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Human Papillomavirus E6 interaction with cellular PDZ domain proteins modulates YAP nuclear localization

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Abstract

HPV E6 oncoproteins associate with cellular PDZ proteins. In addition to previously identified cellular PDZ proteins, we found association of the HPV16 E6 PBM with the Dystrophin Glycoprotein Complex, LRCC1, and SLC9A3R2. HPV18 E6 had additional associations when lysates from adenomatous cell lines were used including LRPPRC, RLGAPB, EIF3A, SMC2 and 3, AMOT, AMOTL1, and ARHGEF1; some of these cellular PDZ proteins are implicated in the regulation of the YAP1 transcriptional co-activator. In keratinocytes, nuclear translocation of YAP1 was promoted by the complete HPV-16 genome, or by expression of the individual E6 or E7 oncoproteins; the activity of E6 required an intact PBM at the carboxy-terminus. This work demonstrates that E6 association with cellular PDZ proteins promotes the nuclear localization of YAP1. The ability of E6 to promote the nuclear transport of YAP thus identifies an E6 activity that could contribute to the transformation of cells by E6.

Keywords

polarity; adherens junctions; tight junctions; TAZ; Hippo

Introduction

Papillomaviruses induce benign squamous epithelial neoplasms (papillomas) in vertebrates, and replicate within the differentiating cell layers of the papilloma. Although papillomas are initially benign, some papillomas may evolve over time into malignancies; notably a subset of alpha genus human mucosal papillomaviruses are associated with anogenital and head and neck cancers (reviewed in (zur Hausen, 2009)), and the cotton tailed rabbit papillomavirus with cutaneous cancers (CRPV, (Rous and Beard, 1935)). The subset of viruses associated with human alpha genus anogenital mucosal cancers is referred to as “high-risk” HPV types; HPV-16, HPV-18, and HPV-31 are model systems for the high-risk types, and the related mucosal alpha genus viruses that do not cause malignancies are called “low-risk” types (the prototypes are HPV-6 and HPV-11).

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An observation in the biology of small DNA tumor viruses is the association between the targeting of the retinoblastoma protein (RB) by viral oncoproteins such as Adenovirus E1A or high-risk HPV E7, and the targeting of both TP53 and cell polarity proteins by viral oncoproteins of the same virus. For example, the Adenovirus E1A protein interacts with retinoblastoma (RB), while E1B and E4 gene products target TP53, and the E4ORF4 protein targets cellular PDZ proteins (James and Roberts, 2016; Javier, 2008). Similarly, among polyoma type viruses, SV40 TAg associates with RB and TP53, and SV40 small t antigen disrupts the integrity of cellular tight junctions (Nunbhakdi-Craig et al., 2003). In parallel, high-risk papillomavirus E7 targets RB for degradation, high-risk E6 associates with a cellular ubiquitin ligase (UBE3A or E6AP) to target the degradation of TP53, and high-risk E6 also associates with certain cellular PDZ proteins (reviewed in (Ganti et al., 2015)). In contrast, the low risk alpha genus HPV-11 oncoproteins have not yet been described to target the degradation of RB or TP53, nor have these E6 proteins been shown to associate with cellular PDZ proteins. Why all three of these activities are consistently found in high-risk alpha genus HPV and not in low-risk HPV is as yet unclear.

The PDZ domain (named for the proteins PSD95, DLG, and ZO1) is a small folded protein structural domain that binds to peptide ligands (termed PBMs for PDZ Binding Motifs) on target proteins. The PBMs can be internal, but are most typically carboxy-terminal peptide ligands. Type I PBMs have a consensus sequence (S/T)X Φ , type II PBMs Φ X Φ , and type III (E/D)X Φ (Sheng and Sala, 2001). High-risk HPV E6 proteins have a type I PBM at the c-terminus of E6. PDZ domain adapter proteins can contain multiple PDZ domains, thereby forming large multi-protein complexes built through the association of multiple PDZ domain proteins and their binding partners. Affinities of PDZ-PBM interactions are typically in the low micro-molar range, and can be modulated by phosphorylation of the PBM or the PDZ domain (reviewed in (Lee and Zheng)). There are over 200 different PDZ domain-containing human proteins, and they have diverse functions in signal transduction and the building of protein complexes.

E6 interactions with PDZ proteins have phenotypes that are revealed by comparison of wild-type E6 to E6-mutants deleted of the PBM. In mice, HPV-16 E6 (16E6) transgenic expression in the skin or eye causes aberrant differentiation; this phenotype requires the PBM of 16E6 (Nguyen et al., 2003a; Nguyen et al., 2003b). Truncation of the E6 PBM in the context of the entire HPV-31, HPV-16, or HPV-18 genome results in viral genomes that transiently replicate in keratinocytes, but where the viral plasmid is lost upon prolonged cell passage (Delury et al., 2013; Lee and Laimins, 2004). It has recently been shown that neutralization of TP53 by either shRNA or dominant-negative TP53 expression can improve the plasmid maintenance of HPV genomes in which the E6 PBM was deleted (Brimer and Vande Pol, 2013), but another study found some plasmid maintenance of HPV-16 genomes when the E6 PBM was deleted (Lorenz et al., 2013). Diverse phenotypes have been described in tissue culture systems where E6 deleted of the PBM compared to wild-type E6 was assessed in cancer cell lines or under overexpression conditions. Under low expression conditions, 16E6 reduces growth factor dependence in human keratinocytes, and this requires the PBM of 16E6 (Jing et al., 2007). In SV40 immortalized keratinocytes, the E6 PBM function promotes epithelial to mesenchymal transitions (Watson et al., 2003), and in

human keratinocytes the E6 PBM promotes co-operation with mutated ras oncogenes and anchorage independent colony formation (Spanos et al., 2008a).

Which PDZ associations with E6 mediate these phenotypes? E6 only associates with a small fraction of the total number of cellular PDZ proteins, but E6-interacting proteins include PDZ-containing tyrosine phosphatases (PTPN3 and PTP13), (Jing et al., 2007) (Topffer et al., 2007) (Spanos et al., 2008b), PDZ adapter proteins (DLG1 (Lee et al., 1997) , DLG4 (PSD95)(Handa et al., 2007), MPDZ (MUPP1) (Lee et al., 2000) , SLC9A3 (NHERF1), MAGI 1, 2, and 3 (Glaunsinger et al., 2000; Thomas et al., 2002) , PATJ (Storrs and Silverstein, 2007), and SCRIB (Nakagawa and Huibregtse, 2000). These associations were published as resulting in the targeted degradation of the PDZ protein, often in an E6AP and proteasome dependent manner, analogous to the previously described and widely replicated targeted degradation of TP53 by high-risk E6 proteins. However, the targeted overall degradation of the PDZ proteins by E6 in vivo has more recently been challenged (Choi et al., 2014; Kranjec and Banks, 2010). DLG1, which is degraded by high-risk 16E6 in vitro, does not show reduced expression or re-localization in the context of E6 expressed from the HPV-31 episomal genome in primary keratinocytes, or in organotypic raft cultures (Lee and Laimins, 2004), but is reduced in primary keratinocytes expressing HPV-18 (Delury et al., 2013). Some studies have proposed that only certain subcellular fractions or subcellular phosphorylated fractions of high-risk E6-associated PDZ proteins are degraded (Massimi et al., 2004; Massimi et al., 2006; Narayan et al., 2009), but these experiments typically involved expression levels higher than produced by episomal genomes. Further, there is inconsistency as to the mechanism by which E6 may reduce expression of PDZ proteins, with some studies showing E6AP and proteasome dependence (Handa et al., 2007; Jing et al., 2007; Kuballa et al., 2007) and others observing neither ubiquitin nor proteasome dependence (Ainsworth et al., 2008; Grm and Banks, 2004). The phosphorylation of the high-risk E6 PDZ ligand enables interaction of E6 with 14-3-3 proteins to tether E6 in the cytoplasm, and thereby excludes interaction with PDZ proteins (Boon and Banks, 2013; Massimi et al., 2001). Thus, particular culture conditions or cellular differentiation states may be required for high-risk E6-PDZ interactions to occur, or for protein degradation to occur in vivo. Finally, tandem affinity purification (TAP) of HPV-16, 18, and 31 E6 from retrovirally transduced keratinocytes, failed to identify a consensus cellular PDZ domain protein consistently associated with all the tested high-risk HPV types (White et al., 2012). In summary, although the importance of the interaction between high-risk E6 and PDZ proteins is clear, neither the critical cellular target(s), nor the mechanisms or consequences of the interactions that are necessary for the HPV life cycle have been resolved.

There is sequence variation in PBM ligands of high-risk E6 proteins. In order to identify common sets of associated cellular PDZ proteins, cellular proteins that associate with either HPV-16 E6 (with a c-terminal sequence of SSRTRRETQL) or HPV-18 E6 (ERLQRRRETQV) were isolated and identified. Interestingly, the two PDZ ligands associate with a common core set of cellular PDZ proteins from squamous epithelia that are also found in cell lines derived from glandular cervical epithelia. But compared to HPV-16, the HPV-18 E6 PBM associated with an additional set of cellular proteins found in adenomatous cell lines but not squamous epithelial cells. A number of the proteins found to complex with the E6 PBMs have been recently implicated in the activation and nuclear

translocation of the YAP and TAZ transcriptional coactivators. YAP and TAZ when activated regulate the size of tissues and are drivers of cancer phenotypes (Zanconato et al., 2016). We show here that 16E6 and 18E6 promote the nuclear translocation of YAP in squamous epithelia, with this activity requiring the E6 PBM.

Results

Because high-risk E6 oncoproteins may target the degradation of some E6 associated proteins, we decided to purify E6-associated PDZ proteins from cellular lysates that did not express E6. To identify cellular PDZ proteins that associate with HPV-16 E6 (16E6) in a PDZ dependent manner, chitin binding domain (CBD)-TEV-FLAG-16E6 was expressed by vaccinia virus transduction, purified on chitin beads and used as a substrate for binding to cellular proteins provided in HaCat squamous cell lysate. HaCat and later NIKS cell lines were used as lysates because both are capable of forming stratified squamous epithelia, and NIKS support the full HPV replication cycle (Allen-Hoffmann et al., 2000). Control binding reactions used were BPV-1 E6 (BE6), and 16E6 mutants Y79N that decreases the association of 16E6 with UBE3A (E6AP) (Cooper et al., 2003; Zanier et al., 2013) or 16E6 deleted of the carboxy-terminal 2 amino acids (16E6 PBM), thereby disrupting the PBM. Cellular E6-associated protein complexes were TAP purified and resolved by SDS-PAGE; silver stained bands that were PBM dependent were excised and the proteins identified by mass spectroscopy as shown in Fig. 1. Cellular PDZ proteins associated with 16E6 included SCRIB, DLG family members (DLG1, DLG2, DLG4) and DLG1-associated proteins CASK and MPP7 were identified, which we refer to as the core set of 16E6 PBM associated proteins. E6AP bound both wild type and 16E6 PBM but not 16E6 Y79N, as expected (Fig. 1). All E6-associated PDZ proteins identified in this experiment bound to 16E6 independently of association with E6AP, which differs from the association of E6 with TP53 (Ansari et al., 2012).

To simplify and enhance the sensitivity of the analysis, bacterially-expressed GST fusions of the last 10 amino acids of 16E6 (PBM), or the same peptide but deleted of the last 2 amino acids (PBM⁻) were used for in vitro binding to HaCat lysates, and bound proteins released by TEV protease cleavage. Silver-stained proteins found in the PBM lane but not in the PBM⁻ lane were identified as indicated in Fig. 2.

Silver-stained gel bands represent the most prevalent and most stable complexes formed with the PBM. In order to rule out the co-migration of identifiable PDZ proteins that might have co-migrated with non-specific cellular bands, affinity purification on the 16E6 PBM and PBM⁻ was performed using equal amounts of lysate from NIKS squamous cells lysed either in NP40 based cell lysis buffer or RIPA cell lysis buffer and the associated proteins are shown in Table 1 Exps. 2 and 3. Compared to NP40 cell lysis buffer, RIPA buffer yielded greater numbers of peptides as well as additional PDZ domain cellular proteins not identified in the NP40 lysis buffer, in particular the dystrophin glycoprotein complex (comprised of Dystrophin, Dystrobrevin-beta, beta-Syntrophin), LRRC1, NHERF2, and PTPN3.

In order to determine sets of cellular PDZ proteins that associate with 16E6 compared to 18E6, GST-TEV-FLAG fusions to the 16E6 or 18E6 PBM's or the mutated PBM's (PBM⁻) were TAP purified using either squamous epithelial cell lysates (NIKS keratinocytes) or adenomatous cell lysates (293 cells). Silver-stained gel bands of cellular proteins that associated with either the 16E6 or 18E6 PBM appeared identical when the lysate source was from NIKS squamous epithelial cells (and contained the same proteins identified by LC-MS) as shown in Fig. 3. Fig. 3 shows that the 16E6 PBM bound the same set of core cellular PDZ proteins in 293 lysate as in NIKS lysate, while the 18E6 PBM bound the same core PDZ proteins from NIKS lysates as the 16E6 PBM plus an additional set of cellular PDZ proteins (MPP4, MAGI3), indicating that the 18E6 PBM had an expanded range of associated proteins observed in adenomatous cell lysate.

To repeat and confirm the results of Fig. 3 in a cervical adenomatous cell type, 18E6 and 16E6 PBMs and the corresponding PBM⁻s were used to TAP purify protein complexes from C33a cervical adenocarcinoma. Both 16E6 and 18E6 PBMs bound to the core set of PDZ proteins (scribble, DLG family proteins (DLG1 and 3), and DLG1-associated MPP7) (Table 1 Exp.4 and Table 2 Exp. 3). Unlike in squamous cell lysate, 16E6 bound slightly to MAGI1, while 18E6 retained larger numbers of peptides from MAGI1, MAGI3, and the Utrophin-glycoprotein-complex (Utrophin, Syntrophin, and Dystrobrevin). By LC_MS peptide counts, 16 and 18E6 PBMs bound a very similar amount of SCRIB, DLG1, and MPP7, while 18E6 bound more MAGI1 (138 versus 8 peptides), and only 18E6 PBM bound MAGI3, DLG3, and the Utrophin-Glycoprotein-Complex. In this experiment, no peptides bound to the 16E6 PBM that did not also bind to the 18E6 PBM while the converse was not the case. These experiments comparing the PBMs of 16E6 and 18E6 in binding to proteins from either squamous or adenomatous lysates are shown in Table 2 and Fig. 3.

The TAP purification protocol has prolonged purification and washing steps, so more labile binding proteins could be lost. We repeated the pull down using three very quick washes only and repeated the analysis of proteins associated the 18E6 PDZ and not the 18E6 PBM ligand as shown in Table 2 Exp. 2. Additional PBM-associated proteins, some previously observed as E6-associated proteins, notably included AMOT, AMOTL1, GTPases regulatory proteins, (ARHGEF26, ARHGEF1 (Krishna Subbaiah et al., 2012), RALGAPB), a ubiquitin ligase (PDZRN3) (Thomas and Banks, 2015), and a translation initiator factor (EIF3A).

A number of the E6-PBM associated proteins identified here have been implicated in regulation of YAP/TAZ transcription factors, including DLG1, SCRIB (Yang et al., 2015) and AMOT (Chan et al., 2011; Chan et al., 2013). YAP is localized to the cytoplasm when inactive, but is translocated to the nucleus when the Hippo signaling pathway is repressed. There are many inputs to YAP activation, including cell-matrix and cell-cell interactions, cell shape, and soluble growth factor signaling (reviewed in (Yu et al., 2015)). We hypothesized that activation of YAP/TAZ transcription factors might be an effector of some of the diverse activities attributed to E6 PDZ interactions (such as transformation, inhibition of apoptosis and stem cell attributes, recently reviewed in (Ganti et al., 2015)). In order to determine YAP1 localization in keratinocytes, NIKS keratinocytes were cultured in the presence or absence of feeder cells and growth factors overnight on coverslips. When cultured in the presence of growth factors and serum, YAP1 was predominantly localized in the nucleus of

vector-transduced NIKS cells, as expected. However, when cultured overnight in the absence of these growth factors, YAP1 was predominantly (but not exclusively) localized in the cytoplasm (Fig. 4). Fig. 4 shows that in comparison to cells cultured in complete media, starved NIKS cells were less well spread on the coverslips. In contrast to vector-transduced NIKS cells, NIKS cells transfected with the complete HPV16 genome were both more spread and YAP1 was predominantly localized to the nucleus when cultured without growth factors (Fig. 4).

In order to determine if HPV-16 E6 or E7 promoted the nuclear translocation of YAP1, NIKS cells were transduced with retroviruses expressing either E6 or E7 and assayed for YAP1 localization in the absence of exogenous growth factors. The retroviral expression of HPV-16 E7 individually significantly contributed to YAP1 nuclear localization in the absence of growth factors (Fig.5).

In order to assay the role of E6 and E6-PDZ interactions and possible differences between 16E6 and 18E6 PBMs, NIKS keratinocytes were transduced with either vector, 16E6, 16E6 PBM, or 16E6 where the PBM had been substituted with the 18E6 PBM (16E6.18). In the absence of serum and growth factors, cultures expressing 16E6 with intact PBMs showed enhanced nuclear localization compared to vector-transduced cells (Fig. 6). Deletion of the 16E6 PBM ablated the effect of 16E6 upon nuclear localization of YAP1, and the 16E6 PBM could be substituted by the 18E6 PBM (Fig. 6).

Nuclear localization of YAP1 from experiments parallel to those shown in Figs 4–6 were quantified as shown in Fig. 7. 16E6 deleted of the PBM (E6 pbm) did not statistically differ from vector-transduced cells, indicating that the effect of E6 upon YAP1 nuclear localization was mediated through E6-PDZ interactions (Fig. 7). 18E6 showed a slightly higher activity in this assay than 16E6, which was affirmed by the substitution of the 18E6 PDZ PBM onto 16E6 (termed 16E6.18); the 16E6.18 sample was somewhat more potent in this assay than native 16E6. When cultured in the presence of serum and growth factors, all samples, including vector-transduced NIKS cells showed predominantly YAP1 nuclear localization, as expected, and did not statistically differ in this assay (Fig. 7).

Discussion

The invariant finding of a type I PBM at the carboxy-terminus of human alpha genus high-risk E6 proteins has been a long-standing observation and puzzle. Although the E6 PBM influences certain E6 biological functions such as epithelial hyperplasia in E6 transgenic mice (Nguyen et al., 2003a), and the maintenance of the episomal viral plasmid in cultured keratinocytes (Delury et al., 2013; Lee and Laimins, 2004), identifying effector pathways that could connect to those biological phenotypes has been difficult. Since YAP1 is an oncogenic transcriptional co-activator (Zanconato et al., 2016), the nuclear translocation of YAP1 by cancer-associated E6 proteins is a plausible activity to contribute to the biological activities attributed to the E6 PBM. HPV-16 has been shown to activate YAP1 in organotypic cell culture (He et al., 2015). Supporting the in vivo significance, shRNA to YAP1 has been shown to reduce the growth rate of cervical carcinoma tumors in mice (He et al., 2015). We show here that 16E6 and 16E7 independently promote the nuclear localization of YAP1, and

that E6 interaction with cellular PDZ proteins is required. The mechanism by which HPV16E7 might influence YAP1 localization was not studied here, but recently, the degradation of the cellular phosphatase PTPN14 has been described by the Banks, Howley, and Munger labs (Szalmas et al., 2017; White et al., 2016). Preceding those studies, PTPN14 was found to negatively regulate YAP1 (Liu et al., 2013; Michaloglou et al., 2013).

Identification of PDZ proteins in this study

Analysis of cellular PDZ proteins associated with the E6 PBM in our study is similar to a recently performed study that also showed differences between the 16E6 and 18E6 PBMs in the isolation of PDZ proteins from either HaCat or NIKS lysates (Thomas et al., 2016). Newly identified in our present study include LRRC1 (overexpression of which enhances hepatocellular carcinoma (Li et al., 2013)), SLC9A3R2 (similar to SLC9A3R1 AKA NHERF1, that is targeted for degradation by E6 (Accardi et al., 2011)), RALGAPB (implicated in mitotic regulation (Personnic et al., 2014)), LRPPRC, EIF3A (a component of the translation initiation complex eIF3), chromatin remodeling proteins (AMC, SMC2, NCAPG2, SMARPP1, PARP1), APBA1 (AKA MINT1, a DLG1 associated protein (Leonoudakis et al., 2004) , AMOT, AMOTL1 (YAP1 regulatory proteins (Chan et al., 2011; Cordenonsi et al., 2011; Zhao et al., 2011)). The Thomas et al study identified tight junction proteins ZO1 and ZO2 as associating with the 16E6 and 18E6 PBMs, and we did not observe those peptides in any of our experiments.

Clustering of PDZ proteins in multiprotein complexes

While ours and the Thomas et al study both newly identified beta-syntrophins, we identified both SNBT1 and SNBT2, and the additional syntrophin associated proteins Utrophin (UTRN), Dystrophin (DMD), and Dystrobrevin (DTNB) that together are referred to as the dystrophin-glycoprotein complex (DGC; in adenomatous (glandular) tissues, UTRN replaces DMD). The DGC is essential for mechanical stability of muscle cells, and is involved in the regulation of calcium flux from the sarcoplasmic reticulum of muscle. While the DGC and Utrophin glycoprotein complex are widely expressed in epithelia, a clear function related to cancer is not yet evident. In addition to the dystrophin-glycoprotein complex discussed above, a number of the identified PBM-associated proteins are part of other described multi-protein complexes. DLG1 associates with other cellular proteins through L27 domain interactions with CASK or MPP7, and LIN7C to form a tripartite complex that stabilizes the members of the complex and targets DLG1 to adherens junctions, and participates in the formation of tight junctions (Bohl et al., 2007; Stucke et al., 2007). The other DLG family proteins also identified here (DLG2, and 4) also have L27 domains that may complex with MPP7, MPP4, CASK, and LIN7C. APBA1 (MINT1) is described as a DLG1 associated protein (Leonoudakis et al., 2004). So, it is not surprising that all these components were isolated in our experiments.

Purification of PDZ proteins

Differences in the cells used to prepare lysates and techniques of lysate preparation may account for differences in identified proteins between studies as is indicated by the differences observed when lysates were prepared with either non-ionic detergents or RIPA buffer in the current study (Table 1, Exps 2 and 3), and differences in DLG family proteins

identified in the different experiments in this study using differing cell lines. An important factor may also be the extent of washing of bead associated proteins prior to elution, as an extended array of 18E6 PBM associated proteins were identified when rapid washes were employed (Table 2, Exp. 2). It also may be that important targets of E6 are not the directly interacting cellular PDZ proteins, but rather proteins that associate with cellular PDZ proteins in a labile or indirect manner; the Banks lab identified ARHGEF1 as one such case, where the association of ARHGEF1 with DLG1 regulated invasiveness (Krishna Subbaiah et al., 2012). In our study, the finding of AMOT and AMOTL1 was only observed after rapid washes and was not observed in our TAP experiments. It would be tempting to speculate that the extended range of cellular PDZ proteins found from adenomatous cell lysates in association with the 18E6 PBM compared to the 16E6 PBM might correlate with the greater propensity of HPV-18 in the causation of adenocarcinomas compared to HPV-16; however, this seems unlikely as most other high-risk E6 proteins also have PBM's ending in a valine (quite similar to the HPV-18 E6 PBM) and are predominantly associated with cervical squamous cell cancers.

YAP1 regulation is responsive to multiple inputs, and is associated with cellular PDZ proteins

YAP1-activated transcription is responsive to a wide variety of exogenous and intrinsic regulatory inputs, including soluble growth factors, matrix interactions including matrix stiffness (Dupont, 2016), as well as inputs such as cell geometry, contact inhibition through cadherin engagement (Kim et al., 2011; Zhao et al., 2007), and tight junction assembly (Oka et al., 2010; Zhao et al., 2011). Under routine culture conditions where subconfluent cells are cultured with soluble growth factors on plastic surfaces, YAP1 and TAZ are activated and localized to the nucleus. This required us to assay for YAP nuclear localization in the absence of growth factors. YAP is negatively regulated by the Hippo pathway, whereby a kinase cascade (activation of MST1/2 resulting in the activation of LATS1/2) results in the phosphorylation of serine 127 of YAP1, and thereby tethering of YAP1 in the cytoplasm via interaction with cellular cytoplasmic 14-3-3 proteins (reviewed in (Yu et al., 2015)). However, YAP1 pS127 is not the only mechanism for cytoplasmic localization, in that genetic knock-in experiments of YAP1 S112A (the mouse homolog of human YAP1 S127) have ruled out an essential role of tethering of pS127-YAP1 to 14-3-3 proteins for the negative regulation of YAP1 activity (Chen et al., 2015). Further, double knockout of MST1/2 in mice, although promoting the development of carcinomas, does not ablate YAP1 pS127 phosphorylation, indicating that there are additional mechanisms for regulation of YAP1 phosphorylation. YAP1 abundance is also known to be regulated by phosphorylation-dependent proteasome degradation (Zhao et al., 2010). In preliminary experiments not shown here, we have not observed that YAP1 S127 phosphorylation status closely correlates with the presence of a PBM at the carboxy-terminus of E6 in E6 transduced NIKS cells.

Because YAP1 is responsive to so many disparate inputs, it is highly plausible that a papillomavirus oncoprotein such as E6 would influence the activation of YAP1, possibly by regulating YAP1 localization. Our results show that the influence of E6 upon YAP1 localization is dependent upon the carboxy-terminal PDZ binding motif, although other activities of E6 (such as E6AP association) might also play a role. Examination of the

cellular proteins found in association with the 16E6 and 18E6 PBMs revealed a set of cellular proteins bound in common that were isolated from NIKS cells, and an additional set that were only associated with the 18E6 PBM when adenomatous lysates were used (293 and C33a lysates). Although both 16E6 and 18E6 promoted nuclear YAP1 localization, the 18E6 PBM had stronger activity in this assay. The additional set of cellular PDZ proteins that interact with the 18E6 PBM (MAGI1,2,3) have been previously described as associating with 18E6, and are proteins that localize to tight junctions. Interestingly, a portion of the YAP1 cellular pool localizes to tight junctions in other cell types (Paramasivam et al., 2011).

Because YAP1 is activated under typical in vitro culture conditions in complete media, the effect of high-risk E6 proteins upon YAP1 localization was evident only under conditions of growth factor depletion. As differentiated squamous cells detach and move progressively further from the basement membrane during stratification, it may be that loss of matrix attachment and exposure to exogenous growth factors diminishes, and under these conditions, E6 may help sustain YAP1 nuclear localization in HPV infected keratinocytes

E6-associated PDZ proteins regulate YAP1 localization and activity

Among the PDZ targets of the 18E6 and 16E6 PBMs, several participate in YAP1 pathway regulation and additional interactors are plausible regulators. The E6 PBM associated with SCRIB, which interacts with both LATS1/2 and MST1/2, and loss of SCRIB disrupts the inhibition of the YAP1-related protein TAZ by the Hippo pathway kinases MST and LATS (Cordenonsi et al., 2011; Mohseni et al., 2014). The association of 16E6 with SCRIB results in E6AP dependent degradation of SCRIB in vitro (Nakagawa and Huibregtse, 2000), but overall 16E6 dependent degradation of SCRIB in vivo is not consistently observed (Kranjec and Banks, 2010). The E6 PBM associated with DLG1, which is part of the DLG-SCRIB-LGL complex that regulates cell polarity, which is also an input to YAP and TAZ nuclear localization (Yu et al., 2015). The E6 PBM associated with AMOT, and AMOT family members AMOTL1 and AMOTL2 are negative regulators of YAP, and are SCRIB-associated proteins that sequester YAP and TAZ to cell junctions; this binding indirectly activates LATS1/2 (Zhao et al., 2011). The role in E6 regulation of AMOT family protein's expression is currently under investigation. The E6 PBM associated with PTPN3, which is a PDZ-containing tyrosine phosphatase that was discovered as an E6 associated protein targeted for degradation by high-risk E6 proteins; knockdown of PTPN3 reduces the growth factor dependence of keratinocytes, which is a phenotype also conferred by 16E6 expression and is dependent upon the E6 PBM (Jing et al., 2007). PTPN3 has recently been implicated in the degradation of the EGF receptor (Li et al., 2015); EGFR is both a target and an input to YAP1 localization (Zhang et al., 2009). PTPN13 is another PDZ containing tyrosine phosphatase that associates with high-risk E6 proteins, and where the targeted loss of the phosphatase is associated with oncogenic consequences (Hoover et al., 2009; Spanos et al., 2008b).

Thus, multiple E6 PBM-PDZ interactions have already been shown to be connected to YAP1 nuclear localization in several different ways. Which particular E6-PDZ interactions are most important for influencing YAP1 nuclear localization in particular stages of the viral life cycle is as yet unresolved. Given the many cellular proteins that associate with the PBMs of

18E6 and 16E6, and the known direct or possibly indirect linkages of several of those proteins to YAP1 nuclear localization, it seems likely that 16E6 could influence YAP1 nuclear localization by multiple different PBM-PDZ associations in parallel, making further dissection challenging. One could speculate that the particular E6 PBM sequences found on high-risk E6 proteins may have evolved under that selection.

Materials and Methods

Cells and cell Culture

293T and C33A cells were obtained from ATCC (Manassas, Virginia) and cultured in DMEM supplemented with 10% fetal bovine serum. NIKS were obtained from ATCC, co-cultured with mitomycin C treated 3T3 cells in F-media as described previously (Lambert et al., 2005). NIKS were retrovirally transduced with replication defective murine retroviruses or transfected with the HPV-16 genome as previously described (Lambert et al., 2005). To investigate YAP localization on coverslips, NIKS cells were treated by cell starvation and stimulation assays as described (Strickland and Vande Pol, 2016). Briefly, 4×10^5 trypsinized NIKS cells were plated on glass coverslips in 6-well plates (Day 1) together with mitomycin C treated feeder cells. Approximately 48 hours later 3T3's were removed by light trypsinization and the remaining keratinocytes were cultured in F-media for another 10 hours (Day 3, morning). The subconfluent, transduced NIKS were then placed into starvation media (F-media without growth factors) for 15–18 hours.

Plasmids

Replication defective murine retroviruses expressing papillomavirus oncogenes were based upon pLXSN or pBabe-puro. The last 10 amino acids (aa 141–151) or the same segment deleted of the last 2 amino acids (aa 141–149) of HPV-16 and HPV-18 were cloned as GST fusions with intervening TEV protease and FLAG epitopes (i.e., GST-TEV-FLAG fusions). Epitope tagged HA-NHERF1 originated from Vijaya Ramesh's Lab (from Addgene, plasmid 11635).

Antibodies, western blots and immune fluorescence analysis

Transfected mammalian cells were lysed in 1% SDS, equilibrated for protein content (Biorad assay kit), resolved by SDS-PAGE electrophoresis and transferred to PVDF membranes. Antibody sources: YAP monoclonal antibodies (CST D8H1X and CST 1A12) and CDH1 (BD biosciences, clone 36) were used for localization. For immune fluorescence, NIKS cells were cultured in complete F media or starved as described above. At the end of the 12 hr. starvation period, cells on glass cover slips were chilled on ice, rinsed twice with ice cold PBS and then fixed with 4% formalin for 10 min, rinsed with PBS, permeabilized with PBS containing 0.1% SDS for 5 min, rinsed with for 15 min each with Tris-buffered saline containing 0.05% tween (TTBS) prior to blocking overnight in TTBS containing 2% BSA. Antibodies were diluted in block buffer and incubated for 1 hr, followed by 30 min of washes. Fluorescent-coupled secondary antibodies were coupled to Alexa 488 or Rhodamine, were incubated for 30 min and washed for 1 hr prior to mounting of the coverslips in Fluorogel (Electron Microscopy Sciences, Hatfield, PA.). All images were captured at equivalent exposure times and were shown in the figures at equal magnification.

Protein affinity purification and identification

Cells expanded in tissue culture were washed three times with PBS, and lysed on ice with 0.5X NP40 lysis buffer (1X NP40 lysis buffer is 150mM NaCl; 50mM Tris pH 7.5; 50mM NaF; 5mM NaPPi; 1% IPEGAL; 0.01% phenylmethylsulfonyl fluoride; 1mM sodium vanadate; 1ug/ml leupeptin/aprotinin) or RIPA lysis buffer. Lysates from approximately 5X10⁸ 293T, C33A or NIKS cells were centrifuged at 15000 X G 4 C for 20 minutes. The cleared lysates were precleared by passing through a 2.0 ml column of Sepharose CL4B then a 1.0 ml column of glutathione agarose coupled to glutathione-s-transferase. The eluate was then rocked with 20 ug of GST-TEV-FLAG-PDZ ligand or GST-TEV-FLAG-PDZ-mutated ligand bound to Glutathione agarose beads for 1 hr at 4 C. The beads were collected, washed three times and associated proteins eluted by cleavage at room temperature with TEV protease. Where further purification was pursued, the TEV released protein fraction was applied to 20 ug FLAG M2 antibody beads for 1 hr on ice, washed three times, and then eluted three times for 30 minutes each with 2 ug FLAG peptide in 0.25X NP40 lysis buffer. Eluted proteins were freeze-dried, re-suspended in SDS PAGE sample buffer, and applied to 5–20% Tris-glycine SDS PAGE gels (Invitrogen). Gels were stained with silver to visualize protein bands (Shevchenko et al., 1996). Excised bands were reduced, alkylated and digested with trypsin in the gel. The peptides formed in the digestion were extracted, concentrated, and characterized by capillary column LC-tandem MS. Database searches using the program SEQUEST were used to identify the protein by matching the collision induced dissociation (CID) spectra. These matching spectra were verified by manual inspection of the CID spectra. Inclusion as an identified protein required three or more peptides unique to the listed protein isoform associated with the 16E6 PBM and not found in samples or co-migrating gel bands derived from protein associated with PBM ligands.

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Highlights

- Cellular PDZ proteins that associate with HPV16 and HPV18 were purified and identified
- HPV18 associated with more cellular PDZ proteins than HPV16 when lysates from glandular epithelia were used
- Several of the purified cellular PDZ proteins have been implicated in regulation of YAP1 localization
- HPV16 E6 and E7 each promoted the nuclear localization of YAP1 and for E6, this required association with cellular PDZ proteins.

Fig. 1. In vitro association of cellular PDZ proteins with 16E6

GST-TEV-FLAG fusions to the indicated E6 proteins BPV-1 E6 (BE6), HPV-16E6 wild type (16E6) and the 16E6 mutants Y79N and PBM (deleted of the last 2 amino acids of 16E6) were expressed in CV1 cells by vaccinia virus overexpression and purified on glutathione agarose beads. Squamous cellular lysate from HaCat cells was incubated with the bead-bound GST-TEV-FLAG_E6, beads were washed and the fusion protein cleaved from the beads with TEV protease, repurified on anti-FLAG antibody beads, and then released by incubation with excess FLAG peptide. Samples were resolved on 5–20% gradient SDS-PAGE gels, stained with silver, selected bands excised and subjected to in-gel trypsinization and peptide analysis by mass spectrometry. The position of PDZ dependent cellular proteins associated with 16E6 is indicated with arrows and black dots.

Fig. 2. Association of cellular PDZ proteins with bacterially expressed 16E6 PBM or mutated PBM

GST-TEV fusions to the last 10 amino acids of 16E6 (PBM) or the 16E6 PBM deleted of the last 2 amino acids (PBM Δ) were expressed in bacteria, purified on glutathione-agarose beads, incubated with cell lysate from HaCat cells, washed extensively and then released from the beads with TEV protease. Samples were resolved on 5–20% gradient SDS-PAGE gels, stained with silver, and the indicated bands excised and subjected to in-gel trypsinization and peptide analysis by mass spectrometry. The position and identity of PDZ dependent cellular proteins associated with 16E6 are indicated with black dots.

Fig. 3. TAP-affinity purification of cellular PDZ complexes with 16E6 or 18E6 PBMs
 GST_TEV_FLAG fusions to the last 10 amino acids of 16E6 (16-PBM) or 18E6 (18-PBM) the respective PBMs deleted of the last 2 amino acids (PBM Δ) were expressed and purified as detailed in Fig. 2. Cell lysates were obtained from either NIKS keratinocytes or 293T cells. TEV released proteins were repurified on anti-FLAG beads, and then released by incubation with excess FLAG peptide. The identity of LC-MS identified PBM-associated cellular proteins is indicated.

Fig. 4. Confluency and growth factors influence YAP nuclear localization in keratinocytes
The indicated cell lines were seeded onto glass coverslips and feeder cells removed and starved overnight as described in the methods. NIKS/HPV-16 cells contain the complete episomal HPV-16 genome by transfection of circularized HPV-16 DNA. At the end of the assay cells were fixed, and prepared for immunostaining using monoclonal anti-YAP (green), anti-E-cadherin (CDH1) in red, and DNA was stained with DAPI as detailed in the methods. The left column of pictures shows areas of confluency and the right column of pictures shows areas of subconfluency. All the pictures were acquired at the same exposures and show equal magnification.

Fig. 5. HPV-16 and HPV-16 E7 enhances YAP nuclear localization

A. NIKS cells containing episomal HPV-16 were plated on glass coverslips and maintained in F-media for 48 hours. Feeder cells were removed and keratinocytes were starved (No serum, EGF or Insulin) overnight and then either unstimulated or stimulated for 15 mins with complete F-media. Cells were formalin fixed and then immunostained for YAP (green), red for actin (Alexa-468-Phalloidin) and counterstained with DAPI (blue). Cell outlines were determined by phalloidin staining and the nucleus with DAPI; individual cell YAP cytoplasmic / nuclear ratios were then calculated using Image J. Statistics were calculated from quantified cells collected from 4 separate images (***) $p < 0.001$. **B. E7 enhances YAP nuclear localization.** NIKS cells that were retrovirally transduced with vector or 16E7 were plated on glass coverslips and starved overnight as described in part A and then either remaining unstimulated or stimulated for 15 mins with full F-media. Cells were formalin fixed and then stained for YAP (green), Phalloidin (red) and DAPI (blue). YAP nuclear /

cytoplasmic ratios were calculated as in part A. and results from 4 separate images quantified using ImageJ. (*= $p < 0.05$, ***= $p < 0.001$). Results are representative of three independent experiments.

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Fig. 6. The PDZ ligand of 16E6 or 18E6 is required to promote nuclear YAP localization by E6 in the absence of growth factors

NIKS cells retrovirally transduced with vector, 16E6, 16E6 where the carboxy-terminal PDZ ligand (PBM) was deleted (16E6 Δ PBM) or 16E6 where the PBM was replaced with the 18E6 PBM (16E6_18PBM) were plated on glass coverslips and maintained in F-media for 48 hours. Feeder cells were then removed and keratinocytes were starved (No serum, EGF or Insulin) overnight in the absence of growth factors. All the panels show equal magnification with views of confluency (left panels) or less confluency (right panels).

Fig. 7. The E6 PDZ ligand influences the nuclear localization of YAP

Early passage NIKS cells retrovirally and stably transduced with 16E6, 18E6, 16E6 deleted of the carboxy-terminal PDZ binding motif (16E6 Δ PBM), or 16E6 where the PBM was substituted with the PBM from 18E6 (16E6.18) were cultured on coverslips in complete media in the absence of feeder cells or in media without growth factors or feeder cells overnight and then fixed and immunostained for YAP and E-cadherin. Approximately 400 nuclei from each culture condition were counted from at least 5 photographs of randomly selected fields and scored for nuclear localization. Shown is percent of cells with nuclear localization from an experiment representative of four independently replicated experiments with bars representing the mean and standard deviation between counted fields. Student t test results: * = < 0.05, ** = < 0.01, *** = < 0.001.

Table 1

Proteins associated with the HPV-16 E6 PBM, but not associated with HPV-16 E6 PBM . Number of total peptides and peptides unique to the listed Gi isoform are shown. Inclusion in the table required three or more peptides unique to the listed Gi isoform. Proteins identified in experiment 3 were excised as a single band in a gel run only 1 cm that migrated larger than 25 kDa in order to exclude GST and TEV peptides from attenuating the sensitivity of the LC-MS analysis.

Gene ID. Protein names Protein Gi	Protein size aa.	Total/Unique peptides HaCat. Exp. 1.	Total/Unique peptides NIKS NP40. Exp. 2.	Total/Unique peptides NIKS RIPA. Exp. 3.	Total/Unique peptides NP40 C33 lysate. Exp. 4.
DMD. Dystrophin. Gi 503231	3677	6/6	8/8		
SCRIB. Scribble. Gi 45827731	1655	19/19	67/67		237/40
MAGI1. Gi 74272284	1462				8/3
DLG1. Discs large 1. SAP97. Gi 148539578	926	54/41	18/15	42/34	321/28
CASK. Calcium/calmodulin- dependent serine protein kinase. Gi 4502567	921	37/36	5/5	39/39	
PTPN3. Protein tyrosine phosphatase non- receptor type 3. Gi 219520091	913			3/3	
DLG2. Disc large 2. Chapsyn 110. Gi 218156338	817		3/0		
DLG4. Discs large protein 4. PSD95. Gi 4557529	767	8/2			
MPP4 Membrane protein, palmitoylated 4. gi 14780902	637				3/3
DTNB. Dystrobrevin. Gi 37577099	597			8/7	
MPP7. Membrane protein, palmitoylated 7. gi 111154074	576	22/21	9/8	31/30	66/19

Gene ID, Protein names Protein Gi	Protein size aa.	Total/Unique peptides HaCat. Exp. 1.	Total/Unique peptides NIKS NP40. Exp. 2.	Total/Unique peptides NIKS RIPA. Exp. 3.	Total/Unique peptides NP40 C33 lysate. Exp. 4.
SNTB2. Syntrophin basic beta 2 . Gi 5803177	540	12/6	2/2	21/18	
LRRC1. Leucine rich repeat containing 1. Gi 95113664	524			3/3	
PDZK1. PDZ domain containing 1. NHERF3. Gi 21361142	519	5/5			
SLC9A3R2 solute carrier family 9 isoform 3 regulator 2. NHERF2. Gi 194018553	326	4/4		3/3	
LIN7C. Gi 8922944 Lin7A Gi 4759306	197	13/7		12/8	

Table 2

Proteins identified by LC_MS associated with the HPV-18 E6 PBM, but not associated with HPV-18 E6 PBM .

Gene ID Protein Name Gi identifier	Protein size aa.	Unique/ total peptides. 293T lysate Exp. 1.	Unique/total peptides. 293T lysate Rapid washes. Exp. 2.	Unique / total peptides. C33 lysate. Exp. 3.
DYNC1H1. dynein1. Gi 33350932.	4646		38/21	
DMD (BMD). Dystrophin. Gi 5032315	3677		12/3	
UTRN. Utrophin. Gi 110611228	3433		2077/194	3/3
MPDZ (MUPP1). Multiple PDZ domain protein. Gi 148746189	2041		22/11	
KIF13B (GAKIN). Kinesin 13B. Gi 46852172	1826		/6	
SCRIB (Vartul). Scribble. Gi 45827731	1655		2013/97	244/40
RALGAPB. Ral GTPase-activating protein subunit beta. Gi 34787409	1494		/3	
MAGI1. Gi 74272284	1462		746/70	138/30
MAGI2. Gi 27436957	1455		74/26	
LRPPRC. Leucine-rich PPR motif- containing protein Gi 31621305	1394		3/12	
EIF3A. Eukaryotic translation initiation factor 3. Gi 4503509	1382		19/10	
SMC3. Structural maintenance of chromosomes 3. Gi 4885399	1217		43/18	
SMC2. Structural maintenance of chromosomes 2-like 1. Gi 110347420	1197		74/26	
NCAPG2. Leucine zipper protein 5. Gi 116812586	1143		3/3	
MAGI3. Gi 155969695	1125	5/3	1341/84	181/33
SMARCC1. SWI/SNF-related matrix- associated actin- dependent regulator of chromatin c1. Gi 21237802	1105		11/6	
PDZRN3 (SEMCAP3). PDZ domain containing RING finger 3 ubiquitin ligase. Gi 57529737	1066		3/3	

Gene ID Protein Name Gi identifier	Protein size aa.	Unique/ total peptides. 293T lysate Exp. 1.	Unique/total peptides. 293T lysate Rapid washes. Exp. 2.	Unique / total peptides. C33 lysate. Exp. 3.
PARP1. Poly (ADP-ribose) polymerase family, member 1. gi 156523968; Accession# NP_001609)	1014		272/39	
DLGAP4 (DAP4, DLP4). Disks large-associated protein 4 isoform a. Gi 34335253	989		7/3	
DLG2 (PSD93, Chapsyn-110). Discs large 2. Gi:218156338	975	8/1		
AMOTL1 (JEAP). Angiomotin like 1. Gi 22027646	956		37/2	
DLG1 (SAP97). Discs large 1. Gi 148539578	926	27/7	1838/69	434/31
CASK. Calcium/calmodulin- dependent serine protein kinase. Gi 4502567.	921	16/9	1890/60	
ARHGEF1 (RHOGEF, SGEF). Src homology 3 domain-containing guanine nucleotide exchange factor (for gi 21361487; Accession# NP_056410)	871		245/33	
APBA1 (MINT1, LIN10). Amyloid beta A4 precursor protein binding, family A, member. Gi 22035548	837		21/7	
DLG3 (SAP102). Discs large 3. Gi 119943106	817		133/22	18/9
DLG4 (PSD95). Discs large 4 Gi 4557529	767			
AMOT. Angiomotin. Gi 19111150	675		648/41	
DTNB. Dystrobrevin, beta isoform 2 Gi 37577099	597			9/5
MPP7. Palmitoylated membrane protein 7. Gi 111154074	576			57/18
SNTB2 (SNT3, SNTL). Basic beta 2 syntrophin. Gi 5803177.	540			18/4

Number of total peptides and peptides unique to the listed Gi isoform are shown. Inclusion in the table required three or more peptides unique to the listed Gi isoform. Proteins identified in experiments 1 and 2 were identified from individual excised silver-stained bands associated with the 18E6 PBM compared to a analogous control band from proteins associated with 18E6 PBM . Proteins identified in experiment 3 were excised as a single band in a gel run only 1 cm that migrated larger than 25 kDa in order to exclude GST and TEV peptides from attenuating the sensitivity of the LC-MS analysis.