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Human papillomavirus E5 oncoproteins bind the A4 endoplasmic reticulum protein to regulate proliferative ability upon differentiation

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ABSTRACT

Human papillomaviruses (HPV) infect stratified epithelia and link their life cycles to epithelial differentiation. The HPV E5 protein plays a role in the productive phase of the HPV life cycle but its mechanism of action is still unclear. We identify a new binding partner of E5, A4, using a membrane-associated yeast-two hybrid system. The A4 protein co-localizes with HPV 31 E5 in perinuclear regions and forms complexes with E5 and Bap31. In normal keratinocytes, A4 is found primarily in basal cells while in HPV positive cells high levels of A4 are seen in both undifferentiated and differentiated cells. Reduction of A4 expression by shRNAs, enhanced HPV genome amplification and increased cell proliferation ability following differentiation but this was not seen in cells lacking E5. Our studies suggest that the A4 protein is an important E5 binding partner that plays a role in regulating cell proliferation ability upon differentiation.

Introduction

Human papillomaviruses (HPVs) are small DNA viruses that infect stratified epithelia at various body locations (Hebner and Laimins, 2006). Over 120 HPV types have been identified and nearly one-third of these infect epithelia in the genital tract. Genital HPV viruses are further subdivided into the high risk types (such as HPV-16,–18, −31) that are associated with cancer development, and low-risk types (such as HPV-6, −11) which cause benign lesions (Lowy et al., 1994; zur Hausen and de Villiers, 1994).

The HPV productive life cycle is coupled to the differentiation of the host cell. Following entry, HPV genomes are established in the nucleus at about 50–100 copies per cell that replicate in synchrony with cellular chromosomes. When normal basal cells divide, one of the daughter cells detaches from the basement membrane, stops proliferating and undergoes differentiation in suprabasal layers (Watt, 1998). In contrast, in HPV infections, the daughter cell that migrates away from the basement membrane remains active in the cell cycle as it undergoes differentiation in suprabasal layers. HPV genomes are replicated to high levels in highly differentiated cells in a process called amplification that depends on host cell replication "machinery" (Dollard et al., 1992; Hummel et al., 1992; Frattini et al., 1996; Ruesch and Laimins, 1998).

In the high-risk HPV types, three viral proteins have been reported to promote oncogenic activities: E6, E7 and E5. The high-risk E6 proteins are approximately 18 kDa in size, and are localized to both the nuclear and cytoplasmic compartments. (Howie et al., 2009). E6 interacts with a variety of cellular factors including the tumor suppressor p53 in complex with the cellular ubiquitin ligase E6AP leading to rapid degradation of p53 (Scheffner et al., 1990; Werness et al., 1990). The E7 oncoprotein, which is similar in size to E6, is localized primarily to the nucleus (McLaughlin-Drubin and Munger, 2009) and targets the retinoblastoma (Rb) proteins for degradation (Dyson et al., 1989). The combination of E6 and E7 can efficiently immortalize a variety cell types (Hawley-Nelson et al., 1989; Munger et al., 1989).

While the transforming activities of high risk HPV E6 and E7 are well characterized, the role of E5 in tumor progression remains poorly understood. The high-risk E5 proteins are small hydrophobic peptides of approximately 83 amino acids in size that localize to the membranes of the endoplasmic reticulum (ER) and nuclear envelope (Leechanachai et al., 1992; Conrad et al., 1993). E5 has been shown to potentiate the immortalization activity of E6 and E7 in *in vitro* assays (Bouvard et al., 1994; Valle and Banks, 1995; Stoppler et al., 1996). Importantly, targeted expression of HPV16 E5 to the skin of transgenic mouse model rapidly induces cutaneous tumors (Maufort et al., 2007). In estrogen treated E5 transgenic mice, E5 expression alone was sufficient to induce cervical tumors indicating that it can act as

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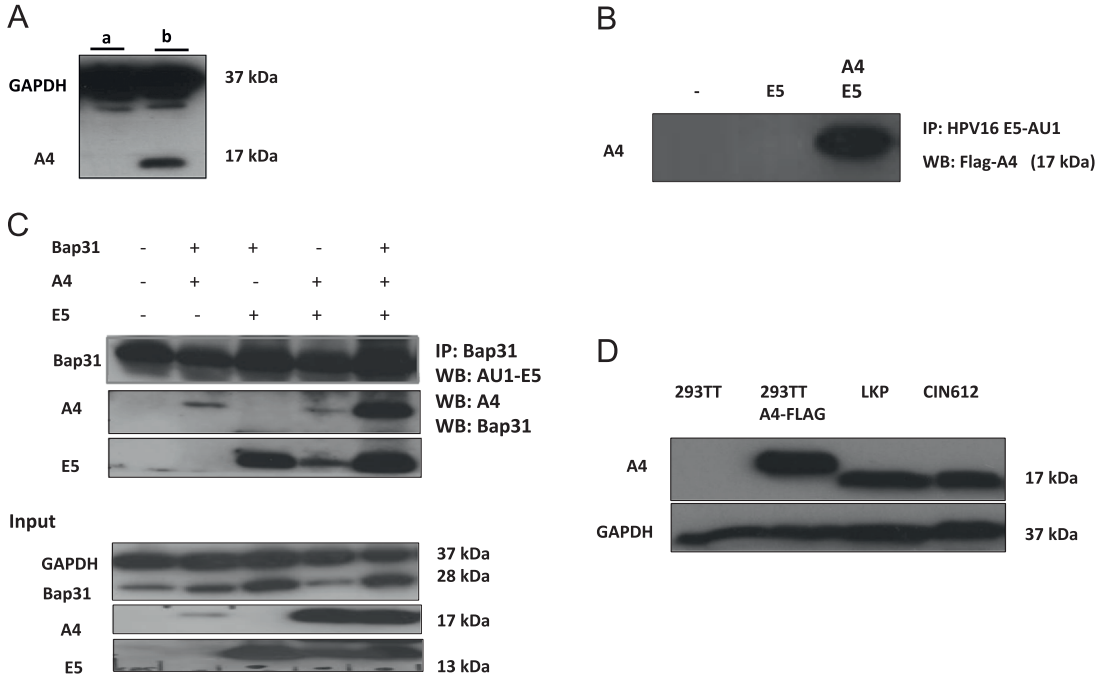
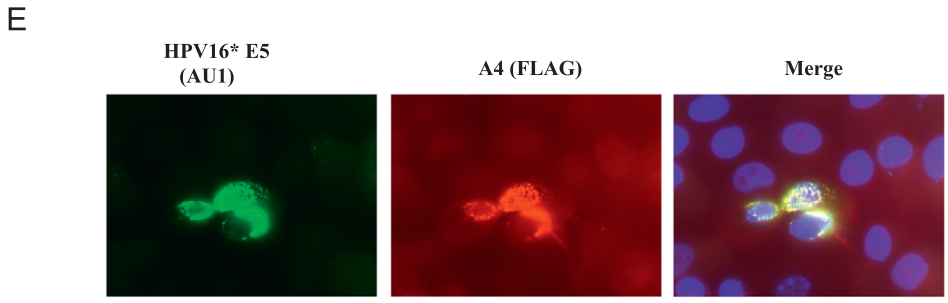
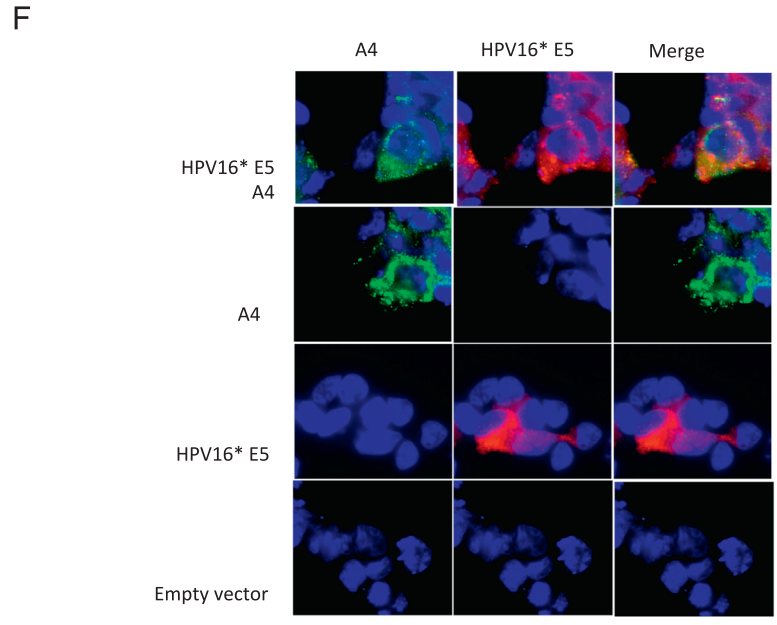
an oncogene (Maufort et al., 2010). In addition, E5 has been shown to transform several rodent fibroblast cell lines and this appears linked to enhanced EGFR (epidermal growth factor receptor) activity in a ligand-dependent manner (Straight et al., 1993). In keratinocytes transfected with either wildtype or E5 mutant HPV 31 genomes, no change in the levels of phosphorylated or total EGFR was seen suggesting that E5 has additional targets in these cells. The HPV 16 E5 oncoprotein has also been shown to interact with the 16 kDa







subunit of vacuolar ATPase proton pump, however, HPV E5 proteins are localized to endoplasmic reticulum membranes and not endosomes suggesting this may not be significant interaction in keratinocytes (Straight et al., 1995; Disbrow et al., 2005). Recent studies have shown that HPV 31 E5 interacts with Bap31 (B-cell-associated protein 31) (Ladasky et al., 2006; Regan and Laimins, 2008), a ubiquitously expressed 28-kDa membrane protein that is highly enriched in the ER (Annaert et al., 1997; Ng et al., 1997). Importantly, this interaction is important for regulation of differentiation-dependent late viral functions (Regan and Laimins, 2008).

In this study we identify another binding partner of E5, the A4 protein. A4 is a small transmembrane lipoprotein of 152 amino acids that was initially found in differentiated human colonic epithelium (Oliva et al., 1993). This hydrophobic lipoprotein is localized in the endoplasmic reticulum and A4 has been reported to interact with Bap31 (Wang et al., 2003). HPV E5 enhances the differentiation-dependent expression of A4 and this contributes to regulating the proliferation ability of cells upon differentiation which is necessary for the viral life cycle.

Results

A4 is a novel target of HPV E5 identified by yeast two-hybrid screen

To identify novel binding partners of HPV 31 E5, we used a membrane-associated yeast two hybrid system (Regan and Laimins, 2008) (DUAL membrane system) screen initially developed by Johnsson and Varshavsky (1994). In this system, the C-terminal half of ubiquitin (Cub) is linked to the transcription factor LexA-VP16 fused to a codon-modified HPV16 E5 protein. The N-terminal half of ubiquitin (NubG) is fused to prey proteins from HeLa cDNA library and a positive lac Z signal identifies associations that occur on cell membranes. Using a quantitative LacZ assay, positive clones with the highest expression were isolated and A4, a membrane protein localized to the ER, was identified as a candidate for binding partner of E5.

A4 form complexes with HPV16 E5 and Bap31

Prior to screening if E5 and A4 formed complexes in human cells, we first investigated if A4 protein was expressed in a series of cell lines using Western blot analysis. The A4 protein was found to be expressed at high levels in Cos7, HFKs and HPV positive keratinocytes such as LKPs and CIN 612 cells that maintain HPV 31 as stable episomes. In contrast, no A4 protein was found expressed in 293TT cells that are derived from human embryonic kidney cells (Fig. 1A).

Since A4 was identified as a potential binding partner of E5 by yeast two hybrid analyses, we examined whether these two proteins could form a complex in human cells. For this analysis, 293TT cells, which do not express A4, were transfected with plasmids expressing A4 and AU1 tagged HPV16 E5 genes. At 48 h posttransfection, cells were harvested and suspended in CHAPS lysis buffer followed by immunoprecipitation with an antibody to the AU1 tag. Precipitates were then analyzed by Western blot analyses for A4 protein and showed that E5 protein forms a complex with the human A4 protein (Fig. 1B). Our previous studies identified the Bap31 protein as a binding partner of E5 (Regan and Laimins, 2008), and it has also been reported that Bap31 can associate with A4 (Wang et al., 2003) as both are localized to the endoplasmic reticulum. We therefore investigated if E5 protein could potentially bind both, A4 and Bap31. To address this question, several transient transfections were performed in 293 TT cells using HPV16 E5, A4 and Bap31 expression vectors. Protein lysates were prepared 48 h posttransfection as described above, and tested by immunoprecipitation with an antibody to Bap31 following by Western blot analyses for A4, AU1 tag of E5 and Bap31 proteins. Our data (Fig. 1C) demonstrate that E5 binds A4 in the presence of endogenous and exogenous Bap31, indicating that all three proteins can associate in complexes. Similar levels of A4 proteins are seen in 293 T cells transfected with A4 expression vectors as are present in cells such as LKP or CIN 612 (Fig. 1D). In our yeast two-hybrid assays, no endogenous Bap31 are expressed yet we detected binding of A4 to E5. This indicates that E5 can bind directly to A4 in the absence of Bap31. Finally, we consistently observed an increase in A4 levels due to the presence of E5 suggesting it may regulate levels of its expression (Fig. 1C).

A4 and HPV16 E5 co-localize to perinuclear regions consistent with the endoplasmic reticulum

Having confirmed that HPV E5 binds to the A4 protein, it was important to determine whether these two proteins localize to the same region inside the cell. The E5 protein has been shown to localize to the perinuclear region consistent with the endoplasmic reticulum and A4 has been reported to exhibit a similar perinuclear localization pattern (Goldstein and Schlegel, 1990; Conrad et al., 1993; Breitwieser et al., 1997; Disbrow et al., 2003; Wang et al., 2003; Disbrow et al., 2005; Regan and Laimins, 2008). Since it was possible that other viral proteins affected localization, HPV-31 positive CIN 612 cells were transiently co-transfected with vectors expressing AU1-tagged HPV16 E5 and FLAG-tagged A4. At 48 h post-transfection immunofluorescence analyses were performed and showed that E5 and A4 co-localized in the same perinuclear regions (Fig. 1E). Similar perinuclear localization of E5 and A4 is seen in 293TT cells transfected with expression vectors (Fig. 1F).

A4 expression is downregulated upon differentiation of HFKs but is maintained at high levels in HPV positive cells

We next investigated whether expression of A4 protein changes upon differentiation of normal human keratinocytes. For this analysis, HFKs were differentiated in methylcellulose for 24 h and 48 h as this allows for isolation of uniformly differentiated populations of cells. Cell lysates were harvested and screened for A4 levels by Western blot analyses. As shown in Fig. 2A, A4

Fig. 1. HPV E5 interacts with human A4 protein. (A) Expression of A4 protein in different cell types. In order to determine the expression of A4 proteins in different cell lines the cell lysates from (A) 293TT and (B) Cos7 cells were prepared and analyzed by Western blot using antibodies against A4. Similar levels of A4 are seen in HPV positive cells. (B) HPV16 E5 binds the human A4 protein. Total protein lysates were isolated from 293TT cells transfected with expression plasmids for HPV16 E5 and A4, and 1 mg of protein lysate was immunoprecipitated with an AU1 tag E5 antibody. Precipitates were analyzed by Western blot using FLAG tag A4 antibody. (C) HPV16 E5 interacts with A4 and Bap31. 293TT cells were transiently co-transfected with pSG5 empty vector and DNA vectors expressing HPV16 E5, A4 and Bap31 genes. Cell lysates were immunoprecipitated with an anti-Bap31 antibody and further analyzed by Western blotting using AU1, A4 and Bap31 antibodies. (D) Levels of A4 proteins are similar in transfected and stable lines. Cell lysates from 293TT cells transfected with expression vector for A4 were compared to endogenous levels in LKP and CIN 612 cell lines by Western blot analysis. (E) Colocalization of A4 and HPV16 E5. HPV31 positive keratinocytes (CIN612) were transiently co-transfected with FLAG-tagged A4 and AU1-tagged HPV16 E5 expression vectors. Cells were immunostained with an anti-AU1 (FITC) and anti-FLAG (Texas Red) antibodies. Nuclei are visualized by staining with DAPI (blue). Yellow signals in merged image represent co-localization of A4 and HPV16 E5 within the ER. Panels in middle show E5 alone and A4 alone localization in the absence of transfection of the other vector. (F) Colocalization of A4 and transfected HPV16 E5. 293TT cells were transiently transfected with AU1-tagged HPV16 E5 and FLAG-tagged A4 expression vectors by themselves selves and in combination as indicated. Top panels show co-transfections while middle panels show A4 and E5 by themselves. Cells were immunostained with an anti-FLAG and anti-AU1 (Texas Red) antibodies. Nuclei are visualized by staining with DAPI (blue). Yellow signals in merged image represent co-localization of A4 and HPV16 E5 within the ER.

proteins are expressed at high levels in undifferentiated normal keratinocytes (HFKs) but expression decreases dramatically upon differentiation. Similar effects were seen in HFKs isolated from different donors (not shown). We next sought to investigate if A4 expression was altered in HPV positive keratinocytes. For these studies, HFKs that stably maintain HPV31 genomes (LKP) were differentiated in 1.5% methylcellulose for 24 h and 48 h. Western blot analyses of whole cell lysates demonstrated high and constant levels of A4 upon differentiation (Fig. 2B). Similar high levels of expression following differentiation were seen in other human epithelial cell lines that maintain HPV31 episomes generated either by transfection (HFKg31) or derived from a cervical biopsy of a CIN lesion (CIN612). After differentiation in semi-solid media for 24 h and 48 h we again observed high expression of A4 protein upon differentiation (Fig. 2C). The same expression pattern of A4 in normal and HPV31 positive keratinocytes was also observed in cells differentiated in high calcium (data not shown).

To confirm the activation of A4 expression in monolayer and differentiated HPV positive cells, we grew organotypic raft cultures and screened for expression by immunofluoresence using an anti-A4 antibody combined with an anti-Cytokeratin antibody (Fig. 2D and E). These immunofluoresence analyses confirmed high level expression of A4 protein in the basal layer but low expression in the upper differentiated layers of the normal keratinocyte raft cultures. In contrast, high levels of the A4 protein were observed throughout all layers of HPV positive raft cultures. These data confirm our findings that HPV31 increases expression of A4 protein during epithelial differentiation.

HPV31 E5 oncoprotein may modulate A4 levels in human epithelium

Since HPV31 E5 forms complexes with the A4 protein, we investigated if E5 was responsible for the increased levels of A4

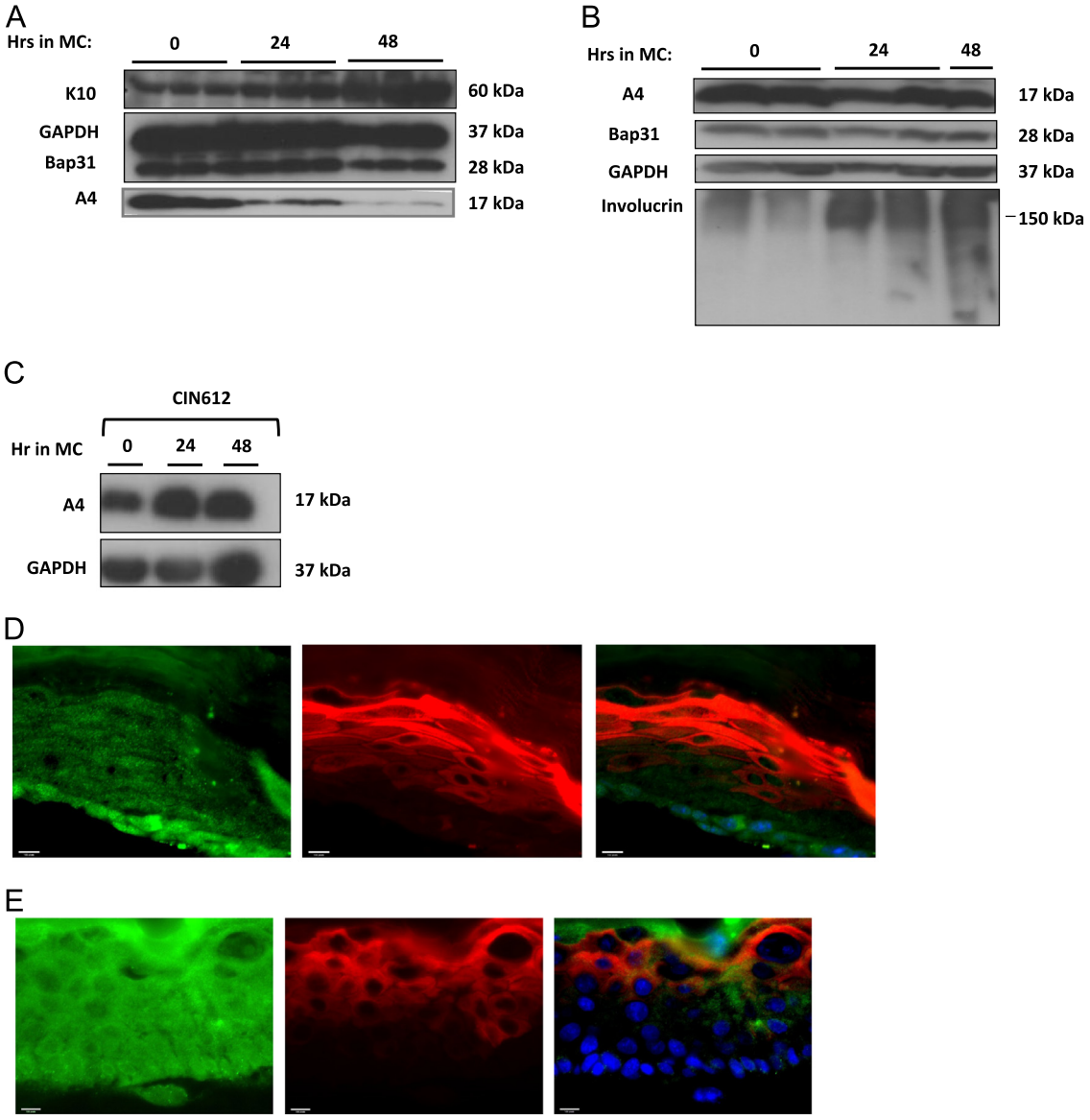


Fig. 2. HPV increases the levels of A4 protein in HPV positive keratinocytes upon differentiation. (A–C) A4 expression in keratinocytes induced to differentiate by suspension in methylcellulose for 24 and 48 h. (A) Normal human keratinocytes (HFK) (B) HPV 31 positive LKP cells and (C) biopsy derived HPV 31 positive CIN612. Cell lysates were prepared and analyzed for protein expression by Western Blot. *Legend*: MC: methylcellulose. Experiments shown in panels (A) and (B) were reproduced in three independent experiments. (D and E) Immunostaining of organotypic raft cultures of normal (D) and LKP HPV 31 positive keratinocytes (E). Cell differentiation was induced by growing keratinocytes in organotypic raft cultures for two weeks. Histological cross-sections were examined by immunohistochemistry using an antibody to A4 (FITC) and Cytokeratin (Texas Red). Left panel: A4 staining; middle panel: K10 staining; right panel: merge. Nuclear staining is DAPI (4′,6-diamidino-2-phenylindole).

upon differentiation. Initially, we attempted to establish stable cell lines overexpressing codon modified 16E5 or 31E5 from retroviral vectors but in multiple experiments the cells failed to survive selection, which contrasted with cells transfected with control plasmids. This indicated that high-level expression of E5 could be toxic in our cells. As an alternate approach to study the effect of E5 on A4 levels, we generated HFKs stably transfected with HPV31 genomes in which a translation termination stop codon had been inserted into the E5 ORF (HPVg31 E5KO) and compared expression to that seen in normal keratinocytes or cells with wild type genomes. All sets of cells were examined for A4 expression by Western blot analyses using cell lysates of undifferentiated and differentiated keratinocytes. As shown in Fig. 3, the amount of A4 protein decreases upon differentiation in HPVg31E5KO cells, similar to that seen in differentiated normal keratinocytes. We have previously demonstrated that effects seen in keratinocytes induced to differentiate in high-calcium media accurately recapitulate differentiation seen in raft cultures (Moody and Laimins, 2009; Bodily et al., 2011; Gunasekharan and Laimins, 2013). We conclude that E5 is necessary for enhanced levels of A4 upon differentiation.

HPV31 E5 induces high A4 levels to support cell proliferative ability upon differentiation

Our data indicate that E5 may control A4 levels upon differentiation and it was previously shown that cells that maintained HPV31 genomes in which E5 was mutated exhibited decreased viral amplification as well as cell proliferation ability in differentiating cells (Fehrmann et al., 2003). The knockdown of Bap31 knockdown similarly inhibits cell proliferation ability upon differentiation of HPV positive keratinocytes (Regan and Laimins, 2008), but has no effect on HPV amplification of stable replication. We therefore investigated whether A4 as a binding partner of E5 had any effect on HPV amplification and proliferation ability. To examine the effect of reducing A4 levels, we infected HPV 31 positive cells with lentiviruses expressing shRNAs against A4 (Fig. 4A) and screened for HPV31 genome amplification upon differentiation by Southern Blot. Interestingly, cells in which A4 was knocked down (LKPshA4) showed increased levels of viral episomes in undifferentiated cells and these were maintained at high levels upon differentiation. The level of HPV episomes in undifferentiated and differentiated cells in which A4 was knocked down was increased over that seen in undifferentiated wt cells and similar to that seen upon differentiation (Fig. 4B). This suggests that knock down of A4 has a positive effect on HPV replication. In contrast to stable knockdowns in HPV positive cells, we were unable to obtain viable cells following knockdown of A4 in normal keratinocytes.

It was next important to investigate if A4 knockdown had any effect on cell proliferation of HPV positive keratinocytes following differentiation. Wildtype and shA4 knockdown HPV 31 positive cell lines were differentiated in 1.5% methylcellulose for 48 h and cells were then re-plated to new dishes. After 5 days the number of cells was determined and we consistently observed a significant increase of cell growth in the A4 knock down cells as compared to the wild type HPV 31 positive cells (Fig. 4C and D). Since HPV31 genomes containing mutated E5 exhibit decreased cell proliferation following differentiation (Fehrmann and Klumpp et al., 2003) we wanted to test whether A4 knockdown could restore this ability in cells containing mutant E5 proteins. For this analysis, we knocked down A4 with shRNAs in cells that maintain HPV 31 genomes with mutated E5 (HFKg31E5KOshA4). In these cells only partial (54%) A4 knockdown was achieved as determined by Western blot assay (Fig. 4A). The same re-plating assay described above was performed following cell differentiation in semi-solid media and showed that knockdown of A4 did not restore cell proliferation ability to that seen in wt cells (Fig. 4C, D). This suggests that the effects of A4 on proliferative ability are significant only in the presence of a functional E5 protein.

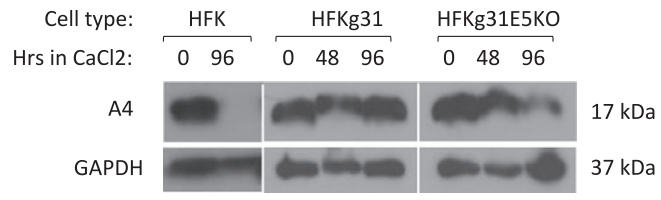


Fig. 3. HPV31 E5 viral oncoprotein modulates expression of A4 protein in differentiated keratinocytes. A4 levels are increased by HPV E5 oncoproteins in human keratinocytes upon differentiation. The protein levels of A4 protein were analyzed by Western blot methods using the following cell lines: wild type human foreskin cells (HFKs), HFKs infected with HPV31 (HFKg31) and HFKs infected with E5 knockout HPV31 genomes (HFKg31E5KO). All sets of cells were differentiated in high calcium and harvested for detection of A4 protein levels using an antibody to A4.

Discussion

Our studies identify the proteolipid protein, A4, as a binding partner of HPV 31 E5. The 17 kDa A4 protein has characteristics of a four transmembrane ion channel (Breitwieser et al., 1997) and co-localizes with E5 to the endoplasmic reticulum. A4 was identified as a binding partner of HPV 16 E5 through the use of split-ubiquitin membrane associated yeast two-hybrid screen (Johnsson and Varshavsky, 1994; Regan and Laimins, 2008) and complex formation was confirmed in human cells through co-immunoprecipitation studies. The A4 protein can also form complexes with another endoplasmic reticulum protein, Bap31, that we previously reported also bound to E5 (Regan and Laimins, 2008). Since yeast cells do not contain a Bap31 homolog, this indicated the interaction of A4 with E5 maybe direct. In our studies we attempted to confirm that the two factors bound independently to E5 using human cell lines. All cells we examined were found to express Bap31 and this is consistent with previous reports (Quistgaard et al., 2013). While many cells express A4, 293TT embryonic kidney cells were found to be A4 deficient. Using these cells Bap31 binding to E5 was found to be independent of A4 binding. Since all human cells express Bap31, we tried to abrogate BAP31 expression by use of shRNAs but were never able to reduce amounts by more than 50% suggesting Bap31 may be an essential protein. Future studies involving a detailed mutagenesis of E5 binding sequences may help to determine if these factors bind the same sequences or act through different binding sites on E5.

The A4 protein is normally expressed in a differentiation-dependent manner. In primary keratinocytes, A4 expression is restricted to undifferentiated basal cells and is absent from differentiated suprabasal layers. This contrasts with Bap31 expression, which is expressed in both basal as well as suprabasal cells and its expression is not changed by HPV proteins. Using organotypic rafts as well as methylcellulose induced differentiation, we observed that A4 was uniformly expressed in both undifferentiated and differentiated layers of cells that maintain HPV episomes. The E5 protein is responsible for inducing expression in suprabasal layers. At present we are unsure if this effect is mediated at the level of transcription or through post-transcriptional effects. In epithelial cancers that lack the ability to differentiate yet retain stratification ability, A4 is also found in suprabasal cells suggesting that A4 expression is linked to proliferative ability of cells (Oliva et al., 1993; Breitwieser et al., 1997).

Upon differentiation of cells that maintain HPV viral DNA as episomes, genome amplification is induced in suprabasal layers. This process requires differentiating cells to remain active in the cell cycle as host replication proteins such as polymerases and PCNA are needed for viral replication (Bodily and Laimins, 2011).

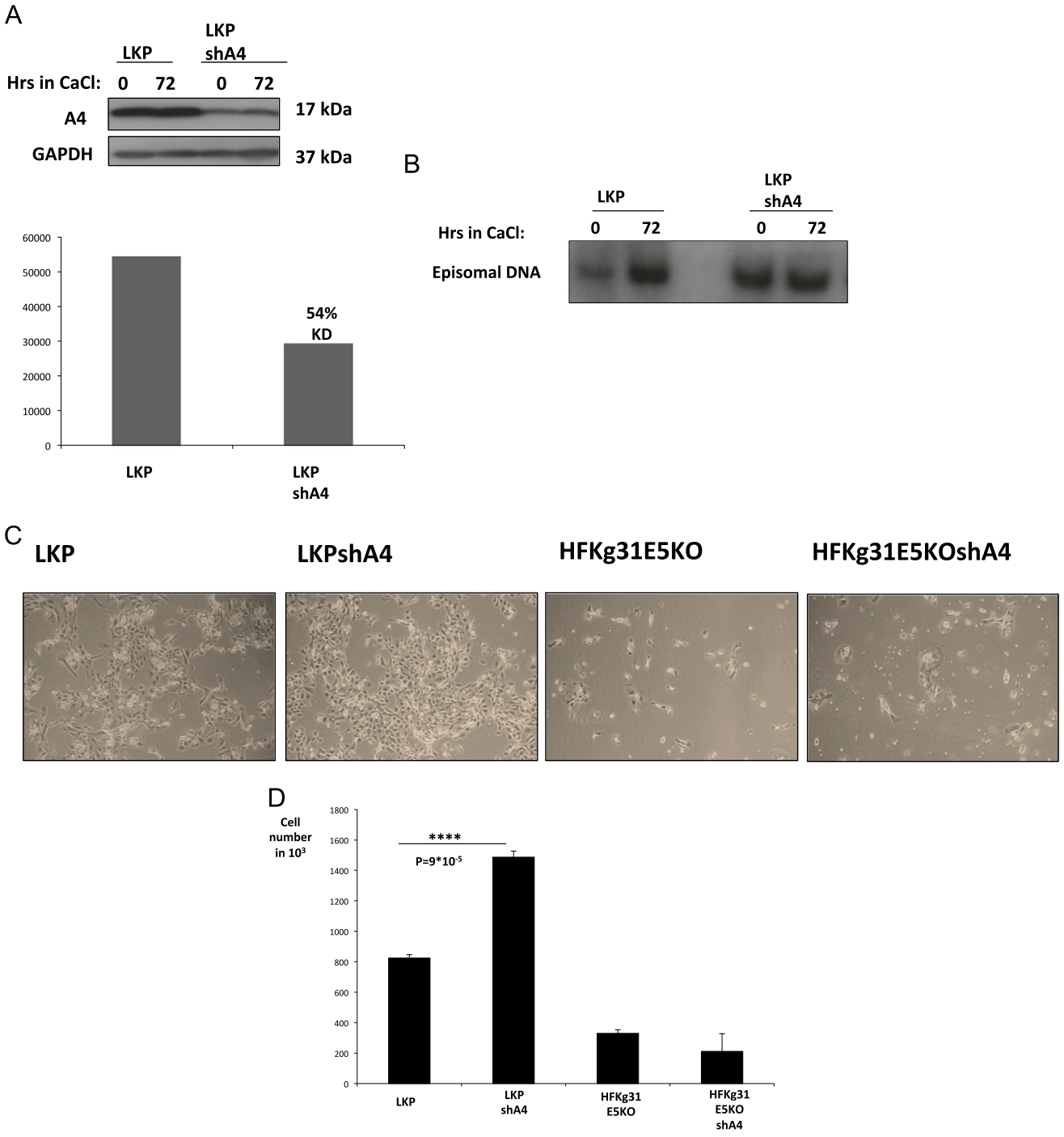


Fig. 4. HPV31 replication and cell proliferation are enhanced in A4 shRNA knock down keratinocytes. (A) Western blot analyses of A4 stable knock down in keratinocytes. HPV31 positive keratinocytes LKP were infected with lentiviral particles carrying shRNAs against the A4 gene. Infected cells were selected with puromycin for two weeks. ImageJ program was used to quantify knockdown of A4 in cell line LKPshA4. (B) Southern blot analysis of LKP and LKPshA4 keratinocytes. Total genomic DNA was harvested from monolayer and differentiated cultures and digested with DpnI to remove residual input DNA and XbaI to linearize the HPV31 genomes. The Southern blot was hybridized with a probe that contains the complete HPV31 genome. (C, D): Cell proliferation assay. LKP, LKPshA4, HFKg31E5KO and HFKg31E5KOshA4 cell lines were differentiated by suspension culture in methylcellulose. 48 h later cells were re-plated into new dishes and cultured for few days. Colonies were visible under microscope on day 5 (C). The total cell number was estimated on day 5 using TC10 automated cell counter (BioRad) (D). Data are means $\pm$ standard deviations (error bars), $n=3$. ****$P < 0.0001$ indicates values significantly different from the control (LKP).

HPV genome amplification occurs as cells transit through S phase into G2/M. This ability to retain proliferative capability upon differentiation is mediated by both E7 and E5 proteins (Fehrmann et al., 2003; Regan and Laimins, 2008; Banerjee et al., 2011). Our studies demonstrate that A4 expression in suprabasal cells is important to regulate this proliferative capability in differentiating cells. In contrast to studies with Bap31, knockdown of A4 enhanced proliferative ability rather than decreased it. Our studies suggest that A4 can modulate proliferative capacity and that is dependent on the presence of a functional E5 protein. A4 has been shown to associate with PI3K resulting in activation of Akt phosphorylation, which is important for cell proliferation and future studies will determine if this is the critical target of A4 action. Our studies indicate that the action of A4 is central to regulating this activity, however, it appears to act in

an opposite manner to Bap31. We also observed that knockdown of A4 lead to an increase in genome copy number in undifferentiated cells and that this enhanced level was maintained throughout differentiation. A4 may thus be important for helping to restrict high level viral replication to differentiated suprabasal cells. Our studies indicate that the interactions of both A4 and Bap31 are critical to E5 functions.

A4 and Bap31 are both co-localized to the endoplasmic reticulum together with E5 proteins. Previous studies had suggested that E5 could form a complex with the subunits of the vacuolar ATPAse (V-ATPase); however, the V-ATPAse is localized to late endosomes and not the endoplasmic reticulum indicating it is likely not a physiological target of E5 (Suprynowicz et al., 2010). Interestingly, A4 shares an amino acid sequence similarity with the V-ATPAse in the first transmembrane region that could be a potential interacting domain with E5 and explain why previous studies implicated V-ATPase as a binding partner of E5. Future mutational analyses of A4 should elucidate if this is the E5 interacting region (Breitwieser et al., 1997).

In summary, our studies demonstrate that high risk HPV E5 proteins bind A4 and maintain high levels of A4 during differentiation. The binding of A4 as well as interactions of Bap31 are necessary to regulate proliferation ability of HPV positive cells upon differentiation. Further analyses of A4 and Bap31 functions will help to provide critical new insights in the role of the E5 in HPV pathogenesis.

Materials and methods

Cell culture

Human foreskin keratinocytes (HFKs) were derived from neonatal human foreskin epithelia as described in Wilson and Laimins (2005) and were maintained in serum-free keratinocytes growth medium supplemented with bovine pituitary extract, insulin, hydrocortisone and epidermal growth factor (Lonza, Walkersville, MD). Stable cell lines expressing HPV E6 and E7 were generated by transduction of retroviral vectors and selected in G418 (Sigma) as previously described (Melar-New and Laimins, 2010).

To create cell lines containing HPV31 genomes (HFKg31) and E5 knockout HPV31 genomes (HFKg31E5KO), HFKs were transfected with HPV type 31 genomes and genomes of HPV type 31 lacking the E5 gene followed by selection with antibiotics as described previously (Wilson and Laimins, 2005).

CIN 612 cells are derived from a biopsy and maintain HPV 31 episomes while LKP cells are derived from normal human foreskin keratinocytes transfected with HPV 31 DNA and maintain episomes (Frattini and Lim et al., 1996) HFKs, all stably transfected HFK cell lines along with LKP and CIN612 cell lines that maintain HPV 31 episomes were grown in the presence of 3T3 J2 fibroblast feeders treated with mitomycin C and cultured in E medium supplemented with mouse epidermal growth factor (EGF) (5 ng/mL (Frattini et al., 1996); BD Biosciences, San Jose, CA) as described (Fehrmann and Laimins, 2005).

293TT, Cos7 and HaCat cells were grown in DMEM medium supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY). For transient transfection assays, 293TT cells were transfected using ployethylenimine reagent at a final concentration of 0.2 mg/mL (Polyscience, Inc., Warrington, PA) at 30% confluence. Cells were harvested 48 h postransfection for further analyses.

Differentiation of keratinocytes

To induce differentiation in high calcium LKP, CIN612 and HPV 31 stably transfected HFKs were cultured in the absence of 3T3 J2 feeders in EGF free E medium for 24 h prior to differentiation. After 24 h cells were switched to EGF free E medium containing $CaCl_2$ at a final concentration 1.5 mM for 48 h, 72 h and/or 96 h. Alternatively, keratinocytes were differentiated in semisolid media as described in (Melar-New and Laimins, 2010).

Western blot analyses

Total cell protein extracts were prepared with RIPA lysis buffer containing a cocktail of protease inhibitors (Roche, Branchburg, NJ) and quantitated using the NanoDrop ND2000 Spectrophotometer (Thermo Fisher, Suwanee, GA). Protein samples were electrophoresed on 15% sodium dodecyl sulfate-polyacrilamide (SDS) gels and subsequently transferred to polyvinylidene difluoride membranes (EMD Milipore, Darmstadt, Germany). The membrane was then blocked in a 5% nonfat milk solution (0.1% Tween 2 in PBS), followed by incubation with primary antibodies: anti-A4, anti-Flag (Sigma-Aldrich, St. Louis, MO), anti-AU1 (Covance, Madison, WI), anti-GAPDH (Santa Cruz, Dallas, TX), anti-Involucrin (Santa Cruz), anti-Cytokeratin (Santa Cruz) and anti-Bap31 (AbCam,, Cambridge, MA). Proteins were visualized via enhanced chemiluminescence (GE, Healthcare, Waukesha, WI).

Immunoprecipitation analyses

Cell lysates of transiently transfected 293TT cells expressing various proteins (A4 and/or E5 and/or Bap31) were prepared with CHAPS (15 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) buffer solution (CHAPS, 30 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl [pH7.4]) (Sigma-Aldrich) 48 h posttransfection as described in Regan and Laimins (2008). Antibodies used in the analyses include: anti-AU1 (Covance) and anti-Bap31 (AbCam).

Immunofluorescence

The expression pattern of A4 proteins were examined by immunofluorescence of cross sections of raft tissue. Paraformaldehyde-fixed, paraffin-embedded tissue on silanized slides were heated at 50 °C for 30 min, washed in Xylenes, 100% and 95% Ethanol and finally in PBS. The slides were then incubated in 10 mM Citrate Solution (pH 6.0) at 95 °C for 30 min, followed by an additional 20 min incubation at room temperature. Subsequently slides were washed with PBS and blocked in Normal Goat Serum (Life Technologies) and 1% Triton X-100 in humid chamber. Incubation with primary antibodies was performed at room temperature for 2 h. Slides were then washed with PBS solution containing 1% triton and incubated with secondary Alexa-Fluor antibodies in light protected humid chamber for 30 min. After staining with DAPI, sections were mounted and analyzed under inverted fluorescent microscope.

Yeast two-hybrid screen

To detect possible protein interactions with E5 protein a yeast two-hybrid screen that is based on the split-ubiquitin technique was performed as described (Johnsson and Varshavsky, 1994; Regan and Laimins, 2008).

Plasmids

To construct A4 expression plasmids, the A4 open reading frame isolated from the yeast two-hybrid positive clones was PCR amplified and cloned into the pSG5. The Bap31 and HPV31 E5 expression vectors were previously described (Regan and Laimins, 2008). A codon optimized HPV16 E5* with an AU1 tag was a generous gift from R. Schlegel and was subcloned in a pSG5 vector (Disbrow and Sunitha et al., 2003).

Southern blot

Stable A4 knockdown cell line expressing shRNA against A4 were established as described (Mighty and Laimins, 2011). Cells were differentiated in high calcium as described above and total DNA was isolated from harvested cells using DNeasy Blood & Tissue Kit (Qiagen Hilden, Germany). Ten micrograms of each total DNA sample was digested with DpnI and XhoI and electrophoresed in a 0.8% agarose gel overnight and transferred to Gene Screen nylon membranes (Bio-Rad, Des Plaines, IL) using vacuum transfer according to the manufacturer's protocols. The DNA HPV31 probe was labeled with [^{32}P]dCTP using Ready-To-Go DNA-labeling beads (Amersham Biosciences, Buckinghamshire, England) and column purified. The membrane was hybridized with labeled probe in hybridization solution overnight, washed and visualized by autoradiography.

Proliferation test

HPV positive cells were grown in the presence of mitomycin C-treated J2 3T3 fibroblasts. At the approximately 80% confluence, feeder cells were removed, keratinocytes were harvested and number of cells was determined by TC10 automated cell counter (BioRad). Two and a half of millions of cells were resuspended in 1.5% methylcellulose for 48 h to induce differentiation. The keratinocytes were then collected, washed with PBS, and replated as monolayer cultures into new dishes. The total number of cells was determined after 5 days in culture by TC10 automated cell counter.

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