Molecular interactions of 'high risk' human papillomaviruses E6 and E7 oncoproteins: implications for tumour progression

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The aetiology of cervical cancer has been primarily attributed to human papillomaviruses (HPVs). These are characterized by the persistent expression of the two oncogenes, E6 and E7. Experimental studies show that *E6* and *E7* genes of the high risk HPVs deregulate key cell cycle controls. Recent work has uncovered new cellular partners for these proteins that throw light on many of the pathways and processes in which these viral proteins intervene. This review focuses on the regulation of host proteins by the viral oncoproteins and consequence of such interactions on cell survival, proliferation, differentiation and apoptosis.

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1. Introduction

Papillomaviruses are a heterogeneous group of viruses. They are small double stranded DNA viruses that infect mucosae and cutaneous surfaces, causing warts and epithelial tumours (zur Hausen 1996). Human papillomaviruses (HPVs) are the primary cause of cervical cancer, the second most prevalent cancer in women worldwide. The HPVs can be divided into two groups, the 'low risk' (such as type 6 and 11) and the 'high risk' (such as type 16 and 18) (zur Hausen 1985). Persistent HPV infection with a high risk virus is essentially observed in cervical intraepithelial neoplasia (CIN) II and III stages (Ho *et al* 1995; Koutsky *et al* 1992). There is sufficient morphological and epidemiological consensus for the assumption that CIN III is a dynamic disease in which some cases regress spontaneously, whereas others progress to the invasive state, invasive cervical carcinoma (ICC) (Gustafsson and Adami 1989; Ostor 1993; Mitchell *et al* 1994; Moreno *et al* 1995). The rate of progression of the disease from CIN III to ICC varies from approximately 12–69%, according to various reported studies. This tumour formation is not an inevitable consequence of viral infection; it rather reflects the multi-step nature of oncogenesis where each step constitutes an independent (reversible or irreversible) genetic change that cumulatively contributes to deregulation of cell cycle, cell growth and survival. HPV infections represent one of these steps and only if the other steps occur in the same cell, it might develop into a cancer (figure 1).

The two early HPV genes, *E6* and *E7*, play crucial role in tumour formation. Both *in vitro* and *in vivo* studies show that the function of E6 and E7 proteins, particularly of the 'high risk types', are essential for cellular transformation (Bedell *et al* 1987; Matlashewski *et al* 1987; Vousden *et al* 1988; Watanabe *et al* 1989; Herber *et al* 1996;

Keywords. E6; E7; regulation of cellular proteins; transformation

Abbreviations used: Ad, Adenovirus; AP, associated protein; APC, adenomatous polyposis coli; CBP, CREB binding protein; CIN, cervical intraepithelial neoplasia; HDAC, histone deacetylases; HPVs, human papillomaviruses; hTERT, human telomerase catalytic subunit; ICC, invasive cervical carcinoma; IGFBP-3, insulin-like growth factor binding protein-3; IRF-3, interferon regulatory factor-3; M2-PK, M_2 pyruvate kinase; Rb, retinoblastoma; TAF-110, TBP-associated factor-110; TGF, transforming growth factor; TNF-α, tumour necrosis factor-α.

Song *et al* 1999). E6 and E7 from 'low risk' types are weaker transforming oncogenes (Farr *et al* 1991; Mansur and Androphy 1993). The best evidence for the involvement of E6 and E7 in malignancy is from biochemical studies. These oncogenes from the 'high risk' viruses have been shown to alter pathways involved in cell cycle control, interacting with and neutralizing the regulatory functions of two important tumour suppresser proteins, p53 and Rb (Vousden 1993), and also in conjunction with cellular genes, like *Notch1* (Rangarajan *et al* 2001), deregulating key signal transduction pathways. This review will focus on the ability of E6 and E7 from the 'high risk' viruses to alter cellular proliferation, resulting from apoptosis evasion and efficient survival mechanisms.

2. E7 protein

HPV 16 E7, a nuclear protein of 98 amino acids, has casein kinase II phosphorylation sites at serine residues 31 and 32. It is divided into three domains, CR1, CR2 and CR3, based upon homology with adenovirus E1A. CR1 is composed of amino acids 1–20, CR2 contains residues 21–39, and CR3 has 40–98 amino acid residues. E7 dimerizes in the CR3 domain via a zinc-finger motif, this motif being essential for its proper folding and viability (figure 2).

E7 interacts with various proteins, most of which are important regulators of cell growth, especially the transition of a cell from the G1 to S-phase of mitosis. Biochemical studies show that E7 associates with retinoblastoma tumour suppresser family proteins (Rb, p107, p130, histone deacetylases (HDAC), AP-1 transcription factors, TATA box binding protein (TBP), cyclins, cyclin-dependent kinases (cdks) and cdk inhibitors, M_2 pyruvate kinase (M2-PK). These interactions are the likely ways in which E7 deregulates cell cycle and hence leads to increased cell proliferation, immortalization and finally transformation. The plausible ways in which E7 affects cell cycle and survival will be discussed in this review (figure 2).

Studies in rodent and human primary keratinocytes have shown that expression of 'high risk' HPV E7 is sufficient for immortalization of these cells, while the expression of E7 along with E6 in primary human foreskin keratinocytes (HFKs) causes cell transformation (Barbosa and Schlegel 1989; Munger and Phelps 1993). *In vitro* studies also show that E7 can overcome several growth arrest signals including DNA damage, serum starvation, anoikis induced cell death, inhibition by transforming growth factor-β (TGF-β) mediated signalling and suprabasal differentiation.

3. Evasion of tumour suppression by E7

3.1 *Association with retinoblastoma family proteins*

The ability of E7 to bind to members of the retinoblastoma (Rb) protein (p107, p130) is perhaps the most characterized property of this viral oncoprotein (Dyson *et al* 1989a,b; Munger *et al* 1989; Davies *et al* 1993). Evasion of cell cycle arrest by binding to Rb is a property common to various viral oncoproteins, such as adenovirus E1A and simian virus SV40 large T antigen (Munger 1995), this underscores the importance of Rb binding in the natural history of virus infection. Rb protein family members play a pivotal role in eukaryotic cell cycle regulation. Hypophosphorylated Rb binds to transcription factors of the E2F family and represses the transcription of particular cell cycle genes. When cells progress in mitosis from G0 through G1 to S-phase, Rb gets hyperphosphorylated by G1 cyclin–cyclin dependent kinases, releasing the transcription factor E2F, which in turn activates genes

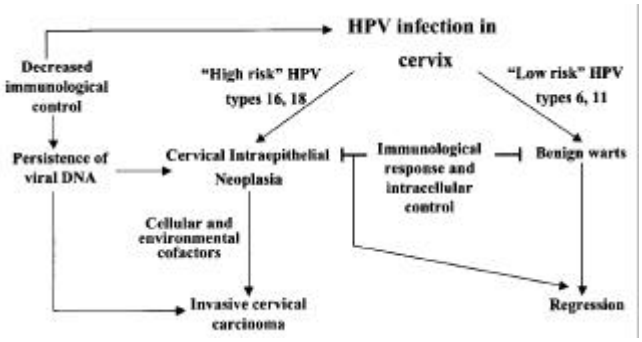

Figure 1. Host response to HPV infection. 'High risk' and 'low risk' HPVs cause different epithelial changes. While former are responsible for invasive carcinomas, the latter form benign warts which regress over time.

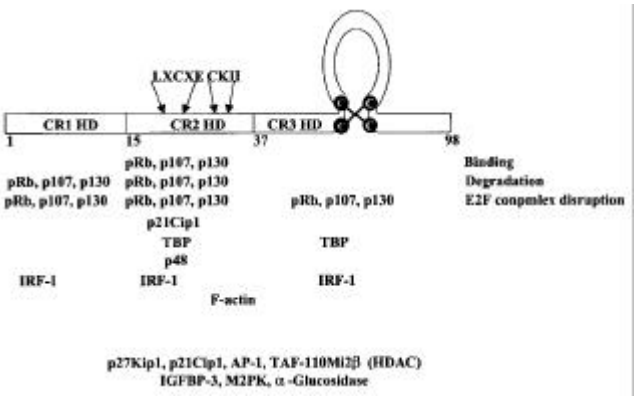

Figure 2. HPV 16 E7 protein. The sequence of the protein along with its various cellular targets has been shown.

involved in DNA synthesis and cell cycle progression (Dyson 1998). E7 binds to hypophosphorylated Rb and thereby induces cells to enter into premature S-phase by disrupting Rb-E2F complexes (Huang *et al* 1993; Wu *et al* 1993; Patrick *et al* 1994).

Recent reports in the Rb-E2F literature highlight the complexities of these interactions and hence the importance of members of these families in transition through cell cycle. p130/E2F complexes are present most abundantly in quiescent or differentiated cells, while p107/E2F and Rb/E2F are more predominant in cells entering G1 and S-phase (Dyson 1998). Both p130 and p107 are required for the regulation of different genes (Hurford *et al* 1997). E7 interacts with both these genes via the CR2 domain (Dyson *et al* 1992; Davies *et al* 1993), hence it is involved in both differentiation and cell proliferation, suggesting that this oncoprotein can uncouple the processes of differentiation and cell cycle progression by transcription of different subsets of genes. Thus providing an environment more conducive to viral replication.

Recent reports suggest that though abrogation of Rb function by E7 is important for cellular transformation, it may not be an absolutely essential requirement, thus suggesting the involvement of other cellular factors in the process of immortalization and cellular transformation.

3.2 *Association with histone deacetylase complexes*

An important mechanism of regulation of gene transcription is by chromatin remodelling through histone acetylation. Actively transcribed genes have a high level of histone acetylation (Grunstein 1997). Recent reports suggest that Rb can associate with HDAC-1 and then both co-operate to repress the E2F mediated transcription machinery (Brehm 1998; Magnaghi-Jaulin *et al* 1998). E7 interacts with HDAC-1 through the CR3 zinc-finger domain. This association is independent of Rb-interaction. E7 associates with HDAC-1 indirectly through Mi2β, a component of the NURD histone deacetylase complex (Brehm *et al* 1999). Mutations in the zinc finger domain which affect Mi2β/HDAC complexes that do not affect the direct Rb binding of E7, nevertheless abrogate the ability of E7 to relieve the Rb mediated repression through yet unclear mechanisms (Brehm *et al* 1999). Targeting of histone deacetylases thus provides another method by which E7 can de-repress gene transcription and may also explain the essential nature of the CR3 domain in activating E2F-regulated genes as well as immortalizing keratinocytes.

3.3 *Interaction with AP-1 transcription factors*

Other Rb independent functions of E7 involve the binding of E7 with members of the AP-1 transcription factors, like c-Jun, JunB, JunD and c-Fos, all crucial in mediating early mitogenic effects and keratinocyte differentiation. Mutational analysis studies indicate that the zinc finger domain of E7 is required for these binding interactions (Antinore *et al* 1996). E7 binds to c-Jun and transactivates transcription from the c-Jun promoter. Effect of E7 on cell cycle progression via AP-1 transcription factors can be Rb-independent (as already mentioned) or dependent. In a Rb-dependent mechanism, c-Jun promoter, itself containing AP-1 sites, gets regulated by Rb/c-Jun complexes (Nead *et al* 1998). Recruitment of c-Jun by Rb to AP-1 sites, leads to activation of transcription from c-Jun responsive promoters. In the presence of E7, the activation of this transcriptional machinery is inhibited. Hence it is evident that a fine balance exists between these that govern cell cycle progression and differentiation. On one hand, E7 binds to c-Jun independently of Rb, potentiates transactivation of genes involved in early cell cycle progression and hence mitosis. On the other hand, through an Rb-dependent mechanism, E7 deregulates keratinocyte differentiation by disrupting Rb/c-Jun complexes.

3.4 *Interaction with TBP and TAFs*

E7 not only interacts with proteins that are important regulators of transcription during cell cycle, it also binds to members of the basal transcriptional machinery, for example TBP and TBP-associated factor-110 (TAF-110) (Mazzarelli *et al* 1995; Massimi *et al* 1996; Phillips and Vousden 1997). The significant of these interactions and their effect on gene transcription are yet unclear, but still suggest that E7 can have a more global effect on the general transcription factors.

3.5 *Effects of E7 on cyclin-Cdks*

Though HPV infects the dividing basal layer of cells, viral genome amplification occurs only in differentiating cells. Hence, the virus must devise ways to manipulate the host DNA replication machinery. In proliferating cells, the G1/S-phase cyclins and the cyclin-dependent kinases (cyclin D-cdk 4, cyclin E-cdk 2, cyclin A-cdk 2) are the key regulators of DNA synthesis and cell cycle progression (Sherr 1993; Sherr and Roberts 1999). In a dividing cell, mitogenic stimulation leads to synthesis and assembly of cyclin D-cdk 4 complexes which contribute to the phosphorylation and hence inactivation of Rb, increased expression of cyclin E, and sequestration of cdk 2 inhibitors of the Cip/Kip family. Cyclin E-cdk 2 continues to inactivate Rb and also phosphorylates substrates important for DNA synthesis and S phase entry. Cyclin A-cdk 2 is assembled during S phase and remains active through the G2 phase. E7, in the expected way affects these cyclin-

cdk complexes. The effects of E7 on each of these complexes are discussed below.

Cyclin E-cdk 2 is essential for DNA synthesis (Ohtsubo *et al* 1995). *In vitro* studies in primary human foreskin keratinocytes show that E7 expressing cells express increased levels of cyclin E-cdk 2 activity (Funk *et al* 1997; Jones *et al* 1997; Ruesch and Laimins 1997, 1998). When these cells are subjected to stress signals, like serum deprivation or anchorage independence, cyclin E-cdk 2 activity is maintained. Mutations in the CR1 domain of E7 abolishes this phenotype and, hence, leads to impaired cellular immortalization and transformation (Ruesch and Laimins 1998; Schulze *et al* 1998). E7 deregulates transcriptional levels of cyclin E due to loss of E2F mediated repression; it can also affect the post-transcriptional levels of cyclin E (Zerfass *et al* 1995; Botz *et al* 1996; Martin *et al* 1998). But, deregulation of cyclin E primarily by transcriptional upregulation is not the only way in which E7 affects this protein. There is also an increase in the levels of cdk 2-associated $p21^{CIP}$, an inhibitor of cdk 2. Biochemical studies show that E7 can physically associate with p107-cyclin E-cdk 2 complexes. *In vitro* and *in vivo* studies also suggest that E7 can bind to $p21^{CIP}$ and $p27^{KIP}$ (Funk *et al* 1997; Jones *et al* 1997), though it is yet unclear whether these interactions yield active complexes *in vivo*. The significance of such interactions is yet to be established fully.

E7 protein has been shown to bind to cyclin A-cdk 2 in a cell cycle dependent manner, with maximal activity in the S and G2 phases (Tommasino *et al* 1993). E7 may interact with this kinase complex through p107, though *in vitro* data also suggests that E7 can physically interact with cyclin A (Tommasino *et al* 1993). E7 expressing cells under stress synthesize cyclin A. E7 can also bind to E2F, with or without the involvement of p107 (Krek *et al* 1994; Tommasino and Crawford 1995). It is possible that E7 diverts the cyclin A dependent kinases away from the normal cellular substrates. Cyclin A-cdk 2 interacts with and phosphorylates E2F, leading to its loss of DNA binding capability. Hence it can be speculated that when E7 interacts with cyclin A-cdk 2, it prevents inactivation of E2F, permitting the cell to bypass normal cell checkpoints with consequent loss of DNA replication fidelity. This might also explain for the increased chromosomal abnormality in E7 expressing cells (White *et al* 1994).

Effect of E7 on cyclin D-cdks depends upon cellular contexts as cyclin D can associate with various cdks and its levels do not vary like the other cyclins during the cell cycle.

3.6 *Interaction with M2-PK*

Though E7 is known to primarily bind to nuclear proteins, mutational studies show that transformation deficient E7 is also defective in its interaction with a cytoplasmic enzyme M2-PK (Zwerschke *et al* 1999). Proliferating cells express M2 type isoenzyme of pyruvate kinase (PK), the key enzyme controlling the exit of the glycolytic pathway. *In vitro* experiments show that E7 expressing cells have a significant increase in the total glycolytic rate, and an increased rate of conversion of glucose into lactate (Zwerschke *et al* 1999). This implies that E7 ensures channelling of glucose carbons to synthetic processes, and at the same time reduces the cell's requirement of oxygen: two important properties of tumour cells.

3.7 *Activation of α-glucosidase*

Transformation competent, 'high risk' E7 acts as an allosteric activator of acid α-glucosidase (Zwerschke *et al* 2000). Changes in the cellular carbohydrate metabolism are hallmarks of malignant transformation; represent one of the earliest discernible events in tumorigenesis. In the early stages of certain epithelial cancers, a metabolic switch is regularly observed, in which slowly growing glycogenotic cells are converted to highly proliferating basophilic cells. This step is accompanied by a rapid depletion of the intracellular glycogen stores; which results from the activation of the enzyme acid alpha-glucosidase by an as yet unknown mechanism. The catalytic activity of this glycogen-degrading enzyme is directly controlled by E7 protein.

4. Effect of E7 on apoptosis

Although there is substantial evidence to support the oncogenic potential of E7, and also that E7 by itself can immortalize cells, there are also reports which show that E7 can strongly sensitize keratinocytes to apoptosis. E7 is known to uncouple E2F function from Rb regulation. Deregulated E2F activity in turn induces apoptosis, possibly by a p53 independent mechanism. When expressed alone, E7 induces p53 expression in post M1 crisis ('extended life span') cells (Demers *et al* 1994). Along with this, expression of E7 alone is sufficient to induce susceptibility to lysis by activated tumoricidal macrophages (Banks *et al* 1991).

There are contradictory results regarding the effect of E7 on tumour necrosis factor-α (TNF-α)-mediated apoptosis. While it sensitizes primary human keratinocytes to TNF-α-induced apoptosis (Stoppler *et al* 1998), it impedes the process of cell death in normal human fibroblasts (Thompson *et al* 2001).

E7 has also been shown to interact with insulin-like growth factor binding protein-3 (IGFBP-3). This p53-inducible gene, overexpressed in senescent cells, can suppress cell proliferation and induce apoptosis. E7 binds to

and triggers proteolytic cleavage of IGFBP-3, hence overriding senescence (Mannhardt *et al* 2000).

5. E6 protein

HPV 16 E6 is a 151 amino acid protein with two zinc finger domains. E6 is one of the primary oncogenes of the virus; causes immortalization of cells and along with E7 brings about transformation. Hence, it is obvious that E6 induces several important changes in the host cell by interacting with a plethora of cellular proteins. *In vitro* and *in vivo* studies show that E6 expression causes hyperproliferation of epithelial cells, loss of differentiation in epithelial layers, and benign and malignant tumour formation. E6, one of the earliest expressed genes in the viral life cycle, blocks apoptosis, alters the transcription machinery, disturbs cell–cell interactions (a crucial step towards malignancy), increases life span of the cells. E6 physically binds to p53, CBP/p300, E6 associated protein (E6-AP), E6 binding protein (E6-BP), paxillin, mammalian homologue of disc large (hDLG), Bak, Tyk2, E6 targeting protein 1 (E6TP1), protein kinase N (PKN), Gps2, interferon regulatory factor-3 (IRF-3). E6 also alters the rate of transcription of human telomerase catalytic subunit (*hTERT*), vascular endothelial growth factor (*VEGF*) genes. The interaction of E6 with the huge repertoire of cellular factors and the biological significance of such interactions are mentioned below (figure 3).

6. Effect of E6 on cell proliferation

6.1 *Alteration of telomerase activity*

E6 can activate telomerase, a ribonucleoprotein enzyme important for the maintenance of telomeric structures at the ends of chromosomes (Greider 1996). Telomerase is active in more than 90% of immortal and cancer cells, but is absent in normal somatic cells (Kim *et al* 1994). Lack of telomerase activity in normal cells leads to the progressive erosion of the telomeric DNA at the ends of the chromosomes, due to incomplete end DNA replication (Harley *et al* 1990). This ultimately leads to chromosomal instability and senescence. Shortening of telomere serves as a 'mitotic clock' which is responsible for the regulation of the normal life span of the cell. Telomerase utilizes an RNA molecule (TER) as a template and a reverse transcriptase protein component (TERT) for synthesis of DNA (Greider and Blackburn 1987). Hence telomerase activity is closely associated with the expression of the catalytic subunit, hTERT.

Recent reports show that E6 can induce telomerase activity but the exact mechanism for the transcriptional activation is yet to be identified. One possible mechanism for this being increase in Myc protein levels in cells expressing E6 and c-Myc, in turn, can directly activate hTERT transcription (Klingelhutz *et al* 1996; Veldman *et al* 2001). Although E6 increase telomerase activity, telomere length need not get increased. E6 expressing immortal cells may have short telomere but the length at which the telomere is maintained is sufficient for cell survival, increase in DNA content and chromosomal aberrations: hence, finally immortalization (Strahl and Blackburn 1996; Stoppler *et al* 1997).

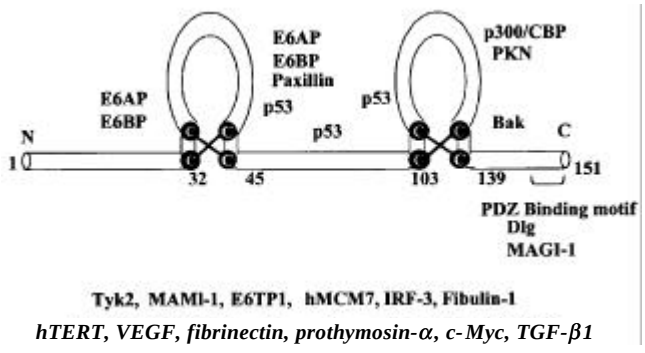

Figure 3. HPV 16 E6 protein. The sequence of the protein along with its various cellular targets has been shown. The transcriptional targets of E6 are mentioned in italics.

7. Effect of E6 on apoptosis

7.1 *Regulation of p53*

E6 can associate with the product of the tumour suppresser gene, *p53* (Werness *et al* 1990). Analyses of human tumours reveal that *p53* is one of the most commonly mutated genes (Vogelstein 1990). Conditions of cellular stress such as UV-irradiation, hypoxia or viral infections cause induction of cellular p53 protein: the amount of the protein can be increased by post-translational stabilization, leading to blocking the cell in G1 (Kuerbitz *et al* 1992). This p53-G1 block allows cells to repair damaged DNA before proceeding in the cell cycle (Lane 1992). Wild type p53 is capable of inducing this block.

p53 is a transcription factor. It stimulates the expression of genes involved in cell cycle arrest and apoptosis, for example the cyclin-dependent kinase inhibitor ($p21^{CIP}$). E6 binds to p53 and leads to ubiquitin mediated degradation of the latter (Scheffner *et al* 1990). E6 mediated degradation of p53 involves another cellular protein, E6 associated protein (E6-AP) (Huibregtse *et al* 1993). E6-AP acts as an E3 ubiquitin ligase (Scheffner *et al* 1993). Reports suggest that it is only in the presence of E6 that E6-AP degrades p53; otherwise, E6-AP by itself is not

involved in normal regulation of p53 (Talis *et al* 1998). E6-AP is also essential for p53 degradation in an infected cell. Studies elucidate that E6 mutants, defective in binding to E6-AP, but retaining p53 binding, are incapable of p53 degradation. E6 binding to p53 can also lead to retention of p53 in the cytoplasm, blocking its translocation to the nucleus and hence abrogating its function independently of degradation (Mantovani and Banks 1999). This prevention of p53 translocation inhibits the ability of p53 to activate or repress target genes (Mietz *et al* 1992).

E6-p53 interaction is important for cell cycle progression. $p14^{ARF}$, an alternate gene product of $p16^{INK4a}$, a tumour suppresser induces arrest in a p53-dependent manner causing elevated levels of $p21^{CIP}$ and finally resulting in G1 or G2 arrest (Stott *et al* 1998). Unlike normal cells, E6 expressing human foreskin fibroblasts (HFF) can bypass this arrest and continue to proliferate despite high levels of $p14^{ARF}$. Reports also show that the ability of E6 to overcome this arrest signal is by a p53-dependent mechanism which involves repression of the cyclin B and cdc2 promoters (Passalaris *et al* 1999). E6 also prevents serum starvation induced apoptosis, through yet uncharacterised mechanisms (Mythily *et al* 1999).

7.2 *Interaction with Bak*

E6 has been implicated to prevent apoptosis in both a p53-dependent and a p53-independent pathway. Adenovirus E1B 19 K protein has been shown to act an anti-apoptotic member of the Bcl-2 family, and has been shown to be functionally equivalent to proteins like Bcl-2 and Bcl-XL (Huang *et al* 1997). Search for on an equivalent function for a HPV oncogene revealed that E6 binds to Bak (a proapoptotic member of the Bcl-2 family) in a p53-independent manner and degrades it – most probably recruiting E6-AP – via ubiquitin-mediated pathway (Thomas and Banks 1998, 1999). Biochemical studies also show that Bak can bind to E6-AP in the absence of E6, unlike in the case of p53 (Thomas and Banks 1998). HPV, strictly an epitheliotropic virus, replicates in the differentiating keratinocytes: recent reports show that Bak protein is abundantly expressed in the upper layers of the epithelium (Krajewska *et al* 1996); hence, it is an obvious target which gets modulated by the HPV oncogene *E6* for the successful survival of the virus.

8. Effect of E6 on gene transcription

HPV E6 is capable of altering transcription of target promoters in a positive or a negative manner. E6 can transactivate the HPV early promoter in the upstream regulatory region (URR) (Gius *et al* 1988). It can also bind to and inhibit the transcriptional co-activators like CREB-binding protein (CBP) and p300 (Patel *et al* 1999; Zimmermann *et al* 1999).

8.1 *Repression of transcription, targeting of CBP/p300*

CBP and the closely related protein p300 are large transcriptional regulators that play a variety of roles in signal modulated cellular responses (O'Connor 2000). While proteins of adenovirus E1A (Ad E1A) and SV40 TAg proteins have been shown to bind to the transcriptional adapter CBP/p300 (Arany *et al* 1995; Eckner *et al* 1996; Lundblad *et al* 1995), it is only recently that such a function as been shown for HPV E6. E6 binds to CBP/p300 and abrogates its activation of p53, but the exact mode of action is not yet characterized. HPV E6, like Ad E1A and SV40TAg, bind to the same site on CBP as p53 (Patel *et al* 1999). CBP activates transcription by at least two mechanisms: by acetylation of histone and non-histone proteins (Bannister and Kouzarides 1996; Gu and Roeder 1997; Ogryzko *et al* 1996; Yang *et al* 1996); and by involving bridging of DNA-bound transcription factors to components of the basal transcription machinery (Nakajima *et al* 1997). E6 binding to CBP can potentially abrogate both these mechanisms. When E6 binds to CBP, it can prevent the acetylation of p53 mediated by CBP and thus reducing its affinity for its cognate DNA-binding sites (Gu *et al* 1997). E6 may also target the bridging mechanism for CBP/p300-dependent activation. CBP/p300 activates CREB-dependent transcription by recruiting RNA helicase A, a component of RNA polymerase II complex, to a promoter containing functional CRE site (Nakajima *et al* 1997). E6 might compete for the same biding site with RNA helicase A, thus preventing its activation. A similar possibility of disruption of CBP/p300-TFIIB interactions by E6 can also be speculated (Kwok *et al* 1994). The repression of the transcriptional machinery hence affects several target genes, especially those involved in cytokine production and immune signalling. Control of IL-6 and IL-8 promoters is largely dependent on the NF-κB transcription factor, which in turn is co-activated by CBP/p300 (Sheppard *et al* 1999). E6 has been shown to inhibit NF-κB mediated transcription. Therefore, the interaction of E6 with CBP/p300 might modulate these signals to the immune system – equipping the virus with, yet, another mechanism for survival (Patel *et al* 1999).

8.2 *Activation of transcription*

As already mentioned, E6 induces transcription of hTERT, through a c-Myc dependent or independent mechanism.

E6, like several other oncogenes (for example, mutant ras, EGF receptor, ErbB2/Her2, c-myc and v-src) upregulates the activity of the VEGF promoter, and hence VEGF transcription (Lopez-Ocejo *et al* 2000). VEGF is one of the most important inducers of angiogenesis: a process essential for the recruitment of new blood vessels and a fundamental prerequisite for the progressive expansion of tumours (Hanahan and Folkman 1996). The stimulatory effect of E6 on the VEGF promoter may predominantly be from its positive impact on the activity of other transcription factors, such as Sp-1 (Gille *et al* 1997). E6 oncoprotein may also contribute to the increase in VEGF message by interfering with the ubiquitin-mediated degradation of the hypoxia inducible factor-1 alpha (HIF-α): a transcription factor involved in the activation of VEGF gene promoter in response to hypoxia (Ravi *et al* 2000). E6 can also increase cellular fibronectin gene expression, transactivate the prothymosin α, c-Myc, TGF-β promoters (Dey *et al* 1997, Kinoshita *et al* 1997).

9. E6 interactions with other cellular proteins

E6 interactions with a wide range of other cell proteins also contribute vitally towards tumour progression. As already mentioned, E6 binds to E6-AP and this interaction plays an indispensable role in the degradation of the tumour suppresser, p53.

E6 binds to the mammalian homologue of the *Drosophila* disc large tumour suppresser (hDLG) protein, a PDZ protein (Kiyono *et al* 1997). PDZ is a septate junction protein. Studies in *Drosophila* show that mutations in this tumour suppresser result in relaxation of cell–cell contact, and neoplastic transformation. E6 binds to/and degrades hDLG, independently of E6-AP interaction (Pim *et al* 2000; Mantovani *et al* 2001). Further, hDLG forms complexes with the product of the adenomatous polyposis coli (APC) tumour suppresser gene: a frequently mutated gene in colon cancers. *In vitro* studies show the binding between APC and hDLG is disrupted by E6. Although the exact contribution of the binding of hDLG with APC is not clear, studies show that most cancers harbour APC mutations in its hDLG-binding domain (Kiyono *et al* 1997). True significance of hDLG interaction with E6 is yet to be illustrated clearly. 'High risk' HPVs also associate with other DLG related, PDZ proteins, namely MAGI-1 which is a member of membrane associated guanylate kinase (MAGUK) protein family. E6 targets this protein for degradation (Glaunsinger *et al* 2000). Recent report shows that 'high risk' HPV E6 can also target another PDZ protein – human homologue of the *Drosophila* Scribble (hScrib), a tumour suppresser – for ubiquitination and degradation mediated by the E6–E6 AP complex (Nakagawa and Huibregtse 2000). This also says that E6 can affect large signalling involving many PDZ proteins.

E6 physically interacts with the focal adhesion protein, paxillin (Tong and Howley 1997). Paxillin is a cytoplasmic protein involved in actin organisation, attachment of cells to the extracellular matrix via focal adhesion proteins like vinculin (Wood *et al* 1994), and transduces signals from the plasma membrane to focal adhesions and the actin cytoskeleton. Only E6 from 'high risk' HPVs bind to paxillin. This results in disruption of the actin cytoskeleton, suggesting that this interaction may play a vital role in tumorigenesis. The protein levels of paxillin and focal adhesion kinase (FAK) are upregulated in cervical carcinoma cell lines and in cells immortalized by 'high risk' HPV (McCormack *et al* 1997). The physiological consequence of the interaction between E6 and E6 binding protein (E6-BP) – a Ca^{2+} binding protein – is unknown (Chen *et al* 1995).

'High risk' E6 has been shown to interact with Fibulin-1, another calcium-binding plasma and extracellular matrix protein (Du *et al* 2002). Fibulin-1 has been implicated in cellular transformation and tumour invasion. Hence E6 might exert its inhibitory roles on cell differentiation through interactions with calcium-binding proteins.

E6 interacts with interferon regulatory factor-3 (IRF-3) which a transcriptional regulator (Ronco *et al* 1998). IRF-3 is activated in the presence of double-stranded RNA or by virus infection to form a stable complex with other transcriptional regulators that bind to the regulatory elements of the interferon (IFN)-β promoter (Au *et al* 1995; Schafer *et al* 1998; Wathelet *et al* 1998; Weaver *et al* 1998). E6 binds to IRF-3 and inhibits its transactivation function. But this binding does not result in ubiquitination or degradation of IRF-3. Instead, it results in marked reduction of the IRF-3 transactivation function (Ronco *et al* 1998). This interaction may be related to the oncogenic potential of the virus, affecting either the regulation of cell proliferation or apoptosis, or through perturbation of the ability of the immune system to recognize an infected cell.

E6 of 'high risk' viruses can also affect IFN-α induced JAK-STAT signalling pathway. E6 physically associates with Tyk2 at the same site that is necessary for Tyk2 and interferon-α receptor1 (INFAR1) interaction. Thus impairing Tyk2 activation and in turn abrogating downstream JAK-STAT signalling (Li *et al* 1999).

'High risk' HPV E6 also binds and degrades E6 targeted protein (E6-TP1) which is a GTPase activating (GAP) protein. The physiological implications of such an interaction are still under investigation (Gao *et al* 1999, 2001).

Together E6 and E7 can also interact with other cellular proteins, like an activated allele of the transmembrane receptor, Notch1 to bring about transformation *in vitro*. These proteins do not physically interact with one other, but may activate various signalling pathways to inhibit apoptosis and promote cell proliferation. For example,

Notch1 can activate the PI3kinase-PKB/Akt pathway, a signalling cascade that is active in diverse cancers–, and this along with the multifunctional HPV oncoproteins (E6 and E7) synergise with each other in tumorigenesis (Daniel *et al* 1997; Rangarajan *et al* 2001).

10. Delicate balance between E7 and E6 functions

The most characterized functions of E6, to prevent p53 mediated growth arrest and apoptosis, are very important because of two reasons. First being that HPV E7 alone can lead to an increase in the amount of p53 in the cells (Demers *et al* 1994). Secondly, HPV E2 protein, involved in the viral genome replication and transcription of its genes, can induce apoptosis via a p53-dependent mechanism when expressed alone in HPV positive/negative cell lines (Webster *et al* 2000). If this were left unperturbed, an infected cell would die even before there is viral replication. Hence, the ability of E6 to modulate p53 protein levels may be an integral event during productive infection (figure 4).

11. Transcriptional regulation of E6 and E7

These two primary oncoproteins can regulate a plethora of cellular genes during the progression of the disease. One more crucial event in this process is the transcriptional regulation of these oncogenes during the transition from a pre-malignant lesion to a malignant tumour. Very low E6/E7 transcripts are detected in basal cells in low grade dysplastic lesions, while an increase in their levels is observed in differentiated upper layers of the epidermis (Durst *et al* 1992; Stoler *et al* 1992) In contrast, high grade lesions and cervical carcinomas show high levels of E6/E7 throughout all layers of the epithelium, including the basal layers. These observations indicate that the progression of a lesion from CIN I/II stage to CIN III/ ICC is accompanied by deregulation of the intracellular control mechanisms of HPV transcription in the basal cells, resulting in an increase in the expression levels of E6/E7 (zur Hausen 1986). Both viral and cellular factors have been implicated to play a role in this increase in E6/E7 transcription providing the host cell with a proliferation advantage necessary for tumour growth (Sang and Barbosa 1992). The increase in the transcript levels in the basal cells normally occurs during epithelial differentiation. This activation of E6/E7 transcription could be the result of an increase in activity of the transcriptional activator or decrease in activity of the transcriptional repressor or a combined effect of both mechanisms. The breakdown of the intracellular surveillance systems in controlling E6/E7 transcription in the basal epithelial cells is instrumental in progression of the dysplastic lesions to invasive carcinomas along with other genetic events (zur Hausen 1986, 1989, 2002).

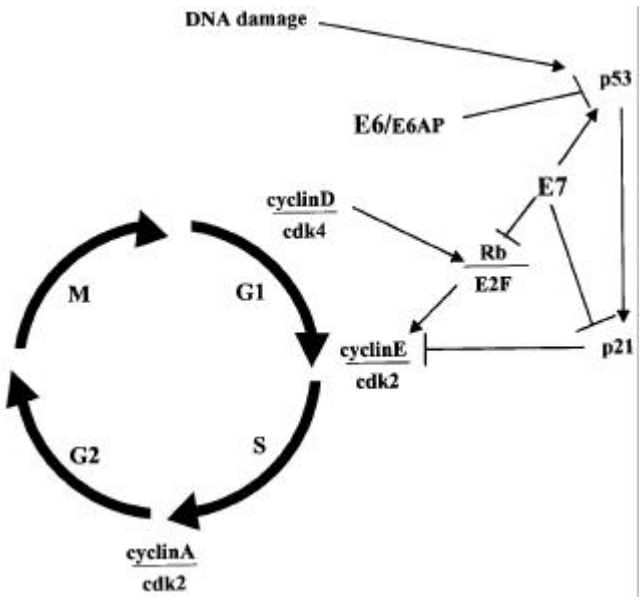

Figure 4. A model for the interaction of HPV E6 and E7 with the important players of the DNA damage pathway. A complex interplay exists between E6, E7 and the regulators of cell cycle that can circumvent all inhibitions and eventually result in cell proliferation.

12. Conclusions

The consequence of a 'high risk' HPV infection is the progression of a proliferative lesion to malignancy. The two 'early' viral proteins, E6 and E7 play an important role in this transformation process. These two proteins can modulate and manipulate the function of a battery of cellular proteins to induce continued cell division and evade apoptosis. However several questions still remain unsolved. For instance why do only a small percentage of the CIN III lesions progress to ICCs? Whether these processes of aberrant proliferation and apoptosis combine with the efficiency of circumventing host defences in each individual remain to be seen. Lasting immuno-suppression through modifications of cellular genes influencing antigen presentation or signalling cascades affecting suppression of viral transcription and oncoprotein function, represent risk factors for persistent expression of viral DNA and progression of the disease (Petry *et al* 1994). However, the actual factors that determine the viral persistence only in a very small subset of women are yet to be understood. A correlation of malignancy and genetic pre-disposition of the host also remains to be identified. Answers to these questions will contribute not only

to a better understanding of the disease but also help target therapeutic approaches more effectively.

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