[Frontiers in Bioscience 13, 121-134, January 1, 2008]

Structure and function of the papillomavirus E6 protein and its interacting proteins

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1. ABSTRACT

Infection by high risk papillomavirus causes various forms of anogenital cancer including squamous cell carcinoma of the cervix. A primary step in the carcinogenesis includes formation of a complex of viral E6 protein and a human E3 ubiquitin ligase. This complex is competent to cause degradation and inactivation of several target proteins including human tumor suppressors which contributes to hyperproliferation of infected cells. Great insight on the mechanism by which E6 binds target proteins has recently been provided by determination of structures of interacting peptides and a E6 domain. These data have also provided a basis for the discovery of small molecules that can inhibit E6. However, there is still a need to further solve the structures of additional interacting complexes to identify the structural relationship that exists between proteins that simultaneously bind E6, such as E6AP and p53 or E6AP and PDZ domain-containing proteins, and to provide a clear picture of the interface between E6 and its ubiquitin ligase.

2. INTRODUCTION

Infection of epithelial tissues by human papillomavirus (HPV) causes cutaneous, mucosal, and anogenital warts. There are more than 100 genotypes of HPV, a subset of which are associated with the development of malignant lesions and classified as "high-risk" for their ability to promote cancer. DNA from high-risk HPV has been found in over 95% of cervical cancers. Among these, about 50% contain the HPV-16 genotype and another 20% have the -18, -31 or -45 types (1). High-risk HPV also plays a role in the pathogenesis of a subset of head and neck cancers (2). The low-risk viruses, such as HPV-6 and HPV-11 that are found in genital warts, or HPV1a that is found in common plantar warts, are rarely associated with cancer.

Papillomavirus infection is thought to begin with infection of the basal epithelium. In undifferentiated basal cells, the viral genome is maintained extra-chromosomally at low copy number. The viral genome is small, and

therefore its protein coding capacity is low; the virus hijacks cellular factors in order to replicate. As the daughter cells originating from the basal epithelium begin to differentiate and normally become non-permissive for DNA synthesis, the virus induces the G1 to S-phase transition to initiate synthesis of viral DNA and expresses early viral genes to prevent cellular stress responses such as p53 activation (3). Unscheduled cellular proliferation such as that caused by viral infection is normally a signal for cell death via apoptosis. The pro-apoptotic protein p53 is central to this defense mechanism by up-regulating expression of apoptotic proteins in response to cellular stress.

The high-risk papillomaviruses have evolved a mechanism to block the cellular stress response. The HPV-16 E6 protein in combination with the human ubiquitin ligase E6AP (E6-associated protein) forms a complex that specifically targets p53 for ubiquitin-mediated degradation (4-6). Additional E6AP-independent mechanisms are also likely to be utilized (7, 8). The E6 protein is known to be important for this process because high-risk HPV episomes encoding p53 degradation-deficient E6 proteins cannot replicate in skin keratinocytes (9, 10). Interestingly, low-risk E6, which binds but does not degrade p53, is also required for episomal maintenance (11). Neither (high-risk) E6 or E6AP alone binds p53 protein efficiently, although the E6/E6AP complex does. The minimal size for interaction comprises nearly full-length E6 (residues 1-142), amino acids 280 to 781 of E6AP (12, 13) and the central DNA-binding domain of p53 (residues 112-290) (14-16). Formation of the trimeric complex of E6AP, E6 and p53 is key for the promotion of tumorigenesis.

In addition to targeting p53 for degradation, the E6 protein plays other roles to promote viral replication (17). E6 disrupts cell cycle checkpoints to promote cellular proliferation (18-20). E6-expressing cells display increased telomerase activity that delays cellular senescence (21, 22). E6-induced transcription of the catalytic component of telomerase (hTERT) appears to involve E6AP, which may induce degradation of a repressive factor at its promoter (23-26). E6 protein also alters the transcriptional pattern at a variety of cellular and viral promoters, which seems to be mediated by its ability to form a bridge between E6AP and various target proteins (27, 28).

HPV-16 E6 uses a second, distinct pathway to promote cellular proliferation that is mediated by binding to members of the PDZ (PSD95/Discs Large/ZO-1) domain family of proteins including hDlg, MAGI-1, 2, and 3, CAL, MUPP1, and hSCRIB (29-33). The PDZ containing protein is then subsequently degraded by E6AP or another unidentified ubiquitin ligase (34-36). The PDZ interaction region is located in the C-terminus of high-risk HPV E6, which is not required for p53 binding or degradation (34, 37).

Reflecting the compact coding capacity of the viral genome and the requirement of each of its proteins to carry out multiple functions for successful establishment, maintenance, and proliferation in a host cell, E6 has been found to bind to many other cellular factors and carry out biochemical functions not necessarily related to ubiquitin-mediated protein degradation. These include ERC-55 (38), paxillin (39), E6TP1 (40, 41), Bak (42), AP-1 (43), Fibulin-1 (44), tuberin (45, 46), CBP/p300 (47), tumor necrosis factor receptor 1 (48), PKN (49), Tyk2 (50), IRF-3 (51), hAda3 (52, 53), FADD (54), and Pitx2a (55). While the significance of many of these interactions is not well understood, many of the factors share a homologous binding region with E6AP (56). The consensus sequence, also known as the charged leucine motif, is LxxLLGh, where L is leucine, G is glycine (sometimes alanine), and h is usually aspartate, asparagine, glutamate, or glutamine and xx is a dipeptide where one of the residues is aspartate, asparagine, glutamate, or glutamine (57). Some tolerance for any hydrophobic residue appears to be allowed for the first and second leucines in the binding motif for most cellular factors (45, 56, 57). More tolerance is allowed for substitutions of the hydrophilic residues of the charged leucine motif, although most mutations reduce binding. The compactness of the recognition sequence suggests that E6 binds a helical partner in the same way that MDM2 protein has been shown to engage a compact helical binding motif from p53 (58) or the way that BCLxL interacts with a short helix from the Bak protein (59).

The precise steps by which an HPV-induced papilloma induces the development of cancer are unclear. Nonetheless, persistent high-risk HPV infection is necessary and is associated with development of cancer. The fact that malignancies emerge after years to perhaps a decade or two implies that stochastic cellular mutations accumulate with persistent HPV. Malignancy may arise from stimulation of cell division by the E6 and E7 oncogene products, which are always expressed in cervical cancers. The E7 protein targets members of the Retinoblastoma (Rb) protein family and induces entry into the cell cycle (reviewed in (60)). As a result of E6 inhibition of normal p53 growth arrest prior to DNA repair, injured cells are hypothesized to accumulate DNA damage and pathogenic mutations. In late stages of dysplasia, the HPV genome is integrated in cervical cancer cells, and viral genes, except for E6 and E7, are disrupted or lost (61). This loss is claimed to release E6 and E7 from E2-mediated transcriptional repression but this has not been proven, as E2 does not repress E6 and E7 in the context of intact viral genomes and levels of E6 proteins are low in cervical cancers and derived cell lines and therefore difficult to measure (62, 63). Decreased expression of E6 mediated by RNAi results in growth arrest, senescence, and in some cases, apoptotic cell death of HPV positive cervical cancer cell lines (64, 65).

Vaccine-based strategies are based on the fact that the foreign HPV proteins represent targets for immune surveillance (66). Prophylactic HPV vaccines have shown exciting results in clinical trials (67). An effective therapy for HPV may also be beneficial against HIV, as HPV and HIV infections have long been recognized to co-exist (68). The immunosuppression associated with AIDS and the effects of HIV proteins on keratinocytes are thought to promote HPV infection (69). In turn, the warts associated

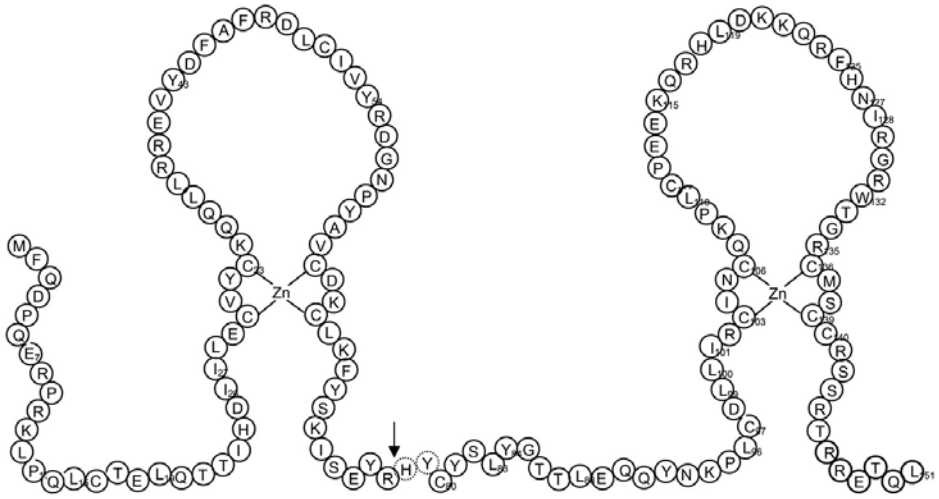

Figure 1. Domain structure and sequence features of the E6 protein of papillomavirus. The sequence and numbers are from papillomavirus type 16 (HPV-16). Amino acid residues are indicated by one-letter codes (143). The coordination between the zinc ions and the coordinating cysteines are indicated by short lines. A proteolytically sensitive site between the two sub-domains is indicated by an arrow. The structure for a fragment comprising residues 80 to 151 was solved by NMR methods (86). Residues described in the text are numbered by sequence number. The six residues at the C-terminus (residues 146-151) are re-numbered relative to the C-terminus (residues -5, -4, -3, -2, -1, 0) when described with respect to interaction with PDZ-domain containing proteins.

with HPV promote the formation of micro-abrasions of genital skin that are thought to effect HIV infection (69-71). A vaccine (or any inhibitor) effective against papillomavirus would therefore be predicted to decrease transmission of HIV (72). At the same time, an effective anti-HPV vaccine may also reduce the development of cervical and anal squamous carcinomas in the HIV-infected patient although a lack of immune competency may reduce its effectiveness.

There are major challenges to widespread acceptance and use of an expensive vaccine (73, 74) and a prophylactic HPV vaccine is not designed to provide benefit for the millions of women and men already infected. A therapeutic vaccine or inhibitor could take advantage of the fact that warts develop slowly and that progression of dysplasia to malignancy takes several years, providing a window of opportunity for an effective medical treatment. While therapeutic vaccines against E6 and E7 proteins have been developed, their efficacy in clinical trials has been modest (75-77). Because the activities of E6 are essential features of HPV-induced infection and oncogenesis, the inhibition of E6 function would appear to represent an attractive target for a therapeutic anti-viral drug to complement the currently available prophylactic vaccine. In addition, HPV has long been used as a model system for the study of fundamental processes in many cells. Therefore, the ability to manipulate the viral E6 protein with small inhibitory molecules also promises to provide new insights for understanding cell homeostasis (78). Understanding the mechanism by which E6 engages its molecular partners is clearly of value in the development of an antiviral agent that targets E6. This review describes the known structures of E6 and the molecules that interact with it.

3. E6 STRUCTURE AND FUNCTION

HPV E6 proteins contain about 150 amino acid residues characterized by two conserved internal sequence repeats. Each repeat is of the pattern $CXXCX_{29}CXXC$, where C represents cysteine and X represents any amino acid (Figure 1). Under reducing conditions, the cysteines chelate zinc atoms (79), which is required for activity (80, 81). Endogenous E6 in HPV-containing cells is expressed at very low levels (63). Consistent with its ability to target proteins located in either the nucleus (82) or the cytoplasm, E6 is subject to both nuclear import (83) and export processes (84).

Obtaining sufficient purified and homogeneous E6 protein for structural studies has been challenging. One problem has been associated with the high-cysteine content of the protein (14 cysteines in HPV-16 E6). Although mutation of the non-conserved cysteines to serine does not affect the ability the protein to participate in *in vitro* or *in vivo* p53 degradation (85, 86), the substitutions help only marginally with solubility of the protein (87). Protein solubility appears to be very sensitive to the level of zinc present in solution (88, 89). Treatment of E6 with the chelating agent EDTA results in an inability to degrade p53 (90), presumably by chelation of the zinc ions and disruption of the structure. However, treatment with EGTA yields monomeric protein capable of full activity when measured for p53 degradation in the presence of cellular extracts (88). Partially misfolded E6 protein fused to maltose-binding protein forms soluble material characterized by strong light scattering, consistent with large, but soluble, aggregates (91-94). The expression of E6 without a fusion tag, or separation from the maltose-binding protein fusion tag, tends to result in a precipitate

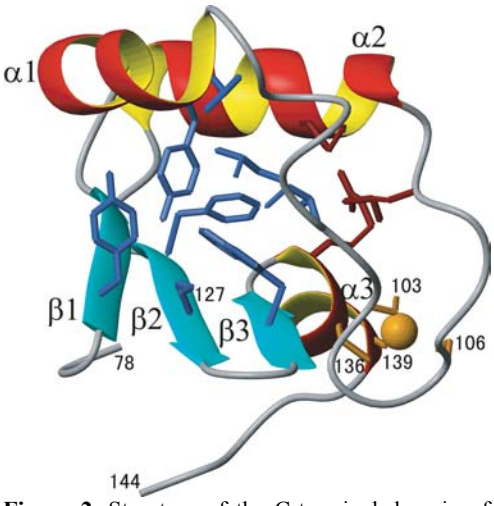

Figure 2. Structure of the C-terminal domain of HPV-16 E6 protein. Coordinates were reported for residues 80 through 144 and two of three N-terminal residues derived from the cloning construct (86). The side-chains of hydrophobic residues of the core are shown in blue and residues that extend the core are shown in red. Mutations at particular residues that are described in the text are indicated by sequence number and their amino acid side-chain. The zinc ion, shown as a space-filling sphere, is liganded by cysteine residues 103, 106, 136, and 139.

(94, 95). Some groups have had success using an N-terminal fusion to the B1 subdomain of protein G (88), although this fusion has not been characterized extensively. The oligomerization state of E6 may have important functional implications. In the prototypic HPV-containing HeLa and CaSki cells, monomeric forms of E6 were localized to the nucleus, whereas stable large oligomers containing approximately 60 protein molecules were found in the cytoplasm (96).

The E6 protein comprises two separately folded domains that function together as a whole (97). Evidence for a domain structure originated from an observation that each half of E6 had sequence similarity with each other (17 % identity), including preservation of the spacing of the CXXC motifs (98). The second observation was that full-length E6 is readily cleaved by proteases between residues 76 to 79 to form two protease-resistant domains (97, 99, 100). Unlike the difficulties associated with full-length E6, the separated domains are sufficiently well behaved in solution to be characterized biophysically. Circular dichroism measurements on each domain suggested a significant proportion of α-helix (97, 99).

Subsequently, the three-dimensional structure of the C-terminal domain of HPV-16 E6 comprising residues 80 – 151 was determined using NMR methods (PDB entry 2FK4) (86). The construct contained some non-native amino acids whose presence are unlikely to affect protein function. The substitution of four non-conserved cysteines (C80, C97, C111, and C140) to serine does not alter E6 function (86, 87, 91), and three amino acids derived from the cloning vector were added at the N-terminus which is unstructured (Figure 2). Relative to protein domains of similar size studied using NMR methods, the reported structure is relatively imprecise compared to other structures of similar size, but nonetheless has revealed much about E6 biology. The structure has roughly equal amounts of α-helix and β-sheet in an α/β topology (Figure 2), which was predicted by early secondary structure prediction (101). Two of the helices, α1 and α2, pack against one side of the small β sheet, while a short third helix, α3, provides two of the four cysteine side-chains (C136 and C139) that ligand a zinc ion. The other two cysteine side-chains (C103 and C106) are provided by a long loop connecting α1 and α2. The main part of hydrophobic core is located between the sheet and helices α1 and α2 and is constituted by the side-chains of residues L83, L96, L119, F125, N127, and W132. The hydrophobic core is extended somewhat by residues I101, L110, K115, and R135, which are contributed by the α3 helix and the loop of the zinc-binding region. The C-terminal residues (C140 to L151), which bind PDZ domains, are unstructured. The N-terminal 6 amino acid residues are absent in some human papillomaviruses, suggesting that this region, which is present in HPV-16 E6, is also unstructured (101).

HPV-16 shares approximately 55% sequence identity with the E6 proteins from the high-risk HPV types and about 35% identity with those of the low-risk types. In addition, the residues identified in HPV-16 E6 to constitute the hydrophobic core of the protein are conserved. Therefore, one would expect conservation of three-dimensional structure for the E6 across all HPV types. What differs among the E6 proteins, however, is their surface charge. The estimated isoelectric points of the different proteins range from pH 7.3 to 10.6, with the E6 proteins from all the high-risk types of viruses being the most basic. The mechanism by which large clusters of basic residues on the surface of E6 contribute to the oncogenicity of the virus is unknown, but presumably reflects the recruitment of negatively-charged molecules.

The structure of the N-terminal domain of E6 was modeled using the shared sequence similarity to the C-terminal domain of E6 (86). After allowing for an insertion near the N-terminus, hydrophobic residues in the N-terminal domain align with the hydrophobic residues found in the core of the C-terminal domain. Two hydrophobic residues in each domain are exposed to solvent (P13 and L15 in the N-terminal domain, and Y84 and L88 in the C-terminal domain), implying that they may form contact points between the two domains. A modeled structure of full-length E6 was created by burying these residues while arranging the 3-stranded β-sheets from each domain in an antiparallel manner to create a 6-stranded sheet. In the model, L15 contacts L99, L100, and N127 of the C-terminal domain and L88 contacts I26, I27, and Y54 of the N-terminal domain. In support of these contacts, resonances for residues S80 to S82 of the β1 strand and L100 at start of the loop connecting the helices α1 and α2 are selectively broadened upon addition of the N-terminal domain (86). The final details of the inter-domain

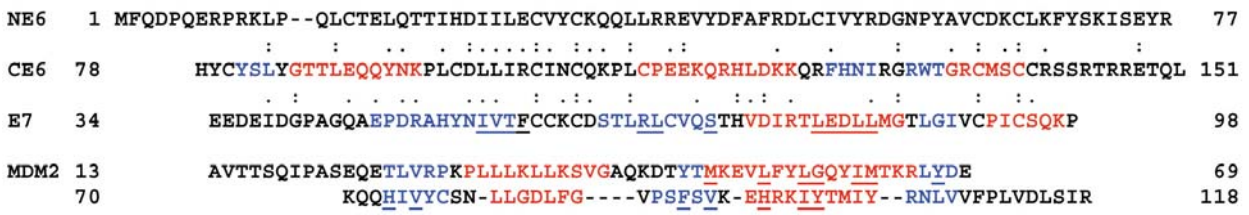

Figure 3. The sequences and secondary structures of the N-terminal domain of HPV-16 E6 (NE6), the C-terminal domain of HPV-16 E6 (CE6), the homologous region of HPV-16 E7, and human MDM2. The secondary structure assignment for HPV-16 E6 was taken from the NMR solution structure (86), that for HPV-16 E7 from the crystal structure of HPV1a E7 (106) and that for MDM2 from its crystal structure in complex with a peptide from p53 (PDB entries 1YCQ and 1YCR) (58). Alpha helices are shown in red whereas beta sheet regions are shown in blue. The E6 and E7 sequences were aligned based on their conserved CxxC motifs, with identities indicated by ":" and similarities by ".". The two symmetric halves of MDM2 and E7 were aligned to each other by centering the secondary elements, with no attempt made to align amino acid sequences. Amino acid residues in MDM2 that contact the helical p53 peptide are underlined. Amino acid residues in E7 critical for interaction with target proteins ($p21^{CIP1}$, Rb, or E2F1) are also underlined.

arrangement will most likely be found to be different when and if the structure of the full-length protein is determined as some mutations and residue conservations cannot be readily explained by the current model. For example, the relatively polar asparagine (N127) provides relatively little hydrophobic surface and is poorly conserved. In HPV18 E6, residue 127 is glutamate, which in the model would be placed near another glutamate at residue 27. In addition, although mutation of tyrosine 54 to aspartate or histidine would place a charge in a hydrophobic dimer surface and would be expected to disrupt structure and function, the mutant still degrades p53 and hADA3 (102, 103). Extensive site-directed mutagenesis modeled on this structure did not reveal an E6AP binding site using a GST pull-down assay as a read-out, suggesting that more than one mutation may be needed to disrupt binding. These mutants included surface residues (L19 to C33 and K121 to I128) in regions previously suggested to bind E6AP (102, 104). A much clearer understanding of E6-E6AP interaction may be obtained from structure determination of full-length E6 in complex with the E6-binding region of E6AP.

The E6 protein appears to have a relatively plastic structure adaptable to binding its target proteins. The structured portion of the C-terminal domain comprises residues 80 to 140. Assuming the alignment to the residues of the C-terminal domain shown in Figure 3, the N-terminal domain spans residues 9 to 67. If the homology is extended to the residues C-terminal to the structured portions, residues 68 to 79 (C-terminal to the N-terminal domain) might be expected to contain unstructured portions, which is consistent with the known site of inter-domain cleavage. Each of the structured domains is likely to show significant internal motion, as well as motion with respect to one other. In the C-terminal domain, a subdomain comprising portions of the β1 and β2 strands and the α1 and α2 helices show significant line broadening (89). Initial relaxation dispersion measurements indicate motion on the high μsec to low msec time scale. The model for the N-terminal domain structure shows that two of its residues (A46 and P59) are not as hydrophobic as the corresponding residues in the hydrophobic core of the C-terminal domain (L119 and W132), suggesting that the N-terminal domain is less stable than the C-terminal domain and therefore has significant mobility. On the other hand, the dynamic behavior observed in the C-terminal domain and that predicted for the N-terminal domain may not be reflected in the intact, full-length protein as the sequence for a portion of the inter-domain linker suggests an α-helical structure that may contact the β sheet and suppress the internal motions of the separated domains (86).

E6, which is a pseudodimer, and the CR3 domain of E7, which crystallizes as a dimer (PDB entry 2B9D) are of roughly the same size. They also function as oncoproteins by binding proteins and targeting them for degradation through the ubiquitin proteasome pathway (105). Because the E7 protein of papillomavirus shares 12% identity in amino acid sequence with HPV E6, including a preservation of the spacing of the cysteine residues in the zinc fingers, one may expect that the two proteins have similar structures (98, 101). Comparison of the E6 structure with the crystal structure of E7 reveals that they do not (106). Comparison of the secondary structures, for example, show little common in the way of shared features except that in both the first two liganding cysteines are on a loop and the last two liganding cysteines are on a short α-helix (Figure 3). Topologically, while the C-terminal domain of E6 is a small sheet with small alpha helices packed against one side, the crystal structure of monomer of the E7 CR3 domain is a two-stranded β sheet with a single α-helix packed against one side. The structural differences between E6 and E7 suggest an extreme case of divergent evolution. The solution structure of HPV-45 E7 protein (PDB entry 2F8B) shows similar structural features as HPV1a E7 (107).

The question may arise whether other proteins that have related functions to E6 or E7, such as the cellular oncoprotein MDM2, have similarity in structure. MDM2 directly binds to a helix in the transcriptional activation region of p53, leading to proteolytic degradation through the ubiquitin proteasome pathway (58, 108), with different

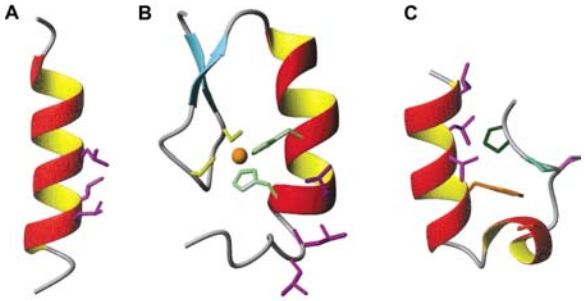

Figure 4. The structures of peptides that inhibit the ability of E6 to associate with E6AP. A. The structure of the 18-residue binding region (residues 391-408) of E6AP protein (PDB entry 1EQX) determined in the presence of 40% trifluoroethanol. The side-chains of the leucine residues of the consensus binding sequence are shown in magenta (116). B. The structure of the binding region of E6AP fused to the C-terminus of a zinc finger (PDB entry 1RIM) solved in aqueous solution (111). Side-chains are shown for the residues (cysteine in yellow; histidine in green) that ligand the zinc ion and the side-chains of leucine residues of the consensus binding sequence grafted onto the zinc finger scaffold are shown in magenta. C. The structure of the binding region of E6AP fused to the N-terminus of Trp-cage structure (PDB entry 1RIJ) solved in aqueous solution (111). Side-chains are shown for the central tryptophan and proline residues of the cage. The side-chains of leucine residues of the consensus binding sequence grafted onto the Trp-cage scaffold are shown in magenta.

patterns and requirements for ubiquitination than HPV E6/E6AP (109, 110). Like the E6 protein, MDM2 is a pseudo-dimer of repeating structure. However, the secondary structural elements are different in MDM2, with each structure comprising a β strand, a short helix, a second β strand, a longer helix, and a third β strand, with the first and second strand forming a sheet and the long helix packing against one face (58). On the other hand, the secondary structure elements of MDM2 can be aligned quite well with those of E7, except that the amino acids corresponding to the first helix of MDM2 form a loop in E7 (Figure 3). There is little sequence similarity, except for a general pattern of hydrophobic residues. Most of the contacts made by MDM2 to its interacting peptide from p53 are made by the long α helix and the immediately preceding β strand. The proposed binding sites of E7 for three of its interacting proteins ($p21^{CIP1}$, E2F and Rb) have also been mapped to its α-helix and the immediately preceding β-strand. The significance of this observation is unclear, but may merely reflect the available surface features of a small domain for binding to target peptides.

4. PEPTIDE INTERACTORS

The structure of several peptides containing the "charged leucine" E6-binding motif have been determined in the absence of E6 (56, 87, 111). The simplest peptide contains residues 391 to 408 of the 865 amino acid E6AP protein and contains the essential binding motif (a LQELLGE sequence). In both aqueous medium and in media containing trifluoroethanol, the E6-binding motif forms an alpha helix (PDB entry 1EQX) (87). The three leucines of the binding motif form a hydrophobic surface on one face of the helix and the charged amino acids lie on the opposite face (Figure 4A). Replacement of any leucine in the binding motif by alanine disrupts binding to E6, as evidenced by the inability of mutated E6AP to interact with GST-E6 (87). Mutations in polar residues that reside on the helix opposite the hydrophobic surface weaken binding, indicating that these residues are also somewhat important for function (112). It should be noted that the three-dimensional structure for the C-terminal region of E6AP (residues 495 to 852) containing the HECT (Homologous to the E6AP Carboxyl Terminus) domain with its ubiquitin E3 ligase activity has also been determined by crystallographic methods (PDB entry 1C4Z) (113). This C-terminal region is not involved in complex formation with HPV E6.

The structures of peptides homologous to the E6-binding region of E6AP have also been determined. One is derived from E6BP/ERC-55 which forms a classic calcium-binding EF-hand structure (56). The E6 binding motif comprises the sequence LEEFLGD. As in the case of E6AP, mutation of any of the hydrophobic residues within the core abolishes binding, whereas mutation of hydrophilic residues to alanine decreases interaction. The effect of mutations observed in another binding protein, paxillin, which contains the binding sequence LDALLAD, was qualitatively the same as in E6AP or E6BP, except that quantitatively the loss in binding was larger (57). E6-binding peptides have also been derived from a random peptide library optimized on an initial hit from an earlier screen (45). The optimal peptide, WEGVFDELLGM, contained the E6 binding motif FDELLGM, with the leucine and glycine residues being strongly selected for binding. NMR analysis indicated a helical structure extending from the N-terminus to the glycine within the binding motif (114).

The exact conformation of the E6 binding motif appears to be flexible. Short, poorly structured peptides of only 8 amino acid residues containing only the core binding sequence retain the ability to inhibit the interaction of E6 with E6AP (111). A recent molecular dynamics simulation shows a fluctuating helical structure in the E6AP 18mer peptide (115). Adaptability of the structures is suggested by the success of one-shot protein grafting that were used to create monomeric E6-binding ligands that are structurally stable in aqueous solution without significantly losing activity. The core binding sequence was added to two scaffolds: an exposed helix present in C-terminus of a zinc finger (PDB entry 1RIM) (Figure 4B) and a helix in the N-terminus of a Trp-cage peptide (PDB entry 1RIJ) (Figure 4C) (111). The structures are slightly different suggesting that conformational changes in peptides are required for binding E6. For example, in the Trp-cage structure, the side-chains of the key "E6-binding" L5 and L6 residues extend inside the structure, sandwiching P22 between them. The side-chain of L6 also contacts the side-chain of W9 in a hydrophobic interaction. A similar phenomenon was observed in the structure of E6BP, for which the side-

chains of the three key hydrophobic residues also reside inside the structure, in this case forming part of a dimer interface (56) (unpublished results). One explanation is that the leucine residues interact to stabilize the internal structure of E6AP or E6BP and that the initial binding to E6 actually occurs on the opposite face. The mutagenesis data argue against this hypothesis because replacing a single leucine does not appreciably change the structure (116), but abrogates binding, whereas mutagenesis of residues on the opposite face has much less of an effect. A more likely explanation is that binding of E6AP to E6 protein is not executed by single event involving binding to the three leucine residues but instead requires two or more steps. Initially, one or more residues may interact with E6 protein to induce a conformational change that exposes the leucine residues and matches the binding interface. Additional experiments are needed to determine which residue (or residues) in the binding motif makes contact and how conformational change corresponds to the binding of ligands with E6 protein.

Additional peptides have been described that bind HPV E6 and antagonize its function. An alternatively spliced variant of E6 comprising residues 1 to 43 plus an additional 14 amino acids (E6*I) has been observed to inhibit the functions of full-length E6 (104, 117-120). E6*I contains the residues of the β1 strand of E6 (as well as the first helix and part of the second helix) which represent a part of the dimer interface predicted to exist between the two domains of E6. Therefore, its proposed mode of action is to mimic the N-terminal domain of E6 and form a pseudo-dimer with the C-terminal domain of the protein. Using HPV-16 E6 for an interaction screen against a constrained peptide library, about one third of the peptides contained the single CxxC motif found in E6*I (121), and may inhibit using a similar mechanism. While some peptides in the library contained binding motifs resembling the binding motif consensus sequence of E6AP and E6BP, others did not, which may represent novel modes of interaction with E6. These latter sequences are rich in glycine and leucine and valine, and are similar to the amino acid sequence at the C-terminus of Bak, which binds to HPV-18 E6 (122). These peptides may function by sequestering E6 into inactive aggresomes (123). Others have generated intracellular single-chain Fv antibodies against HPV-16 E6 that were specific for the N-terminal domain (124).

5. SMALL MOLECULE INHIBITORS THAT TARGET E6 PROTEIN

The structural and functional features of E6-binding peptides have been used to select inhibitory non-peptidic compounds (125). In this study, the three-dimensional structures of E6AP and E6BP inhibitory peptides (56, 116) were transferred to 3D queries needed for database searches of chemical structures. The critical points in space predicted to be necessary for activity (the pharmacophore) were derived by coupling structural information with evolutionary conservation and structure-function data for each amino acid side-chain of the peptides. The static pharmacophore comprised three lipophilic points, two hydrogen-bonding points, and one exclusion sphere. Queries of the National Cancer Institute (NCI) open chemical database and the Sigma-Aldrich Library of Rare Chemicals yielded 73 available compounds within the selection criteria. Testing for the inhibition of HPV-16 E6 binding to E6AP, using variations of methods described earlier (126) and p53 degradation resulted in 10 inhibitory compounds with *in vitro* activity. The respective IC_{50} values were as low as 10 μM. One of the compounds, a polyaromatic dye, showed modest inhibitory activity of E6-mediated p53 degradation in a keratinocyte cell line (NIKS) (125).

A second approach to inhibit E6 took advantage of E6's requirement for zinc (90). Several dithio or azo compounds were observed to effectively eject zinc from E6 and inhibit *in vitro* binding with an IC_{50} of about 10 μM. The inhibition of E6 would be expected to suppress ubiquitin-mediated degradation of p53 and increase its level. A dithiodimorpholino compound showed increased p53 levels in HPV-containing HeLa and SiHa cell lines, but minimal increases in control, non-HPV containing HaCat and MCF7 cell lines. A more extensive survey of organic disulfides confirmed the inhibitory activity, but did not result in the identification of a more inhibitory compound (127). The mechanism by which these organic disulfides eject zinc from E6 is not fully understood. One possibility is that the disulfide may cleave to generate a free radical monomer that reacts further to form a mixed disulfide with a zinc-coordinating cysteine of E6 protein. Another possibility is that the disulfide acts as an electrophile to directly attack the sulfur atoms of a zinc-coordinating cysteine.

6. PDZ DOMAIN INTERACTORS

High-risk E6 proteins use their C-terminal PDZ-binding motif (Figure 1) to bind certain PDZ domain containing proteins and target them for subsequent proteasomal degradation. The ability to degrade PDZ domain containing proteins correlates with the oncogenic potential of E6 (128). Through the degradation of hDlg, for example, the high-risk HPV E6 proteins alter cell growth and cell polarity in response to cell contact (129). A PDZ domain generally contains six β-strands and two α-helices. In structures of PDZ domains in complex with an interacting peptide, the peptide typically lies in a groove formed by the longest α-helix and the second β-strand (β_2) of the PDZ domain and forms an additional β strand antiparallel to the β_2 strand (130). The binding is predominantly contributed by the C-terminal 3 or 4 residues suggesting that a short peptide is a good mimic for the interacting protein.

Structures of individual PDZ domains of hDlg/SAP97 and MAGI-1 have been determined recently in complex with the PDZ-binding region of HPV-18 E6 by crystallographic methods (PDB entries 2I04, 2I0L, and 2I0I) (131) and by NMR (PDB entry 2OQS). Of the three PDZ domains of hDlg, the PDZ2 domain appears to be most important for binding both HPV-16 E6 and HPV-18 E6, and therefore the discussion here is focused on the crystal structure of PDZ2 bound to a peptide from HPV-18

E6 (131). E6, containing a Class I PDZ-binding sequence (-S/T-x-ϕ, where ϕ is a hydrophobic residue), binds using the canonical binding mode. There are, however, significant differences in structure. The six conserved amino acid residues of HPV E6 were observed to interact with the PDZ domain, instead of the usual three or four residues observed in other peptide-PDZ complexes. A novel conformation was observed for a loop (the BC loop) connecting two β strands that allowed contact to the highly conserved arginine at the -5 position of the peptide representing HPV E6 (R147 in HPV-16 E6, R154 in HPV-18 E6). High-risk HPV E6 proteins also contain a consensus protein kinase A (PKA) recognition site (RXX[S/T] or RX[S/T]) that overlaps with the PDZ-binding motif in high-risk HPV E6, but not low-risk E6. The arginine at the -5 position is likely to contribute to E6 function as its mutation (to leucine, valine, or glycine) suppresses the ability of E6 to degrade hDlg (128, 131). Residue T149 at the -2 position of HPV16 E6 (T156 in HPV18 E6) is highly conserved in almost all high-risk HPV E6 proteins and its replacement by valine prevents phosphorylation by PKA (132). Although no direct binding measurements of phosphorylated and non-phosphorylated E6 peptides have been made, a free hydroxyl on the threonine appears to be important for binding the PDZ domain, as phosphorylation by PKA disrupts E6-mediated turnover of hDlg (132). In addition, the overlapping binding sites indicate that the binding of HPV E6 to PKA and hDlg may be competitive and thus regulate critical biological functions.

7. MISCELLANEOUS INTERACTORS

Unlike the detailed information known for several complexes described in the preceding sections, several other complexes have been identified for which the molecular partner that binds E6 protein is known, but the roles of individual amino acids in the interaction have not been yet well defined. Together, they illustrate the multitude of cellular functions that the HPV E6 protein must make in order for the virus to successfully propagate itself. Brief descriptions on the domain level for protein interactors of known structure have been selected to be included here based on their relevance to E6 biology.

High-risk HPV E6 proteins may function by forming a quaternary complex comprising the E6 protein (about 150 amino acid residues), a PDZ-domain containing protein (the domain comprising about 100 amino acid residues), a domain of E6AP (about 500 amino acid residues), and the central core of p53 (residues 112-290). The core of p53 is its DNA-binding region, and its structure (residues 102 to 292) has been determined using crystallographic methods (PDB entry 1TSR and 1TUP) (133). E6 in complex with p53 fails to recognize its usual DNA sequence (134, 135), thus suggesting that the regions of p53 that contact or are near DNA are also important for binding E6. In addition, mutation of at least one surface amino acid residue (C277) that makes a direct contact to DNA (133) also disrupts the ability of E6 to bind E6AP (136).

As observed with other viral proteins, E6 associates with CBP and the highly related protein p300, which are co-activators that function in regulation of the cell cycle and in differentiation (137, 138). The C/H3 domain of CBP, the C-terminal region of CBP and the C/H1 domain of p300 interact with E6 (138). Known structures for the C/H1 domain (PDB entries 1UDN and 1L3E), and the C/H3 domain (PDB entry 1F81) contain novel helical folds and bind helical peptides (139). The individual amino acid residues needed for the E6/p300 interaction are not well characterized. E6 inhibits p300-mediated acetylation of p53 which requires the E6-interacting regions of p300 (140). Thus E6 can down-regulate the activity of p53 by either acting as a bridging protein that allows the ubiquitin ligase activity of E6AP to mediate degradation of p53, or by antagonizing p53-mediated activation of cellular target genes by repressing acetylation through p300.

E6 is known to alter transcriptional patterns, most likely by the targeted degradation of specific transcription factors (24, 28, 52, 138, 141), which can, for example, result in activation of telomerase (21, 24-26). However, in keeping with the positive charge of its C-terminal domain, the direct and specific binding of E6 to DNA has also been observed (95) and appears to be specific for cruciform or four-way junction DNA (86, 100, 142). The *in vivo* significance of this *in vitro* observation has not yet been demonstrated.

8. REMARKS AND PERSPECTIVES

Although we do not yet have the structure of any complexes with HPV E6, the determination of ligand structures has been useful for the discovery of small drug-like compounds (125) and inhibitory peptides (121). Obstruction of the E6AP-binding pocket on E6 appears to be an attractive strategy for the development of antiviral agents as E6AP appears to be the primary protein needed to dock to E6 in order to direct the degradation of PDZ domain-containing proteins, p53, and others. Although one might imagine blocking the binding pockets on cellular proteins, such as that of the PDZ domain, compounds that target a pocket on a viral protein would appear to provide a better opportunity for development as viral-specific inhibitors.

The mobile nature of E6 that is emerging may be a double-edged sword. Because of mobility, large differences in protein conformation will likely be observed as structures of different molecular complexes with E6 are determined. The docked structure of a particular ligand may thus provide little utility for guiding modifications to the ligand, as the ligand-protein interface may change for the complex with each new ligand, thus complicating the ability to create a tight-binding inhibitor of E6. On the other hand, the different binding modalities would then provide a rich and interesting field of research for many structures to come.

9. ACKNOWLEDGEMENTS

This work was supported in part by NIH grant AI38001.

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Abbreviations: E6AP, E6-associated protein; HECT, Homologous to the E6AP Carboxyl Terminus; HPV, human papillomavirus; hTERT, catalytic component of telomerase; NMR, nuclear magnetic resonance; PDZ, PSD95/Discs Large/ZO-1; Rb, Retinoblastoma

Key Words Virus, Cancer, Tumor, Carcinogenesis, Papillomavirus, Three-Dimensional Structure, NMR spectroscopy, Review

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