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Papillomavirus E6 and E7 proteins and their cellular targets

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1. ABSTRACT

The mucosal human papillomaviruses (HPVs) infect human genital and oral epithelial cells and cause lesions ranging in severity from benign to malignant. HPV associated malignancies include cervical and other anogenital cancers as well as a subpopulation of head and neck cancers. Viral infection of epidermal stem or transit amplifying cells can result in long term viral persistence, and the development of carcinogenesis over a significant amount of time then requires additional cooperating genetic hits. Only the so-called high risk HPV types mediate human carcinogenesis, whereas the low risk HPVs have been linked to benign epithelial lesions that are not generally life threatening, but nonetheless are a major health burden. Expression of the high risk HPV E6 and E7 oncogenes is sufficient for primary human keratinocyte immortalization and is required for initiation and all subsequent stages of carcinogenic progression. Together with the finding that high levels of E6/E7 are a unifying hallmark of HPV positive cancers, these two genes are presumed to be the relevant virus-derived transformation stimuli in humans. E6 and E7 proteins do not possess intrinsic enzymatic activities, but instead function though a number of direct and indirect interactions with cellular proteins, a number of which are well known cellular tumor suppressors. We will summarize here current insights into E6 and E7 interactions with specific cellular targets that stimulate aspects of the viral life cycle, interfere with cell cycle controls and promote carcinogenic processes.

2. INTRODUCTION

2.1. HPV infection and carcinogenesis

A large body of evidence has accumulated over the past few decades to identify high risk human papillomaviruses (HPVs) as sexually transmitted pathogens that are responsible for anogenital and to a lesser extent head and neck cancers (1, 2). With regard to cervical cancer, fifteen high risk types are carcinogenic, with HPV16 and 18 by far the most predominant (3). DNA from the high risk HPV types is detected in over 90% of cervical cancers, thus reaffirming the notion that HPV infection is a necessary cause (4, 5). HPV infection is common in sexually active young women (6), however, the risk of associated carcinogenesis is rare in industrialized countries due to effective screening and prevention programs. In the absence of such programs, the risk of cervical cancer development is high in underdeveloped countries. Worldwide, cervical cancer represents the second most frequent cancer in women, claiming approximately 230,000 lives each year (7). Promising approaches to reduce cancer incidence through HPV vaccination are currently ongoing, and offer much hope for future prevention of HPV associated cancer (8).

2.2. Major transforming activities

Within the group of mucosal HPVs, the so-called high risk types are distinguished from the low risk types depending upon the risk of an infected individual to develop carcinogenic lesions. This clinical classification of

HPVs into high versus low risk types correlates well with the abilities of the corresponding viral genomes to immortalize primary human keratinocytes (HFKs) (9-13). Whereas the high risk HPV types are fully functional in cell culture based immortalization assays, the low risk HPV types are largely deficient. Keratinocytes are the appropriate cell type for these experiments since they are the host of HPV infection and the cell type from which HPV associated squamous cell and adenocarcinomas arise. HPV immortalized keratinocytes display altered growth and differentiation properties, but do not exhibit the classical features of transformation, i. e. anchorage independent growth and/or tumorigenesis in nude mice. Subsequent transformation can be achieved in such cells through extensive passaging *in vitro* or the co-expression of an activated oncogene such as ras (14-17). High risk HPV E7 alone can induce immortalization in primary human keratinocytes, but functions much more potently when co-expressed with E6 (18). Cooperativity *in vitro* and the universal detection of E6/E7 expression in human HPV positive cancers, have defined them as the primary viral oncogenes (12, 18, 19). However, a dominant role for E7 has emerged in cell culture studies and holds up in E6 and E7 transgenic mouse models of cervical as well as head and neck cancers (20-22). Importantly, additional genetic hits within the cellular genome are also required for carcinogenic progression. This is consistent with many years of latency in infected women before the development of clinically recognizable lesions and conversion towards malignancy.

2.3. The HPV life cycle

The HPV infectious life cycle involves viral access to the dividing basal cell layer through microtrauma, initial amplification of viral episomal DNA and subsequent maintenance in the stem and/or transit amplifying cell compartment, a process that can be reflected in long term viral latency. During upward migration and concomitant differentiation of infected keratinocytes, the productive phase of the viral life cycle is initiated. High levels of viral DNA and proteins are produced, intact capsids assemble in the granular and cornified cell layer and viral particles are then released in association with cornified squamous flakes (23). In HPV positive cancers, the viral DNA is almost always found integrated within the cellular genome, a configuration which is associated with high level expression of E6 and E7 (24, 25). Since E6 and E7 clearly evolved to support viral replication in infected epithelium rather than to cause cancer, we will emphasize in the remainder of this review E6/E7 functions in HPV carcinogenesis as well as the viral life cycle.

3. THE HPV E7 PROTEIN

High risk HPV E7 proteins are approximately 100 amino acids in size and share distinct amino acid sequence homologies and biological activities with the Adenovirus E1A and the SV40 large T antigen. A multitude of cellular interacting partners have been described in the literature, and the biological significance for many of the observed interactions remains to be explored. Figure 1 depicts a schematic of HPV16 E7 and specific mutations that are reported to disrupt binding to a number of cellular targets. Three conserved regions are referred to as CR1, CR2 and CR3, and are critical for viral oncogenic activities. An LXCXE motif within CR2 mediates binding to the retinoblastoma protein family (26-28), and is required for viral DNA maintenance during the infectious cycle. Both high and low risk HPV E7 proteins can interact with Rb, however, relative Rb binding affinities are more than 10-fold increased in the high risk compared to the low risk E7 proteins (29). The primary determinant for the observed difference in Rb binding as well as transformation activity maps to an aspartic acid versus glycine residue in position 21 (for the high risk) and 22 (for the low risk) HPV E7 proteins (30). Only high risk HPV E7 binding is followed by the rapid ubiquitin-mediated Rb degradation. This function requires sequences in addition to the LXCXE motif, and Rb destabilization correlates tightly with E7 transformation (31-36). The CR3 region within the E7 C-terminus consists of a metal binding domain composed of two CXXC motifs separated from each other by 29 amino acids. The C-terminal zinc-binding regions are important for E7 dimerization (37, 38) and intracellular stabilization (39, 40), as well as for the formation of high-molecular mass oligomers with apparent chaperone holdase activity (41). Multiple cellular proteins can bind domains within the E7 C-terminus including a second, weaker Rb interaction domain, an E2F binding domain (42) and residues that directly interact with the cyclin/cdk kinases $p21^{CIP1}$ and $p27^{KIP1}$. The latter are two well characterized activators of Rb with important roles in cell cycle progression, differentiation and cell death (43-45). C-terminal sequences that are conserved within high risk HPV16 and 31 E7 proteins have also been shown to bind class I histone deacetylases (HDACs) (46). This binding is in a manner that is independent of Rb interactions and likely mediated through an association with the Mi2beta protein, a component of the mammalian NuRD chromatin remodeling complex.

Structural information on the viral E7 proteins is scarce, and has long been limited to the structure of a nine residue peptide containing the LxCxE motif bound to the Rb pocket domain (47). Two recent reports, however, have contributed significant insight into additional structural features of E7. The E7 C-terminus of cutaneous low risk HPV1A was examined by high resolution x-ray crystallography in the first report (48). HPV1 E7 protein is unusual in that it exhibits strong Rb binding activities, yet is unable to mediate subsequent proteasomal Rb degradation (32, 49). The results of these studies support a dynamic model, whereby the E7 CR2 and CR3 domains cooperate for E2F displacement from Rb: initial high affinity contact between the Rb B domain and E7 CR2 results in the exposure of two patches within the E7 CR3 domain, allowing for additional contacts with the Rb C-domain and with the marked box region of E2F, respectively. In a second report, the nuclear magnetic resonance (NMR)-based solution structure of a high risk HPV45 E7 homodimer was described (50), and contains a unique, well structured C-terminal Zn-binding domain. The latter bears remarkable homology to the above HPV1a E7 C-terminus. According to the data, the C-terminal $p21^{CIP1}$

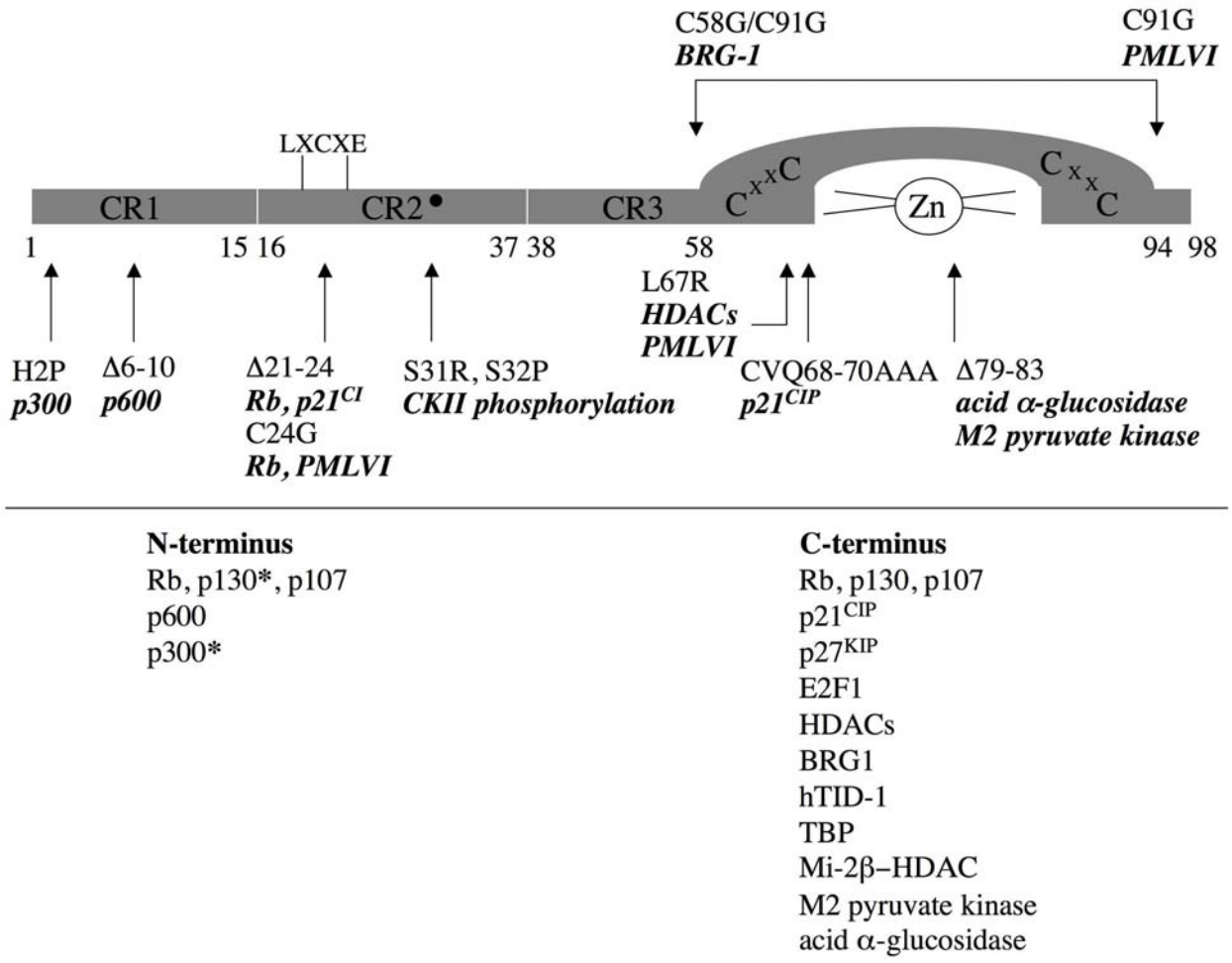

Figure 1. Schematic representation of the HPV 16 E7 protein. Conserved regions (CR) 1-3 are indicated and exhibit homologies with Adenovirus E1A and SV40 large T antigen. A consensus casein kinase phosphorylation site within CR2 is denoted by a black dot. Regions that harbor binding domains for cellular proteins are indicated. These include within the N-terminus a strong interaction domain for the retinoblastoma protein family as described in the text, as well as domains for the binding of p300 (86) and p600 (166, 167). The C-terminus contains a weak interaction domain for the retinoblastoma protein family and an E2F binding domain, as well as domains for the binding of the $p21^{CIP1}$ and $p27^{KIP1}$ cyclin/cdk inhibitors as described, hTID-1 (168), BRG1 (169),TATA binding protein (170), Mi-2beta (46), M2 pyruvate kinase (171) and acid alpha-glucosidase (172). Asterisks indicate cellular proteins that interact with both high and low risk HPV E7. Mutations that affect binding to specific cellular targets are depicted.

interaction domain within E7 overlaps the low affinity binding site for Rb. Whether mutually exclusive $p21^{CIP1}$ and pRb C domain binding to E7 occurs intracellularly, and whether such competition might carry biological significance, remains to be determined.

3.1. Cellular targets of HPV E7 proteins: the retinoblastoma protein family

The Rb, p107 and p130 members of the cellular retinoblastoma family have been extensively studied as targets of the high risk E7 proteins and are collectively referred to as "pocket proteins". These nuclear phosphoproteins interact preferentially with distinct groups of E2F family members and differ in expression during cell cycle progression and cellular differentiation. Pocket protein activity is regulated at the level of phosphorylation (and consequent inactivation) by cyclin-dependent kinases (cdks). Important roles for Rb have been demonstrated in the suppression of cellular proliferation (51), stimulation of differentiation and senescence (52, 53), cellular survival (54) and the maintenance of stem cell quiescence (55). While Rb family members can functionally compensate for each other in various settings, the Rb protein has emerged as the predominant tumor suppressor based on the results of knockout mouse models and the finding that inactivation of Rb, but not p130 and p107, is a general hallmark of sporadic human cancer (56). In agreement with this view, skin cancer phenotypes resulting from the transgenic, basal cell specific expression of E7 are almost entirely mirrored by the loss of Rb expression in those same cells (57). Many, but not all, of the above Rb functions involve the repression of E2F transcription factor activities. E2F

transcription factors are best known for their ability to stimulate cell cycle progression. They function as heterodimers composed of one member of the E2F and one member of the DP1 protein family. Rb mediated repression of E2F transcriptional activities occurs through direct binding and blocking of the E2F transactivation domain and indirectly through recruitment of histone deacetylases (HDACs), histone methyltransferase SUV39H1, and chromatin remodelling factors such as SWI/SNF and Polycomb protein silencing complexes (58). Consequently, Rb inhibition by E7 is expected to result in the activation of E2F transcriptional activity for subsequent progression through the cell cycle (59), inhibition of differentiation and cell death, and stimulated rates of proliferation in basal stem and progenitor cells (55).

While Rb inhibition by E7 is clearly important for human tumorigenesis, surprisingly little is known about the role(s) of specific retinoblastoma controlled pathways in the viral replicative cycle. HPV replication is dependent upon an active cellular DNA replication apparatus, and E2F mediated transcriptional activation of the cellular DNA replication machinery in differentiated cells serves to provide the required replication competent milieu. At least in the mouse epidermis, specific roles for Rb, only partially redundant with p107, involve the maintenance of quiescence in stem and differentiated cells, whereas p107 and p130 are important for terminal differentiation (55). It is therefore likely that retinoblastoma protein family members support the HPV life cycle at different stages via multiple mechanisms. Three-dimensional human epidermal model systems can be utilized for studies of the viral life cycle (60) and will be helpful for the dissection of retinoblastoma protein functions. Within these organotypic raft cultures – as in intact epidermis - the proliferative basal cell layer is geographically distinct from the differentiating suprabasal layers. Suprabasal postmitotic cells are driven into S-phase in the presence of both high and low risk HPV E7 proteins in a manner that requires Rb interactions (61). HPV maintenance occurs in basal cells, whereas vegetative DNA replication is restricted to the suprabasal compartment. Depending upon interactions between specific Rb family member(s) and E7, distinct aspects of the replicative life cycle might be regulated. For instance, a recent study has revealed that regulation of individual Rb family members by low and high risk HPV E7 proteins differs, in that pRB and p107 are destabilized only by high risk HPV E7, whereas destabilization of p130 is mediated by low and high risk HPV E7 proteins (62). Given a role for p130 in terminal differentiation, p130 may therefore play a unique role in the productive phase of the viral replicative cycle. In contrast, Rb (and p107) may exhibit distinct roles in viral maintenance. Interestingly, whereas inhibition of E2F transcription factors by Rb has historically been associated with the downregulated expression of G1/S-progression mediators, more recent studies have highlighted distinct Rb functions in S-phase and thus potential direct effects on HPV replication by E7 (59). Within S phase, Rb inhibition involves two temporally and mechanistically distinguishable processes. The first occurs acutely through reduced PCNA association with the processive cellular DNA replication machinery and in a more chronic fashion through reduced accumulation of a number of critical DNA replication proteins such as DNA polymerase delta as well as the MCM7 replication protein (63). Both processes are likely to be dependent upon E2F activities, although direct protein-protein interactions between Rb and DNA replication factors such as MCM7 have also been described in the literature (64, 65). With regard to HPV replication, E7 expression is critical for viral maintenance in the basal cell layer (66, 67). A recent report has demonstrated that viral DNA maintenance in basal cells can proceed randomly or once per cell cycle (68). The authors have postulated that the choice of mechanism may depend upon the levels of the viral E1 replication protein as well as the availability of cellular DNA replication proteins components such as MCMs. Given Rb's role in the regulation of MCMs and other cellular replication factors, and its binding – albeit with different affinities - to both high and low risk HPV E7 proteins (30), it is worth considering whether E7-Rb interactions may directly regulate HPV replication in the basal cell layer. Given a role for Rb in the maintenance of the epidermal stem cell pool in the basal cell layer (55), such putative E7 effects may ultimately relate to the particular replication machineries available to the HPV genome in infected stem and transit amplifying cells.

3.2. Cellular targets of HPV E7 protein: cyclin-dependent kinase inhibitors

E7-Rb interactions are not sufficient for the abrogation of cell cycle arrest in human epithelial cells, an activity which also requires independent binding to the Rb-activating cdk inhibitor $p21^{CIP1}$ (44, 45, 69). Suggestive of importance to the HPV life cycle, $p21^{CIP1}$ interactions were observed with representative E7 proteins encoded by both high and low risk HPVs. While this may simply ensure complete inactivation of the Rb protein family, it is also possible that $p21^{CIP1}$ specific, Rb independent activities might be at play. Targeted disruption of the $p21^{CIP1}$ gene in mouse models has clearly identified this molecule as a tumor suppressor following chemical or genetic oncogenic insults (70-73). However, the role of $p21^{CIP1}$ is complex and must be viewed in the context of stratified epithelium. At early stages of keratinocyte differentiation, $p21^{CIP1}$ is critical for induction of cell growth arrest (74), whereas its repression occurs later on as a prerequisite for terminal differentiation (75). Another important function relates to the maintenance of the stem cell compartment in a relatively quiescent state, where $p21^{CIP1}$ acts as a molecular link between Notch and Wnt signalling pathways (74, 76). With multiple $p21^{CIP1}$ functions in various strata of the human epidermis, E7 may thus regulate a variety of processes ranging from viral persistence in stem cells to vegetative replication in terminally differentiated cells. Finally, a number of reports have uncovered coactivator properties within $p21^{CIP1}$ as an interacting partner for multiple transcription factors such as c-Myc, STAT3 and Estrogen Receptor ERalpha (77-79). It will be interesting to determine whether such binding activities may in fact contribute to the effects of known carcinogenic risk factors like chronic estrogen exposure following HPV infection.

3.3. Cellular targets of HPV E7 proteins: chromatin modifying factors

Certain mutations within the C-terminal zinc finger like motifs retain the ability to degrade Rb yet are unable to facilitate immortalization (36). This finding has sparked the search for other important cellular E7 targets, and class I HDACs, consisting of HDACs 1, 2, 3, and 8, have recently emerged as viable candidates. HDACs are well known to remove acetyl groups from histone lysine tails, leading to the compaction of chromatin and local transcriptional repression of many cellular promoters including E2F-dependent ones. HDACs are found associated with a number of other co-repressors such as mammalian SIN3 proteins, and can also act directly upon transcription factors such as p53 to contribute to the transcriptional repression of p53 target genes (80, 81). HDAC activity has been associated with cancer, and multiple HDAC inhibitors are currently in clinical trials based on their anti-proliferative activities (82). The significance of complex formation between E7 and HDAC to HPV carcinogenesis is still unknown, but perhaps de-emphasized by the finding that both high and low risk HPV E7 proteins bind HDACs (83). Instead, the observed interaction may support shared aspects of the viral life cycle. Indeed, structure-function studies of HPV31 E7 in the context of viral genomes revealed importance for stable viral maintenance and replication in raft cultures in a manner that does not involve global changes in HDAC levels and activity but implicates specifically the regulation of E2F2 (67, 83). Upon keratinocyte differentiation, E2F2 levels decreased markedly in correlation with HDAC binding to the E2F2 promoter region. In the presence of E7, bound HDAC was released from the promoter with subsequent transcriptional E2F2 induction. In line with a functional role in the life cycle, E2F2 knockdown experiments revealed a decrease in viral DNA replication without any obvious effects on cellular proliferation. In contrast to the observed upregulation of E2F2 expression, E7 has also been shown to utilize HDAC for the repression of the interferon beta promoter through interaction with interferon response factor 1 (IRF1) (84, 85). Such positive and negative regulatory effects on specific target genes may also extend to the observed ability of high and low risk HPV E7 genes to bind the histone acetylase p300 (86). In that way, rather than mediating extensive genome-wide transcriptional changes through the observed interactions with chromatin modifiers, HPV might have evolved to reprogram these proteins in a fine-tuned manner for the deregulation of a specific subset of target genes. The differentiation status of infected keratinocytes and availability of chromatin regulators may then help further define the choice of subsets to support aspects of the viral life cycle that range from genome maintenance to the evasion of host immune responses.

4. THE HPV E6 PROTEIN

The HPV E6 proteins are approximately 150 residues and contain two zinc finger motifs composed of four C-X-X-C motifs which are required for E6 function (87, 88). Figure 2 depicts HPV16 E6 and some of its cellular targets. HPV E6 plays important roles in the HPV viral life cycle, as well as in cellular immortalization and transformation. High-risk HPV E6 cooperates with HPV E7 to immortalize primary human keratinocytes and additionally with the ras oncogene to promote transformation in rodent cells. Interestingly, high-risk HPV E6 alone can also immortalize primary human mammary epithelial cells (89) and individually transform established rodent cell lines (90-92). Studies in which HPV E6 was expressed in the ocular lens of transgenic mice resulted in abnormal cell differentiation, a function which may contribute to E6 mediated immortalization (93). Additionally, transgenic expression of E6 in mouse epithelium proved sufficient for the induction of malignant skin tumors (94).

Transforming functions of high risk HPV E6 proteins involve the targeted degradation of the p53 tumor suppressor by E6 in association with cellular ubiquitin ligase E6-associated protein (E6AP) (95, 96). In addition to p53 interactions, a highly conserved 5 amino acids C-terminal domain within high risk HPV E6 proteins mediates direct binding to PDZ proteins. The name PDZ proteins as a collective designation refers to the first three members identified within the group: PSD-95 (a post-synaptic density signalling protein), Dlg (the *Drosophila* disc large protein), and ZO1 (the zonula occludens 1 protein with functional roles in epithelial cell polarity). PDZ domain proteins serve critical roles in a variety of molecular processes including cell polarity and signal transduction. PDZ binding by E6 is clearly important for HPV associated carcinogenesis, since deletion of the relevant domain inhibits E6 driven transformation of rodent cells (97) and epithelial hyperplasia in mouse model systems (98). Finally, HPV E6 protein expression results in the distinct activation of the telomerase reverse transcriptase (TERT) promoter allowing for increased telomerase activity and extension of the cellular life-span through elongation of telomeric repeats (99).

The structure of HPV E6, much like HPV E7, has proven difficult to analyze due to the unstable nature of the full length proteins in solution. A recent publication, however, has reported structural properties of the C-terminal 75 amino acids of HPV16 E6 engineered to contain specific stabilizing cysteine mutations that do not appear to affect protein function (100, 101). Using NMR, a unique alpha/beta zinc binding fold was identified which is composed of a three-stranded beta sheet and two short helices (101). Based on existing similarities between the E6 N- and C-termini, perhaps a consequence of an evolutionary gene duplication event (88), the authors formulated a new model for a pseudodimeric E6 arrangement and provided a solid foundation for interpretation of previous, and design of future structure-function studies of HPV E6 proteins.

4.1. Cellular targets of HPV E6 proteins

One of the first identified and best characterized interacting partners of E6 is the p53 tumor suppressor. The p53 transcription factor is the most frequently inactivated tumor suppressor gene in human cancer and is involved in the control of cellular proliferation in response to stress (for reviews see (102-104)). Inactivation of p53 by HPV E6

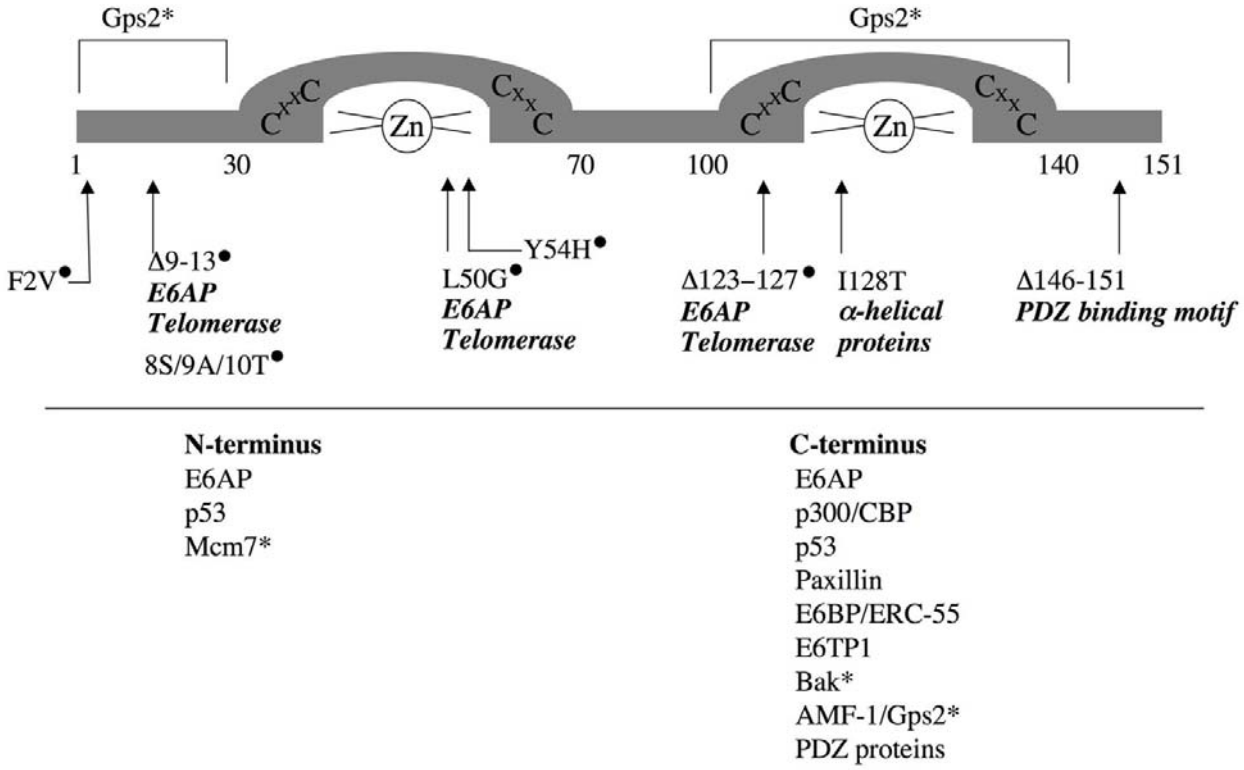

Figure 2. Schematic representation of the HPV 16 E6 protein. The E6 proteins contain two zinc finger regions as indicated. Mutants of the HPV E6 proteins are indicated by arrows and loss of binding to individual cellular proteins is shown. Black dots represent a loss in the ability of the mutant E6 proteins to degrade p53. Cellular binding partners are shown in relation to the respective E6 domains in the table below including AMF-1/Gps2 (155), Mcm7 (156, 157), p300/CBP (173, 174), PDZ proteins (hDlg, MAGI-1,-2,-3, MUPP-1, hScrib, and TIP-2/GIPC) (118-124), Bak (158), paxillin (153), E6-BP/ERC-55 (175), and E6-TP1 (151). Asterisks represent binding partners shared between high and low risk HPV E6 proteins. IRF-3 (176), p73 (159), and c-Myc (177, 178) are additional interacting proteins whose binding has not yet been mapped to a particular domain of HPV E6.

may serve multiple functions relating to apoptosis, or programmed cell death, cell-cycle arrest, senescence, and differentiation. Inactivation of p53 by the high risk HPV E6 protein occurs through recruitment of the cellular E6AP ubiquitin ligase. Together, HPV E6 and E6AP bind p53 in the core region, facilitating its polyubiquitination and proteasomal degradation and resulting in low baseline levels of the tumor suppressor in HPV positive cell lines (95, 105). E6AP repression through antisense oligonucleotides (106) or dominant negative mutant proteins (101, 107) resulted in upregulated p53 protein levels in HPV positive, but not in HPV negative cells, thus supporting the notion that E6AP is a critical player. The regulation of p53 degradation by HPV E6 may be further attenuated by alternative splice products of high risk HPV E6 termed E6* (108). E6* binds to full length E6 proteins and blocks proteosomal degradation in a manner that may compete with intramolecular associations between the E6 N- and C-terminus (101). Little is known about the significance of E6 functions to the viral life cycle, but the ability of p53 to repress viral DNA amplification *in vitro* without obvious effects on episomal maintenance may be important (109). Perhaps virally encoded, dominant negative E6 polypeptides stimulate DNA replication at late, productive stages through the repression of p53 degradation.

Degradation of p53 is specific to the high risk E6 proteins, and relies upon E6 protein binding to the core region of p53 as well as to E6AP. Interestingly, all HPV E6 proteins have been shown to bind to the C-terminal region of p53 (105). This interaction alone does not result in p53 degradation, thus providing a molecular basis for the difference between low and high risk HPV E6 in their ability to degrade p53 (110). However, the observed ubiquitous p53 binding might apply to a recent report demonstrating E6 effects on p53 that do not involve p53 degradation: complexed with the transcriptional coactivator p300 and p53, low and high risk HPV E6 proteins inhibit p53 as well as histone acetylation, thus mediating the repression of p53 target genes such as $p21^{CIP1}$ (111). Physiological significance for this novel E6 function remains to be uncovered, but E6 mutant proteins defective in the degradation of p53 retained p53 transcriptional repression activities (112). Other reports have also supported E6 mediated interference with p53 functions by inhibition of p53 DNA binding (113, 114) and cytoplasmic sequestration of p53 by HPV E6 (115).

4.2. Cellular targets of HPV E6 proteins: PDZ proteins

The C-terminal domain of high-risk HPV E6 proteins is highly conserved and contains a PDZ-binding motif (XT/SXV) which mediates specific interactions with a variety of PDZ proteins. PDZ proteins only bind mucosal high-risk HPV E6 proteins. Functions of the PDZ proteins include cell signaling, cell adhesion, tight-junction integrity, molecular scaffolding for protein complex assembly, and possibly tumor suppressor activity (116)(117). The membrane-associated guanylate kinase (MAGUK) proteins including Dlg, Scribble, and MAGI-1,-2, and -3 (118-121), MUPP1 (122), cystic fibrosis transmembrane regulator associated ligand (CAL) (123) and TIP-2/GIPC (124) are PDZ proteins that interact with high-risk HPV E6 proteins. Their PDZ domains consist of a stretch of 80-90 amino acids which are contacted by four amino acids at the extreme carboxy-terminus of HPV E6. The sequence XTXV/L for HPV18 and 16 E6 proteins, respectively, allows binding of a single PDZ protein and in turn mediates its degradation via the 26S proteasome. Decreasing PDZ protein levels by HPV E6 is thought to contribute to the progression of cervical carcinogenesis as exemplified by numerous *in vitro* and *in vivo* experiments.

The first reported PDZ target of high-risk HPV E6 was Dlg (97, 125). Dlg is involved in cell adhesion, apicobasal polarity and proliferation of epithelial tissues. Loss of Dlg is known to cause aberrant morphology and invasive growth of the mouse epithelium causing embryonic lethality (125, 126). Loss of Dlg in the HPV context correlates with the ability of HPV E6 to cause proliferation and dysplasia in the progression of cervical cancer. The low risk viruses do not harbor the PDZ binding motif and do not affect Dlg protein levels. Dlg targeting, however, can be conferred to a low risk HPV E6 protein by transfer of the relevant C-terminal motif encoded by a high risk HPV E6 protein (127). Interestingly, the use of E6 mutants that are unable to bind E6AP, but retain Dlg binding, indicates that Dlg degradation does not require E6AP function and perhaps implicates a novel cellular ubiquitin ligase in this process (127). Other PDZ proteins including hScrib, and MAGI-1 are also targeted for proteosomal degradation by HPV E6 and are involved in forming epithelial tight junctions (119, 128). MAGI-1 is found in a complex with beta-catenin (129), a molecule that is downregulated in a number of human cancers. MAGI-2 and -3 are involved in the regulation and activation of the PTEN tumor suppressor, specifically by binding PTEN and down-regulating the PKB/Akt kinase pathway, with a decrease in cellular survival and proliferation as the end result (130). Another PDZ protein, MUPP1, is also a binding partner of HPV E6 and functions in signal transduction (131). HPV E6 destabilizes MUPP1 (122) for presumptive interference with signaling through the plasma membrane. Finally, TIP-2/GIPC, which was first characterized as a PDZ protein interacting with human T cell lymphotrophic virus (HTLV-1) Tax and the GTPase activating protein, GAIP, which is involved in TGF-beta signaling and cellular transformation (132, 133), also represent bona fide E6 targets (124). Functionally, Dlg, MAGI-1 and MUPP1 were all strong inhibitors of cell transformation when co-transfected with common cooperating oncogenes such as HPV E7/ras. As predicted, the addition of high risk, but not low risk, HPV E6 alleviated this inhibition by degrading these proteins (121). Deletion of the C-terminal PDZ binding domain resulted in a loss of of E6 transforming abilities (97). In transgenic mice, a PDZ binding defective mutant HPV $E6^{delta146-151}$ protein did not result in hyperplasia of the lens epithelium in comparison with wild type and mutant controls (134), thus demonstrating PDZ protein tumor suppressor activity *in vivo*. Since HPV $E6^{delta146-151}$ retains p53 degradation activity, this function is likely independent of p53. An intriguing recent report also implicated the activation of NF-kappaB pathways by E6 in a manner that required the PDZ binding motif and that provided resistance to TNF-alpha induced cell death (98). Other tumor viruses such as human T cell lymphotropic virus and adenovirus have evolved functions that target PDZ proteins, and it will therefore be important to determine the relative contributions of individual PDZ family members to the inhibition of human carcinogenesis and viral replication (118, 122, 133).

4.3. Cellular targets of HPV E6 proteins: telomerase

Elevated telomerase activity is detected in many immortalized and cancer cells but absent in most normal somatic cells, suggesting that telomerase activation is an important event in human carcinogenesis (135, 136). Activation of telomerase, an enzyme that caps and thus protects telomeres at the end of chromosomes, is sufficient to overcome replicative senescence checkpoints in primary human cells such as fibroblasts and retinal pigment epithelial cells (137, 138). However, primary human keratinocytes require additional genetic hits to achieve immortality such as inactivation of the retinoblastoma tumor suppressor pathways (139, 140), even though a requirement for telomerase induction by E6 has been challenged (141). Telomerase activation by E6 is primarily regulated at the level of transcription of the limiting catalytic TERT subunit (19). Two distinct and p53 independent mechanisms have been described for the observed TERT induction by E6 (99), i) direct transcriptional activation of the hTERT promoter by an E6/c-Myc complex (142), and ii) indirect promoter activation through E6AP dependent degradation of a natural repressor of the hTERT promoter, NFX1-91 (143-145). With regard to the former mechanism, the presence of intact E-boxes is required within the hTERT promoter (143). E box sequences are bound by c-Myc and Max heterodimers for subsequent promoter activation (146). It was hypothesized that HPV E6 may allow c-Myc access to the E-boxes either by elimination of a repressor or by altering chromatin architecture. More recent data support E6 mediated increases in c-Myc protein levels. Significance for c-Myc activity was further emphasized by the finding that a dominant-negative form of c-Myc abolished the activation of hTERT by HPV E6. Specific hTERT repressors, USF1 and USF2, were also implicated in this process, and RNA interference experiments resulted in enhanced transactivation of the hTERT promoter in the presence of HPV E6 (147). These experiments helped produce a model where HPV E6 upregulation of c-Myc is followed by c-Myc competition with the repressors USF1 and USF2 for

subsequent activation of hTERT transcription. This model was supported by chromatin immunoprecipitation assays in E6 expressing keratinocytes, which demonstrated an E6 dependent switch to increased c-Myc and decreased USF1 and USF2 binding to the proximal E box of the TERT promoter (147). While c-Myc is an important hTERT transactivator in some experiments, other studies showed a relative lack of correlation between upregulated c-Myc protein levels and levels of hTERT expression (142, 143, 148). Using a panel of HPV E6 mutant proteins and RNA interference targeting E6-AP, an elegant set of experiments demonstrated that E6/E6AP interaction was required for telomerase induction (144) and implicated the degradation by E6 of a novel transcriptional hTERT repressor, NFX1-91, in the observed telomerase activation. It is important to emphasize that these two distinct E6 dependent mechanisms are by no means mutually exclusive. Indeed, it is likely hTERT transcription is subject to complex regulatory controls. The concomitant activation by E6 of stimulatory factors such as c-Myc, and inactivation of repressing factors such as NFX1-91 and USF-1 and –2 would therefore allow for maximal hTERT activation and cellular immortality.

4.4. HPV E6 and other interacting partners

A number of other E6 binding partners have been identified, and await characterization in the context of HPV infection and carcinogenesis. ERC-55 (E6-BP), a calcium binding protein that may be involved in cellular differentiation, might be important for HPV viral replication (149). A Rap1 GTPase-activating protein, E6-TP1, serves as a potential growth factor responsive signalling protein and is degraded by HPV E6 in a manner which correlates with the immortalization of mammary epithelial cells (150-152). The focal adhesion protein paxillin binds high-risk HPV E6 proteins and causes a disruption in the actin cytoskeleton with potential importance for human carcinogenesis (153). Low risk HPV E6 proteins bind zyxin, while both high and low risk types bind Gps2, Bak, MCM7 and possibly p73, although the respective roles of these proteins in viral pathogenesis have not yet been characterized (154-159).

4.5. HPV E6 and the viral life cycle

Functions of HPV E6 proteins in human carcinogenesis have been extensively studied, but their roles in the viral life cycle are still poorly understood. In this regard, transfection of human keratinocytes with HPV genomes in conjunction with E6 structure function studies have demonstrated a distinct E6 requirement for the long term maintenance of viral episomes (160). A similar role was also identified for low risk HPV11 E6 and mapped to four conserved residues (66). High risk HPV E6 mutant proteins exhibiting defects in the maintenance of episomes included L110Q (defective in the binding and degradation of p53, E6-AP and E6-BP) (159, 161, 162)), CC66/137GG (defective in p53- or p73-dependent transactivation) (159)), W133R (defective in p53 binding and degradation (163)), and R78A (unknown loss of function). This indicates that p53 degradation as well as the targeting of other factors by E6 are important contributors to the genome maintenance stage of the infectious cycle. Similar experiments with HPV 31 genomic DNA containing mutations in the E6 PDZ binding domain revealed decreased cellular proliferation and a reduction in viral genome copy number (164), thus demonstrating that E6 binding to PDZ proteins is necessary for optimal support of viral maintenance. Finally, E6TP1 is likely another important target. Stable transfection of human keratinocytes with HPV 31 mutant genomes whose E6 product is unable to bind E6TP1 but retains the ability to bind another alpha helical protein E6AP, resulted in decreased genome copy numbers over time (165). Whether these functions in the life cycle are shared by the low risk viruses remains to be determined as do the relevant mechanisms.

5. CONCLUDING REMARKS

It has been hypothesized in the past that inhibiting p53 by HPV E6 and the retinoblastoma protein family by E7 were key steps in viral replication. However, the low risk viruses which similarly replicate their genomes to induce pathogenesis are clearly not as efficient at eliminating p53 and RB functions. It is therefore imperative to discover new targets, or define new roles for already identified targets, that relate to the many shared aspects of the infectious viral life cycle. While it is possible that high and low risk viruses have evolved different mechanisms to replicate their DNA, the likelier scenario might be our historical focus on cancer-specific differences at the expense of uncovering infection-specific commonalities. Characterization of such viral functions and their exploitation towards antiviral therapies will be an exciting task for the future.

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Abbreviations: Gps2: G protein pathway suppresssor 2; Mcm7: minicopy maintenance protein 7; p300/CBP: CREB binding protein; Dlg: *Drosophila* disc large protein; MAGI-1-2,-3: membrane-associated guanylate kinase homology proteins with an inverted domain structure; MUPP-1: multi-PDZ domain protein-1; TIP-2; Tax interaction protein 2/ GIPC: GIAP (GTPase-activating protein for G-alpha-i) interacting protein C terminus; E6-BP: E6 binding protein; E6-TP1: E6 targeted protein; IRF-3: Interferon regulatory factor 3; TNFalpha: tumor necrosis factor alpha; HTLV: human T-lymphotropic virus;; TGFbeta: transforming growth factor beta, DSD-95: post-synaptic density signaling protein; NFX1-91: nuclear transcription factor, x-box binding 1; USF-1, 2: upstream stimulatory factor 1, 2.

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