, MCM, and cyclin A. The dependency of amplification on the presence of E1^E4 is supported by knock-out experiments in HPV16, HPV31, and cotton tail rabbit PV (CRPV) in which

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lack of E1^E4 expression results in a reduced ability to amplify genomes (Nakahara et al., 2005; Peh et al., 2004; Wilson et al., 2005). The knock-out studies also showed that expression of the viral capsid proteins is abrogated in the absence of E1^E4. It is interesting to note that 16E1^E4 is able to bind at least one factor involved in the regulation of protein expression, E4-DBP (Doorbar et al., 2000). E1^E4 has also been implicated in the release of new virus particles. 16E1^E4 has been shown in cell culture (Doorbar et al., 1991) and in vivo (Wang et al., 2004) to associate with keratin networks and cause them and their attached 16E1^E4 to re-organize. It is thought that the decrease in integrity of the keratin/16E1^E4 network may aid viral egress. A similar role has been proposed for the 11E1^E4 protein which appears to disrupt the assembly of cornified envelope (Bryan and Brown, 2000). Although PV are non-lytic viruses and are shed from the surface of the epithelium as part of the differentiation process of the tissue, 16E1^E4 has been shown to bind to mitochondria, thus altering their membrane potential and inducing apoptosis (Raj et al., 2004), although the significance of this is not known.

Recently, it has become apparent that like many other PV proteins, E1^E4 proteins can affect the cell cycle. Expression of 16E1^E4 in cell culture causes G2 arrest as a result of 16E1^E4 holding Cdk1/cyclin B1 in the cytoplasm (Davy et al., 2002, 2005; Nakahara et al., 2002). The G2 arrest phenotype appears to be a property of E1^E4 proteins from other PV types including HPV11 (Davy et al., 2002), HPV18 (Nakahara et al., 2002), and HPV1 (Knight et al., 2004). For both 16E1^E4 and 18E1^E4, arrest appears to be mediated by a central region of the protein (between amino acids 17 to 45 for 16E1^E4 and between amino acids 21 to 59 for 18E1^E4). G2 arrest induced by 16E1^E4 is inhibited by mutation of residues T22 and T23 to alanines (Davy et al., 2002), and mutation of the corresponding residue, T13, in the 1E1^E4 protein similarly abrogates arrest (Knight et al., 2004). Despite the reliance of the type 1 and 16 E1^E4 proteins on similar residues, there appears to be some disparity in their mechanisms of arrest. Unlike 16E1^E4 which prevents nuclear entry of cyclin B1 (Davy et al., 2005), 1E1^E4 does not affect the subcellular localization of cyclin B1 but instead decreases the number of cyclin B1-positive cells (Knight et al., 2004).

Given that cell cycle progression is regulated by many different Cdk and cyclin proteins, we were keen to determine whether 16E1^E4 could affect other family members in addition to Cdk1 and cyclin B1. In particular, the close correlation between 16E1^E4 and viral DNA amplification suggested to us that 16E1^E4 may have some impact on S phase proteins. In the work presented here, we have further investigated the effect of 16E1^E4 on these key cell-cycle regulators. We find that as with cyclin B1, 16E1^E4 associates with cyclin A2 (from here on referred to as cyclin A) but does not appear to affect cyclins D or E. Cyclin A is believed to have roles during S phase and at the G2/M transition, but the interaction of 16E1^E4 with cyclin A appears predominantly in cells that are in G2. As a result of association, cyclin A becomes redistributed from the nucleus to the cytoplasm, where it colocalizes with 16E1^E4. This can be seen in cell culture and also in HPV16-induced lesions in the layers within which viral DNA amplification is occurring. Association with cyclin A is dependent on threonine residues 22/23 located within the proline-rich region of 16E1^E4, but the residue that is phosphorylated by Cdk2/cyclin A is serine 32.

## Results

### *Analysis of 16E1^E4 during the S and G2 cell cycle phases*

As 16E1^E4 had already been found to bind and affect the distribution of cyclin B1-containing complexes (Davy et al., 2005), we investigated whether 16E1^E4 could affect other cyclin complexes. Previous work investigating the interaction of 16E1^E4 with cyclin B1 used cells that had been released from a G1/S block for 16 h (Davy et al., 2005), in which cells that express 16E1^E4 were found to have blocked in G2/M. In the present work, to allow investigation of cyclins important for earlier stages of the cell cycle, analysis was additionally carried out at 4 h post-block release, i.e., when the cells are in S phase. SiHa cells, a cell line derived from an HPV16-induced cervical cancer, were synchronized at the G1/S boundary using serum starvation followed by aphidicolin treatment and infected with recombinant adenoviruses that expressed either β-gal or 16E1^E4. Cells were released from the block into medium containing 10% FCS, and the cell cycle profile of the populations was analyzed using propidium iodide staining and flow cytometry. Fig. 1A shows that following our synchronization protocol, the majority of cells infected with β-gal- or 16E1^E4-expressing recombinant adenoviruses arrest with G1 DNA content (0 h). Fig. 1 also shows that at 4 h post-block release, the cells that express β-gal and those that express 16E1^E4 are both in S phase. As expected, by 16 h post-block release a difference in cell cycle profiles can be seen. This is in agreement with previous work, which showed that cells that express high levels of 16E1^E4 arrest in G2, while those that express β-gal return to G1 (Davy et al., 2002, 2005).

### *16E1^E4 decreases the solubility of cyclin A and Cdk2 in a cell-cycle-dependent manner but does not affect Cdk4, cyclin D, or cyclin E*

The association of 16E1^E4 with cyclin B1 and Cdk1 was found to result in a reduction in solubility of these proteins (Davy et al., 2005). To determine if 16E1^E4 has the ability to reduce the solubility of other cyclin and Cdk proteins, detergent fractionation was used (Fig. 1B). Cells expressing β-gal or 16E1^E4 were lysed in a buffer containing 0.5% NP-40 and centrifuged to obtain the NP-40-soluble and -insoluble fractions. As expected, the controls tubulin and GAPDH are found exclusively in the NP-40-soluble fraction and at equivalent levels in the β-gal and 16E1^E4 samples. Similarly histone 2B is found almost exclusively in the NP-40-insoluble fraction, again at equivalent levels in the β-gal and 16E1^E4 samples. At 16 h, it was found, as before, that cyclin B1 and Cdk1 as well as being present in the NP-40-soluble fraction were additionally present in the NP-40-insoluble fraction. This was not the case for cyclin E, cyclin D, or Cdk4, which were all

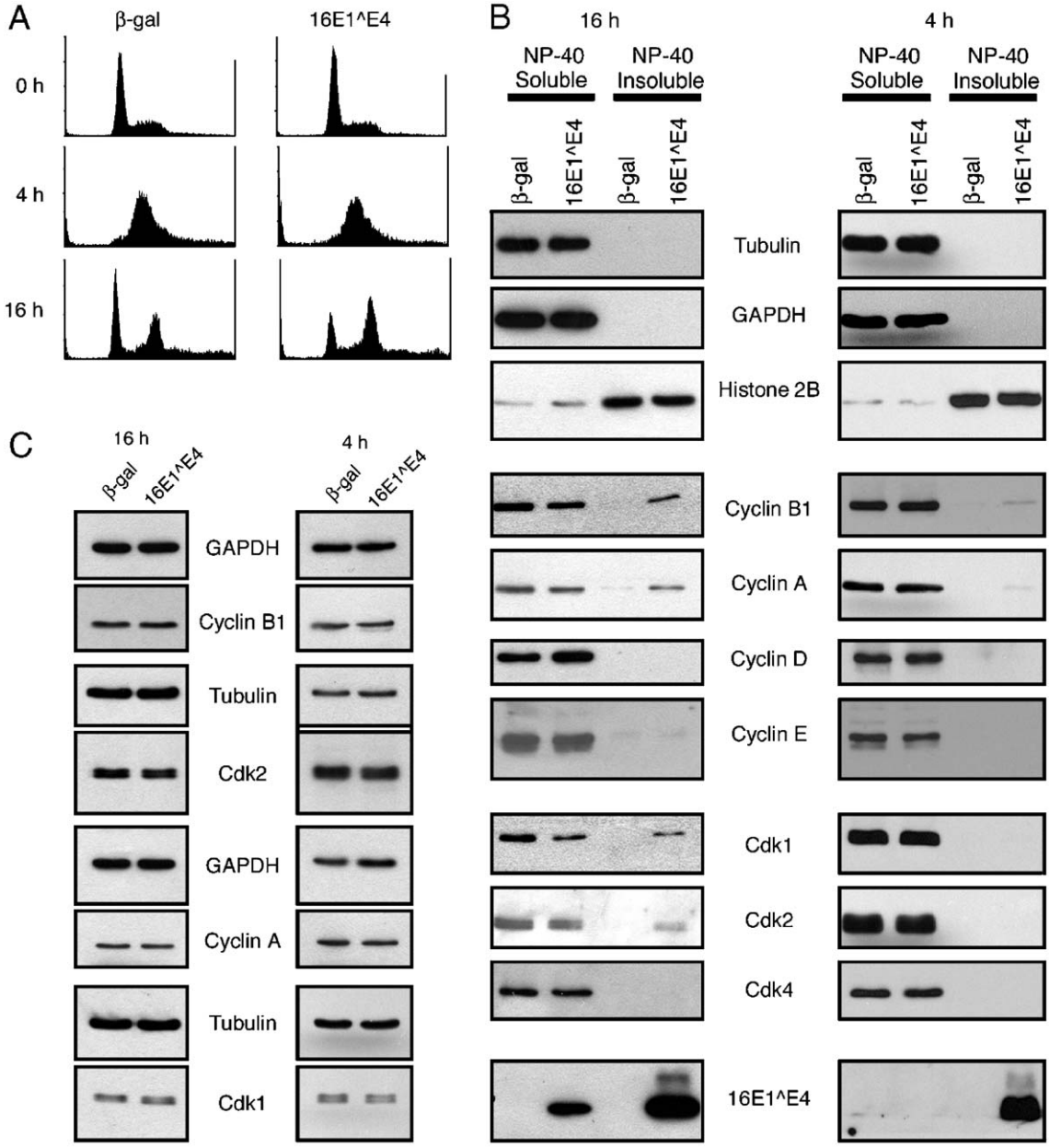



Fig. 1. The effect of 16E1^E4 on cell cycle regulators in S phase and G2/M. G1/S synchronized SiHa cells infected with recombinant adenoviruses that express β-gal or 16E1^E4 were harvested at 0, 4 and 16 h post-block release. (A) Cells were stained with immunofluorescent antibodies and with propidium iodide and were analyzed by flow cytometry. Plots show cell number versus DNA content. (B) Cells were harvested at 16 h and 4 h post-block release. The cells were fractionated into 0.5% NP-40-soluble and -insoluble fractions. Fractions were Western blotted for 16E1^E4; cyclins A, B1, D, and E; Cdk1, Cdk2, and Cdk4; and (as controls for fractionation), tubulin, GAPDH, and histone 2B. (C) Cells were harvested at 4 and 16 h post-block release. Cell extracts were Western blotted for cyclins A and B1, and Cdks 1 and 2, using GAPDH and tubulin loading controls as appropriate.

found exclusively in the NP-40-soluble fraction. Cyclin A and Cdk2 however were found to partially redistribute to the NP-40-insoluble fraction, suggesting that as with Cdk1 and cyclin B1, Cdk2 and cyclin A have the ability to interact with 16E1^E4 at 16 h, i.e., in G2. In contrast when the same fractionation was carried out at 4 h, i.e., in S phase, Cdk1 and Cdk2 were not desolubilized. To emphasize this point, the 4-h extracts are shown at much greater exposure than the 16 h, and while insoluble bands of Cdk1 and Cdk2 appear at 16 h, they are not present at 4 h. In addition, the proportions of cyclins A and B seen to be desolubilized were much smaller than at 16 h, and again, the 4 h extract was exposed for longer to highlight this difference. As at 16 h, no desolubilization of Cdk4 or cyclins D or E was observed at 4 h. Interestingly, while at 16 h 16E1^E4 was observed both in the soluble and insoluble fractions, at 4 h, the 16E1^E4 was completely insoluble.

### *16E1^E4 does not affect the levels of cyclin A or Cdk2*

The interaction of 16E1^E4 with cyclin B1 did not result in a change in the levels of cyclin B1 or Cdk1 (Davy et al., 2005). To examine whether this was similarly the case for cyclin A and

Cdk2, total cell extracts from 4 and 16 h were Western blotted and compared to levels of control proteins on the same blot. Fig. 1C shows that as for Cdk1 and cyclin B1, there is no difference in the levels of cyclin A or Cdk2 between β-gal- and 16E1^E4-expressing cells at 4 and 16 h.

*In G2, 16E1^E4 causes relocalization of cyclin A from the nucleus to the cytoplasm where it associates with 16E1^E4*

Analysis of cells expressing 16E1^E4 had shown that cyclin B1 was relocalized from a diffuse cytoplasmic pattern to one in which it colocalized with 16E1^E4 (Davy et al., 2005). Cyclin A is a protein that normally displays both cytoplasmic and nuclear staining patterns (Pines and Hunter, 1991). To determine the effect of 16E1^E4 on the intracellular distribution of cyclin A, immunofluorescence microscopy was carried out on synchronized cells that express β-gal or 16E1^E4 harvested at 16 h. As shown in Fig. 2A, while the distribution of β-gal in cells is fairly uniform, 16E1^E4 appears with a number of different distribution patterns, and the significance of these will be considered in more detail in Figs. 4 and 5. Fig. 2A shows that as expected, cells at 16 h that express 16E1^E4 show discrete colocalization with cyclin B1 but not with the tubulin control. Immunostaining of control cells that express β-gal shows cyclin A predominantly in the nucleus, with some diffuse cytoplasmic staining and no discrete colocalization between β-gal and cyclin A. In contrast, in cells that express 16E1^E4, there appears little cyclin A in the nucleus. Instead, the cyclin A in 16E1^E4-expressing cells is often found associated with 16E1^E4 in the cytoplasm. In fact a number of different staining patterns were observed (see Fig. 2A). To quantify the proportions of cells showing different staining patterns, every cell containing 16E1^E4 and cyclin A was scored according to the degree of colocalization. It was found that only 1% of cells showed no colocalization of cyclin A with 16E1^E4 (e.g., cell "1", Fig. 2A). Of the 99% of cells that showed some colocalization, 19% of cells showed less than 50% colocalization (e.g., cell "2", Fig. 2A), and 80% of cells showed greater than 50% colocalization (e.g., cell "3", Fig. 2A). This was not significantly different from observations of cyclin B1 colocalization with 16E1^E4; 3% showing no colocalization, 16% showing less than 50% colocalization, and 81% showing greater than 50% colocalization. Analysis of optical sections obtained using confocal immunofluorescence microscopy suggested that despite most of the cyclin A in these 16E^E4-expressing cells being found colocalized with 16E1^E4 in the cytoplasm, some cyclin A does remain in the nucleus (Fig. 2B).

*Cells in S phase show little association of cyclin A with 16E1^E4*

To assess whether association of cyclin A with 16E1^E4 was independent of cell cycle stage, immunofluorescence microscopy was carried out on cells that express β-gal or 16E1^E4 harvested at 4 h as they pass through S phase. In contrast to the good colocalization observed at 16 h post-block release, at 4 h cyclin A rarely appears to colocalize with 16E1^E4 (Fig. 3). The patterns were quantified as above, and now, the majority of the cells (63%) showed no colocalization. Of the remaining cells, most (34%) showed less than 50% colocalization, with only a few (3.5%) showing a degree of colocalization greater than 50%. Colocalization was not observed in control cells. The decrease in colocalization seen in S phase compared to G2 is in agreement with what was found regarding the solubility of cyclin A (Fig. 1B). The solubility of cyclin A is reduced by 16E1^E4 at 16 h, but this effect is not so apparent at 4 h. Since cyclin A does not colocalize well with 16E1^E4 during S phase, it is perhaps not surprising that changes to its solubility are not detected. Confocal immunofluorescent microscopy showed again that in cells in which some of the cyclin A was associated with 16E1^E4 in the cytoplasm, a fraction of the cyclin A was still free and found in the nucleus (Fig. 3B).

In addition to the lack of 16E1^E4 colocalization with cyclin A, a difference was also noted between 4 h and 16 h cells in the integrity of their 16E1^E4 networks. The trend is illustrated in Fig. 3C, which shows that at 4 h, the majority of cells have filamentous 16E1^E4, while at 16 h, the majority of cells no longer show filaments as a result of network re-organization.

*Comparison of the colocalization of cyclin A with 16E1^E4 during S and G2 phases*

To investigate the reasons why cyclin A might colocalize with 16E1^E4 in G2 but not in S phase, we first compared the levels of cyclin A, Cdk1, Cdk2, and 16E1^E4 in 16E1^E4-expressing cells at 4 and 16 h by Western blot (Fig. 4A). While levels of cyclin A, Cdk1, and Cdk2 are not significantly different at 4 h compared to 16 h, there is a large increase in the expression of 16E1^E4 between these time-points. This occurred even though the cells had been infected with the rAd.16E1^E4 for the same length of time. Analysis of the β-gal expression from the control recombinant adenovirus showed only a modest increase in levels at 16 h compared to 4 h. This suggests that the differential expression of 16E1^E4 at different stages of the cell cycle may be an inherent feature of the protein, a characteristic that is apparent when other expression systems are used (data not shown). To examine whether the lower levels of 16E1^E4 at 4 h might explain the lack of cyclin A colocalization, cells were selected from the 4-h population that showed abundant 16E1^E4 by immunofluorescent microscopy. These were compared with cells from the 16-h population that appeared to express lower levels of 16E1^E4, by equivalent exposure digital imaging (using IP Lab imaging software). It can be seen from Fig. 4B that the level of 16E1^E4 in the cell does not appear to correlate with the ability to colocalize with cyclin A. This is apparent from cells 1 and 2 of the 4 h panels which show abundant 16E1^E4 yet lack cyclin A colocalization while cells 5 and 6 in the 16-h panels show low-level 16E1^E4 yet good colocalization with cyclin A. Cell 4 in the 4-h panel and cell 8 in the 16-h panel show similar levels and extent of collapse of 16E1^E4, yet there is better cyclin A colocalization at 16 h than at 4 h.

As noted earlier, the cells at 4 and 16 h show differences in the integrity of their 16E1^E4 networks. It was also noticed that

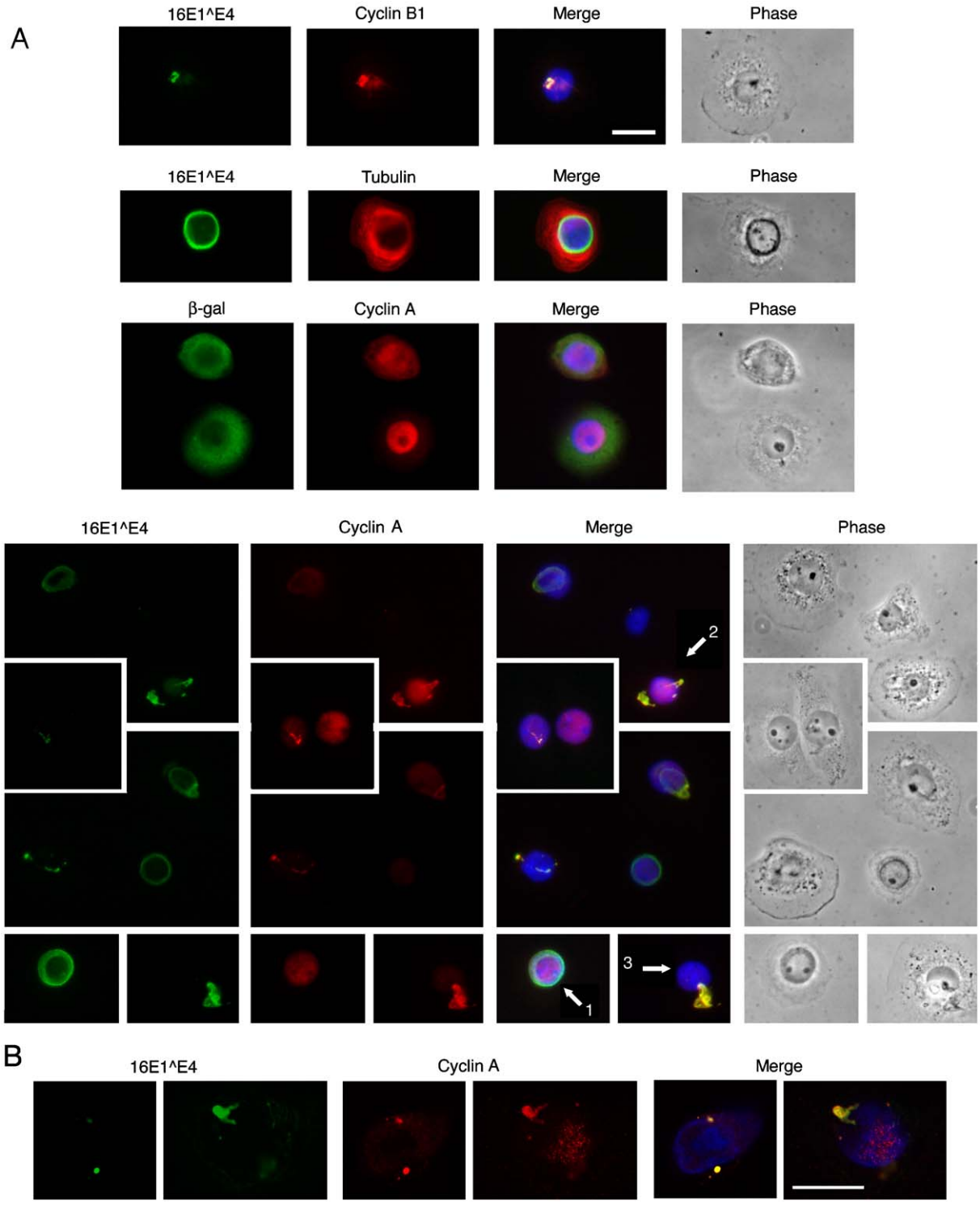



Fig. 2. 16E1^E4 association with cyclin A in G2. G1/S synchronized SiHa cells expressing β-gal or 16E1^E4 were fixed at 16 h post-block release. Cells were stained for β-gal or 16E1^E4 (green) and cyclin B1, tubulin, or cyclin A (red). A DAPI nuclear stain was included in the merged image (blue). Scale bars equal 10 μm. Cells were analyzed (A) using the 40× lens on a standard immunofluorescent microscope or (B) using confocal immunofluorescent microscopy. Cell "1" shows no 16E1^E4-cyclin A colocalization, cell "2" shows less than 50% colocalization, and cell "3" shows greater than 50% colocalization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this appeared to correlate with cyclin A colocalization. In cells in which 16E1^E4 was completely filamentous, e.g., cell 3 in the 4-h panel of Fig. 4B, cyclin A was rarely seen to colocalize with 16E1^E4. Such purely filamentous cells are a common feature of the 4 h time-point but are not often found at 16 h. Conversely, in cells in which 16E1^E4 existed as small perinuclear bundles, e.g., cells 5 and 6 in Fig. 4B, cyclin A was seen to colocalize well (such cells are typical of 16 h, not of

4 h). The data lead us to hypothesize that the cell cycle dependency of the cyclin A interaction may be due to the degree of network re-organization. To determine if this is the case, the integrity of the 16E1^E4 network was compared with the extent of cyclin A colocalization (Fig. 4C). Although it appears that the greater the degree of network re-organization, the greater the amount of colocalization, when cell populations with equivalent degrees of re-organization were compared at 4 and 16 h, statistically significant differences were found between their levels of cyclin A colocalization ($P \leq 0.001$, see Fig. 4). This suggests that while the cell-cycle-related network re-organization may be a factor contributing to cyclin A colocalization, other factors that vary between S phase and G2 are also important.

One way in which 16E1^E4 is potentially modified during the cell cycle is by phosphorylation. This can be seen by SDS-PAGE, where monomeric 16E1^E4 appears as two bands, the upper band being more phosphorylated than the lower band (Wang et al., 2004). Western blotting shows that the ratio of the two bands is similar at the two time-points (Fig. 4A), suggesting that the phosphorylation event that results in the migration change is unlikely to be involved in the differential cyclin association A.

### *Cdk2 and Cdk1 colocalize with 16E1^E4 in a cell-cycle-dependent manner*

It had already been shown that Cdk1, like cyclin B1, colocalizes with 16E1^E4 during G2 (Davy et al., 2005). To determine if this was similarly the case for Cdk2, we used immunofluorescence microscopy. As we had already observed a difference in the desolubilization of Cdk2 by 16E1^E4 between S phase and G2, we used cells from both stages. The proportions of cells showing no colocalization, low-level colocalization, or high-level colocalization with 16E1^E4 were determined for Cdk2 and Cdk1 at 4 and 16 h (Fig. 5A), and examples of different cell types are shown (Fig. 5B). The data show that Cdk2, like Cdk1 colocalizes with 16E1^E4. To examine whether as with cyclin A, the degree of filament re-organization was not the sole determinant of colocalization, the integrity of the 16E1^E4 network was compared with the extent of Cdk colocalization. When cell populations with equivalent degrees of re-organization were compared at 4 and 16 h, statistically significant differences were found between their levels of Cdk colocalization ($P \leq 0.001$, see Fig. 5). This supports the data obtained for cyclin A (Fig. 4), which suggested that some other cell-cycle-related factor, in addition to re-organization of the filaments, controls the ability of 16E1^E4 to colocalize with these cell cycle proteins.

### *Cyclin A association is dependent on threonine residues within the proline-rich region of 16E1^E4*

It had previously been shown that residues T22 and T23 within the proline-rich region of 16E1^E4 are important for binding cyclin B1 in vitro (Davy et al., 2005). To determine if these residues were also important for binding cyclin A, cells transfected with plasmids that express β-gal, wild-type 16E1^E4 or 16E1^E4-T22A,T23A were immunostained. Fig. 6A shows that while the wild-type protein colocalizes with cyclin A (as it does when expressed by adenovirus vectors), the T22A,T23A mutation inhibits this interaction (Fig. 6B). As expected, cyclin A does not colocalize with β-gal. The reduction in binding ability of the T22A,T23A mutant was also observed by GST pull-downs (Figs. 6C and D). Wild-type 16E1^E4 and T22A,T23A-16E1^E4 GST fusions were produced along with GST alone and coupled to glutathione beads. Proteins eluted from different volumes of beads were separated by SDS-PAGE and Coomassie stained to assess the relative efficiencies of coupling (Fig. 6C). Equivalent amounts of GST and GST fusion proteins on beads were used in pull-downs of cell extracts, and the eluted proteins were Western blotted for cyclin A (Fig. 6D). While GST-wild-type 16E1^E4 is able to pull-down cyclin A, GST alone cannot. The ability of GST-T22A,T23A-16E1^E4 to pull-down cyclin A is reduced compared to wild type, and this reduction in affinity may explain the lack of colocalization between T22A,T23A-16E1^E4 and cyclin A in cells. Together, these data suggest that interaction with cyclin B1 and cyclin A is dependent on the same site on 16E1^E4. As yet it is not clear for either cyclin protein whether the interaction is direct or indirect. Type 11 E1^E4 protein has also been shown to be capable of inducing G2 cell cycle arrest (Davy et al., 2002) and contains a region similar to that found to be important for 16E1^E4-cyclin A binding. To determine if 11E1^E4 can also associate with cyclin A, cells transfected with a plasmid that expressed 11E1^E4 were stained for cyclin A and 11E1^E4 (Fig. 6E). As with 16E1^E4, the 11E1^E4 protein is able to associate in cells with cyclin A. This suggests that low risk and high risk HPV types can have E1^E4 proteins that interact with cyclins.

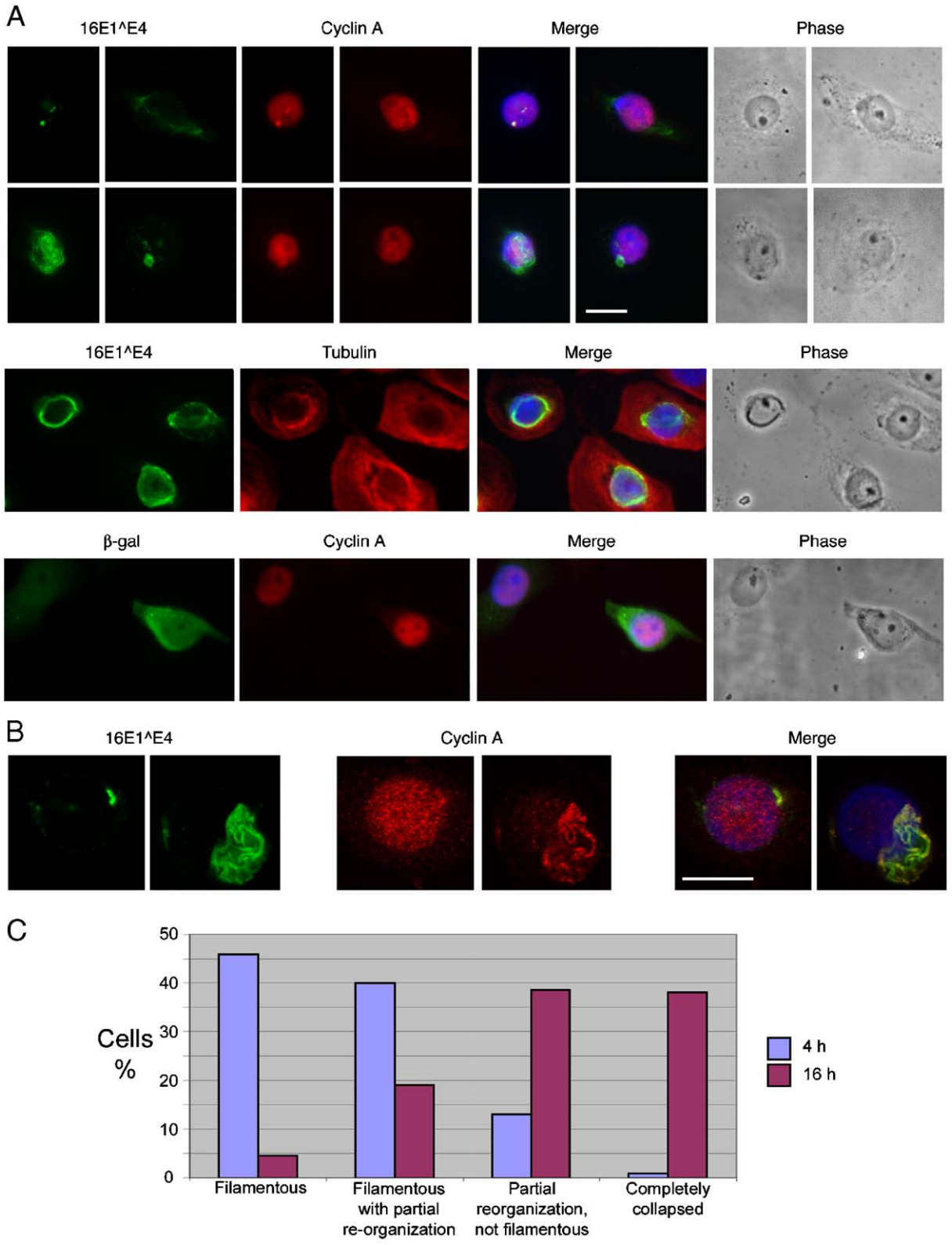
### *16E1^E4 Ser32 is a substrate for Cdk2/cyclin A*

To determine whether 16E1^E4 is a substrate for phosphorylation by Cdk2/cyclin A, bacterially expressed 16E1^E4 was used in an in vitro kinase assay with Cdk2/cyclin A. Fig. 7A shows that in the presence of 16E1^E4 and Cdk2/cyclin A, phosphorylated bands of the correct size for 16E1^E4 and multimers of 16E1^E4 appear. These bands are absent when either the kinase complex or 16E1^E4 are omitted from the reaction. This suggests that Cdk2/cyclin A can phosphorylate 16E1^E4 in vitro.

16E1^E4 contains a number of sites that match the consensus for phosphorylation by Cdk2/cyclin A. To determine which residue is phosphorylated by Cdk2/cyclin A, the phosphorylated 16E1^E4 protein was analyzed by mass spectrometry. Nanospray mass spectrometry coupled with collision-induced fragmentation (data not shown) suggested that the residue phosphorylated was found within a fragment consisting of residues 32 to 34. Contained within this region is a site matching the Cdk2/cyclin A consensus site for phosphorylation, i.e., S32P33 (Fig. 7B). To determine if serine 32 is the residue

phosphorylated by Cdk2/cyclin A, a S32A mutant of 16E1^E4 was tested in the in vitro kinase assay. Fig. 7C shows that mutation of serine 32 to alanine abolishes the majority of phosphorylation by Cdk2/cyclin A, suggesting that it is this residue that is the primary target of the Cdk2/cyclin A kinase complex.

To determine if the S32 site was phosphorylated in cells, extracts from cells that express wild-type and S32A-16E1^E4 proteins were analyzed by 2D electrophoresis; iso-electric focusing in the first dimension and SDS-PAGE in the second dimension. Fig. 7D top panel shows that in extracts from cells that express wild-type 16E1^E4, two spots are apparent, and



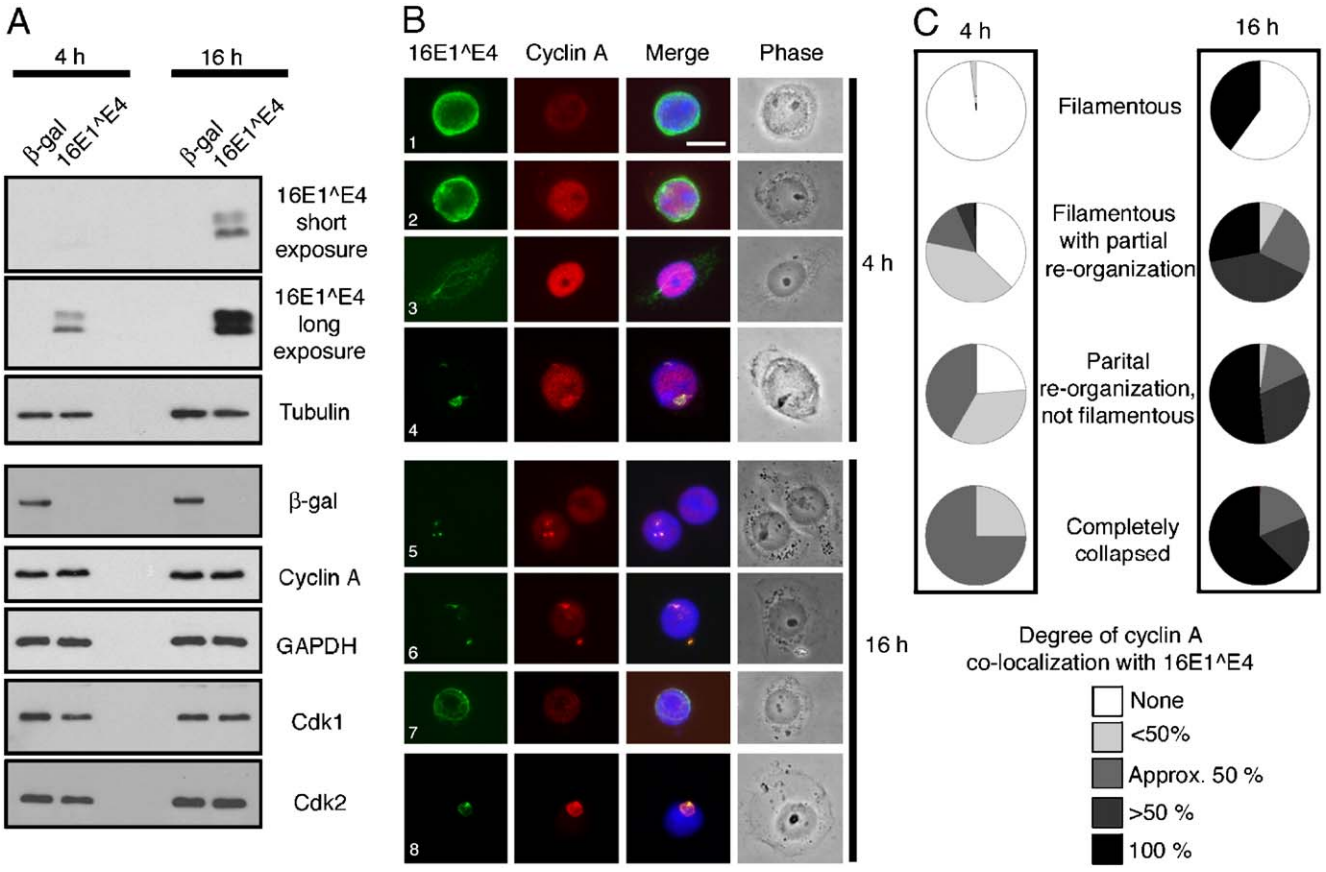



Fig. 4. Comparison of 16E1^E4-cyclin A association at 4 h and 16 post-block release. G1/S synchronized SiHa cells simultaneously infected with recombinant adenoviruses expressing β-gal or 16E1^E4 were harvested at 4 and 16 h post-block release. (A) Cell extracts separated on the same gel were Western blotted for 16E1^E4, β-gal, cyclin A, Cdk1, and Cdk2 using GAPDH and tubulin as loading controls. (B) Cells were double stained for 16E1^E4 (green) and cyclin A (red). A DAPI nuclear stain was included in the merged image (blue). Immunofluorescent images were captured using equal exposure times to allow comparison between 4-h and 16-h cells. Scale bar equals 10 μm. (C) Immunofluorescent microscopy was used to analyze approximately 600 cells from multiple experiments that were visually positive for both 16E1^E4 and cyclin A. Cells were categorized according to the extent of filament reorganization and the degree of cyclin A colocalization. Pie charts show the proportions of cells at 4 h and 16 h having particular degrees of 16E1^E4 reorganization and cyclin A colocalization. For all categories of 16E1^E4 pattern, a greater degree of colocalization with cyclin A is observed at 16 h compared to 4 h. The $\chi^2$ test gave $P$ values of ≤0.001 for the "filamentous with partial reorganization" category and of ≤0.001, for the "partial reorganization, not filamentous" category. These $\chi^2$ values suggest that the null hypothesis, which states that for any given degree of re-organization, there is no difference in ability of cyclin A to colocalize with 16E1^E4 at 4 h or 16 h, should be rejected. The number of 4 h cells showing "complete collapse" and the number of 16 h cells being "filamentous" were too small for statistical analysis to be appropriate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

these correspond to 16E1^E4 modified by the addition of one (approximately p*I* 8) and two (approximately p*I* 7) phosphates (as predicted by Scansite, http://scansite.mit.edu). No completely unphosphorylated protein is present. In contrast, in extracts from cells that express S32A-16E1^E4, the p*I* of the two spots has been increased with one of the spots now corresponding to singly phosphorylated 16E1^E4 and the other (approximately p*I* 9), representing unphosphorylated 16E1^E4 (Fig. 7D, middle panel). This suggests that in wild-type 16E1^E4, the protein can be phosphorylated on two sites; the two spots correspond to 16E1^E4 that is phosphorylated on S32 only and 16E1^E4 that is phosphorylated on S32 and another site. Mutation of serine 32 to alanine removes one of the phosphorylation sites such that now the protein only has the potential to be phosphorylated on one site, and some of the protein remains unphosphorylated.

To investigate if the kinase in cells responsible for phosphorylation of 16E1^E4 on serine 32 is Cdk2/cyclin A, we treated cells expressing wild-type 16E1^E4 with the Cdk inhibitor roscovitine and analyzed the phosphorylation status of

Fig. 3. 16E1^E4 association with cyclin A in S phase. G1/S synchronized SiHa cells expressing β-gal or 16E1^E4 were fixed at 4 h post-block release. Cells were stained for β-gal or 16E1^E4 (green) and cyclin B1, tubulin, or cyclin A (red). A DAPI nuclear stain was included in the merged image (blue). Bars equal 10 μm. Cells were analyzed (A) using the 40× lens on a standard immunofluorescent microscope or (B) using confocal immunofluorescent microscopy. No discrete colocalization is observed between 16E1^E4 and tubulin or β-gal and cyclin A. (C) Immunofluorescent microscopy was used to analyze approximately 600 16E1^E4-positive cells from multiple experiments harvested at 4 and 16 h. Cells were categorized as "filamentous" if they contained 16E1^E4 filaments with no apparent re-organization; "filamentous with partial re-organization" if 16E1^E4 filaments were present but regions of re-organization could also be seen; "partial re-organization, not filamentous", if no filaments were present and the reorganized 16E1^E4 had not yet attained smooth edges; and finally "complete collapse" if the only 16E1^E4 apparent was in small, smooth edged regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

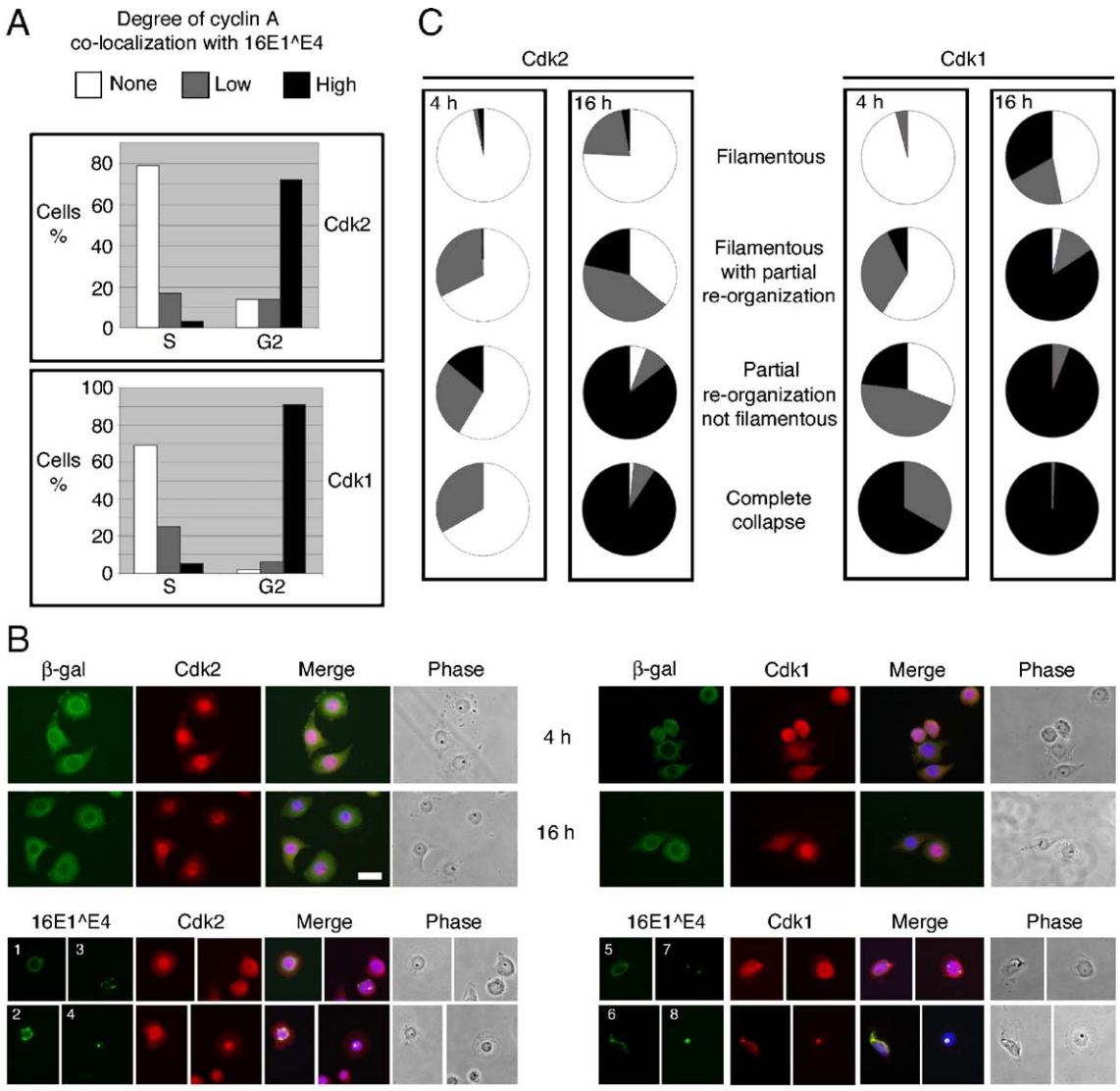



Fig. 5. Association of Cdk1 and Cdk2 with 16E1^E4. G1/S synchronized SiHa cells simultaneously infected with recombinant adenoviruses expressing β-gal or 16E1^E4 were harvested at 4 and 16 h post-block release. (A) Cells were double stained for β-gal or 16E1^E4 (green) and Cdk1 or Cdk2 (red). A DAPI nuclear stain was included in the merged images (blue). Bar equals 20 μm. Immunofluorescent microscopy was used to analyze approximately 600 cells from multiple experiments that were visually positive for 16E1^E4. (A) Cells were categorized according to their degree of Cdk colocalization with 16E1^E4. (B) Examples of typical staining patterns. (C) Cells were categorized according to the degree of Cdk colocalization with 16E1^E4 and the extent of filament reorganization. Pie charts show the proportions of cells at 4 h and 16 h having particular degrees of 16E1^E4 reorganization and Cdk colocalization. For all categories of 16E1^E4 pattern, a greater degree of colocalization with Cdk is observed at 16 h compared to 4 h. The $\chi^2$ test gave $P$ values of $\leq 0.001$ for the "filamentous with partial reorganization" (e.g., cells 2 and 6) category and of $\leq 0.001$, for the "partial reorganization, not filamentous" category (e.g., cells 3 and 7). These $\chi^2$ values suggest that the null hypothesis, which states that for any given degree of reorganization, there is no difference in ability of Cdk to colocalize with 16E1^E4 at 4 h or 16 h, should be rejected. The number of cells showing "complete collapse" (e.g., cells 4 and 8) and the number of cells being "filamentous" (e.g., cells 1 and 5) were too small for statistical analysis to be appropriate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

16E1^E4 using 2D electrophoresis (Fig. 7D, bottom panel). Roscovitine treatment results in loss of the doubly phosphorylated form of 16E1^E4 which suggests that this drug is able to inhibit a kinase that phosphorylates 16E1^E4 in cells.

As with all experiments of this type, these results do not conclusively show that 16E1^E4 is phosphorylated in cells by Cdk2/cyclin A because roscovitine can directly inhibit a number of other kinase complexes and also indirectly inhibit other kinases through downstream signaling pathways. However, the combination of data derived from in vitro kinase assays, mass spectrometry, 2D electrophoresis, mutational analysis, and inhibitor analysis provides strong support that 16E1^E4 serine 32 can be phosphorylated in cells by Cdk2/cyclin A.

### *Localization of cyclin A in 16E1^E4-expressing cells of HPV16-induced lesions*

In addition to observing cyclin B1 associating with 16E1^E4 in cultured cells, cyclin B1 was also seen to partially colocalize with 16E1^E4 in HPV16-induced lesions and raft cultures (Davy et al., 2005). To determine whether the association of cyclin A with 16E1^E4 is also found in vivo, lesions induced by

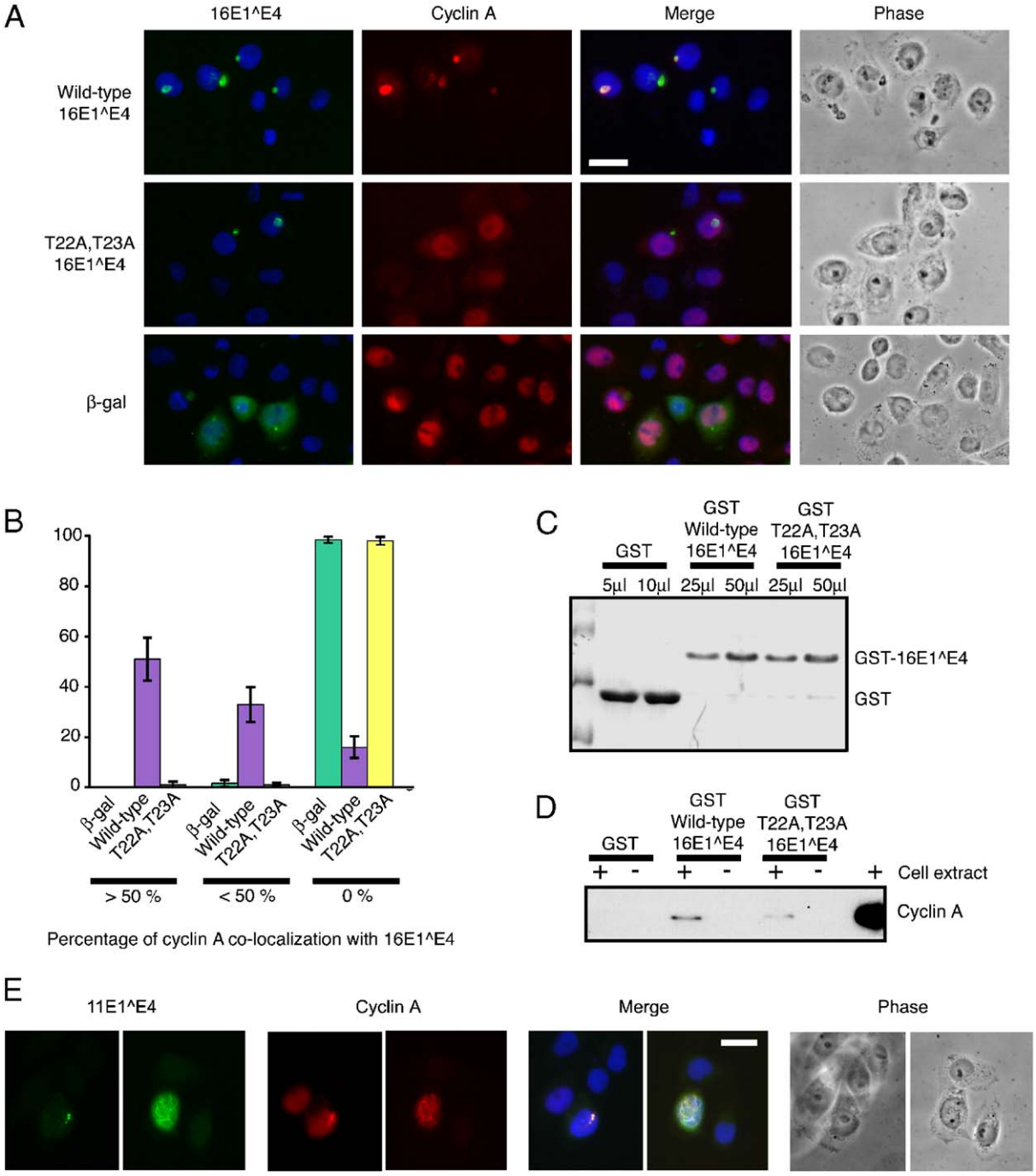



Fig. 6. Association of cyclin A with mutant 16E1^E4. (A) Plasmids that express β-gal, wild-type 16E1^E4 or T22A,T23A 16E1^E4 proteins were transfected into cells and were fixed at 48 h post-transfection. Cells were double stained for β-gal or 16E1^E4 (green) and cyclin A (red). A DAPI nuclear stain was included (blue) in the 16E1^E4 and merged images. Bar equals 20 µm. (B) Colocalization between β-gal or 16E1^E4 and cyclin A was quantified visually by immunofluorescent microscopy. Cells that expressed β-gal or 16E1^E4 and cyclin A were classified as having 0%, less than 50% or greater than 50% colocalization. Results shown are the mean of three experiments, ±SEM. (C) GST, GST-wild-type-16E1^E4 and GST-T22A,T23A proteins were prepared from bacterial extracts and coupled to glutathione beads. Proteins were eluted from varying volumes of the beads, separated by SDS-PAGE and stained using Coomassie. (D) GST beads (5 µl plus 45 µl of uncoupled beads), GST wild-type 16E1^E4 beads and GST-T22A,T23A beads (50 µl each) were used in pull-down experiments, with and without cell extracts derived from unsynchronized SiHa cells. The precipitated proteins were Western blotted for cyclin A and are shown compared to 50% of the input amount of cyclin A. (E) A plasmid that expresses wild-type 11E1^E4 protein was transfected into cells and fixed at 48 h post-transfection. Cells were double stained for 11E1^E4 (green) and cyclin A (red). A DAPI nuclear stain was included (blue). Bar equals 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HPV16 were immunostained. It has previously been shown that such lesions have regions of overlap between expression of 16E1^E4 and cell cycle proteins such as MCM, PCNA and cyclin A (Middleton et al., 2003). Such regions are the sites of viral genome amplification which is seen to correlate with 16E1^E4 expression (Doorbar et al., 1997). Fig. 8A illustrates

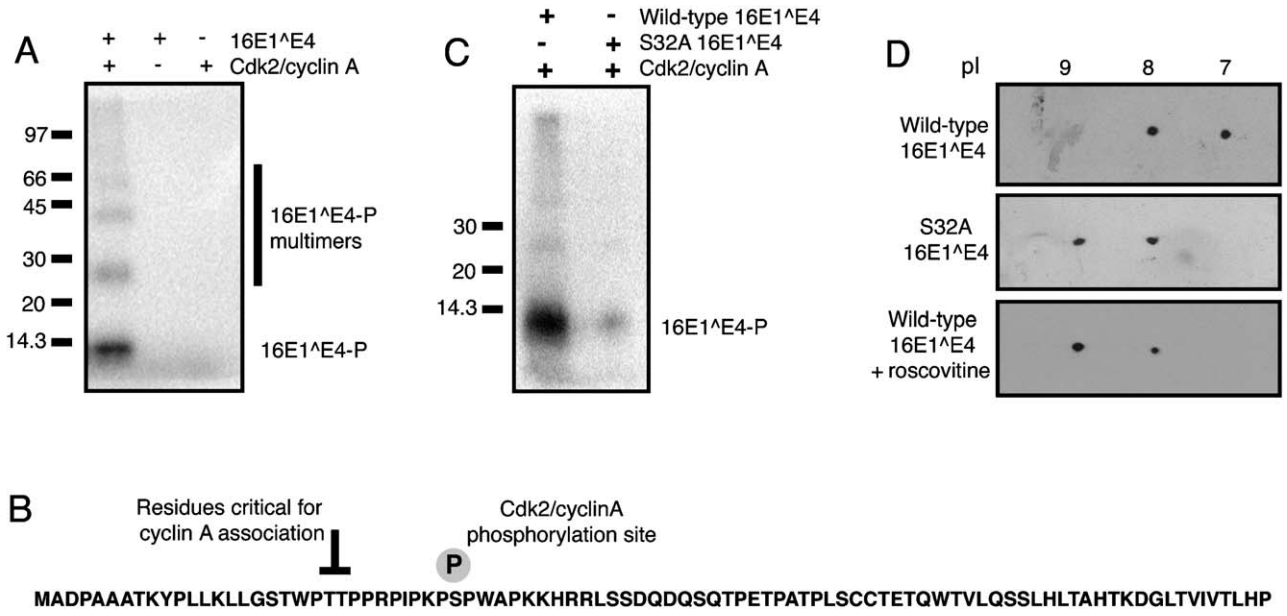



Fig. 7. Phosphorylation of 16E1^E4 S32 by Cdk2/cyclin A. (A) Bacterially expressed and purified 16E1^E4 was used as a substrate in an in vitro kinase reaction with Cdk2/cyclin A. The reaction was separated by SDS-PAGE and analyzed using a phosphorimager. (B) The amino acid sequence of wild-type 16E1^E4 showing the location of the residues identified as critical for cyclin binding and the residue phosphorylated in vitro by Cdk2/cyclin A. (C) In vitro phosphorylation by Cdk2/cyclin A of bacterially expressed wild-type and S32A-16E1^E4 proteins was compared. (D) Extracts from cells expressing wild-type 16E1^E4 (in the absence and presence of roscovitine) or S32A-16E1^E4 (only in the absence of roscovitine) were separated using 2D gel electrophoresis, in the first dimension by iso-electric focusing and in the second dimension by SDS-PAGE.

the regions where previously events within the life-cycle have been found to occur. Cells within the lesion contain varying amounts and distributions of cyclin A. Those in the basal and parabasal layers have predominantly nuclear cyclin A. In comparison, cells in the upper layers of the epithelium that express 16E1^E4 have some cytoplasmic cyclin A. Further magnification of these cells is shown in Fig. 8B, and cyclin A can be seen to localize closely with the sites of 16E1^E4 accumulation, suggesting that the phenomena we have observed in cell culture may also occur in vivo.

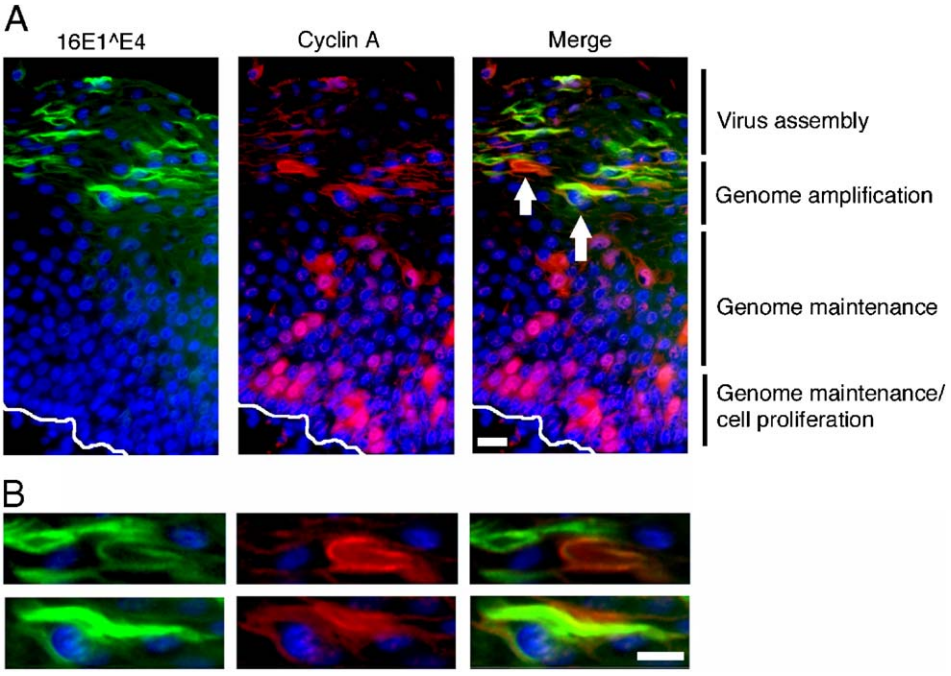



Fig. 8. Association of 16E1^E4 with cyclin A in lesions induced by HPV16. (A) An HPV-16-induced lesion was double stained for the presence of 16E1^E4 (green) and cyclin A (red). A DAPI nuclear stain was included (blue). The regions where events in the life-cycle are expected to occur are shown on the right of the figure. Scale bar equals 20 μm. (B) The cells highlighted by arrows in panel A are shown enlarged. Scale bar equals 10 μm.

## Discussion

It is shown here that the HPV16 E1^E4 protein is able to bind to cyclin A complexes and hold them in the cytoplasm. It is also shown that 16E1^E4 is a substrate for the Cdk2/cyclin A kinase complex, and that at least in cell culture, the residue targeted by Cdk2/cyclin A in vitro is phosphorylated in vivo.

Cyclin A is believed to be a key cell cycle regulator that binds both Cdk1 and Cdk2 and has roles in both S phase and in the G2/M transition, although its precise functions are not fully understood. The activity of cyclin A-dependent kinases during S phase and the localization of cyclin A to sites of DNA replication (Sobczak-Thepot et al., 1993) implicate cyclin A in the control of cellular DNA replication. This is supported by experiments in which either cyclin A anti-sense constructs or anti-cyclin A antibodies were able to inhibit entry into S phase (Girard et al., 1991; Zindy et al., 1992). Further experiments have shown cyclin A to have at least two functions during S phase, the first in activating DNA replication complexes preassembled on the chromatin and the second in preventing re-replication by inhibiting the assembly of new replication complexes (Coverley et al., 2002). However, whether cyclin A is absolutely required for all cell cycles is not clear as it has been observed that cyclin A null mouse embryonic cells are able to both replicate their DNA and divide (Winston et al., 2000), and the situation may actually involve a certain degree of functional redundancy between the different cyclin types. While we cannot rule out completely that 16E1^E4 may have some effect on the ability of cyclin A to perform its S phase roles, it seems unlikely since at this stage of the cell cycle, 16E1^E4 is not observed to associate significantly with cyclin A. Moreover, we observe no difference in the 4-h flow cytometry profiles of 16E1^E4-expressing cells compared to β-gal cells, providing further evidence that 16E1^E4 is not acting on cyclin A during S phase.

In addition to its role in S phase, cyclin A also seems to be involved in controlling the G2/M boundary because inhibition of Cdk2/cyclin A activity in HeLa cells can prevent mitotic entry (Furuno et al., 1999). This stage of the cell cycle is likely to be more relevant for 16E1^E4-induced effects because it is here and not in S phase that good association with Cdk2/cyclin A is observed. Cdk2/cyclin A activity operates ahead of cyclin B1 translocation to the nucleus, and it is thought that while the Cdk1/cyclin B1 complex triggers nuclear membrane breakdown, it is Cdk2/cyclin A activity that induces chromosome condensation (Furuno et al., 1999; Hagting et al., 1999). Our previous work demonstrated that cells expressing 16E1^E4 failed to translocate cyclin B1 into the nucleus and did not enter mitosis (Davy et al., 2005). However, it was not clear why condensation of the chromosomes did not occur since this event does not require cyclin B1. From the work presented here, it appears that the association of 16E1^E4 with cyclin A may prevent chromosome condensation, and it may be that by targeting both A and B cyclins, 16E1^E4 is able to more efficiently block cell cycle progression. Targeting more than one cyclin may be necessary in light of the functional redundancy of cyclins. While the data shown here do not support the idea that 16E1^E4 also interacts with other Cdk/cyclins, e.g., cyclin E, D, Cdk4, neither do the data rule this out. It may be that 16E1^E4 is indeed also able to interact with these proteins but not at the points in the cell cycle which were investigated here, i.e., 4 and 16 h post-release of an aphidicolin block. Further work will be required to determine if this is indeed the case.

One protein that is thought to be phosphorylated by Cdk2 kinase complexes is the HPV E1 protein (Ma et al., 1999). Phosphorylation of E1 on Cdk phosphorylation sites results in its accumulation in the nucleus (Deng et al., 2004), and prevention of phosphorylation results in down-regulation of E2/*ori*-dependent replication (Ma et al., 1999). It may be that by altering the intracellular distribution of Cdk/cyclin complexes, E1^E4 may be able to alter phosphorylation of E1 and hence viral DNA replication. Given the dependence of viral DNA amplification on the presence of E1^E4 (Peh et al., 2004; Wilson et al., 2005), it will be interesting to see whether our T22A,T23A mutation affects genome amplification. Although not themselves cyclin A-dependent kinase targets, the E7 proteins from HPV types 6, 16, and 31 are able to associate with and increase the activity of Cdk2/cyclin A (He et al., 2003). The presence of 16E1^E4 may be able to modulate this effect. Interestingly, it has been shown that the abnormal centrosome duplication that is induced by HPV16 E7 is critically dependent on cyclin/Cdk2 activity (Duensing et al., 2004).

The difference in ability of 16E1^E4 to associate with cyclin A at 4 h and 16 h post-block release is dramatic. How is it that 16E1^E4, a cytoplasmic protein, is able to sequester in the cytoplasm, cyclin A, a protein that until a few hours previous had appeared predominantly nuclear? The answer perhaps lies in the shuttling activity of cyclin A which although normally appearing nuclear is actually translocating between the nucleus and the cytoplasm with its import vastly exceeding its export (Jackman et al., 2002). Thus, progressive interaction of cyclin A with 16E1^E4 as the former passes transiently through the cytoplasm could eventually lead to depletion of cyclin A from the nucleus. Other potential reasons why association occurs at 16 h but not at 4 h include differences in the levels or modification of proteins. Our data suggest that differences in the levels of 16E1^E4, cyclin A, Cdk1, and Cdk2 are not responsible for the cell-cycle specificity, but they do not rule out differences in levels of some other as yet unknown factor required for interaction. Similarly although the modification of 16E1^E4 that results in its slower migration on SDS-PAGE does not seem to facilitate association with cyclin A, there may be other modifications of 16E1^E4, cyclin A, or another factor that facilitate association at 16 but not 4 h. Whatever the event is in G2 that promotes association, it may be related to the re-organization of the 16E1^E4 networks since this too appears to be a phenomenon primarily found in G2.

Cytoplasmic accumulation of cyclin A has previously been observed in response to UV irradiation (Hiromura et al., 2002). Under these conditions, the level of active Cdk2/cyclin A complexes in the cytoplasm builds up and acts

upstream of caspase 3 to induce apoptosis. 16E1^E4 is already known to promote apoptosis (Raj et al., 2004), and it may be that its ability to hold cyclin A in the cytoplasm contributes to this.

The interaction of 16E1^E4 with active Cdk/cyclin A complexes appears to result in phosphorylation of serine 32 of 16E1^E4. It has already been observed, at least in lesions caused by HPV1 and 11, that in vivo phosphorylation of the E1^E4 protein can occur (Bryan et al., 2000; Grand et al., 1989). Although it is not clear what kinases are responsible, phosphorylation is thought to alter the intracellular distribution and multimerization abilities of 11E1^E4. It will be interesting to determine what, if any, effect serine 32 phosphorylation has on the activity of the 16E1^E4 protein.

Given the significance of cyclins for cell cycle progression and the ability of 16E1^E4 to bind at least two members of this protein family, it will be important to investigate the effect of abrogating this interaction in the HPV16 organotypic raft culture system.

## Materials and methods

### *Cell culture, transfection, and recombinant adenovirus (rAd) infection*

SiHa cells were synchronized and infected with rAd as previously described (Davy et al., 2002). Briefly, cells were synchronized at the G1/S border by 48-h incubation in low serum and then aphidicolin for 22 h. Cells were infected with recombinant adenovirus (rAd.β-gal expressing β-galactosidase or rAd.16E1^E4-expressing wild-type 16E1^E4) at a multiplicity of infection of 100, for 6 h beginning 22 h prior to harvest. Four or sixteen hours prior to harvest, the cells were released from the aphidicolin block.

pMV11.16E1^E4 was mutated to pMV11.S32A-16E1^E4 using Quikchange Site Directed Mutagenesis (Stratagene). This plasmid and plasmids that express β-gal (pCDNA6.β-gal), wild-type 16E1^E4 (pMV11.16E1^E4), T22A,T23A-16E1^E4 (pMV11. T22A,T23A 16E1^E4), and wild-type 11E1^E4 (pMV11.11E1^E4) were transfected into SiHa cells as previously described (Davy et al., 2002). Briefly, DNA was prepared using the EndoFree Plasmid Maxi Kit (QIAgen) and transfected into cells using Effectene (QIAgen). The cells were harvested at 24 h post-transfection for 2-D SDS-PAGE analysis and at 48 h post-transfection for immunostaining. Roscovitine (30 μM; Calbiochem) was added to some cells for 16 h prior to harvest.

### *Fractionation*

To fractionate the cells for Western blotting, pellets of $5 \times 10^5$ cells were resuspended in 400 μl of NP-40 fractionation buffer (0.5% NP-40, 10 mM EDTA, 50 $Uml^{-1}$ benzonase [VWR] and protease inhibitor cocktail [Roche] in phosphate buffered saline [PBS]). The cells were lysed in this buffer by pipetting up and down and then were immediately centrifuged at 10,000 × *g* for 5 min at 4 °C. The supernatant was harvested to obtain the NP-40-soluble fraction. The NP-40-insoluble pellet was resuspended in 400 μl of 1% SDS, 5 mM dithiothreitol (DTT) and incubated for 10 min at 95 °C. The extracts were centrifuged at 10,000 × *g* for 5 min at room temperature. The supernatant was harvested to obtain the NP-40-insoluble (SDS-soluble) fraction. Equal volumes of NP-40-soluble and insoluble fractions were loaded for Western blotting.

### *GST pull-downs*

Vectors based on pGEX that express glutathione S transferase (GST), wild-type 16E1^E4 fused to GST (GST-16E1^E4) and T22A,T23A16E1^E4 fused to GST (GST-T22A, T23A16E1^E4) have been previously described (Davy et al., 2005; Doorbar et al., 1992). Expression and purification of GST, GST-16E1^E4, and GST-T22A,T23A16E1^E4 proteins were essentially as previously described (Keen et al., 1994). The integrity of the proteins bound to beads was confirmed by Coomassie stain of SDS-polyacrylamide gels. These gels were used to determine the volumes of beads having equivalent levels of protein coupling. Unsynchronized SiHa cells were harvested and lysed in 0.5% NP-40, 50 $Uml^{-1}$ benzonase, protease inhibitor cocktail (Roche); PBS. The lysate was centrifuged at 13 krpm for 5 min at 4 °C, and the supernatant was diluted 1:10 in pull-down buffer (0.05% NP-40, 10 $\mu gml^{-1}$ BSA, protease inhibitor cocktail; PBS). Diluted supernatant (1 ml) was added to each of 5 μl of GST beads plus 45 μl of uncoupled beads, 50 μl of GST-16E1^E4 beads, or 50 μl of GST-T22A, T23A16E1^E4 beads. Control tubes were set up containing beads and buffer alone. The mixtures were rotated at 4 °C for 1 h and washed four times with pull-down buffer. Proteins were eluted from the beads by boiling for 5 min at 95 °C in 30 μl 1 mM DTT, 10% SDS.

### *Kinase assays, mass spectrometry, 1D and 2D electrophoresis and Western blotting*

For in vitro kinase reactions, bacterially expressed wild-type 16E1^E4 or S32A-16E1^E4 both fused at the C-terminus to 6 histidine residues were used. Purified 16E1^E4 proteins (1 μM) were incubated with 50 U Cdk2/cyclin A (NEB) in 1× reaction buffer (NEB) containing 1 mM ATP/γ-$^{32}$P-ATP (250 μCi/μmol) at 30 °C for 30 min, in a total volume of 25 μl. The reactions were stopped by addition of 12.5 μl of 3× SDS sample buffer plus DTT (NEB). Samples were separated on 15% SDS-polyacrylamide gels. Phosphorylation was analyzed using a Storm Phosphorimager and ImageQuant software (Molecular Dynamics).

In vitro phosphorylated wild-type 16E1^E4 fused at the C-terminus to 6 histidine residues was purified, digested with chymotrypsin, and analyzed by mass spectrometry. Using MALDI-mass spectrometry an ion of mass to charge ratio 2082 was observed, corresponding to mono-phosphorylated peptide of residues 17–34. Nanospray mass spectrometry coupled with collision-induced fragmentation of peptide ion 17–34 produced an ion of mass to charge ratio 469.15 corresponding to the mono-phosphorylated peptide of residues 32–34.

Samples for 2D electrophoresis were prepared from cells by lysing in 2% SDS, 1 μg/ml okadaic acid and benzonase. Cell extract (10 μl) plus 90 μl of buffer containing 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3–10, 65 mM DTT, 20 mM Tris pH 9.5, and 0.1 mM EDTA were centrifuged at 100,000 × *g* for 50 min at 16 °C. The supernatant plus 0.8 μl of IPG buffer (GE Healthcare) was filtered through a 0.22 μM membrane before loading onto a pH 6–11 IPG strip (GE Healthcare). Iso-electric focusing was performed using the Multiphor II system (GE Healthcare), a MultiTempII thermostatic circulator set to 20 °C and an EPS 3501 Xlpower supply for 60,000 Vh. The IPG strip was equilibrated in equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 2% DTT for 15 min, followed by equilibration buffer containing 2.5% iodoacetamide and a trace of bromophenol blue for another 15 min. SDS-PAGE was performed by electrophoresing from the equilibrated strip into a 15% polyacrylamide gel.

For Western blotting, samples were separated by SDS-PAGE and transferred to an Immobilon P membrane (Millipore). Detection was with anti-16E1^E4 clone TVG402 (Doorbar et al., 1992), anti-cyclin A clone 6E6 (Novocastra), anti-cyclin B1 clone V152 (Neomarkers), anti-cyclin D clone 5D4 (Upstate), anti-cyclin E (Upstate), anti-Cdk1 Ab3 (Neomarkers), anti-Cdk2 clone 748 (Santa Cruz), anti-Cdk4 clone 749 (Santa Cruz), anti-tubulin clone B512 (Sigma), anti-histone 2B clone 371 (Upstate), or anti-GAPDH clone 374 (Chemicon) then anti-rabbit (NA9340) or anti-mouse (NA931) HRP conjugates (Amersham Pharmacia Biotech), followed by detection using the ECL kit (Amersham Pharmacia Biotech).

*Immunostaining and flow cytometry*

For microscopy, monolayer cells were fixed for 5 min in 5% formaldehyde in PBS and permeabilized in 0.5% Triton X-100 in PBS for 10 min. Sections were fixed in 10% formaldehyde and permeabilized using a microwave/citric acid based method (Southern et al., 2000). Nuclei were stained with 1 $\mu g ml^{-1}$ DAPI (4′-6-diamidino-2-phenylindole; Sigma). 16E1^E4 was detected using TGV405 (Doorbar et al., 1997) directly conjugated to Alexa-488 (Molecular Probes). Other antigens were detected with anti-11E1^E4 (Peh et al., 2002), anti-Cdk1 clone Ab-1 (Oncogene), anti-β-gal polyclonal 55976 (ICN biomedicals), anti-tubulin clone B512 (Sigma), anti-cyclin A clone 6E6 (Novocastra), and anti-cyclin B1 clone GNS1 (Neomarkers). Primary antibodies were detected using Alexa-488- or Alexa-594-conjugated anti-mouse or anti-rabbit antibodies (Molecular Probes). The cells were examined using a fluorescent Labophot II monochrome camera (Nikon) and IP Lab imaging software (Roper Scientific). Confocal images were obtained using a TCS-SP1 DMRXE confocal microscope (Leica) and LCS imaging software (Leica).

Flow cytometry was carried out as previously described (Davy et al., 2002). Briefly, cells were stained as for microscopy and then with propidium iodide (12.5 $\mu g ml^{-1}$). Their DNA content was analyzed using a FACSCalibur and CellQuest software (Becton Dickinson).

## Acknowledgments

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