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Papillomavirus virus-like particles activate the PI3-kinase pathway via alpha-6 beta-4 integrin upon binding

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Abstract

We have previously shown that human papillomavirus virus-like particles (VLPs) are able to activate the Ras/MAP kinase pathway. Ras can also elicit an anti-apoptotic signal via PI3-kinase so we investigated this further. Here we show that binding of VLPs from HPV types 6b, 18, 31, 35 and BPV1 results in activation of PI3-kinase. Activation was achieved by either L1 or L1/L2 VLPs and was dependent on both VLP–cell interaction and correct conformation of the virus particle. VLP-induced PI3-kinase activity resulted in efficient downstream signaling to Akt and consequent phosphorylation of FKHR and GSK3β. We also present evidence that PV signaling is activated via the α6β4 integrin. These data suggest that papillomaviruses use a common receptor that is able to signal through to Ras. Combined activation of the Ras/MAP kinase and PI3-kinase pathways may be beneficial for the virus by increasing cell numbers and producing an environment more conducive to infection.

Keywords: Papillomavirus; Integrin; VLP; PI3-kinase; Akt; FKHR and GSK3β

Introduction

Papillomaviruses (PVs) are non-enveloped dsDNA tumor viruses that cause a range of proliferative lesions upon infection of epithelial cells (Howley, 1996). These viruses are the causative agent of warts (plantar, laryngopharyngeal and genital) (Bosch et al., 1995) and the critical factor in the formation of anogenital cancer (Zur Hausen, 1994). Many aspects of the PV life cycle have been difficult to elucidate due to the lack of robust in vitro replication systems. However, the advent of virus-like particle (VLP) technology has partially overcome this problem and allowed investigation of the early events of infection.

The first step in viral infection is the binding of the virus to its receptor upon a host cell. The quest to identify cellular receptors for papillomavirus has so far yielded a few different candidates. It is known the receptor is a protein and that a wide variety of cells facilitate binding (Qi et al., 1996). Also, virus particles from different PV species or subtypes are able to interfere with another's binding (Muller et al., 1995; Roden et al., 1994) and conservation of the L1 gene between PV species suggests that a common receptor is utilized. The α6β4 integrin complex and heparan sulfate proteoglycans (HSPGs) have been identified as receptors for PV (Bousarghin et al., 2003; Combita et al., 2001; Drobni et al., 2003; Evander et al., 1997; Giroglou et al., 2001; Joyce et al., 1999; McMillan et al., 1999; Selinka et al., 2002, 2003; Shafti-Keramat et al., 2003; Yoon et al., 2001), although conversely there are recent data to suggest not all PVs may use these receptors. Models of infection using PV types 11 and 31 indicate that these viruses are able to infect cells that do not posses α6β4 or heparan sulfate, respectively (Patterson et al., 2005; Shafti-Keramat et al., 2003).

There is mounting evidence that virus receptor attachment is not merely a conduit for entry into the cell but is also a means of activating cellular signaling pathways to promote viral infection. For example, HIV binding is known to cause phosphorylation of Pyk2 (Davis et al., 1997) as well as the activation of calcium signaling pathways (Weissman et al., 1997). Measles virus binding directly activates NFκB (Helin et al., 2001) as well as signaling to stop proliferation (Erlenhoefer et al., 2001). Furthermore, EBV is able to activate NFκB via interaction with its receptor, CD21, to enhance transcription from the EBNA2 promoter, Wp (Sugano et al., 1997). We have

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also shown that PV attachment to cells is able to activate the Ras/MAP kinase pathway (Payne et al., 2001). Other viruses are also able to activate this pathway, including JC virus (Querbes et al., 2004), SIV and HIV (Popik et al., 1998; Popik and Pitha, 1998), CMV (Boyle et al., 1999) and HHV8 (Naranatt et al., 2003). For PV, activation results in a binding-mediated induction of c-*myc* mRNA and induction of DNA synthesis in these cells.

It is well known that activation of Ras not only induces a growth signal to cells via MAP kinases but also elicits an anti-apoptotic signal via the PI3-kinase pathway (Brazil and Hemmings, 2001; Nicholson and Anderson, 2002; Scheid and Woodgett, 2001, 2003). Activated PI3-kinase has been known for some time to rescue cells from a variety of apoptotic insults and was first recognized by Yao and Cooper (1995) as being required to mediate the anti-apoptotic effect of nerve growth factor. Phosphorylation of downstream mediators such as BAD, forkhead transcription factors (FKHR) and glycogen synthase kinase 3β (GSK3β) via Akt/PKB provide a mechanism for this anti-apoptotic effect and play an important role in control of the cell-cycle (for a review, see Liang and Slingerland, 2003).

The PI3-kinase/Akt pathway is known to be stimulated by a number of viruses following infection including polyomavirus (Whitman et al., 1985), EBV (Darr et al., 2001; Swart et al., 2000), HBV (Shih et al., 2000) and cytomegalovirus (Johnson et al., 2001). Viral proteins produced by these viruses during infection activate this important cell regulatory pathway to aid in viral replication, promote cellular survival by inhibiting apoptosis and are also implicated in the reactivation of viruses from latency. However, both adenovirus and HIV are able to induce signaling upon initial binding to their cellular receptors (Briand et al., 1997; Li et al., 1998). Interestingly, adenovirus binds to the αV integrin and viral endocytosis was inhibited by blocking PI3-kinase activation (Li et al., 1998).

We therefore wanted to know whether PV-activated Ras was also able to stimulate PI3-kinase and result in transmission of known survival signals to the cells. Importantly, we also wished to know whether this signal was propagated by PV binding to the α6β4 integrin. This study shows that binding of VLPs from several different PV types, both human and animal, results in activation of PI3-kinase. Both L1 and L1/L2 VLPs were able to activate PI3-kinase and activation was dependent on the ability of the VLP to interact with cells and on the correct conformation of the virus particles. VLP-mediated activation of PI3-kinase resulted in efficient downstream signaling with the activation of Akt and consequent phosphorylation of FKHR and GSK3β. Finally, we examine if PV signaling is activated through the α6β4 integrin.

Results

Papillomavirus virus-like particles activate PI3-kinase

We have previously shown that treating cells with HPV6b L1 VLPs for as little as 5 min results in the activation of the Ras-MAP kinase pathway and stimulation of cell proliferation (Payne et al., 2001). Activation of Ras is also known to activate PI3-kinase to promote cell survival via anti-apoptotic signals (Brazil and Hemmings, 2001; Nicholson and Anderson, 2002; Scheid and Woodgett, 2001, 2003). Therefore, to further elucidate the activation of signaling pathways by papillomavirus, we investigated the possibility that PV-VLPs were able to activate the PI3-kinase pathway.

We initially treated serum-starved A431 cells (5×10^6) with 100 ng of PV VLPs containing either L1/L2 (BPV, HPV-16, -31 and -35) or only L1 (HPV-18 and -6b) to assess the level of PI3-kinase activity. This amount of VLP was found to be the lowest required to give a signal (data not shown) and equates to approximately 600 viral particles per cell, based on VLPs having an MW of 1.98×10^7 (360 L1 molecules/VLP at 55 kDa/L1). Hence, 1.98×10^7 g of VLPs equals 1 mol and therefore 6×10^{23} particles (Avogadro's number). From this we deduce that 100 ng = 3×10^9 particles, which is 600 particles per cell in our assay of 5×10^6 cells. Previous data have shown that there are around 1×10^4 receptors/cell using CV-1 cells (Qi et al., 1996) and 2×10^4 receptors using HeLa cells (Volpers et al., 1995), hence our assay does not reach the point of receptor saturation. Following treatment, cell extracts were obtained, PI3-kinase immunoprecipitated and the purified kinase mixed with bovine brain extract in the presence of [γ-^{32}P]ATP. The conversion of phospholipid substrate to phosphatidylinositol-3,4,5-trisphosphate by PI3-kinase was measured via thin-layer chromatography and phosphorimagery. Quantification of spots was performed using ImageQuant™ software and local average background correction applied. Correction for load was performed using quantified western blot data from immunoprecipitates. This method is a well-established and sensitive way of assaying for PI3-kinase activity with 2- to 10-fold increases seen to be significant (Okkenhaug et al., 2002; Nadler et al., 2001; Street et al., 2005). Exposure of cells to VLPs resulted in the activation of PI3-kinase by 3.3- to 5-fold over mock-infected cells (Fig. 1A), depending on the VLP used. This compares favorably with the 9.6-fold activation observed when cells were treated with 10% serum. The activation of PI3-kinase was not dependent on PV type, or the presence of L2 in the VLPs, as L1-only VLPs (HPV18 and -6b; Fig. 1B) were able to activate PI3-kinase as well as L1/L2 VLPs (BPV, HPV-16, -31 and -35).

PV VLP interaction with cells and VLP integrity is critical for activation of PI3-kinase

To eliminate the possibility that PI3-kinase was being activated by a means other than VLP binding, we undertook a number of control experiments. Pre-treatment of HPV6bL1 VLPs with an anti-HPV6b monoclonal antibody (MC8) resulted in an 80% reduction in PI3-kinase activation whereas treatment with a non-specific, isotype-matched control antibody showed no effect (Fig. 1B). We have previously used this antibody to block VLP-induced cell proliferation (Payne et al., 2001). This suggested that the activation of PI3-kinase required the interaction of VLPs with the cell. To ascertain if VLP structural integrity was required for activation, we boiled VLPs for 5 min. We observed that boiling VLPs reduced the level of PI3-kinase

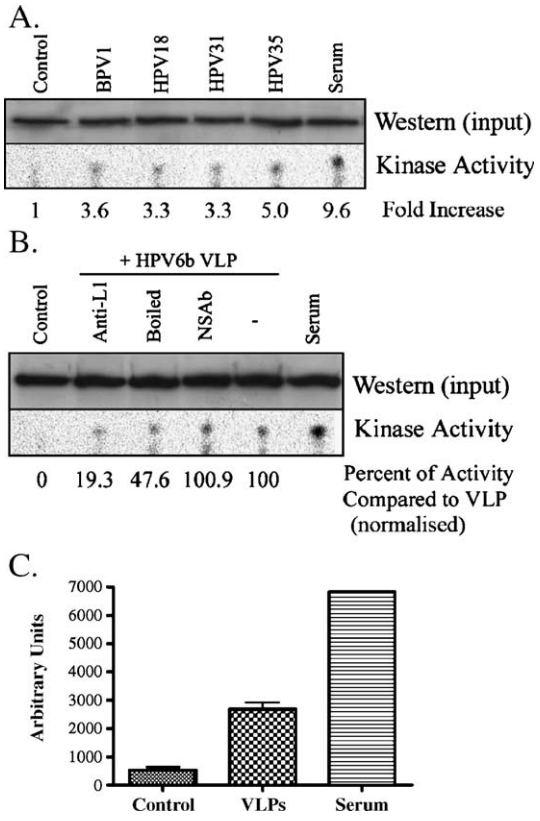

Fig. 1. PV VLPs activate PI3-kinase and integrity and interaction with cells are critical. (A) Serum-starved A431 cells (5×10^6) were treated with VLPs (100 ng) for 60 min at 37 °C. (B) Serum-starved A431 cells were treated with 100 ng of HPV6b VLPs, VLPs pre-blocked with an anti-L1 monoclonal antibody or a non-specific isotype matched antibody, or VLPs boiled for 5 min. For all samples, PI3-kinase was immunoprecipitated from cell extracts and an aliquot was western blotted for PI3-kinase to control for enzyme input. PI3-kinase activity from the remaining immunoprecipitate was measured by assaying lipid substrate conversion to phosphatidylinositol-3,4,5-trisphosphate using incorporated radiolabeled ATP. Phospholipids were separated by thin-layer chromatography and radioactivity measured by phosphorimager analysis. Positive control cells were treated with 10% serum and negative control cells an equivalent amount of VLP diluent (PBS). Data are representative of two or more independent assays. Numbers indicate load-corrected fold increase over control or normalized percent activity compared to VLP. (C) Averaged raw data (background corrected) for all control, serum and VLP spots in panels A and B.

activation by 50% (Fig. 1B). This was consistent with the reduced level of activation of the Ras-MAPK pathway we had previously observed (Payne et al., 2001). We speculate that incomplete denaturation of the VLP by boiling is the reason why we did not see a further reduction, with residual pentameric L1 possibly leading to cross-linking and minor activation of the receptor.

Akt is activated by VLPs

We next wished to ascertain if downstream effectors in the PI3-kinase pathway were activated by VLP treatment. Activation of PI3-kinase has been shown to result in recruitment of the kinase PDK-1 to the plasma membrane, where it is activated and phosphorylates Akt (Alessi et al., 1997; Burgering and Coffer, 1995; Stokoe et al., 1997). Therefore, we treated serum-starved A431 cells with PV VLPs from HPV types 18, 16, 6b and BPV1 for various times before ascertaining Akt activation. For these experiments, we wished to maximize the signal by engaging every receptor with a VLP. Although no data is available to indicate the number of receptors on A431 cells, flow cytometry analysis of α6β4 expression suggests they have approximately the same number as CV-1 cells, around 1×10^4/cell (Payne et al., 2001). Therefore, we used 1 μg VLP ($\sim 3 \times 10^{10}$ particles) per 1.2×10^6 cells. All the VLPs tested were able to activate Akt as indicated using a phospho-specific (S^{473}) Akt antibody, as did 10% serum treatment for 20 min (Fig. 2A). Maximal phosphorylation appeared to be at around 10–20 min although we also noted a somewhat biphasic activation. Activity was observed to decline gradually after 20 min and then rise again with a second peak occurring around 120 min (data not shown and Fig. 3). This temporal regulation was observed with all VLP types tested. We also observed some minor phosphorylation of Akt on T^{308} (data not shown).

Once again, VLP–cell contact was required for this activity as pre-blocking HPV16 or BPV1 VLPs with the H16.V5 (anti-HPV16) or B1.A1 (anti-BPV) monoclonal antibodies, respectively, reduced Akt phosphorylation to background levels (Fig. 2B). These antibodies have both been shown to inhibit VLP binding to cells via the hemagglutination inhibition assay (Roden et al., 1996) and in vitro neutralization of the focus-forming assay (Christensen and Kreider, 1993; Roden et al., 1997). Pre-blocking with a non-specific monoclonal antibody had little impact on VLP-induced Akt activation (89% and 95% binding compared to positive control), whereas blocking with H16.V5 and B1.A1 antibodies reduced activation to background levels. VLP integrity was also required for Akt activation, as denaturing HPV16 and BPV VLPs, by boiling for 30 min, reduced phosphorylation to background levels.

Next, we wished to address the issue of how activation of Akt was occurring as there are several studies suggesting PDK-1-independent activation of Akt is possible (Basso et al., 2002; Yano et al., 1998). Therefore, to ensure that PV VLPs were activating Akt via PI3-kinase, we used the potent and specific PI3-kinase inhibitor, wortmannin. We also decided to use BPV1 L1/L2 VLPs for all subsequent experiments as they were observed by electron microscopy to contain the best quality particles of available preparations. Also, there was no significant difference between L1 and L1/L2 VLPs in terms of signal intensity and duration. Cells were pre-treated with 200 nM wortmannin for 20 min prior to the addition of BPV1 VLPs. As expected, wortmannin completely inhibited the activation of Akt by serum (Fig. 3) and caused potent inhibition of VLP-mediated activation of Akt, thus indicating a requirement for PI3-kinase.

Cell cycle mediators downstream from Akt are activated by VLPs

There are a growing number of proteins that are activated downstream of Akt that regulate cell cycle progression and cell survival. Two of the best studied are FKHR and GSK3β. When phosphorylated by Akt, these proteins have been shown to

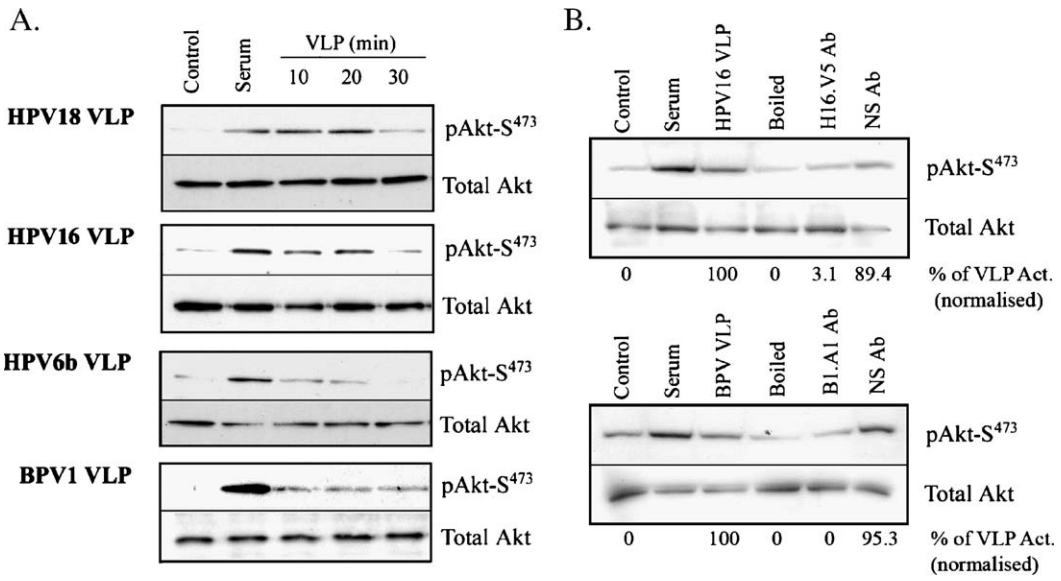

Fig. 2. Akt is activated by PV VLPs. Signaling is removed by VLP denaturation and antibody blockade. (A) A431 cells (1.2×10^6) were serum-starved overnight before treatment with 1 μg VLPs for 10-30 min or 10% serum for 20 min as positive control. (B) HPV16 and BPV1 VLPs were pre-blocked with H16.V5 (anti-HPV16) and B1.A1 (anti-BPV1) monoclonal antibodies, non-specific control monoclonal antibody, or were denatured by boiling prior to treatment of serum-starved A431 cells. Following treatment, cells were solubilized in loading buffer and western blot performed using anti-pAkt (S^{473}) or anti-Akt antibodies. Data are representative of two or more independent assays. Numbers indicate load-corrected normalized percent activity compared to VLP.

promote activation of the cell cycle and induce an anti-apoptotic effect (for a review, see Liang and Slingerland, 2003), thus supporting our previous data that VLP treatment induced cellular DNA synthesis (Payne et al., 2001). We wished to determine the status of these downstream effectors upon VLP stimulation. As expected, treatment of cells with 10% serum for 20 min resulted in a robust activation of both FKHR and GSK3β as determined using phospho-specific antibodies (Fig. 3). Cells treated with BPV1 L1/L2 VLPs were also observed to have increased levels of phospho-FKHR and GSK3β at both 20 min and to a lesser extent at 120 min. To show that activation was specifically regulated via PI3-kinase, we also pre-treated cells with wortmannin. This resulted in the complete loss of activation of both FKHR and GSK3β in all treatments.

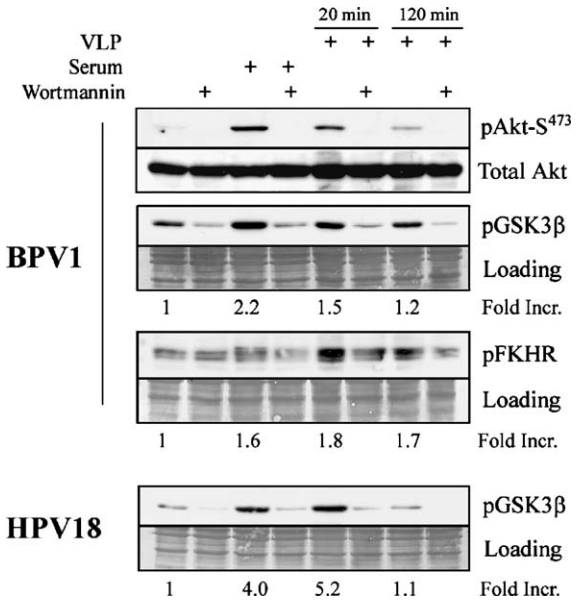

Fig. 3. Akt activation is mediated via PI3-kinase and FKHR and GSK3β, cell cycle regulators downstream from Akt, are phosphorylated. Serum-starved A431 cells (1.2×10^6) were treated with 1 μg BPV1 or HPV18 VLP for 20 or 120 min as indicated. Wortmannin-treated cells were pre-treated with 200 mM wortmannin for 20 min prior to addition of VLP. Cells were solubilized in loading buffer and western blotted using anti-pAkt (S^{473}), anti-Akt, anti-pGSK3β or anti-pFKHR antibodies. After probing, the membrane was Coomassie stained to provide an additional loading control. Numbers indicate fold increase compared to serum-positive control after normalization to negative controls.

Activation of the PI3-kinase pathway by PV VLP is mediated through α6β4 integrin

PV has been shown to readily bind to the α6β4 integrin, and expression of α6 on B cells confers the ability to bind PV VLPs (Evander et al., 1997; McMillan et al., 1999). It was therefore proposed as a PV receptor. However, it is clear that heparan sulfate proteoglycans can also support PV VLP binding, although they have been shown to not be required for infection (Patterson et al., 2005). PV appears to interact with a number of cell surface molecules and so we decided to investigate whether PV signaling through the PI3-kinase pathway was being mediated by α6β4 integrin.

In order to show that α6β4 integrin was required for signaling, we utilized two different methods, RNA interference and specific antibody blocking. Firstly, we designed an siRNA and tested its ability to down-regulate the cell surface expression of the α6 integrin. We achieved a 50% loss of cell surface α6 integrin (Fig. 4B), as determined by mean fluorescence intensity, compared to control stained cells, whereas treatment of cells with a control siRNA resulted in only a 16.6% reduction in α6 expression (Fig. 4C). Thus, we

were able to obtain a significant, but not complete, reduction in the level of cell surface α6 integrin. We next examined the phosphorylation of Akt to determine the signaling capability of the siRNA-treated cells. We observed that the level of pAkt-S^{473} was reduced to background levels in cells treated with α6 siRNA, whereas control GFP siRNA had no effect (Fig. 4A). To ensure this was not due to non-α6 integrin events, we tested a known activator of the α6β4 integrin, laminin, and observed that laminin-mediated activation of Akt was also reduced in α6 siRNA-treated cells. Interestingly, there was also a decrease in Akt activation in α6 siRNA-treated cells stimulated with serum, suggesting that a significant part of serum signaling is via the α6 integrin. However, this reduction did not reach the background levels observed with VLP and laminin treatment.

Secondly, we showed the α6β4 integrin was transmitting the VLP-mediated signal by using antibody blockade. The anti-α6 (GoH3 clone) and anti-β4 (Asc-9 clone) antibodies have previously been shown to block cells expressing α6β4 integrin from binding to laminin-5 (Kikkawa et al., 2000; Stahl et al., 1997). They have also been used as function-blocking antibodies while, importantly, being observed not to block TNFα-induced apoptosis or activate NFκB (Weaver et al., 2002), suggesting an inability to stimulate the integrins. In addition, we have previously shown that the GoH3 antibody is able to block VLP binding to cells by 63% (Evander et al., 1997). Therefore, we pre-blocked A431 cells at 4 °C with both these antibodies before exposure to PV VLPs and observed the relative activation of Akt. Under these conditions, specific blockade of α6 and β4 led to profound loss of Akt activation, whereas isotype-matched control antibodies had no effect (Fig. 5).

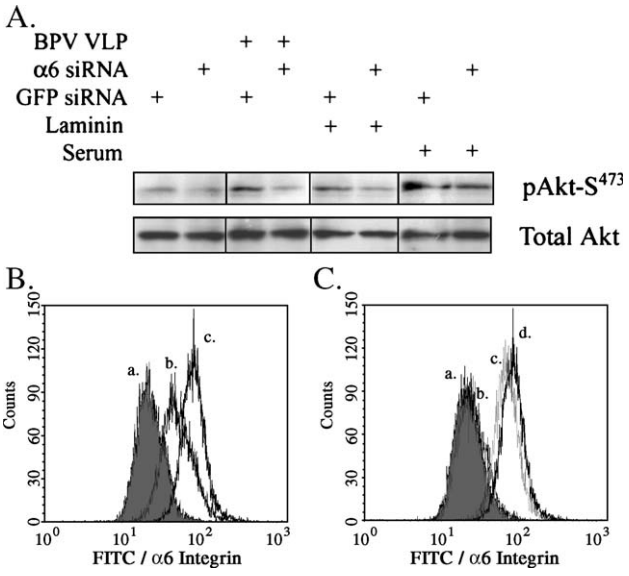

Fig. 4. siRNA against α6 integrin reduces PV VLP-induced activation of Akt. (A) A431 cells were transfected with α6 siRNA or GFP control siRNA as described. BPV VLPs, laminin or serum was added for 20 min before cells were solubilized and western blot performed using anti-pAkt (S^{473}) or anti-Akt antibody. (B) siRNA transfected cells were analyzed by flow cytometry using FITC-labeled antibodies; (a) α6 siRNA transfected cells stained with control antibody; (b) α6 siRNA transfected cells stained with anti-α6 antibody; (c) mock transfected cells stained with anti-α6 antibody. The α6 siRNA reduced surface α6 levels by 50% (b) compared to controls (a and c). (C) siRNA transfected cells were analyzed by flow cytometry using FITC-labeled antibodies; (a) mock transfected cells stained with control antibody; (b) control siRNA transfected cells stained with control antibody; (c) control siRNA transfected cells stained with anti-α6 antibody; (d) mock transfected cells stained with anti-α6 antibody.

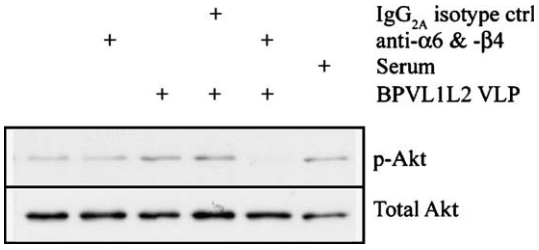

Fig. 5. Pre-treating A431 cells with function-blocking, antibodies against α6 and β4 integrins prevents PV VLP-induced Akt activation. Cells were pre-treated with anti-α6 and anti-β4 integrin antibodies or an equivalent amount of control isotype-matched antibody at 4 °C for 30 min prior to stimulation for 20 min with BPV VLP or 10% serum as positive control. Cells were solubilized and western blot performed using anti-pAkt (S^{473}) or anti-Akt antibody as per previous Akt experiments.

Discussion

This study clearly shows that PV can activate the PI3-kinase pathway and that this occurs via the α6β4 integrin. This phenomenon is not limited to a single PV type and is true for both human and bovine PV VLPs. Indeed, we show that five different PV VLP types were able to induce PI3-kinase and Akt signaling, suggesting a common receptor is utilized. Previously, we and others have proposed the α6β4 integrin as a potential PV receptor (Evander et al., 1997; McMillan et al., 1999; Yoon et al., 2001). This would be consistent with our findings, as the ligation of α6β4 with laminin or polystyrene bead-conjugated anti-β4 antibodies (to promote integrin clustering) has been shown to result in the activation of Ras and PI3-kinase (Mainiero et al., 1997; Tang et al., 1999). Our evidence suggesting that activation of the PI3-kinase pathway by PV VLPs is induced via α6β4 integrin uses both an siRNA knockdown approach as well as function-blocking antibodies against the α6β4 complex. The α6 siRNA also inhibited signaling from laminin, a known ligand of α6β4. Although siRNA treatment only reduced cell surface α6 expression by 50%, signaling was reduced to background levels. This fits with current thinking that for signaling to occur in many systems a critical, threshold concentration of cell surface receptors must be reached in order to allow clustering of the receptor/signaling complex. We speculate that the siRNA-mediated reduction resulted in the concentration of α6β4 falling below this threshold level.

Heparan sulfate (HS) can also act as a primary attachment receptor for PV VLPs (Bousarghin et al., 2003; Combita et al., 2001; Drobni et al., 2003; Giroglou et al., 2001; Joyce et al., 1999; Selinka et al., 2002, 2003; Shafti-Keramat et al., 2003). Heparan sulfate proteoglycans (HSPGs) play a crucial role in growth regulation by assembling signaling complexes and

presenting growth factors to their cognate receptors. For example, syndecan-1 has been shown to enhance the activation of Ras-MAPK and PI3-kinase/Akt pathways by hepatocyte growth factor via the Met receptor in multiple myeloma cells (Derksen et al., 2002). Syndecans are intimately associated with integrins and syndecan-1 has been shown to be involved in PV binding (Shafti-Keramat et al., 2003). At present, it is not known exactly how the PV signal is mediated and our work does not rule out a role for HSPGs in viral signaling. We hypothesize that HSPGs are used for initial attachment and/or concentration of the virus around a more specific receptor such as the α6β4 integrin. Notably, both α6β4 and HS have been shown to be redundant for infection in at least two PV types (11 and 31, respectively), suggesting not all PV may use these receptors (Patterson et al., 2005; Shafti-Keramat et al., 2003) or that this interaction is not critical for overall replication.

Both L1 and L1/L2 VLPs are able to signal cells. This suggests L1 is the viral protein responsible for the activation of signal transduction. Moreover, as we are using VLPs, an early viral replication event cannot be the cause of PI3-kinase activation, but rather a direct interaction with the cell is required. We also show that intact virus particles and virus receptor interactions are required for PI3-kinase and Akt signals to be generated. Denaturing VLPs by boiling significantly reduced PI3-kinase activation and Akt phosphorylation, which fits well with our previous data showing boiling markedly reduced cell proliferation (Payne et al., 2001). We note that 5-min boiling reduced the PI3-kinase signal by 50%. However, the PI3-kinase signal was not completely attenuated, suggesting either denaturation was incomplete or that linear L1 is able to activate the signal, albeit at significantly reduced levels than intact VLPs. Others have shown small linear peptides of L1 and L2 are able to bind to heparan sulfate and block PV infection (Bousarghin et al., 2003, 2004), although we do not know if these small peptides could activate signaling. In support for lack of denaturation, we show that boiling VLPs for an extended period of 30 min was able to completely eliminate Akt phosphorylation. Additionally, it has been observed by electron microscopy that boiling VLPs for 20 min completely removes tertiary structure (Payne et al., 2001). We speculate that complete denaturation of the VLPs and L1 pentamers requires this prolonged treatment with only partial denaturation occurring after 5 min of boiling.

PI3-kinase is known to control a number of different pathways through its ability to convert membrane phospholipids into the active phosphatidyl-inositol 3,4,5 trisphosphate (PtdIns(3,4,5)P3). The blockade of apoptosis, mediated via Akt, being the best described. Here we show that VLP binding is able to induce the phosphorylation of Akt via PI3-kinase, leading to the activation of downstream effectors. Interestingly, Akt activation was observed to be somewhat biphasic. This is similar to the biphasic VLP-mediated Erk activation we have previously noted (Payne et al., 2001). There are a number of proteins signaling from Akt that are involved in the control of apoptosis and which have also been shown to be intimately linked to regulation of the cell cycle. FKHR and GSK3β are two such proteins. Upon phosphorylation, FKHR is translocated from the nucleus by the protein 14-3-3 and is no longer able to act as a transcriptional regulator. This prevents the production of apoptosis inducers such as Fas as well as proteins involved in cell cycle control including p27. GSK3β also plays an important role in cell cycle regulation. It acts by phosphorylating the short-lived cyclin D and c-Myc, promoting their nuclear export and consequent ubiquitin-mediated proteolysis. However, when phosphorylated, GSK3β is unable to act as a kinase and consequently c-Myc and cyclin D levels are stabilized. This promotes progression through G_1 phase. Signaling through the Ras-MAPK pathway also stimulates c-Myc and cyclin D and thus Akt acts in concert to produce a sustained growth signal as well as an anti-apoptotic signal. Therefore, signaling by PV VLPs would provide the cell with both anti-apoptotic and pro-growth signals, via the PI3-kinase and Ras-MAPK pathways, setting the cell up to be receptive for viral replication.

A more speculative possibility is that PV activates PI3-kinase to modulate the immune system and limit proinflammatory responses. Indeed, PI3-kinase/Akt signalling has been suggested to play an important role in limiting immune responses (Fruman and Cantley, 2002). It has been reported that activation of the PI3-kinase/Akt pathway limits the proinflammatory effects of LPS in cultured monocytes (Guha and Mackman, 2002), whereas others have suggested that PI3-kinase activation may provide a negative feedback mechanism that prevents excessive innate immune responses (Fukao and Koyasu, 2003). Moreover, activation of the PI3-kinase pathway has been observed to suppress proinflammatory and apoptotic processes in response to sepsis and/or inflammatory injury (Williams et al., 2004). Indeed, VLPs are unable to activate Langerhans cells, which has been suggested as a reason for their poor immunogenicity (Fausch et al., 2002).

In summary, the results presented here provide the first evidence that PV VLPs are capable of stimulating the PI3-kinase/Akt pathway and can activate key downstream effectors. This supports previous data that demonstrated their ability to induce the Ras-MAPK pathway leading to cell growth. Our results also show that these signaling pathways are mediated via the α6β4 integrin, which further suggests it is the primary PV receptor. Activation of these important growth regulatory pathways could be beneficial by producing an environment more suitable to infection and may be a requirement for the virus to complete its life cycle.

Materials and methods

Cells lines and virus-like particles

A431 (human epidermoid carcinoma) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Melbourne, Australia) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 U/mL penicillin G, 100 U/mL streptomycin and 2.9 mg/mL glutamine (Full DMEM) and maintained in a humidified incubator at 37 °C with 5% CO_2. HPV6b L1 VLPs were produced in Sf9 cells by recombinant baculovirus and purified as previously

described (Qi et al., 1996). To further purify VLPs and remove any contaminating L1 monomers and pentamers in our HPV6b VLP preps, we used an S300 (Sephacryl S300-Pharmacia Biotech) size-exclusion column using gravity flow. The packed column was washed three times with 10 mL equilibration buffer (PBS, 0.1 mM $CaCl_2$, 0.01 mM $MgCl_2$). Buffer level was brought to resin interphase and 800 mL purified VLP from the baculovirus prep was run into column bed. The column was topped with equilibration buffer and 1 mL void volume collected. Fractions of 250 μL were collected and analyzed by Bio-Rad protein assay and western blot using a monoclonal antibody against HPV6b (MC8). The fractions containing the intact virions were pooled and centrifuged at 60000 rpm for 1 h at 4 °C using a TLA100.3 rotor in a Beckman TL-100 ultracentrifuge. The pellet containing the purified VLP virions was resuspended in 100 mL equilibration buffer. BPV1 L1/L2, HPV 16 L1/L2, 31 L1/L2, 35 L1/L2 and 18 L1 VLPs were a generous gift from Ray Viscidi, Johns Hopkins University School of Medicine, Baltimore, MD. They were prepared in *Trichoplusia ni* (High Five) cells (Invitrogen, Carlsbad, CA) from recombinant baculoviruses expressing the L1 and/or L2 genes of the respective virus and were purified by density gradient ultracentrifugation and column chromatography techniques as described previously (Viscidi et al., 2003).

PI3-kinase immunoprecipitations

To immunoprecipitate PI3-kinase, A431 cells were grown to 80–90% confluence in 10-cm dishes before cells were incubated overnight in serum-free DMEM prior to stimulation. VLP blocking was performed by pre-incubating with 1 μL anti-HPV6b L1 monoclonal antibody (MC8) (a gift from Wen-Jun Liu, University of Queensland, Brisbane, Australia) or 1 μL isotype-matched control antibody for 1 h at 37 °C prior to addition to cells. VLP denaturation was performed by boiling for 5 min prior to addition to cells. Normal or blocked/inactivated VLP (100 ng) or 10% serum as a positive control were added to medium and incubated at 37 °C for the required time period. Treated cells were then placed on ice and washed three times with 10 mL ice-cold PBS supplemented with 1 mM DTT, 1 mM PMSF and 1 mM $NaVO_4$. Cells were lysed in 1 mL of lysis buffer (20 mM Tris–HCl pH 7.4, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% NP40, 1 mM DTT, 1 mM PMSF and 1 mM $NaVO_4$) and incubated at 4 °C for 20 min. Extracts were centrifuged at 10,000 × *g* for 15 min at 4 °C and supernatants collected. Extracts were then incubated with 2 μL anti-PI3K (p85 subunit) antibody (New England Biolabs, Beverly, MA) at 4 °C for 2 h before the addition of 40 μL protein-G sepharose (50% suspension in PBS, freshly blocked with BSA) (Amersham Pharmacia, Castle Hill, Australia) and incubation continued overnight. PI3-kinase was collected by centrifuging at 12000×*g* for 5 s at 4 °C and washed three times with 1 mL ice-cold 1% NP40-PBS, 1 mL ice-cold 0.5 M LiCl in 100 mM Tris pH 7.4 and 1 mL ice-cold 100 mM NaCl in 10 mM Tris pH 7.4, respectively, before being resuspended in 1 mL of 100 mM NaCl in 10 mM Tris pH 7.4.

PI3-kinase activity assays

A 100-μL aliquot of freshly immunoprecipitated PI3-kinase was taken for western blot analysis to allow for normalization of enzyme load in the assay. The remaining immunoprecipitate was then resuspended in 30 μL room temperature 20 mM $MgCl_2$, 100 mM NaCl, 10 mM Tris–HCl pH 7.4 containing 1 μCi [γ-^{32}P]ATP and 20 μL of 1 mg/mL bovine brain extract (Sigma, Castle Hill, Australia) (dissolved in 25 mM HEPES pH 7.4, 1 mM EDTA). The reaction mix was incubated at room temperature for 10 min before the addition of 100 μL 1 M HCl and 160 μL chloroform/methanol (1:1). The mixture was vortexed briefly and centrifuged at RT for 1 min at 12,000×*g*. The upper phase was discarded and 20 μL of lower phase was spotted onto a thin-layer chromatography (TLC) plate, dried and TLC was performed.

Thin-layer chromatography

TLC was performed using silica gel 60 pre-coated glass-backed TLC plates (EM Science, Gibbstown, NJ). Plates were prepared by immersion in fresh oxalate solution (1.3% potassium oxalate, 40% methanol, 2 mM EDTA) for 30 min before being baked for 10 min in a pre-heated 100 °C oven. Samples were separated by TLC using 50% chloroform, 40% methanol, 1.7% NH_4OH as solvent. Radioactivity was measured and quantified using a STORM phosphorimager and ImageQuant software (Molecular Dynamics). Background correction using local average was applied.

Activation of Akt, GSK3β and FKHR

A431 cells were grown to 80% confluence in 6-well tissue culture plates before being serum-starved overnight in 1 mL per well serum-free DMEM. Wortmannin-treated cells were pre-incubated with 200 mM wortmannin (Sigma, Castle Hill, Australia) in DMEM for 20 min prior to addition of stimulant. Antibody pre-blocking of A431 cells was performed using mouse anti-human CD49f (α6 integrin) and mouse anti-human CD104 (β4 integrin) function-blocking antibodies (clones NKI-GoH3 and ASC-9, respectively) (Chemicon, Temecula, CA). Cells were treated with 1 μg of each blocking antibody or 2 μg control serotype-matched mouse IgG_{2a} antibody (Bethyl Laboratories, Montgomery, TX) in 1 mL DMEM per well for 30 min at 4 °C. These antibodies were dialysed against PBS for 1 h at RT using a 0.22-μM PES dialysis membrane (Millipore, Sydney) to remove azide and other additives that could affect signaling.

VLP blocking was performed by pre-incubating 1 μg VLP with 4 μL H16.V5 (anti-HPV16) or B1.A1 (anti-BPV1) mouse ascites monoclonal antibodies (gifts from Neil Christensen, Penn State College of Medicine, Hershey, PA), or 4 μL of control anti-PKR mouse ascites monoclonal as a negative control, for 1 h at 4 °C prior to addition to cells. VLP denaturation was performed by boiling for 30 min prior to addition to cells. The desired stimulant was then added to the medium and incubated with cells for the required time, 10%

fetal bovine serum (Gibco BRL) and/or 10 μg laminin (from mouse EHS-sarcoma) (Boehringer Manheim, GmbH, Germany) treatment for 20 min being used as positive controls. Cell extracts were prepared by solubilising cells in 200 μL SDS–PAGE loading buffer and collecting in tubes.

siRNA interference

An siRNA target sequence was identified with the Ambion siRNA Target Finder program (http://www.ambion.com/techlib/misc/siRNA_finder.html) against the human α6 integrin mRNA sequence (NM_000210) as 5′-AAGGTGGCTGCGGTAGCAGCA-3′ and duplex siRNA oligos with 3′ UU overhangs supplied by Proligo (Lismore, Australia). Control siRNAs against GFP (sense 5′-GCACGACUUCUUCAAGUCCUU-3′, AS 3′-UUCGUGCUGAAGAAGUUCAGG-5′) and HPV16 E6 (sense 5′ CACGUAGAGAAACCCAGCUUU-3′, AS 3′-UUGUGCAUCUCUUUGGGUCGA-5′) were also obtained from Proligo. Prior to transfection, A431 cells (90% confluent in 6-well plates) were washed once in PBS and put into 800 μL Optimem (Invitrogen, Sydney) low-serum medium. siRNAs (100 nM) were transfected into A431 cells using Oligofectamine (Invitrogen, Sydney) according to the manufacturer's instructions. After 5 h, cells were washed once, placed into full DMEM medium and grown overnight. The following day, cells were washed and placed into serum-free DMEM overnight prior to analysis of Akt activation or flow cytometry.

Western blotting and flow cytometry

PI3-kinase western blots were performed to control for enzyme amount using 100 μL immunoprecipitate, which was resuspended in 20 μL SDS–PAGE loading buffer. Protein was separated using a 10% SDS–PAGE gel and transferred to PVDF. Akt, phospho-Akt, phospho-GSK3β and phospho-FKHR western blots were performed by separating 20–30 μL of cell extract using a 12% SDS–PAGE gel and transfer to PVDF membrane. Anti-PI3-kinase (p85 subunit), anti-Akt, anti-phospho Akt (Ser473 and Thr308), anti-phospho GSK3β and anti-phospho FKHR antibodies were all sourced from Cell Signalling Technologies (Beverly, MA) and used at 1:1000 dilution in TBS-T/5% BSA overnight at 4 °C. Membranes were washed twice for 5 min with TBS-T before incubation with 1:1000 dilution of secondary antibody (anti-rabbit IgG, HRP-conjugated antibody) (Chemicon, Boronia, Australia) for 1 h at RT. Membranes were washed three times in TBS-T before protein detection by enhanced chemiluminescence.

Flow cytometry was performed on siRNA-treated A431 cells to identify levels of α6 integrin surface expression. siRNA-treated and control cells were trypsinized from the plate and washed once in full DMEM. They were then allowed to re-express surface protein for 1 h at 37 °C in full DMEM with gentle mixing every 15 min to prevent attachment to the tube. Cells were washed once in cold PBS and fixed with 4% paraformaldehyde for 20 min at RT before washing in PBS once again. Staining for α6 integrin was performed by incubating cells in the dark at 4 °C for 30 min with 10 μL neat FITC-labeled mouse-anti CD49f (α6 integrin) GoH3 antibody (Serotec, Oxford, England) per 10^6 cells. Control antibody staining was performed with an equivalent amount of FITC-labeled mouse-anti rabbit IgG antibody (Rockland, Gilbetsville, PA). Cells were then washed four times with cold PBS and fluorescence analyzed using a BD Facscalibur flow cytometer.

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