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The biochemical and biological functions of human papillomavirus type 16 E5 protein

Brief Review

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Summary. Human papillomavirus type 16 (HPV-16) E5 protein, along with the more publicized E6 and E7 proteins of this virus, has been found to be oncogenic. E5 is a highly hydrophobic membrane-bound protein of 83 amino acids associated with the Golgi apparatus, endoplasmic reticulum, and nuclear membrane in infected cells. E5 can activate epidermal growth factor receptor (EGFR) through binding to the 16 kD subunit of protein pump ATPase leading to a reduced downregulation of EGFR receptors. The activation of EGFR can initiate biochemical cascades that lead to overexpression of a variety of protooncogenes and stimulate rapid cell growth. Moreover, E5 can inhibit the expression of tumor suppressor gene $p21^{(WafI/SdiI/CipI)}$ and impair the control of cell cycle checkpoint. E5 protein has been identified as a potential tumor vaccine target antigen.

Introduction

Bovine papillomavirus (BPV) E5 is a hydrophobic protein of 44 amino acids that can induce transformation of rodent and human fibroblasts [4, 23]. BPV E5 is localized primarily in the membranes of the endoplasmic reticulum and Golgi apparatus of transformed cells and exists as a dimer of two subunits linked by disulfide bonds. BPV E5 can form complexes with various cellular proteins. For instance, BPV E5 can interact with β type platelet-derived growth factor receptor (β PDGF-R) as well as the 16 kD subunit of the vacuolar H^+-ATPase. These interactions are thought to be important in cellular transformation induced by this protein. Amino acid analysis has revealed marked differences between human

papillomavirus (HPV) E5 and BPV E5 proteins, however there remain conserved residues such as the potential helix-breaking prolines and hydrophobic residues (leucine, isoleucine, and phenylanine) [3, 7]. Despite the lack of sequence homology, BPV1 E5 and the HPV E5s share several functional similarities, suggesting there might be shared three-dimensional structure features between BPV and HPV E5.

HPVs have been associated with both benign and malignant epithelial lesions [54]. Among the HPVs, HPV type 6 (HPV-6) and HPV-11 are regarded as low-risk types since they are usually found in benign warts and papillomas whereas HPV-16 and HPV-18 are classified as the high-risk types as they are the predominant viruses found in malignant lesions [54]. HPV-16 is the predominant virus type identified in cervical cancers [29, 54] and encodes three transforming oncogenes; E5, E6, and E7. Initial infection with the high-risk HPV-16 causes low-grade disease and the viral DNA exists in episomal form in the cell nucleus. During the progression to malignant disease, HPV DNA frequently integrates into the host cell genome [12, 13, 21, 37, 42]. The integration of viral DNA allows for persistent expression of the E6 and E7 oncogenes. E6 mediates degradation of p53 and E7 inactivates the pRb tumor suppressor protein [24]. In the episomal form of the viral DNA present in the initial stages of HPV-infection, the E5 open reading frame is present in the major abundant viral transcript, E1^E4 mRNA [26, 54]. However, the E5 gene is frequently deleted when the HPV genome is integrated [24, 54]. Therefore, E5 gene expression is often extinguished after integration of the viral genome during the progression from low-grade to malignant disease. For E5 protein to exert effects on carcinogenesis, it should therefore act at a very early stage. In this review, we provide recent information regarding the biochemical and biological aspects of the role of E5 in transformation.

E5 gene expression and E5 variants in HPV-16 infected tissues

The HPV-16 E5 protein, is a strongly hydrophobic protein of 83 amino acids that associates with the Golgi apparatus, endoplasmic reticulum and nuclear membrane in host cells [15, 33, 49]. E5 is composed of three membrane helices and short regions at the N- and C-termini that extended beyond the lipid bilayers [48]. In HPV-positive carcinomas *in situ* of cervix, the most abundant viral transcript is a polycistronic mRNA covering the E5 open reading frame [43]. The E5 mRNA and protein are abundant in the lower third of the epithelium of low grade cervical lesions detected by *in situ* hybridization and immunohistochemistry assay [6, 43].

The E5 gene is frequently deleted when HPV genome is integrated into the host genome [40]. There is compelling evidence that DNA of both the high- and low risk HPV remain in episomal form in benign gynecologic infections [21, 25, 34]. In contrast, integrated HPV DNA is often identified in biopsy specimens and cell lines from cervical cancers in which HPV DNA is detected [25]. Viral integration has been proposed to play a pivotal role in the progression of cervical cancer. Unlike the other high risk HPVs, HPV-16 DNA can exist in episomal, integrated, or episomal/integrated forms in primary cervical cancers [6, 12, 13,

21, 37, 42]. However, E5 is not necessarily responsible for the maintenance of malignancy since some tumors do not contain the E5 gene or protein. Therefore, E5 might contribute to neoplastic proliferation during the early stages of infection, but is not indispensable for malignant transformation. E6 and E7 are necessary to maintain the transformed phenotype [44].

Sequence analysis of HPV-16 E5 gene variants has been examined at all stages of cervical carcinoma [1, 8, 30]. Three mutational hot spots were identified at positions 3979, 4042, and 4077 of the HPV-16 E5 gene. We have found six mutant E5 DNA sequences in the HPV-16 infected human tissues that encode four mutant E5 proteins [27]. However, comparing the biological functions of these four mutants with the wild type E5 proteins, only one E5 mutant has reduced transforming activity (59% of the wild type protein) while the other three E5 mutant proteins exhibit the same transforming activity as wild type E5. These findings suggested that despite the frequent mutations of DNA sequence of E5 gene, the biological function of the encoded mutant E5 proteins is not altered.

EGF-dependent and independent E5-induced activation of epidermal growth factor receptor

To elucidate the oncogenic activity of E5, the significance of the interaction between E5 and epidermal growth factor receptor (EGFR) has been studied. Increased activation of signaling cascade by E5 is EGFR-dependent. In the presence of E5 protein, even long-term exposure to epidermal growth factor (EGF) resulted in increased EGFR number and phosphorylation [46]. This effect is due to an impaired downregulation of receptors and recycling of the receptors to the cell membrane in E5-expressing cells [45]. This enhanced receptor recycling is mediated by binding of E5 to the 16 kD subunit of the proton pump ATPase located in the endosomal membrane [45, 46]; this binding that can be demonstrated *in vitro* [11, 27, 49]. *In vivo* data also revealed that the E5 protein predominantly exists in the perinuclear region of the cytoplasm of E5-transfected cells [6, 15, 39] and the HPV-16 infected tissues [6], locations consistent with the distribution of the 16 kD molecule [15]. Conversely, one study reported that EGF treatment only increased EGFR phosphorylation instead of receptor number in E5-expressing cells [28]. This result suggested that E5 could form a complex with EGFR to induce the phosphorylation of EGFR. However, other researchers could not demonstrate E5-EGFR complexes [16]. EGFR-independent pathways have also been proposed to function in the E5 protein activated signaling cascade [20]. HPV-16 E5 modulates the sorbital-dependent activation of MAP kinase p38 and ERK1/2 in human keratinocytes through an EGF-independent mechanism [20]. Taken together, E5-activating signaling cascades appear to be mediated via either a receptor-dependent or receptor-independent pathways.

The type I family of growth factor receptors include EGFR (erbB1), erbB2, erbB3, and erbB4, which are frequently overexpressed in various human cancers.

In response to ligand binding, the EGFR receptor undergoes homo- or hetero-dimerization resulting in phosphorylation of the receptor tyrosine kinase. The relationship between E5 and EGFR has been widely investigated [18], whereas the relationship with other receptors are rarely reported. Via immunohistochemistry assay, we have demonstrated the association between E5 protein and the EGFR or erbB4 receptor in HPV-16 infected lesions [6]. This finding implies the transforming activity of E5 might activate the signaling pathway by affecting the EGFR homodimer, the erbB4 homodimer, or EGFR/erbB4 heterodimer formation. Transphosphorylation of ligand-induced homo- or hetero-dimers has been reported to modulate the growth regulatory signal [5, 53]. A recent report demonstrated that EGF treatment results in enhanced activation of the erbB2 receptor in E5-expressing cells, whereas treatment with heparin-binding epidermal growth factor-like growth factor (HB-EGF) did not increase phosphorylation of the erbB4 receptor in the E5-expressing cells [18]. Hence, there remains much to be explored regarding the binding of E5 protein with each receptor of the erbB family, not to mention the autophosphorylation or transphorylation between the homodimer and the heterodimer of receptors after E5-protein binding.

E5 and signal transduction

HPV-16 E5 protein can activate EGFR, which in turn can initiate diverse biochemical events that ultimately render overexpression of a variety of proto-oncogenes. HPV-16 E5 might activate MAP kinases via two different pathways: the protein kinase C (PKC)-dependent, and the receptor tyrosine-kinase mediated (PKC-independent) pathway [17, 28]. We also have reported E5 could stimulate the nuclear oncogenes as c-jun, junB and c-fos [9, 10, 47]. The c-jun nuclear oncogene is activated by HPV-16 E5 via PKC and ras-dependent pathways to transmit signals to the nucleus [9]. E5 induction of c-jun is through an activator protein-1 (AP-1) binding site [9] and E5-activation of c-fos is via nuclear factor-1 (NF-1) binding sites in the nucleus [10]. Therefore, E5 may potentiate viral gene expression via the activation of AP-1 and NF-1 [10]. Since several binding sites of AP-1 and NF-1 are located in the regulatory region of the HPV-16 DNA and these transcription factors can activate the enhancer of HPV-16 [14]. Therefore, E5 protein transactivates viral genes and thereby increases viral E6/E7 gene expression, stimulating E7-mediated cell transformation [2, 44]. Thus, E5 may also play role in cell transformation by regulating the expression of other cellular and viral genes.

We also found E5 protein could suppress the expression of the $p21^{(WafI/SdiI/CipI)}$ tumor suppressor gene in NIH 3T3 cells and in immortalized human keratinocytes [47]. The suppression of $p21^{(WafI/SdiI/CipI)}$ gene expression could promote cell proliferation. This observation also suggests a role for E5 in augmenting or supplementing the function of E6 and E7 during immortalization of human genital keratinocytes in the course of primary infection. Colony numbers were found to be higher in primary keratinocytes infected with the full-length HPV-16 genome than in those with interrupted E5 genes-containing HPV-16 genomes. This finding

suggested that E5 present in *cis* to its native promoter could increase the efficiency of cellular immortalization by E6/E7 [44]. In addition, mutations in E7 of HPV-16 genome which inhibit the binding of pRb could not abrogate HPV-16-induced immortalization of primary human keratinocytes [31]. Moreover, intradermal injection of cottontail rabbit papillomavirus DNA, a virus with a natural history of infection quite similar to that of HPV-16, with mutations in the E7 gene sequences critical for the binding of pRb could still induce papilloma formations in rabbits [22]. Hence, the repression of the p21 gene by E5 protein may complement the altered pRb binding activity of mutated E7. Suppression of p21 gene by E5 may facilitate the activation of CDK4-cyclin D complexes, which are known to phosphorylate pRb and inactivate Rb-checkpoint control. It remains uncertain whether E5 can supplement E6 transformation. E6 protein targets the tumor suppressor protein p53, leading to p53 degradation and contributes to malignant transformation [36]. Although p53 can activate p21 gene expression, it is unclear whether HPV repression of p21 gene expression is p53-dependent. Therefore, efforts still are needed to decipher the complicated networks existing between E6, E5, p53, and p21.

E5 in cell growth and apoptosis

It is quite curious how HPV-infected cells can escape from the control mechanisms present in the surrounding environments. Several lines of data suggest E5 may play a role in growth stimulation: (1) The expression of E5 protein in human keratinocytes can impair the gap-junction mediated cell–cell communication; this decrease in gap-junctional intercellular communication is coupled with dephosphorylation of connexin 43, the most abundant connexin in keratinocytes [39]. (2) HPV-16 E5 can induce anchorage-independent growth of the immortalized human keratinocytes by stimulating c-jun and junB expression [9]. (3) EGF treatment of E5-expressing fibroblasts and keratinocytes increased colony size but not the colony numbers [39, 41]. (4) E5 can activate endothelin-1 (ET-1) and ET_A receptor allowing human keratinocytes under growth factor-starved situations to induce DNA synthesis [50].

HPV-16 E5 also appears to render infected cells able to endure physical and chemical insults. E5 can protect primary cultures of keratinocytes from UV β-irradiation-induced apoptosis [52]. Two signal pathway enzymes downstream of the EGFR, phosphatidylinositol 3-kinase (PI3K) and ERK1/2 mitogen-activated protein kinase (ERK1/2 MAPK), have been recognized to participate in two survival signal pathways induced in response to UV β-induced apoptosis [52]. Therefore, E5 might exert protective effects against UV β-irradiation. However, other authors proposed that E5 could act as pro-apoptotic factor under osmotic stress. When expressed in human keratinocytes and mouse fibroblasts, E5 sensitizes these cells to sorbitol-induced apoptosis [32]. The liability of E5-expressing cells to osmotic stress possibly results from modifications of the cellular membranes due to the presence of the strongly hydrophobic E5 which alters the phospholipid component and phospholipase activity of cell membranes [19].

E5 and vaccine development

E5 is expressed in precancerous stages of cervical epithelium during HPV infection. Since precancerous lesions usually contain fewer cells than the invasive malignancies, it can be speculated that early immunological intervention might offer a chance to eradicate tumors while still in a premalignant stage. Furthermore, cells in more advanced stages are found to have very low levels of expression of MHC class I and II mRNA, which consequently might hamper the presentation of antigen and lead to decreased immunosurveillance [38]. Recent reports demonstrated that lymphocyte proliferation in response to HPV-16 E5 is inversely proportional to the severity of the squamous intraepithelial neoplasia lesions (SILs) [26]. Hence, in the E5-expressed precancerous lesions such as SILs and condyloma, using E5 as a vaccine target might be a good strategy to prevent these precancerous lesions from progressing into invasive cervical cancers. Our recent investigation demonstrated that a single muscular injection of the recombinant adenovirus (rAd) encoding the HPV-16 E5 gene into mice with TC-1 tumorigenic cell line [51] engineered to contain the E5 gene could reduce tumor growth of in the lesions containing E5 gene. The effects of E5 vaccine might exert via $CD8^+$ rather than $CD4^+$ T cells [35]. This indicates that E5 might be identified as a tumor antigen by the immune system. Further investigations to identify the cytotoxic T lymphocyte-specific epitope are needed.

Conclusion

For better understanding the pathogenic roles of E5 protein, future studies should be directed toward exploring the three-dimension structure of E5 protein particular in comparison to its BPV homologue, the binding affinity of E5 with erbB family receptors and the resulting autophosphorylation or transphorylation of the homodimer or heterodimer of these receptors, and the effect of E5 on cell proliferation and inhibition of apoptosis. The promise of E5 as a vaccine target antigen also needs to be explored.

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