Human papillomaviruses target the double-stranded RNA protein kinase pathway

Christy M. Hebner,¹ Regina Wilson,¹ Janet Rader,² Miri Bidder² and Laimonis A. Laimins¹

¹Department of Microbiology–Immunology, The Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA
²Department of Obstetrics and Gynecology, Washington University School of Medicine, St Louis, MO 63110, USA

The double-stranded RNA protein kinase (PKR) pathway plays a vital role in the innate immune response to viral infection. Activation of PKR following virus entry can lead to a shutdown in translation, thereby inhibiting viral protein synthesis and replication. Little is currently known about whether human papillomaviruses (HPVs) modulate PKR expression and activity. In this study, normal human foreskin keratinocytes (NHKs) transfected stably with the HPV 31 or 16 genomes and cell lines expressing the HPV 16 E6 and E7 oncoproteins were used to examine effects on the PKR pathway. HPV gene products were found to modulate PKR phosphorylation, activity and localization. The levels of total PKR protein were reduced modestly in cells that maintained HPV 16 or 31 episomes through a reduction in PKR transcription. However, levels of phosphorylated PKR were decreased 4-fold through a post-transcriptional mechanism mediated by E6 and E7 that was independent of the transcriptional downregulation mediated by HPV. In response to infection by vesicular stomatitis virus, phosphorylation of eIF2α was blocked in cells expressing HPV oncoproteins, but not in NHKs. Finally, it was observed that the cellular localization of PKR was altered by HPV gene products in HPV raft cultures, as well as HPV-positive patient biopsies. This effect was mediated by the HPV E6 oncoprotein and leads to the co-localization of PKR with P-bodies. These studies demonstrate that high-risk HPVs target the PKR pathway by multiple mechanisms.

INTRODUCTION

Human papillomaviruses (HPVs) are small, non-enveloped, double-stranded DNA viruses that infect the mucosal or cutaneous epithelium (Howley, 1996). Over 100 types of HPV have been identified, with one-third targeting the genital tract. These viruses can be subdivided further into ‘low-risk’ and ‘high-risk’ categories. Low-risk HPVs, such as subtypes 6 and 11, are rarely associated with malignancies and cause benign warts (de Villiers, 1994; zur Hausen, 2002; zur Hausen & de Villiers, 1994). In contrast, infection by high-risk HPVs, including subtypes 31, 18 and 16, is associated with the development of cervical carcinoma (Monsonego et al., 2004; zur Hausen, 2002).

The productive life cycle of HPV is linked closely to epithelial differentiation. HPV is thought to initially infect cells of the basal epithelium. During the initial stages of infection, viral transcription occurs from the early promoter, directing expression of polycistronic transcripts encoding the multiple viral proteins, including the two major oncoproteins encoded by high-risk HPVs, E6 and E7 (Hummel et al., 1992; Ozbun & Meyers, 1998). E6 forms a complex with the cellular ubiquitin protein ligase, E6AP, leading to the degradation of p53, whilst E7 can bind and inhibit the activities of the Rb family of proteins (Cheng et al., 1995; Dyson et al., 1989; Huibregtse et al., 1991; Martin et al., 1998; Schuessler et al., 1990, 1993; Werness et al., 1990). These functions allow high-risk HPV types to override various cell-cycle checkpoints and facilitate viral DNA synthesis.

During infection, viruses often target the innate immune system in order to facilitate productive replication. One important component of this system is signalling through the type I interferons (IFNs), specifically IFN-α and -β, resulting in transcription of genes involved in growth inhibition, immunomodulation and diverse antiviral effects (Barber, 2001; Brierley & Fish, 2002; Malmgaard, 2004; Stark et al., 1998). Through the use of microarray analysis, HPV 16 and 31 gene products were shown to downregulate the expression of IFN-inducible genes, such as MxA, 2’5’-oligoadenylate synthetase 2 and Stat-1 (Chang & Laimins, 2000; Nees et al., 2001). Additional studies suggested that HPV 16 E6 can bind to IFN-regulatory factor-3 (IRF-3),
thereby blocking IFN expression, whilst HPV 16 E7 can interact with IRF-1 (Park et al., 2000; Ronco et al., 1998; Um et al., 2002). Thus, HPV proteins have been suggested to act at several levels to hinder IFN-responsive genes.

Double-stranded RNA protein kinase (PKR) is an IFN-inducible serine/threonine kinase that is expressed constitutively in cells in an inactive form and is a major component of the innate immune response to viral infection. PKR binding of double-stranded RNA or a cellular protein, PACT, induces a conformational change resulting in autophosphorylation and, thus, activation of the kinase function of PKR (Clemens, 1997; Patel & Sen, 1998; Williams, 1999). PKR activation results in the phosphorylation of multiple substrates, leading to various antiviral effects including caspase activation and the inhibition of protein synthesis. One of the best-characterized substrates of PKR is the alpha subunit of eukaryotic initiation factor 2 (eIF2α) (de Haro et al., 1996). Phosphorylation of eIF2α at Ser51 leads to an inhibition of protein synthesis via sequestration of the guanine nucleotide-exchange factor eIF2B (Hershey, 1991). This results in a block in translation, thereby inhibiting viral protein synthesis. Many viruses, including herpes simplex virus, Epstein–Barr virus and adenovirus, have developed means of circumventing signalling through the PKR pathway to ensure viral survival (Burgert et al., 2002; Elia et al., 1996; Mohr, 2004; Roizman, 1999).

Little is currently known about whether HPV modulates the PKR pathway through either transcriptional or post-transcriptional mechanisms. To investigate the effects of HPV on PKR signalling, we have utilized normal human foreskin keratinocytes (NHKs) transfected with HPV genomes, which reflect the physiological characteristics of early HPV infection. By using this system together with cell lines expressing individual HPV gene products and patient biopsy samples, we have found that HPV gene products modulate PKR by multiple means, including effects on phosphorylation, activity and localization.

METHODS

Cell culture. NHKs were isolated from neonatal human foreskin epithelia and cultured as described previously (Ruesch & Laimins, 1998). HPV genome transfectants and retroviral transductants were cultured with mitomycin C-treated NIH 3T3 fibroblast feeders in E-medium plus mouse epidermal growth factor (5 ng ml⁻¹; Collaborative Biomedical Products). In experiments where untransfected NHKs were compared with transfected cell lines, all cells were grown with fibroblast feeders in E-medium plus epidermal growth factor. Prior to harvesting keratinocytes for analysis, feeders were reblocked for 30 min following secondary-antibody incubation, washed and permeated by using 0.1% paraformaldehyde/PBS, then incubated overnight with anti-PKR antibody in blocking buffer at 4°C, washed and permeated by using 0.1% paraformaldehyde/PBS, then incubated overnight with anti-PKR antibody in blocking buffer at 4°C in a humid chamber. Coverslips were washed, incubated for 1 h at room temperature with a goat anti-rabbit FITC-linked secondary antibody, then DAPI-counterstained. Coverslips were mounted with Vectashield mounting medium, sealed and analysed by using a Leica inverted microscope. For co-localization studies, coverslips were reblocked for 30 min following secondary-antibody incubation, incubated for 1 h at room temperature using anti-GW-182 antibody as described previously (Fehrmann et al., 2003). After selection, pooled populations were expanded and analysed for epimism as described previously (Longworth & Laimins, 2004).

Creation of retroviral cell lines. Creation of the LXSN constructs has been described previously by Halbert et al. (1991). FT67 cells (Clontech) were transfected with retroviral constructs by using FuGENE transfection reagent (Roche Diagnostics). Stable virus-producing cells were selected by using 1000 µg G418 ml⁻¹ for 4 days, followed by an additional 4 days selection with 500 µg G418 ml⁻¹. Cell supernatants were harvested, filtered and used to infect NHKs overnight in the presence of 8 µg polybrene ml⁻¹ (Sigma). Infected cells underwent G418 antibiotic selection to generate stable cell lines.

Generation of E6 mutants. The L375, G134V and I128T mutations in E6 were generated in the LXSN 16 E6E7 construct by using a QuikChange XL mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Northern blot analysis. Total RNA was extracted from cells by using an SV Total RNA kit (Promega). mRNA quantity and purity were measured by spectrophotometric methods. Specific probes were generated from gel-purified PCR fragments by using a Ready-to-go DNA labelling kit (Amersham Biosciences) and purified with ProbeQuant G-50 microcolumns (Amersham Biosciences). Total RNA (10 µg) was separated on a 1% agarose/2% M formaldehyde gel, vacuum-transferred and hybridized as described previously (Wilson et al., 2005). Gels were visualized by using autoradiography (Hyperfilm; Amersham Biosciences) and RNA bands were quantified by using Fluorchem software (Alpha Innotech). 28S rRNA was used as a loading control.

Western blots. Cell lysates were collected from subconfluent cells and quantified by the Bradford method as described previously (Chang & Laimins, 2000). Fifty micrograms of protein was subjected to SDS-PAGE and Western blotting as described previously (Longworth & Laimins, 2004). Primary antibodies used were anti-PKR, anti-phospho-PKR (Thr451), anti-eIF2α (all from Cell Signaling Technology), anti-phospho-eIF2α (Ser51) (Biosource International) and anti-glyceraldehyde-3-phosphate dehydrogenase (Abcam).

Differentiation of cells in raft culture or methylcellulose. Cells were grown on a solidified collagen matrix containing J2 fibroblast feeders and allowed to differentiate by using an air–liquid interface as described previously (Meyers & Laimins, 1994). Rafts were harvested after 14 days, fixed in 4% paraformaldehyde, paraaffin-embedded and sectioned onto silanized slides for immunohistochemistry. Alternatively, differentiation was induced by suspension in 1:5% methylcellulose as described previously (Wilson et al., 2005).

Immunohistochemical analysis and immunofluorescence. Raft culture sections and human cervix tissue on silanized slides were processed, stained with antibody and analysed as described previously (Wilson et al., 2005). Sections were stained by using an anti-PKR antibody (Cell Signaling Technology) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary (Amersham Biosciences). Monolayer cells grown on glass coverslips in six-well plates were versene-treated to remove fibroblast feeders prior to staining. Cells were fixed by using 4% paraformaldehyde/PBS, washed and permeated by using 0.2% Triton X-100. Cells were blocked for 30 min in 0.5% NP-40/1% BSA/PBS, then incubated overnight with anti-PKR antibody in blocking buffer at 4°C in a humid chamber. Coverslips were washed, incubated for 1 h at room temperature with a goat anti-rabbit FITC-linked secondary antibody, then DAPI-counterstained. Coverslips were mounted with Vectashield mounting medium, sealed and analysed by using a Leica inverted microscope. For co-localization studies, coverslips were reblocked for 30 min following secondary-antibody incubation, incubated for 1 h at room temperature using anti-GW-182 antibody.
mimic infected basal cells examined cells that maintained viral DNA as episomes and keratinocytes growing in tissue culture. For these studies, we well as the active phosphorylated form, in undifferentiated products had any effect on the levels of total PKR protein, as proliferation. We first investigated whether HPV gene infection of cells and activating mechanisms to block viral The PKR pathway plays a major role in both detecting viral infection requires phosphorylation of the protein. The ability of PKR to block translation in response to viral transcriptional synergistic effect of E6 and E7 was titrated on CV-1 cells by plaque assay. NHKs and retroviral cells were versene-treated to remove fibroblast feeders and infected at an m.o.i. of 10 for 1 h at 37°C in 1 ml 1 % BSA/Dulbecco’s modified Eagle’s medium in 10 cm tissue-culture plates. Ten millilitres of E-medium was then added to each plate. At various times post-infection, protein was harvested and quantified from cells as described above and analysed by SDS-PAGE/Western analysis.

RESULTS

Levels of total and phosphorylated PKR are reduced in HPV 31- and 16-positive cells

The PKR pathway plays a major role in both detecting viral infection of cells and activating mechanisms to block viral proliferation. We first investigated whether HPV gene products had any effect on the levels of total PKR protein, as well as the active phosphorylated form, in undifferentiated keratinocytes growing in tissue culture. For these studies, we examined cells that maintained viral DNA as episomes and mimic infected basal cells in vivo. NHKs were transfected with HPV 31 or HPV 16 genomes and stable cell lines were selected and analysed for changes in PKR protein levels. A total of seven sets of independently derived HPV 31-transfected cells and three sets of HPV 16-transfected cells were analysed. By using Western blot analysis, we found that levels of total PKR protein were decreased on average by 2-fold in cells transfected with either the HPV 31 or HPV 16 genomes compared with matched NHK controls (Fig. 1a). In addition, we determined that untransfected NHKs were found to express similar levels of PKR and phospho-PKR as neomycin-selected transfected controls (Fig. 1b).

We next sought to investigate whether the reduction in PKR protein levels was due to transcriptional or post-transcriptional events. We performed Northern analysis by using total RNA isolated from HPV-positive and control cells to see whether the effects were mediated at the level of transcription. Cells containing HPV 31 or HPV 16 episomes showed a 2-fold decrease in the 2.5 kb mature PKR mRNA compared with control NHKs, suggesting that down-regulation of PKR transcription may be the primary cause of decreased levels of PKR protein (Fig. 1c).

The ability of PKR to block translation in response to viral infection requires phosphorylation of the protein. The translation-blocking capability of PKR involves autophosphorylation of multiple residues; however, phosphorylation of Thr451 is absolutely essential to this activity (Romano et al., 1998; Taylor et al., 1996). To investigate whether HPV gene products also affected phosphorylation of PKR, we examined extracts of HPV-positive and NHK cells by Western blot analysis using an antibody recognizing PKR phosphorylated at Thr451. Cells containing HPV 31 genomes were found to exhibit, on average, a 3.0-fold reduction in levels of phospho-PKR compared with control NHKs, whilst cells with HPV 16 episomes had a 4.6-fold reduction (Fig. 1a, d). As phosphorylated PKR levels were reduced by approximately 4-fold whilst total proteins levels were only reduced by 2-fold, we conclude that HPV gene products must also act through post-transcriptional mechanisms to decrease levels of phospho-PKR.

Phospho-PKR is reduced by a post-transcriptional synergistic effect of E6 and E7

It was next important to determine whether the effects on PKR phosphorylation were due to the action of the viral

---

**Fig. 1.** Levels of total and phosphorylated PKR proteins in NHKs and HPV-positive cell lines. (a) Lysates from NHKs transfected stably with HPV 31 or HPV 16 genomes and untransfected controls were analysed by Western analysis using antibodies against PKR and PKR phosphorylated at Thr451. (b) Comparison of total and phospho-PKR levels in untransfected NHKs versus pSV2neo-transfected NHKs that have undergone neomycin selection. (c) PKR mRNA levels as determined by Northern analysis for HPV-positive and control NHKs. 28S rRNA was used as a loading control. Similar results were seen for two different HPV 31 and two different HPV 16 cell lines. (d) Quantitative analysis of phosphorylated PKR protein levels, comparing seven independent HPV 31 and three different HPV 16 cell lines. Protein levels for NHKs (filled bar) were set to 1. Phospho-PKR quantities were 0.33±0.12 and 0.22±0.05 for HPV 31 (empty bar) and 16 (hatched bar), respectively, of those seen in NHK controls.
oncoproteins E6 and/or E7 or another HPV early protein. For these studies, we examined levels of phospho-PKR in cell lines generated by infection of NHKs with retroviruses expressing HPV 16 E6 alone, E7 alone or both E6 and E7. NHKs infected with retroviruses containing an empty vector were used as a control (LXSN), although no significant differences in PKR expression or phosphorylation were observed between LXSN and uninfected NHKs (data not shown). We observed that cells expressing both the E6 and E7 oncoproteins exhibited only slight decreases in total PKR protein levels and no changes in PKR transcript levels compared with controls (Fig. 2a, b). In addition, oncogene expression had no effect on the IFN inducibility of PKR expression (Fig. 2a). More importantly, we observed that the cells expressing both E6 and E7 exhibited, on average, a >4-fold decrease in constitutive levels of phospho-PKR (Fig. 2b). Interestingly, only minor alterations in phospho-PKR protein levels were observed in cells expressing either of the oncogenes alone and significant reductions were only seen when both proteins were expressed. This suggests that reductions in the basal levels of phospho-PKR are due to a synergistic effect of E6 and E7.

We next wanted to see what effect mutations in E6 had on the synergistic ability of E6 and E7 to reduce PKR phosphorylation. Mutations in E6 affecting the binding and degradation activities of p53 have been characterized previously and we were interested to see how these mutations affected levels of phospho-PKR. To do so, we analysed three different mutations in E6, each made in the context of a plasmid expressing both E6 and E7 (Fig. 2c, d). The I128T mutant has previously been shown to be incapable of degrading p53, resulting in a loss of degradative capacity (Nguyen et al., 2002; Thomas & Chiang, 2005). A third mutant, G134V, can degrade p53; however, this mutation in E6 abolishes the ability of the oncoprotein to bind CBP/p300, leading to acetylation of p53 (Thomas & Chiang, 2005). Interestingly, all three mutants showed increased levels of phospho-PKR compared with cells expressing E6 and E7 (Fig. 2c). This effect was most pronounced in the I128T mutant, where PKR phosphorylation was nearly identical to that seen in controls. The L37S and G134V mutants displayed phospho-PKR levels intermediate to those seen in LXSN and E6E7 cells. From these results, we conclude that the L37, G134 and I128 residues of E6 play a role in the synergistic effects of E6 and E7 on PKR phosphorylation, with the I128 residue being critical to this function.

Levels of phospho-eIF2α in response to VSV infection

As HPV gene products induced a reduction in basal levels of phospho-PKR protein levels, we wanted to investigate whether this decrease had an effect on downstream regulators of translation. For this analysis, we examined the levels of total eIF2α protein and eIF2α phosphorylated at Ser51 in cells containing HPV 31 or 16 episomes and NHK control cells. Given that phospho-PKR levels were reduced, we were surprised to find that the levels of either total eIF2α or phosphorylated eIF2α in HPV 31 and 16 cells were similar to those seen in NHK controls (Fig. 2e). In addition, no difference was found in either total or phosphorylated eIF2α in cells expressing the E6 and E7 oncoproteins (Fig. 2f). A slight decrease in phospho-eIF2α was observed in cells expressing HPV 16 E6 alone and this has been reported previously with cells expressing HPV 18 E6 (Kazemi et al., 2004).

The lack of an effect of HPV proteins on eIF2α phosphorylation in stable cell lines was puzzling, given the
decreased levels of PKR phosphorylation that were observed. As other cellular factors can alter phosphorylation of eIF2α, it was possible that, in the absence of a PKR pathway inducer, the basal level of eIF2α phosphorylation was not directly dependent on PKR phosphorylation levels. It was thus important to analyse eIF2α phosphorylation in response to an inducer of PKR activity. To do so, we used the RNA virus VSV, a known physiological stimulus of the PKR pathway (Balachandran & Barber, 2004). For these analyses, we infected cells expressing E6 and E7, either separately or in combination, with VSV at an m.o.i. of 10. At 0, 2, 4, 8 and 12 h post-infection, the levels of total and phosphorylated PKR, as well as of total and phosphorylated eIF2α, were examined by Western analysis (Fig. 3 a, b). In LXSN control cells, a modest increase in phospho-PKR levels was observed at 12 h post-infection, whilst no such increase was seen in cells expressing E6 and E7. An approximate 2-5-fold increase in eIF2α phosphorylation was detected in LXSN controls at 2 h post-infection, which increased, on average, to 5-fold at 12 h post-infection. In contrast, cells expressing the E6 and E7 oncoproteins together demonstrated no increase in eIF2α phosphorylation following infection with VSV throughout all time points. Cells expressing E6 alone showed induction of eIF2α phosphorylation that was neither as rapid nor as robust as that seen in LXSN controls. In cells expressing E7 alone, a reproducible decrease in eIF2α phosphorylation was observed at 2 h post-infection, but increased at the 12 h point. The presence of both oncoproteins together was more effective at reducing eIF2α phosphorylation than either oncoprotein alone. Similar results were seen in multiple experiments and suggest that expression of E6 and E7 blocks eIF2α phosphorylation in response to VSV infection.

PKR localization is changed in HPV raft culture and HPV-positive patient samples

The life cycle of HPV is dependent upon epithelial differentiation, with productive replication and late gene expression restricted to suprabasal cells. As PKR could be activated in response to differentiation-induced genome amplification and late gene expression, we wanted to investigate whether HPV gene products blocked PKR activity in suprabasal cells. To induce differentiation, HPV-positive cells were suspended in methylcellulose and protein extracts were isolated as a function of time. PKR and phospho-PKR levels were then examined by Western blot analysis. These studies demonstrated that both total and phosphorylated PKR levels decreased with differentiation (Fig. 4 a). However, similar decreases were seen in NHK control cells induced to differentiate. In addition, similar to observations in monolayer cultures, the levels of PKR and phospho-PKR were reduced in HPV-positive cells compared with NHKs.

Fig. 3. PKR signalling in HPV-positive cells in response to VSV infection. (a) Cells expressing HPV 16 E6 and/or E7 were infected with VSV (m.o.i. =10) as described in Methods. At given times, cell lysates were harvested and subjected to Western analysis using the indicated antibodies. Similar results were seen in experiments performed by using two different sets of independently derived cell lines. A representative experiment is shown. (b) Graph showing mean fold induction of phospho-eIF2α compared to the zero time point at various times post-infection for each cell line. ■, NHKs; □, E6E7; ▲, E6; △, E7.
We next examined the distribution of PKR by immunohistochemical analysis of organotypic raft cultures and, in agreement with our observations with cells differentiated in methylcellulose, we observed a decrease in PKR levels in the suprabasal levels of the rafts compared with the basal layer (Fig. 4b). Interestingly, we observed a change in PKR localization in both differentiated and undifferentiated HPV-positive cells. Immunofluorescence analysis of rafts derived from NHKs indicated that PKR proteins were distributed uniformly throughout the cytoplasm, with only occasional minor clustering (Fig. 4b). In raft cultures of HPV 31-positive cells, we observed more PKR present in the nuclei of cells than was seen in NHK control rafts (Fig. 4c). More importantly, PKR was found to be localized into tight, punctate clusters in the cytoplasm in all cells, in contrast to the homogeneous cytoplasmic staining observed in rafts of normal keratinocytes. This suggested that the localization of PKR was altered by the action of HPV gene products.

It was important to investigate whether these changes in the localization of PKR in raft cultures were also found in HPV lesions in vivo. For these studies, we examined tissue sections from biopsies of both normal cervical tissue and cervical intraepithelial neoplasia (CIN) for PKR localization by immunofluorescence. In normal cervical tissue, PKR was found to be distributed uniformly throughout the cytoplasm and primarily excluded from the nucleus in the majority of cells, similar to what we observed in organotypic rafts of NHKs (Fig. 5a). In contrast to normal cervical tissue, PKR staining in CIN lesions was found in both the cytoplasm and nucleus throughout all layers of the tissue (Fig. 5b). Furthermore, large, bright, cytoplasmic clusters of PKR were observed in CIN lesions and were similar to what had been seen in HPV rafts. These results suggest that HPV gene products alter the localization of PKR in vivo.

The HPV E6 oncoprotein alters PKR localization

We next wanted to see which HPV proteins were responsible for the observed changes in PKR localization. For this analysis, we performed immunofluorescence for PKR in cells expressing E6 and/or E7 grown in monolayers, as we did not observe any differentiation-specific effects of PKR localization in rafts (Fig. 6). In NHK control cells, we observed PKR to be localized primarily to the cytoplasm. Immunofluorescent staining of PKR in cells expressing the E7 oncoprotein showed staining patterns similar to that seen in NHKs, with PKR being distributed homogeneously throughout the cytoplasm. In cells expressing E6 alone, PKR was localized into large, tight clusters in the cytoplasm, similar to patterns observed in rafts of HPV-positive cells and CIN lesions. In cells expressing both the E6 and E7 oncoproteins, PKR was again found to be organized into large clusters distributed throughout the cytoplasm. Similar results were seen in monolayer cultures of cells with episomal copies of HPV 31 or HPV 16 (data not shown). These data suggest that the E6 oncoprotein alters the distribution of PKR significantly.

We next wanted to examine the effect of mutations in E6 that alter various p53-associated activities on PKR localization. By using immunofluorescence of cells grown in monolayer cultures, we found that both mutants deficient in p53 degradation, 1128T and L37S, failed to induce cytoplasmic clustering of PKR and caused PKR to relocalize into the nucleus (Fig. 7). This nuclear localization was most pronounced in the L37S mutant. The G134V mutant, which is defective for p300 binding, leading to acetylation of p53, induced a perinuclear clustering of PKR that was...
intermediate in phenotype to those seen in E6E7 cells or NHKs. Although we cannot rule out the contribution of other E6-associated functions, these results suggest that the ability of E6 to degrade or modify p53 may play a role in PKR localization.

**PKR co-localizes with P-bodies in the presence of HPV E6**

Finally, the punctate, cytoplasmic pattern that we observed with PKR in HPV-positive cells was reminiscent of the appearance of P-bodies, sites of mRNA storage and degradation (Marx, 2005). Thus, we were interested to see whether PKR was localizing to P-bodies in cells expressing the E6 oncoprotein (Fig. 8). By using co-immunofluorescence with antibodies to PKR and GW182, an essential component of P-bodies (Wilson et al., 2005), we observed co-localization between PKR and GW182. In contrast, NHKs co-stained for PKR and GW182 showed homogeneous staining patterns for both proteins throughout the cytoplasm, without any punctate clustering. Thus, we conclude that the presence of the E6 oncoprotein leads to a localization of PKR to P-bodies and may contribute to its effect on PKR activity.

**DISCUSSION**

PKR is an important component of the innate immune antiviral response and is a common target for both RNA and DNA viruses. In our study, we found that HPV gene products modulate PKR at multiple levels by altering expression, activity and cellular localization. In cells that maintain HPV episomes, the levels of total PKR protein were found to be reduced by approximately 2-fold, mediated through decreases in PKR transcript levels. This reduction was not the result of the action of E6 and/or E7, but rather was mediated through another HPV early protein. One leading candidate is the E2 protein, which can both activate and repress gene expression by binding to promoters. This reduction in PKR transcripts is consistent with microarray analyses indicating that HPV gene products repressed expression of several IFN-inducible genes (Chang & Laimins, 2000; Nees et al., 2001). We also observed a reduction in the basal level of phosphorylated PKR in HPV-positive cells. Phosphorylation of PKR is associated with activation of its kinase activity. This reduction could not be explained through a decrease in total PKR protein levels alone and appears to be associated with post-transcriptional events mediated by the synergistic action of E6 and E7, rather than either oncoprotein alone.
The exact mechanism by which the combined action of E6 and E7 is most effective for decreased basal levels of PKR phosphorylation remains unclear. However, our mutational analyses indicate that E6’s interactions with p53 may be important. This includes the ability to bind and degrade p53 through E6AP, as well as effects on p53 acetylation mediated through E6 binding of p300. The synergistic activity of E6 and E7 in mediating effects on p53 and other cell-cycle regulators has been documented previously (Fehrmann & Laimins, 2003; Galloway & McDougall, 1996; zur Hausen, 1996). In addition, PKR has been implicated as a modulator of some p53 functions and two reports suggest that PKR binds to p53 (Cuddihy et al., 1999a, b). It is possible that E6 and E7 modulation of p53 levels could lead to altered

---

**Fig. 6.** PKR localization in cells expressing HPV 16 E6 and/or E7. Monolayer cultures of cells expressing E6 and/or E7 were grown on glass coverslips as described in Methods. Cells were stained by using a PKR antibody and FITC-conjugated secondary as outlined in Methods and visualized at ×63 using a Leica inverted microscope. Staining patterns observed in NHKs were similar to those observed in LXSN control cells. The inset (top right panel) shows E6E7 cells stained with secondary antibody as a control.

---

**Fig. 7.** Localization of PKR in cells expressing various E6 mutants together with E7. Monolayer cultures grown on coverslips were stained with a PKR primary antibody and FITC-conjugated secondary antibody as described in Methods. Cells were visualized at ×63 by using a Leica inverted microscope.
phosphorylation of PKR. Alternatively, E6 could act to degrade the phosphorylated forms of PKR specifically, perhaps through the action of E6AP. However, we have not observed a direct interaction of E6 with PKR in over-expression assays, although we cannot exclude the possibility that they associate weakly in a complex with p53 (C. M. Hebler, unpublished data). Furthermore, we cannot rule out the contribution of other E6-associated functions. Finally, it is possible that E6 and E7 act on separate, but complementary, upstream mediators of PKR function.

One well-defined downstream target of PKR is eIF2α, which, when phosphorylated, acts to inhibit translation. We observed little change in the basal levels of eIF2α phosphorylation in HPV-positive cells and this was unexpected, as we did observe reductions in the levels of phospho-PKR. However, as other kinases affect levels of eIF2α phosphorylation, the correlation between basal levels of phospho-eIF2α and phospho-PKR may not necessarily be direct. Additionally, it is possible that HPV gene products may actually activate eIF2α phosphorylation, but that this is counteracted by the reduction in levels of PKR phosphorylation induced by E6 and E7, resulting in net levels of phospho-eIF2α comparable to those seen in normal cells.

More significantly, we observed major effects of E6 and E7 on the level of eIF2α phosphorylation in response to infection with VSV. Interestingly, either oncoprotein alone could inhibit eIF2α phosphorylation, although the combination was most effective. It is possible that the action of E6 in this process could be explained in part by the observations that HPV 18 E6 can reduce phosphorylation of eIF2α in the presence of activated PKR (Kazemi et al., 2004). However, as cells expressing E6 alone did not show any significant increase in PKR phosphorylation in response to VSV infection, it is possible that E6 may also modulate PKR function directly. In addition, the action of the individual oncoproteins on eIF2α phosphorylation appeared to be additive, as cells expressing both E6 and E7 displayed the most efficient blockage.

Another mechanism by which the E6 protein may modulate PKR function is by altering its cellular localization. In organotypic raft cultures of HPV-positive cells, we observed a relocalization of PKR into distinct clumps in the cytoplasm, in contrast to the uniform staining observed in rafts of normal human keratinocytes. Similar or even more extensive clumping was seen in biopsy samples of CIN lesions, but not in those from normal cervix, indicating that the HPV-induced alteration in PKR localization occurs in vivo as well. In addition to inducing clumping of cytoplasmic PKR, significant amounts of PKR were observed in the nuclei of HPV-positive rafts compared with NHK rafts. The clustering of cytoplasmic PKR appears to be due to the action of the E6 oncoprotein, as we observed dramatic relocalization in cells expressing E6 alone or both E6 and E7, but not E7 alone. Furthermore, mutations in E6 altered this effect on PKR localization, leading to perinuclear and nuclear localization of the protein. Interestingly, the clustering of PKR induced by E6 appears to co-localize with GW182, a component of P-bodies. P-bodies are thought to be sites of mRNA degradation and contain members of the RISC complex involved in RNA interference (Marx, 2005). Additionally, the levels of P-body components have been shown to be upregulated in some cancers (Miyaji et al., 2003; Nakagawa et al., 1999). As P-bodies have been implicated as playing key roles in regulating protein synthesis, the relocalization of PKR to P-bodies in HPV-positive cells may be significant and deserves further investigation. Overall, our studies indicate that HPV gene products target the PKR pathway by multiple mechanisms that may be important for the persistence of these virally infected cells.

ACKNOWLEDGEMENTS

We thank Kathy Randell for critical reading of this manuscript. C. M. H. was supported by a National Institutes of Health Immunology and Molecular Pathogenesis training grant (5 T32 AI07476). This work was also supported by a grant from the National Cancer Institute to L. A. L. (CA74202). This study was approved by the Human Studies Committee at Washington University and informed consent was obtained from all patients.
REFERENCES


http://vir.sgmjournals.org