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STUDIES ON MOLECULAR MECHANISMS OF TRANSFORMATION BY HUMAN PAPILLOMAVIRUS-THE ROLES OF E6 AND E5 ONCOGENES

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ABSTRACT

The ability of the HPV-18 E6 gene to impair p53-mediated transcriptional activity induced by DNA damaging agents was investigated. It is demonstrated that E6 can abolish DNA damage induced p53-mediated transcription and that a region from amino acid residue 113 to 117 of HPV-18 E6 protein was necessary for E6 to direct the degradation of p53. The biological importance of the E6/p53 interaction was then directly examined in HPV-16 containing cervical carcinoma derived cells by introducing the monomeric p53 mutant which is resistant to E6 mediated degradation. The two major observations made from this study were: i) loss of p53 activity plays an important role in maintaining the malignant phenotype of these cells with respect to cell proliferation; ii) the monomeric p53 mutant without its C-terminal regulatory region was biologically functional with respect to impairing cell proliferation in HPV-16 containing cervical carcinoma derived cells. Finally, it was revealed that the cellular MAP kinase signal transduction pathway was more active in cells expressing the HPV-16 E5 gene than in control cells or cells expressing E6 and E7. These observations help to define the mechanisms by which HPV oncogenes contribute to the development and maintenance of the neoplastic phenotype.

ABREGE

Nous avons étudié la capacité du gène HPV-18 E6 à inhiber l'activité transcriptionnelle médiée par p53 après induction de celui-ci par des agents génotoxiques. Nous avons démontré que la protéine E6 peut abolir la transcription normalement contrôlée par p53 après un dommage à l'ADN, et que la région de la protéine HPV-18 E6 située entre les acides aminés 113 à 117 est essentielle à la capacité de E6 de diriger la destruction de p53. Nous avons ensuite étudié l'importance biologique de l'interaction E6/p53 chez des cellules dérivées d'un carcinome du col de l'utérus infectées par le HPV-16, en introduisant un mutant de p53 résistant à la dégradation dirigée par E6. Deux observations principales ont été faites: i) la perte de l'activité p53 joue un rôle important dans le maintien du phénotype malin au point de vue prolifération cellulaire; ii) le mutant monomérique de p53 auquel il manque la région régulatrice C-terminale s'est avéré efficace à empêcher la prolifération cellulaire. Finalement, nous avons observé que la voie de transduction du signal de la MAP kinase était plus active chez les cellules exprimant le gène HPV-16 E5 que chez les cellules-contrôle ou chez les cellules exprimant E6 et E7. Ces observations contribuent à éclaircir les mécanismes par lesquels les oncogènes du HPV facilitent le développement et le maintien du phénotype néoplastique.

ACKNOWLEDGEMENTS

I would like, first and foremost, to thank my research supervisor, Dr. Greg Matlashewski for his guidance, support, patience, understanding, availability and for sharing his wonderful ideas during the study.

I also thank my supervisory committee of Drs. Elias Georges and Lawrence Banks for their interest, suggestions and help during the study.

I would like to express my appreciation to my colleagues for their elegant expertise and valuable discussions. I thank Dr. Jim Smith and Haining for help with computers. I am sincerely grateful the professors, students and supporting staffs in the Institute of Parasitology.

Finally, I thank my wife, Shumei, for her understanding and support, and my almost 11-year old son, Kevin, who never fails to ask 'Are you going to School tonight?' I thank my parents for their encouragement.

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STATEMENT OF ORIGINALITY

The following elements are considered contributions to original knowledge.

1. The demonstration that HPV-18 E6 is capable of impairing p53-mediated transcriptional activity under biological relevant conditions where p53 activity is induced by genotoxic stress.

2. The demonstration that a region from amino acid residue 113 to 117 of HPV-18 E6 protein is necessary for the protein to promote the degradation of p53 and to abolish p53 mediated transcriptional activity.

3. The demonstration that restoring p53 in HPV positive cervical carcinoma derived cells impairs their proliferative ability and induces these cells to display a more differentiated morphology.

4. The demonstration that p53 in its monomeric form without its C-terminal regulatory domain is biologically active with respect to suppressing the proliferation of HPV-16 DNA containing cells.

5. The demonstration that expressing the HPV-16 E5 gene induces an increase in cellular MAP kinase activity in both the presence and the absence of epidermal growth factor, suggesting that the MAP kinase pathway is a possible target for HPV E5.

CONTRIBUTIONS OF OTHERS

All the experiments described within were designed and performed by myself under the guidance of Dr. Greg Matlashewski with the exception of the study in Chapter II. In this study the construction of the E6-δF mutant was provided by Drs. Lawrence Banks and David Pim, and the E6 directed degradation of p53 was executed by Ms. Sylvie Labreque.

My supervisor, Dr. Greg Matlashewski, provided advice and feedback on the design and analysis of all studies, and in the preparation of the manuscripts for publication.

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LIST OF ABBREVIATION

Ad	Adenovirus
AP1	Activator protein 1
BPV	Bovine papillomavirus
BRK	Baby rat kidney
°C	degree Celsius
CAT	Chloramphenicol acetyl transferase
cdc2	Cell division cycle 2
cdk	Cyclin dependent kinase
CIN	cervical intraepithelial neoplasia
Cip1	cdk-interacting protein 1
CPM	Counts per minute
CR	Conserved region
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
EGF	Epidermal growth factor
EGF-R	EGF receptor
FCS	Fetal calf serum
HPV	Human papillomavirus
LCR	Long control region
MAP	Mitogen activated protein

MBP	Myelin basic protein
mdm-2	Murine double minute 2
NIH	National Institutes of Health
OA	Okadaic acid
ORF	Open reading frame
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PMA	Phorbol myristate acetate
RB	Retinoblastoma gene
SRE	Serum response element
SV40	Simian virus 40
UV	Ultraviolet

INTRODUCTION

There have been several important advances in understanding the mechanisms by which human papillomaviruses (HPVs) contribute to the development of cervical cancer. Perhaps the most significant are the observations that E7 protein binds to the product of the cellular tumour suppressor retinoblastoma (RB) gene and that the E6 protein interacts with the product of the cellular tumour suppressor gene p53 and promotes its degradation. Because of the important roles RB and p53 play in cell cycle control processes, a common consequence of RB binding and p53 degradation thus appears to be the loss of control at essential points in the cell cycle. As the accumulation of knowledge in defining the biological functions of p53 (in apoptosis, cell cycle control, and DNA repair), the E6 mediated degradation of p53 becomes particularly important in understanding HPV associated diseases. The additional observations that the HPV E5 gene can cooperate with epidermal growth factor (EGF) receptor (EGF-R) to amplify the signal transduction from the receptor in response to ligand provide evidence for another important cellular molecule, in addition to RB and p53, which is targeted by HPV E5.

Taken together, these observations suggest that HPVs have modified the activity of at least three important cellular molecules, RB, p53 and EGF-R, for overriding some of the most important control mechanisms of the cells. The main aim of the work presented in this thesis was to investigate the biological functions of E6-mediated degradation of p53 and the cellular signal transduction pathway that E5 may affect.

CHAPTER I: LITERATURE REVIEW

1. Human papillomavirus and pathogenicity

Human papillomaviruses (HPVs) are a group of small DNA viruses that infect epithelial cells and induce a variety of skin lesions including warts and epithelial tumors (zur Hausen, 1989; Howley, 1991). The most notabe is cervical cancer which is the second most common neoplastic disease affecting women on a world wide basis second only to breast cancer (Howley, 1988; Parkin et al., 1988; 1993).

More than 70 HPV genotypes have been distinguished based on differences in their nucleotide sequences (de Villiers, 1994). HPV can be divided into several subsets on the basis of their ability to infect specific anatomic regions of which a subset including HPV types 6, 11, 16, 18, 31, 33 and others are associated with genital infections (de Villiers, 1989; zur Hausen, 1991). The genital HPV types are grouped into 'low-risk' and 'high-risk' types depending on the frequency with which they appear in cervical cancers. The 'low-risk' HPV types such as type 6 and 11 are commonly associated with benign condylomas (zur Hausen and Schneider, 1987; zur Hausen, 1989). The 'high-risk' HPV types such as type 16 and 18 are commonly associated with cervical cancers, and HPV type 16 is the most prevalent genotype present in these lesions (Durst et al., 1983; Ikenberg et al., 1983; Boshart et al., 1984; Beaudenon et al., 1986).

HPV infection has been implicated in the development of more than 90 per cent of cervical carcinomas (Park et al., 1994; Bosch et al., 1995) including condylomata, intra-epithelial neoplasia and invasive tumours of the cervix (for review see Bucklet,

1994; McLachlin, 1995).

The most common morphological manifestation of HPV infection in the lower genital tract is the condyloma acuminatum or genital wart, which usually results from infection with HPV type 6 and 11. Lesions resulting from infection with these viral types are regarded as 'low-risk' for progression to invasive carcinoma (zur Hausen and Schneider, 1987; zur Hausen, 1989). HPV infection is also associated with intra-epithelial neoplasia. The intra-epithelial neoplasm may affect the squamous and columnar epithelium. Squamous carcinomas constitute about 80 per cent of cervical malignancies (Buckley and Fox, 1992). Cervical squamous intra-epithelial neoplasia (CIN) is recognized by disturbances of cellular maturation, stratification, and cytological atypia. It is customarily divided according to its degree of cytoplasmic maturation into three grades (Anderson et al., 1991). The lesions range from a well differentiated intra-epithelial neoplasm, or CIN1, to a poorly differentiated intra-epithelial neoplasm or CIN3 with CIN2 standing at intermediate position (Figure 1). HPV type 16 and 18 which carry a 'high-risk' of progression to invasive carcinoma are most commonly found in CIN2 and 3 (Franquemont et al., 1989; Willett et al., 1989; Wells, 1992). The carcinomas can be invasive and spread locally into the cervical stroma, the paracervical and parametrial tissues, the body of the uterus, the vagina, and late in the course of the disease, to the bladder and rectum (Bucklet, 1994).

However, infection by specific HPV types is not sufficient for cancer of the cervix to occur. For instance, infection with 'high risk' HPV type 16 does not immediately nor necessarily lead to cancer. Warts and cervical papillomas that are

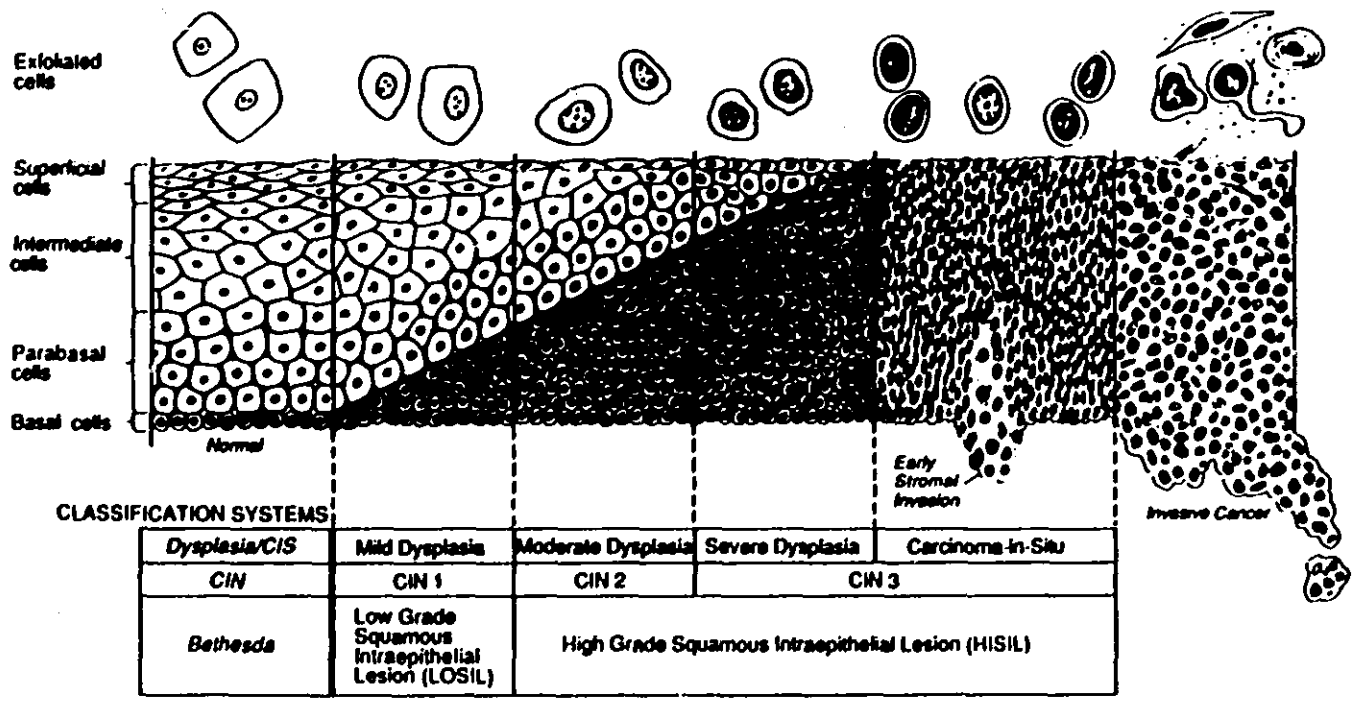

Figure 1. Malignant progression in cervical squamous epithelium (Kistner, 1979). Cervical squamous intra-epithelia neoplasia (CIN) is regognized by disturbances of cellular maturation, stratification and cytological atypia.

infected with HPV type 16 or 18 progress toward a malignant phenotype after a latent period as long as 50 years (zur Hausen, 1986). This long latency of the diseases may reflect the characteristics of the viral life cycle and HPV-associated pathogenicity.

The biological life cycle of the HPV differs from most other viral pathogens. HPV is presumed to infect the basal or germ cells of the epithelium. During the early stage of the disease development, viral genomes are maintained in the episomal state (Lusky and Botchan, 1984). Viral early genes are transcribed in a very low level in these basal cells (Androphy et al., 1987; Banks et al., 1987; Stoler and Broker, 1986). Late gene expression, synthesis of capsid proteins, vegetative viral DNA synthesis, and assembly of virions occur only in the terminally differentiated cells (Amtmann and Sauer, 1982). Because the viral life cycle is not lytic or destructive, this coordination of viral DNA synthesis with host cell differentiation would provide a programmatic means for the virus to transmit into the environment and infect the next host.

During this long latency the viral oncogenes (E5, E6 and E7) may induce cellular events (see following section 2) including inactivation of cellular tumor suppressors (RB and p53) and chromosomal changes in vivo. These events may occur at some low frequency and accumulate with time and thereby facilitate neoplastic progression.

In advanced lesions the viral genomes are often found to integrate into random sites within the host cellular genome, resulting in interruption or deletion of part of the viral genome, normally the E1 and E2 open reading frames (Schwartz et al., 1985; Shirasawa et al., 1987). The major transforming genes (E6 and E7) are selectively retained and expressed in human carcinomas and carcinoma derived cell lines (Schwartz

et al., 1985; Baker and Howley, 1987). Perhaps this integration is significant for the development of the disease in several aspects. Integration can disturb genome stability and create mutations which may contribute to the developing tumor. Integration may also potentiate viral transforming gene expression depending on the site of integration. Integration has also been found to be near cellular oncogenes in some cervical carcinomas (Durst et al., 1987). It has been reported that HPV contributes to the chromosomal instability in HPV transformed cells (Hashida and Yasumoto, 1991).

Taken together, these findings suggested that infection with 'high-risk' HPVs is associated with development of cervical neoplasms (for review see Matlashewski, 1989; DiMaio, 1991; Mansur and Androphy, 1993; zur Hausen and de Villiers, 1994; Tommasino and Crawford, 1995). However, malignant transformation occurs only after a long latency, suggesting that cervical cancer develops through a multistep process. Other co-factors also contribute to the development of the disease including hormone levels, genotoxic agents, cellular oncogenes and accumulation of a number of mutations in an assortment of genes (Matlashewski, 1989; zur Hausen and de Villiers, 1994).

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2. Molecular genetics of human papillomavirus

2.1. *A view of the human papillomavirus genome*

HPVs contain a single circular double stranded DNA genome of approximately 8 kb in size (Gissman et al., 1977; Orth et al., 1977). The viral genome is divided into three functional regions. An early region of about 4.5 kb encodes for several proteins which are involved in transcription and transformation with the exception of the E4 gene. A late region of about 2.5 kb encodes for the capsid proteins and a less than 1 kb long control region (LCR) which contains transcriptional and replication regulatory elements. All of the viral genes are located on one strand of the viral DNA. The organization of all papillomavirus genomes are quite similar. Figure 2 presents the genomic organization of HPV-16 (Shah and Howley, 1990).

2.2. *Functions of the viral genes*

A full and detailed study of the molecular biology of HPV has been covered in several publications and reviews (Broker and Botchan, 1986; Matlashewski, 1989; zur Hausen, 1989; Broker et al., 1989; Arrand 1994). HPV contains approximately 10 designated translational open reading frames (ORF) that have been classified as either early (E) or late (L) ORFs or genes, based on the time when they are expressed during a productive viral infection. The genes (in HPV-16) that are expressed in the early stage of the viral infection, including E1, E2, E4, E5, E6 and E7 genes, are necessary for the viral replication and transformation. The late genes, L1 and L2, code for the structural

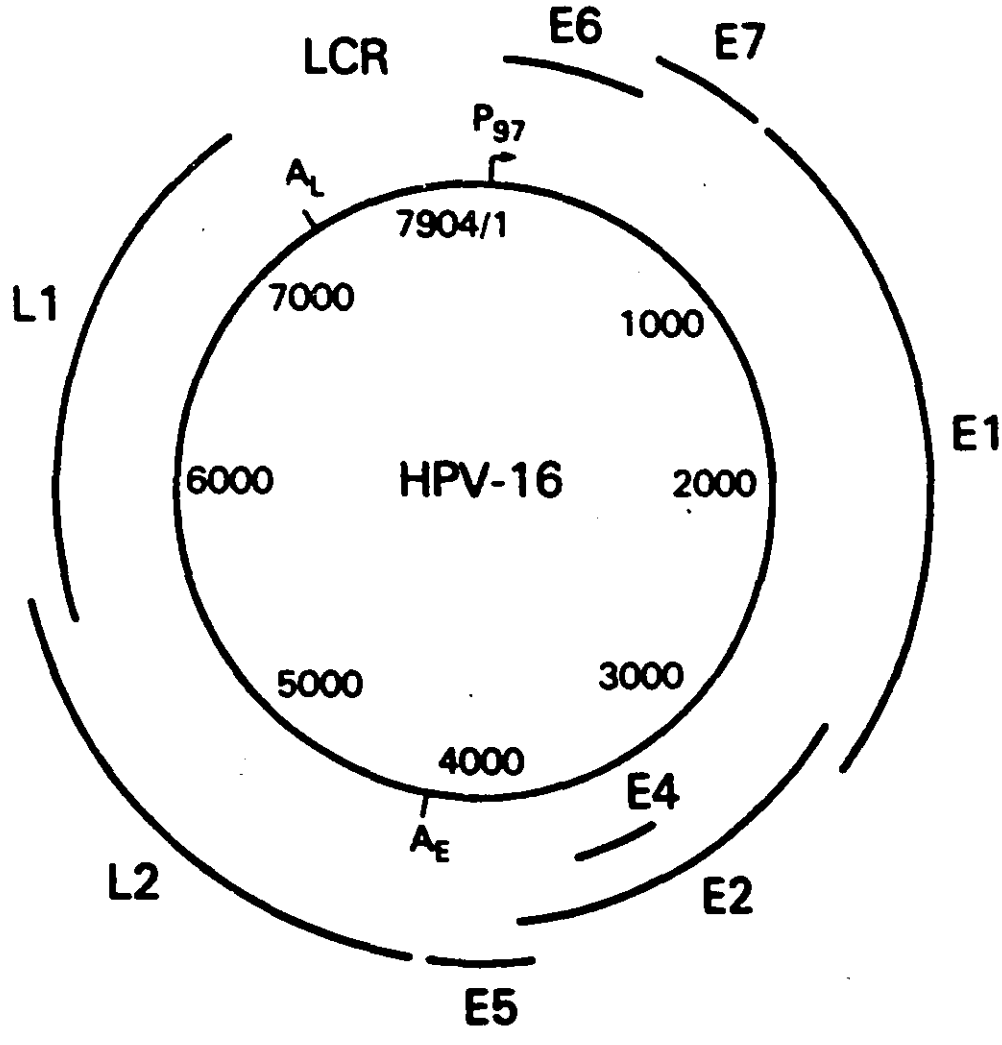

Figure 2. Genetic organization of HPV-16 (Shah and Howley, 1990). The viral genome is divided into three regions designated as Long Control Region (LCR), Early Region (E) and Late Region (L). The ORFs deduced from the DNA sequence are indicated and designated E1 to E7, L1 and L2. Transcription of viral genes is controlled under a major promoter designated as p97 and occurs only in a clockwise manner. AE and AL represent the early and late polyadenylation sites.

proteins of the virion. The biological functions of these genes will be reviewed in a subsequent section below with emphasis on the transforming genes.

2.2.1. *Genes involved in cell transformation*

The HPV genes encoding transforming activity include the E7, E6 and E5 genes. Defining the biochemical and biological properties of these genes has contributed to understanding the mechanisms by which human papillomaviruses contribute to the development of neoplasia. The biochemical characteristics and biological functions of their encoding proteins have been detailed in several recent reviews (for reviews see Mansur and Androphy, 1993; Farthing and Vousden, 1994; Tommasino and Crawford, 1995).

2.2.1.1 *E7 gene*

The 'high risk' HPV DNA can transform established cell lines which are anchorage independent and tumorigenic in nude mice (Tsunokawa et al., 1986; Yasumoto et al., 1986), while they can cooperate with an activated ras oncogene to transform primary rodent and human cells (Matlashewski et al., 1987, 1988; Pirisi et al., 1987; Phelps et al., 1988; Storey et al., 1988). Studies dissecting the functional nature of HPV DNA have confirmed that the transformating activity of the HPVs has been localized to the E6 and E7 genes in both primary and established cell line models (Matlashewski et al., 1987; Yutsudo, et al., 1988), with E7 appearing to be the most potent oncogene (Kanda et al., 1988; Phelps et al., 1988; Crook et al., 1989; Ishiji et al., 1992).

2.2.1.1.1 *The biochemical characteristics of the E7 protein*

The E7 gene encodes for a small acidic protein of 98 amino acids (Smotkin and Wettstein, 1986; 1987) which is a serine phosphoprotein targeted by casein kinase II (CKII) (Firtzlaff et al., 1989; Barbosa et al., 1990). The E7 protein is localised in the nucleus and attached to the nuclear matrix (Greenfield et al., 1991).

It has been demonstrated that there are significant structural and functional similarities between E7 and Adenovirus (Ad) E1A as well as SV40 large T antigen proteins (DeCaprio et al., 1988; Phelps et al., 1988 and 1992; Barbosa et al., 1990). On the basis of the homology with E1A, three functional domains have been identified in the HPV 16 E7 proteins (Dyson et al., 1992). They are the conserved regions 1 (CR1) and 2 (CR2) located in the N-terminal region, and the conserved region 3 (CR3) in the C-terminal region. CR2 is involved in binding to the product of tumor suppressor gene product RB and the RB-related proteins (Münger et al., 1989b; Dyson et al., 1992; Davies et al., 1993), as well as cyclin dependent kinase 2 (cdk2) and cyclin A (Tommasino et al., 1993). The consensus Leu-X-Cys-X-Glu domains for RB binding have been located in the CR2 region of E7 from both "high risk" and "low risk" HPVs (Phelps et al., 1992; and reviewed by Tommasino and Crawford, 1995). However, E7 from "low risk" HPVs display a lower affinity for binding RB (Heck et al., 1992), which suggests that this may partly account for the differences in their immortalising and transforming activities. A CKII site is located in the carboxyl-terminal region of CR2 (Firtzlaff et al., 1989). The biological significance of this phosphorylation is not clear, although there is some evidence that this event may be involved in transformation

(Firtzlaff et al., 1991). Two Cys-X-X-Cys Zn binding motifs that are involved in dimerization of the protein and are necessary for transformation (McIntyre et al., 1993) have been identified in E7 CR3 (Barbosa et al., 1989).

In summary, E7 is a small acidic nuclear protein. The protein can bind to cellular regulatory proteins and self-associate into dimers.

2.2.1.1.2 *The biological functions of the E7 protein*

The E7 gene of HPV has been shown to encode a multifunctional nuclear phosphoprotein possessing both transcriptional modulatory and transformation properties similar to that of adenovirus (Ad) E1A (Barbosa et al., 1990; Firzlaff et al., 1991). E7 can trans-activate the Ad E2 promoter, and can cooperate with an activated ras oncogene to transform primary baby rat kidney (BRK) cells (Phelps et al., 1988; Storey et al., 1988). It is continually expressed both in cell lines derived from cervical tumours (Smotkin and Wettstein, 1986) and in immortalized cell lines generated in vitro (Banks and Crawford, 1988), suggesting that E7 was required for both the development and maintenance of the malignant phenotype.

The E7 gene of HPV-16 was first demonstrated to have transcriptional activating property using the Ad E2 early promoter (Phelps et al., 1988) which is one of the group of promoters to be responsive to the Ad E1A gene (Nevins, 1981). The activation of Ad E2 promoter by the HPV E7 is at the level of transcriptional initiation (Phelps et al., 1988).

Recent studies have demonstrated that E7 could mediate transcriptional

transactivation of Ad E2 promoter through a common pathway (Phelps et al., 1991), that is, releasing the E2F transcription factor to stimulate transcription from Ad E2 promoter and from E2F regulated cellular genes (Bagchi et al., 1990). Several studies have provided evidence that the E2F transcription factor is regulated by RB and p107 as well as cyclin A/cdk2 (Bagchi et al., 1991; Schirodkar et al., 1992). The association between E2F and these cellular proteins prevents transcriptional activation by E2F (Bagchi et al., 1991; Chellappan et al., 1991; Chittenden et al., 1991; Hamel et al., 1992). E7 can disrupt the association of E2F with the cellular proteins including RB (Phelps et al., 1991), resulting in the generation of 'free' E2F capable of binding to and regulating a variety of cellular promoters as described below (Mudryj et al., 1990).

The transforming activity of E7 is likely to be associated with its ability to interact with these cell cycle regulators. Studies on the E7 interactions suggested that any or all of the viral and cellular protein interactions are likely to contribute to the transforming and immortalising activities of E7 (Dyson et al., 1989 and 1992; Davies et al., 1993; Tommasino et al., 1993).

The best understood E7 interaction is that involving the RB protein. It has been shown that RB plays a key role in regulation of mammalian cell replication by inhibiting cell proliferation (Buchkovich et al., 1989; DeCaprio et al., 1989). Biochemical properties associated with RB include binding to viral transforming proteins (E1A, large T and E7) and cellular proteins, such as E2F, which itself functions as transcription factors (Bagchi et al., 1990; Hamel et al., 1992). There is evidence that E2F is involved in controlling transcription of several genes important for entry into the cell cycle and

progress through G1 into S phase (for review see Farnham et al., 1993).

In a normal cell, the RB protein is sequentially phosphorylated throughout the cell cycle and this is likely to be one of the mechanisms by which RB function is controlled (Goodrich et al., 1991). Phosphorylated forms of RB lose the ability to complex with E2F and the released E2F could result in transcription of the genes that are required in DNA replication. These genes may include dihydrofolate reductase (DHFR) (Blake and Azizkhan, 1989), thymidine kinase (Li et al., 1993), DNA polymerase alpha (Nevins, 1992), cdc2 (Dalton, 1992), and c-Myc (Heikkila et al., 1987). DHFR catalyzes the conversion of dihydrofolate to tetrahydrofolate, which is required in DNA synthesis process for the biosynthesis of purines and thymidylate (Lehninger, 1982). Thymidine kinase catalyzes the phosphorylation of thymidine to thymidylate (Lehninger, 1982). DNA polymerase alpha synthesizes the lagging strand of DNA at the replication fork. Cdc2 is a kinase that associates with various cyclins and is required for progression through the proliferative cell cycle (Muller et al., 1993).

The interaction between E7 and RB inhibits binding of E2F to RB (Chellappan et al., 1992), and as such leading to the inappropriate transcription of E2F-responsive genes and release of the normal blocks on progress through the cell cycle. The E7 interaction appears to block the growth suppressive activity of RB and contributes to the transformed phenotype. Although binding to RB may be necessary for transformation, it is not in itself sufficient for transformation since some mutations in the HPV 16 E7 gene fail to cooperate with EJ-ras to immortalize primary BRK cells, but exhibit normal RB binding (Banks et al., 1990). It has been demonstrated that E7 from all the genital-

associated HPVs are able to complex with RB in vitro (Dyson et al., 1989; Scheffner et al., 1991; Münger et al., 1989b). However, the efficiency of the E7 from "low risk" HPVs (eg., HPV-6 and HPV-11) to complex RB is lower than from the 'high risk' HPVs (Münger et al., 1989b; Tommasino and Crawford, 1995).

In addition to RB binding, E7 also interacts with RB-related proteins (p107 and p130) (Davies et al., 1993) and cyclin A/cdk2 (Tommasino et al., 1993). p107 and p130 also regulate the transcription activity of members of the E2F family involved in different checkpoints during cell cycle. p107 exerts its function in the G1 to S transition and in the G2 phase (Schwarz et a., 1993; Shirodkar et al., 1992), while p130 is involved into the transcriptional regulation during the G0 and G1 transition (Cobrinik et al., 1993). E7 binds the cyclin A/cdk2 complex in a cell cycle-dependent manner and the associated kinase activity appeared to be maximal in S and G2 phases when the cyclin A/cdk2 complex is normally active (Tommasino et al., 1993). The physiological importance of the E7 interaction with these cellular kinases is not yet clear. However, it has been hypothesized that the E7 protein may divert these kinases away from their normal substrates (Tommasino and Crawford, 1995). For example, it has been shown that when the cell is in S phase, the cyclin A/cdk2 complex can bind directly to the N-terminal domain of E2F. This interaction results in the phosphorylation of the E2F partner, DP1 (Goodrich and Lee, 1993; La Thangue, 1994), with the consequent shut off of E2F-mediated genes required for the G1/S transition (Krek et al., 1994). This may allow the cell to control the timing of the DNA replication and the progression of the cell cycle to ensure the fidelity of the DNA replication. It has been speculated that interacting with

the cyclin A/cdk2 complex, E7 may prevent the inactivation of E2F/DP1 complex in S phase (Tommasino and Crawford, 1995). This would likely result in the cell escaping the normal cell cycle checkpoints with consequent loss of the DNA replication fidelity and as such contributing to the virus-associated pathogenicity in the early phase of transformation. This hypothesis is consistent with the observation that HPV 16 E7 induces chromosomal abnormalities (Hashida and Yasumoto, 1991; White et al., 1994).

Cellular growth and differentiation are regulated by an intricate network of both positive and negative-acting diffusible factors and regulatory genes. The disruption of these regulatory circuits is thought to constitute an important step in carcinogenic progression. Taken together, the interaction of E7 with the cellular regulatory proteins could result in interference with some of these regulatory circuits as discussed above, thus overriding some of the most important cell cycle control mechanisms.

2.2.1.2 *E6 gene*

The oncogenic activities encoded by the HPV E6 gene include cell transformation, transcriptional transactivation and interaction with cellular proteins.

2.2.1.2.1 *The biochemical characteristics of the E6 protein*

The protein encoded by the E6 gene is a basic protein of about 150 amino acids and is localized to both the nuclear matrix and non-nuclear membrane fraction (Androphy et al., 1987a). The E6 protein levels are extremely low in both HPV containing cervical carcinoma cell lines (Androphy et al., 1987a; Banks et al., 1987) and HPV-immortalized

keratinocytes (Sedman et al., 1991). Analysis of the amino acid sequence of E6 revealed that there are two hypothetical zinc finger motifs (Cys-X-X-Cys), which are conserved throughout all HPV types, although E6 proteins have only moderate homology at the amino acid level. E6 has been shown to bind zinc in vitro, however, the zinc binding function has not been shown in vivo (Barbosa et al., 1989). As with Ad E1B and SV40 LT (Sarnow et al., 1982), E6 can complex with the cellular tumour suppressor p53 protein (Werness et al., 1990). Recently, E6 has been found to bind another cellular protein, called E6-binding protein (E6BP), a putative calcium-binding protein (Chen et al., 1995). The role of E6 binding to E6BP in the viral life cycle or transformation is not known.

2.2.1.2.2 *The biological functions of the E6 protein*

Although studies dissecting the functional nature of the E6/E7 region have confirmed that the E7 gene is the principal HPV-encoded transforming gene, the E6 gene from 'high risk' HPV types has been shown to encode a weak transforming activity. The E6 gene is capable of immortalizing primary human epithelial cells (Band et al., 1990; 1991) and primary mouse cells in cooperation with EJ-ras (Storey and Banks, 1993), and transforming NIH3T3 cells (Sedman et al., 1992). The E6 gene is found to be continuously expressed in tumour cell lines and required for maintenance of the transformed phenotype of cultured cells (Androphy et al., 1987a; Banks et al., 1987). It has been suggested that E6 plays a role in HPV induced cell immortalization and transformation as well as maintenance of the transformed phenotype (for review see

Mansur and Androphy, 1993; Farthing and Vousden, 1994; Tommasino and Crawford, 1995).

Recent studies have suggested that E6 may function through interaction with the cellular tumor suppressor p53 pathway, which functions as a crucial control molecule in the process of cell division (see section 3 for review of p53). The evidence to support this view came from the observation that p53 mutations are found in HPV-negative cervical carcinoma cell lines, while HPV-positive cervical tumour cell lines contain wildtype p53 (Crook et al., 1991b; Scheffner et al., 1991). It has been shown that E6 can interact with the cellular protein p53 both in vitro and in vivo (Werness et al., 1990; Lechner et al., 1992). This interaction is likely mediated with a cellular factor, called E6-AP (E6 associated protein) (Huibregtse et al., 1993), and this association results in the degradation of p53 through the ubiquitin-directed proteolysis pathway (Scheffner et al., 1990; Crook et al., 1991c). However, E6 from 'low risk' HPV types has been shown to interact only weakly with p53 and fails to promote its degradation in vitro (Crook et al., 1991a). This interaction, just as for the E7 and RB interaction, correlates with the oncogenicity of the HPV types and reflects another important difference between 'high risk' and 'low risk' HPV types.

Recent studies have indicated that the tumor suppressor p53 plays an important role in preventing DNA damage induced genetic lesions discussed in Section 3 (for review see Cox and Lane, 1995). It is now clear that p53 can control cellular proliferation by activating and repressing cellular genes involved in cell cycle control. In early neoplastic cells, it is postulated that the loss of p53 will result in cells replicating

following DNA damage and thus allow mutations to be fixed in subsequent replicated cells. This may result in the acquisition of chromosomal abnormalities responsible for the malignant transformation. Based on these data, it has been postulated that E6 interacting with p53 is analogous to an inactivating mutation of the p53 gene.

Several observations suggest that HPV E6 genes also encode p53-independent transforming activities. It has been demonstrated that HPV 16 E6 could transform NIH3T3 cells but trans-dominant p53 mutants could not transform these cells (Sedman et al., 1992), and that HPV 16 E6 could induce anchorage-independent growth of p53 deficient cells (Chen et al., 1995). It has been also shown that wild type p53 in several HPV-positive tumour cell lines is functionally active, however, a low level of the p53 associated trans-activation and DNA binding activities have been detected in these cell lines (Butz et al., 1995). Recently, another cellular target for E6 binding has been identified and it is termed the E6-binding protein (E6BP), a putative calcium-binding protein (Chen et al., 1995). The interaction between E6 and E6BP provides another lead for investigating p53-independent E6 activities. Calcium ion and calcium-binding proteins are involved in cell signalling and differentiation. It has been observed that E6 could alter differentiation of keratinocytes (McCance et al., 1988), and that keratinocytes immortalized by HPV 16 E6 and E7 are resistant to calcium-induced differentiation (Schlegel et al., 1988). Therefore, it is possible that E6 may affect terminal differentiation of epithelial cells by interaction with E6BP to provide the necessary environment for viral DNA replication. Moreover, Ad E1b and Bcl-2 inhibit apoptosis, and these proteins bind to a putative calcium binding protein (Boyd et al., 1994),

suggesting that E6 may similarly prevent apoptosis of HPV infected cells (Pan and Griep, 1994).

In addition to immortalization and transformation, E6 can transactivate several different eukaryotic promoters (Sedman et al., 1991; Desaintes et al., 1992). The equivalent transcriptional capability has been observed for both 'high risk' and 'low risk' E6, suggesting that this function may be of relevance for the viral life cycle rather than correlate with their oncogenic potential (Crook et al., 1991c; Desaintes et al., 1992). So far, a specific DNA element through which E6 exerts this trans-activation has not been defined. It has been shown that mutant E6 unable to bind p53 retained trans-activation activity in 3T3 cells, suggesting that the trans-activation may be a p53-independent activity (Crook et al., 1991c).

Taken together, these findings suggest that the HPV E6 gene encodes a multifunction protein with both transactivating and transforming activity. Its ability to inactivate the cellular tumor suppressor p53 likely plays an important role in neoplastic transformation.

2.2.1.3 *E5 gene*

The HPV E5 gene is usually lost during viral DNA integration which occurs in the advanced carcinoma stage (Baker et al., 1987; Schwartz et al., 1985). Therefore, if the E5 gene is involved in the development of cervical tumours, it would function at the early stage. A significant difference between bovine papillomavirus (BPV) and HPV is that the major transforming activity of BPV is encoded for by the E5 gene (Schiller et

al., 1986; Schlegel et al., 1986), but not the E6/E7 genes which are the main transforming genes in HPV (Martin et al., 1989). However, there is accumulating data suggesting that the E5 gene from HPV has weak transforming activity (for review see Mansur and Androphy, 1993; Banks and Matlashewski, 1993; 1995).

2.2.1.3.1. *The biochemical characteristics of the E5 protein*

The HPV E5 gene encodes a small and very hydrophobic protein (83 aa) with a molecule weight of approximately 10kD (Bubb et al., 1988; Halbert and Galloway, 1988). The E5 proteins are not well conserved among the papillomaviruses, however, the proteins are universally very hydrophobic and specific amino acid residues, including prolines and cysteines are similarly located in E5 from all HPVs. Localisation studies have shown that HPV-6 and 16 E5 proteins are localised to the Golgi apparatus, endoplasmic reticulum, and the nuclear membranes of transfected cells (Conrad et al., 1993). E5 can interact with a 16-kD protein which is a component of the vacuolar proton-ATPase pump complex (Conrad et al., 1993) and a variety of growth factor receptors including EGF-R, PDGF-R, the colony stimulating factor-1 receptor and p185neo (Hwang et al., 1995). The biological activity of E5 is closely associated with its ability to interact with growth factor receptors.

2.2.1.3.2. *The biological functions of the E5 protein*

The first report on the oncogenic potential for the HPV E5 gene came from the observation that expression of the HPV-6 E5 gene could stimulate anchorage-independent

growth of NIH3T3 cells and these cells were then capable of forming tumors in nude mice (Chen and Mounts, 1990). The oncogenic potential of E5 was also reported for HPV-16 (Leechanachai et al., 1992; Pim et al., 1992). These studies have shown that the HPV-16 E5 gene had oncogenic activity and linked E5 activity to the signal transduction from the epidermal growth factor receptor (EGF-R) to the nuclear expression of the c-fos and c-jun genes (Leechanachai et al., 1992; Bouvard et al., 1994a). Fos and Jun are components of the transcription factor AP-1. Transcription of the transforming genes of HPV-16 can also be stimulated through AP-1 binding sites on the viral regulatory region (Chan et al., 1990). As a consequence, E5 may contribute to the regulations of both cellular and viral genes.

The data by Leechanachai et al., 1992 and Pim et al., 1992 suggested that one of the biological functions of E5 may be to amplify or cooperate with the EGF mediated intracellular signal transduction pathway. This view was further supported by the observation that expressing HPV 16 E5 in human keratinocytes resulted in an increase in the number of EGF-Rs at the cell surface and that there was an inhibition of receptor degradation (Straight et al., 1993). Perhaps the demonstration that E5 also had a strong mitogenic activity in these cells was particularly important, because the natural target cells for HPV are human keratinocytes. Consistent with this observation, it was demonstrated that HPV 16 E5 could cooperate with E7 to potentiate a mitogenic response which is enhanced in the presence of the EGF in primary cells (Bouvard et al., 1994a). The possible mechanism by which HPV E5 induced mitogenic activity seems to be associated with its ability to inhibit the down-regulation of the EGF-R. It was further

revealed that E5 could bind to a 16-kD protein, a component of the vacuolar proton-ATPase pump complex (H^+-ATPase) involved in receptor protein degradation (Conrad et al., 1993), resulting in an impairment of acidification of endosomes (Straight et al., 1995). The acidification of endosomes is essential for their proteolysis function, including those involved in the degradation of the EGF-R (Maxfield, 1985; Schneider, 1987). The H^+-ATPase pump is responsible for the acidification, and E5 interaction with 16-kD component may impair this process in HPV infected human keratinocytes.

Based on above observations, a model for HPV E5 induced mitogenic activity has been suggested (Banks and Matlashewski, 1995). E5 interacts with the 16-kD vacuolar H^+-ATPase and impairs its activity for degradation of growth factor receptors, resulting in an increased number of EGF-R recycled back to the cell surface. This provides these cells with a proliferation advantage over non-E5 expressing cells and also has a positive effect on the viral upstream regulatory region.

Given the connection between the E5 and signal transduction from EGF-R to nuclear expression (c-fos and c-jun), it is of interest to elucidate the possible signal transduction pathways influenced by E5. The transcription factor AP-1 is believed to be one of the nuclear targets of several signal transduction pathways, in particular of the EGF-R associated Ras-MAP kinase pathway (reviewed by Hill and Treisman, 1995). A recent study on the mitogenic activity of HPV 6 and 16 E5 in cooperation with E7 provided some evidence for connecting E5 with the Ras-MAP kinase pathway (Valle and Banks, 1995). It has been shown that over-expressing c-Raf, which is a signal mediator between the Ras and MAP kinase, appeared to be capable of functionally substituting for

E5 in these co-mitogen assays (Valle and Banks, 1995).

Based on the above observations, it may be expected that any downstream second messengers which are involved in EGF-R mediated signal transduction would be more active in cells expressing the E5 protein. This was one of the objectives of the study presented within this thesis.

In addition to the regulation of growth factor mediated responses and cell transformation, E5 may also have other activities. There is evidence that E5 may impair the processing of antigen thus making infected cells invisible to the cellular immune response (Cromm et. al., 1994). E5 may also impair gap junction thus reducing cell-cell communication (Oelze et al., 1995).

Taken together, these studies suggest that E5 acts in a multifunctional manner. The activities of E5 contributing to the oncogenic potential include: enhancement of growth factor mediated signal transduction, up-regulation of viral gene expression, and impairment of antigen presentation and cell-cell communication. These activities may contribute to the cell transformation and provide a significant advantage to the virus in term of replication and survival in the host cell.

The above reviewed biochemical and biological evidence all support the causal role of human papillomaviruses in human malignancies, and that the E5, E6, and E7 genes contribute to HPV-associated oncogenicity. However, in addition to these transforming genes, other genes encoded by HPV are involved in viral infection, viral DNA replication and the life cycle. The function of these viral genes are described below.

2.2.2 *Genes with non-transforming activities*

2.2.2.1 *E1 gene-viral DNA replication*

The E1 gene is the largest of the papillomavirus genes and is highly conserved. It is commonly interrupted when viral genomic integration occurs (Schwartz et al., 1985), suggesting a role in the maintenance of the viral genome in the episomal stage (Lusky and Botchan, 1984). Studies using the bovine papillomavirus (BPV) system have established that E1 is necessary for virus DNA replication.

The E1 gene encodes at least two replication functions: the 5' end of the E1 gene encodes a modulator (E1-M) function, and the 3' end two-thirds of the E1 gene encodes a replication (E1-R) function (Lusky and Botchan, 1979; 1986a). Amino acid sequence analysis revealed that its C-terminal portion has some homology with the large T antigens of SV40 and polyomavirus that have a direct role in replication of these viruses (Seif, 1984; Chiang et al., 1991). A BPV-1 E1-M mutant does not disrupt the viral replication, indicating that E1-M is not absolutely required for replication. Instead, it plays a modulator function in a suppressive manner (Roberts and Weintraub, 1986). The BPV-1 E1-R mutant is not able to replicate, suggesting that E1-R plays an essential role in viral DNA replication (Lusky and Botchan, 1986a; 1986b).

More recently it has been reported that HPV-11 containing cells express a spliced mRNA fusing the E1-M domain to the C-terminal portion of E2 (Chiang et al., 1991). The product of this message functions as a modulator in replication similar to BPV E1-M (Chiang et al., 1991).

These studies indicate that the E1 gene is required for maintaining the viral genome in the episomal stage and for viral DNA replication.

2.2.2.2 *E2 gene-multifunction*

The E2 gene is a complex ORF, and like E1, it has been studied principally in BPV. The E2 gene encodes major regulators for viral gene expression and is also required for DNA replication. E2 displays novel features in the mechanism of interaction with the DNA and in alterative splicing which regulates their activity. The E2 protein is present in different forms and exhibits transcriptional activation and repression depending on the form of the E2 proteins (see below) (Lambert et al., 1987; Choe et al., 1989; Stanley et al., 1989; Doorbar et al., 1990; Vaillancourt et al., 1990; Bouvard et al., 1994b).

E2 could stimulate transcription of viral genes through its interaction with enhancers located within the viral LCR. Its ability to regulate viral gene expression appears to be dependent upon the ability to bind to specific sequences in the LCR (Hirochika et al., 1988; Hawley-Nelson et al., 1988). E2 can function as a strong activator of viral gene expression in cooperation with other cellular transcription factors, such as AP-1, Oct-1 as well as Sp-1 (Ushikai et al., 1994). Studies on the predicted amino acid structure of E2 from a variety of papillomaviruses have indicated that the E2 protein consists of two conserved domains, a N-terminal 220 amino acids trans-activation domain (Haugen et al., 1989; McBride et al., 1989), and a C-terminal 100 amino acid domain where specific DNA-binding has been mapped (McBride et al., 1988). Two E2-binding consensus motifs have been recognized as $ACCN_6GGT$ and $ACCN_6GTT$ within

the origins of replication of papillomaviruses, where N is any nucleotide (Androphy et al., 1987b; Moskaluk and Bastia, 1988).

It has been noted, however, that E2 can also activate promoters that do not contain the consensus motif (Heike et al., 1989). It seems that E2 need not necessarily bind DNA to function as trans-activator. Evidence to support this view has come from a recent observation that an E2 mutant that lacks the C-terminal DNA-binding domain retained a low level of transactivation activity (Storey et al., 1992). This general trans-activation could extend the E2 transcriptional regulatory activity to cellular genes. However, to date, there is no evidence that this general E2 mediated trans-activation is of physiologic significance.

Studies have shown that a cDNA, in addition to the full length E2, encoding only the C-terminal DNA binding domain of E2 but lacking the putative trans-activation domain exists in HPV type 16 immortalized keratinocytes (Stanley et al., 1989; Doorbar et al., 1990; Nasseri et al., 1991; Sherman and Alloul, 1992). This smaller HPV 16 E2 protein is similar to the BPV-1 repressor E2 cDNA E8^E2 (Choe et al., 1989; Vaillancourt et al., 1990). It has been demonstrated that this shorter form of E2 protein was capable of repressing the viral promoter and enhancer by interfering with the transcriptional activation of the full length E2 protein (Bouvard et. al., 1994b). It has been suggested that in vivo, the respective amounts of the two forms of E2 are critical for controlling viral gene expression. In addition to its role in transcriptional regulation, E2 is also required for viral DNA replication in conjunction with E1 to bind specific DNA sequences (Bream et al., 1993). Consistent with this view is the observation that

mutations which inactivate E2 result in integration of viral DNA into host genome (Rabson et al., 1986).

These findings indicate that the E2 gene functions as a transcription regulator for viral gene expression and is also necessary for viral DNA replication and maintenance of the viral genome as an episome.

2.2.2.3 *E4 gene-virus release*

The E4 gene is contained entirely within the E2 gene in a different reading frame. Its product does not seem to be a component of the virus particle or to be involved in in vitro transformation (Phelps et al., 1988; Storey et al., 1988), but accumulates in the cytoplasm (Doorbar et al., 1989; Crum et al., 1990; Sterling et al., 1993).

It has been shown that the E4 gene is coexpressed with the late genes coding for the capsid proteins, although the E4 gene is located in the early region of the viral genome,(Baker and Howley, 1987; Crum et al., 1990). In spite of the plentiful abundance of E4 within some infected cells, the function of the protein is still not clear. However, it has been suggested that the E4 proteins may enhance virus release from the productively infected cell. This view is supported by the observation that the E4 proteins are associated with cytokeratins in human keratinocytes, resulting in the collapse of the intermediate filament network in the cell (Doorbar et al., 1991).

2.2.2.4. *Genes encoding structural proteins*

The HPV particles are 52-55 nm in diameter. The viral genome is contained

within a spherical protein coat or 72 pentameric capsomeres (Baker et al., 1991). The capsid consists of at least two structural proteins. The L1 gene codes for a major capsid protein that has a molecular weight of approximately 55,000 daltons and represents 80% of the total viral protein. It has been shown recently that L1 proteins from a variety of HPV types, following expression in recombinant systems, will self-assemble to produce virus-like particles (Hagensee et al., 1993; Rose et al., 1993; Kirnbauer et al., 1993). However, the virus-like particles containing L1 alone appear to have a higher degree of heterogeneity when compared to those consisting of both L1 and L2, suggesting a role for L2 in the stabilization of the capsid structure (Hagensee et al., 1993).

The L2 gene codes for a minor capsid protein that has a molecular weight of approximately 70,000 daltons (Favre et al., 1975; Pfister et al., 1977). Studies on the immunohistochemistry of the L2 gene products (L2) from HPV types indicated that there was less cross-reactivity, suggesting that L2 contains antigenic determinants that are relatively type-specific (Firzlaff et al., 1988). Naturally occurring antisera, at least for HPVs 16 and 18 infection, are frequently directed against this protein and are mostly type-specific (Jenison et al., 1991; Komly et al., 1986). These results have opened a view to producing high titre, type-specific peptide antisera for use as immunological probes (Volpers et al., 1993).

In conclusion, the L1 and L2 genes are both necessary for the virion. L1 is the major structural protein. L2 is also a structural protein and may play a role in stabilization of the capsid structure. They are expressed in terminal differentiated keratinocytes for viral assembly.

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3. The p53 tumour suppressor

p53 is one of the most significant human tumor suppressor proteins. It plays an important role in cell cycle control, DNA repair, and apoptosis (for review see Vogelstein and Kinzler, 1992; Lane, 1994; Cox and Lane, 1995; Liebermann et al., 1995). There is accumulating evidence that inactivation of the p53 tumour suppressor is a common event in the development of human malignancies, occurring in more than 50% of all tumours (Hollstein et al., 1991; Friend, 1994). The continued expression of wild type p53 therefore appears to counteract the tumorigenic process. This realization has spurred extensive investigation into the biochemical and biological nature of this protein.

3.1. *Biochemical characteristics of p53*

The gene for p53 has been isolated from different species (for review see Montenarh, 1992) such as human (Matlashewski et al., 1984), monkey (Rigaudy and Eckhart, 1989) and mouse (Oren and Levine, 1983). Analysis of the structural and evolutionary features of the p53 proteins indicates that some regions of the protein are highly conserved at the amino acid level (for review see Donehower and Bradley, 1993). These evolutionarily conserved regions fall into five widely spaced amino acid clusters termed domains I to V as illustrated in figure 3 (page 55A) and discussed below. p53s from different species share some biochemical and biological similarities. The biochemical characteristics of p53 include protein/protein interaction, DNA binding, transcription regulation, and phosphorylation (for review see Montenarh, 1992; Donehower and Bradley, 1993; Haffner and Oren, 1995).

The human p53 protein is a 53 kilodalton nuclear phosphoprotein. The ability of p53 to form stable complexes with viral oncoproteins was the basis for the discovery of this cellular protein. It forms specific complexes with several viral proteins including SV40 T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979), adenovirus E1b 58Kd protein (Sarnow et al., 1982) and HPV E6 proteins (Werness et al., 1990). The interaction with oncogenic HPV E6 leads to the degradation of p53 through the ubiquitin proteolysis pathway (Scheffner et al., 1990) and this process requires a third cellular protein component termed E6-AP (Huibregtse et al., 1991, 1993).

Recently p53 has been shown to be able to complex with several cellular proteins including two protein kinases, casein kinase II (CKII) (Kraiss et al., 1990) and $p34^{cdc2}$ (Sturtzbecher et al., 1990; Milner et al., 1990), mdm2 (Momand et al., 1992; Oliner et al., 1992), and TATA-binding protein (Seto et al., 1992; Horikoshi et al., 1995; Thut et al., 1995). In addition to its ability to bind heterologous proteins, p53 is able to self-associate to form homodimers and tetramers (Schmieg and Simmons, 1988; Sturtzbecher et al., 1992).

Besides protein/protein interactions, p53 exhibits sequence specific DNA binding property. Several nucleotides specifically recognized by the p53 protein have been reported (Bargonetti et al., 1991; Kern et al., 1991; El-Deiry et al., 1992; Funk et al., 1992; Foord et al., 1993). For efficient DNA binding, p53 requires two copies of the 10-bp motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', separated by 0 to 13 bp (El-Deiry et al., 1992; Funk et al., 1992). p53 is a transcriptional regulator (for review see Vogelstein and Kinzler, 1992; Lane, 1994; Cox and Lane, 1995). It binds specific enhancer

sequences and stimulates transcription of genes with this sequence. Transcriptional activation represents an important component of the tumor suppression functions of p53 (Vogelstein and Kinzler, 1992). p53 can also repress expression of many genes that lack p53 response elements by binding to TATA-binding proteins (Ginsberg et al., 1991; Santhanam et al., 1991; Seto et al., 1992; Subler et al., 1992; Ragimov et al., 1993), however, the biological consequences of repression have not yet been established.

The p53 protein is phosphorylated on multiple sites by several cellular kinases including protein kinase C (Hupp and Lane, 1994), CKII (Kraiss et al., 1990), $p34^{cdc2}$ (Sturtzbecher et al., 1990). Phosphorylation is believed to modulate the function of the protein (for review see Meek and Street; 1992; Cox and Lane, 1995).

Most biochemical properties and the domains for the interaction of p53 with viral and cellular proteins have been mapped on different region of the polypeptide chain of p53 as illustrated in Figure 3. The domains for binding viral and cellular proteins, such as SV40 T antigen and mdm2 have been identified (Jenkins and Sturtzbecher, 1988; Yew and Berk, 1992). Recently the domain from residues 160 to 347 of p53 has been suggested to be necessary for binding HPV E6, however, it is separated from the domain required for E6-mediated degradation (Mansur et al., 1995). p53 contains a domain from amino acids 102 to 292 which is sufficient for sequence specific DNA binding (Bargonetti et al., 1993; Pavletich et al., 1993). The amino-terminal 73 amino acids domain is required for transcription activation (Shaulian et al., 1992). The C-terminal domain is necessary for self-association of p53 into dimers and tetramers (Sturtzbecher et al., 1992) and is involved in allosterically regulating p53 DNA binding activity (Hupp

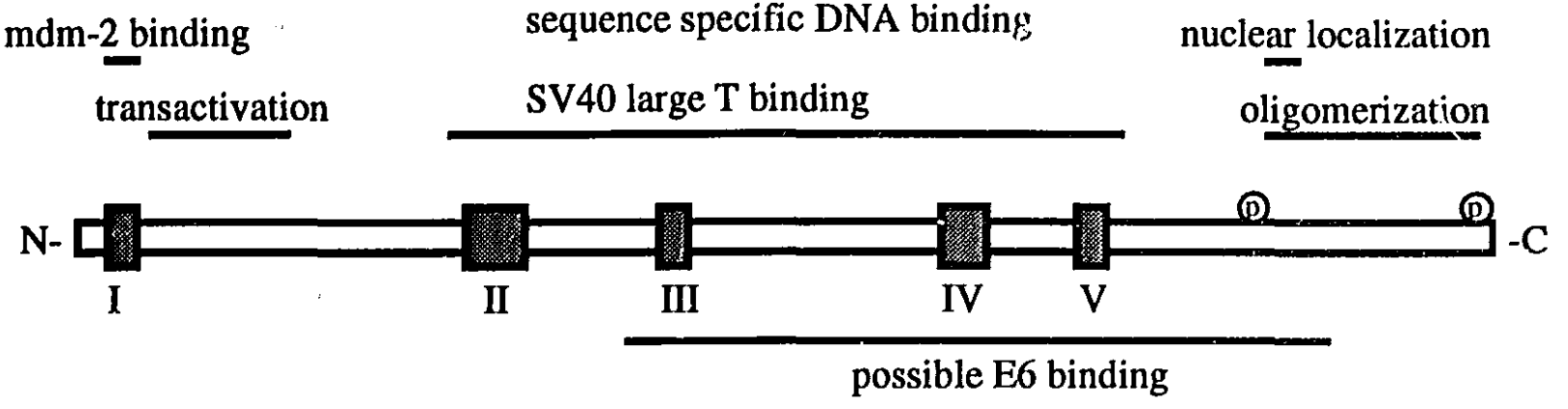

Figure 3. Schematic representation of the p53 protein (Adapted from Crook et al., 1994). Closed boxes represent the evolutionarily converved regions I-V. The sites for phosphorylation by cdc-2 and CKII, and regions of the protein important for oligomerization, nuclear localization, DNA binding, transactivation, and for common protein/protein interaction are indicated.

et al., 1992; Halazonetis and Kandil, 1993).

In summary, p53 is a nuclear phosphoprotein which exhibits sequence-specific DNA binding activity and is a transcription regulatory factor. It can interact with several cellular and viral proteins. The protein itself can form dimers and tetramers. These biochemical properties are localized to different regions of the protein.

3.2. *p53-tumor suppressor*

The evidence for the tumor suppressor function of wild type p53 is now conclusive. Its tumor suppressive function is associated with its ability to regulate a variety of cellular processes, including growth arrest, DNA repair, and apoptosis (for review see Lane, 1992; Cox and Lane, 1995; Liebermann et al., 1995).

It has been observed that the cell cycle can proceed normally in the total absence of p53 protein, and that mice deficient for p53 develop normally, however, they are susceptible to spontaneous tumors at an early age (Donehower et al., 1992). The studies indicate that functional p53 is not critical for the normal cell cycle, but demonstrate that p53 plays an important role in preventing neoplastic transformation. It has been proposed that in response to DNA damage, p53 could induce cell growth arrest and DNA repair, or apoptosis, thus providing cells with a defensive mechanism in response to DNA damage (for review see Lane, 1992; Cox and Lane, 1995; Liebermann et al., 1995).

3.2.1. *Growth arrest-the p53 response to DNA damage*

Cancer develops through a multistep process whereby accumulation of a number

of mutations in an assortment genes is required. A number of genotoxic agents contribute to the genomic instability which could lead to genetic mutations, including tumor viruses, genotoxic drugs, and radiation. Cells with DNA damage respond by undergoing cell cycle arrest for DNA repair or apoptosis possibly dependent on the level and persistence of DNA damage (Cox and Lane, 1995).

There are lines of evidence that p53 is implicated in growth arrest in response to DNA damage. Significantly, increased p53 protein levels and p53-mediated transcription activating activity have been observed in human and mouse cells both in vitro and in vivo following DNA damage (Maltzman and czyzk, 1984; Hall et al., 1993; Lu and Lane, 1993; Gu et al., 1994). Recently, a novel p53 promoter element involved in genotoxic stress-inducible p53 gene expression has been identified (Sun et al., 1995). Moreover, following DNA damage, G1 arrest occurs in cells with wild type p53, but not in cells with nonfunctional mutant p53 (Kastan et al., 1991; McIlwrath et al., 1994). Consistent with these observations are that transfection and over-expression of p53 blocks cell cycle progression from G1 to enter the S phase (Diller et al., 1990; Michalovitz et al., 1990, Lin et al., 1992). These studies demonstrate that p53 induces G1 arrest following DNA damage.

It has been shown that p53 mediated transactivation is induced by DNA damage agents (Lu and Lane, 1993). p53 can transactivate the cyclin-kinase inhibitor, $p21^{Cip1}$, also called CIP1/WAF1, which blocks kinase activity of cyclin E-Cdk2 that acts at the G1/S transition (Koff et al., 1992; El-Deiry et al., 1993). Another p53-up-regulated gene, GADD45, has also been found to suppress cell growth following DNA damage (Kastan

et al., 1992; Zhan et al., 1994). Moreover, p53 is capable of transcriptionally down-regulating growth associated genes such as proliferating cell nuclear antigen (PCNA) (Jackson et al., 1994) and fos (Kley et al., 1992). Thus, p53 likely plays a role in the induction of the G1 checkpoint following DNA damage, and does so by acting as a transcriptional regulatory factor that activates genes involved in negative growth control.

Taken together, in response to DNA damage, the p53 protein level is increased. p53 acts as a transcription regulatory factor for both negative and positive growth control genes, thus inducing cell cycle arrest. The significance is that p53-induced G1 arrest allows cells with DNA damage sufficient time for DNA repair. In addition, p53 is involved in DNA repair process and apoptosis following DNA damage and these will be expanded on below.

3.2.2. *DNA repair-the p53 response to DNA damage*

The long-term survival of a species may be enhanced by changes in its genetic inheritance, but its survival in the short term demands accurate maintenance of the integrity of DNA sequences. DNA integrity can be achieved by the high fidelity of DNA replication and DNA repair processes. Failure of the cell to recognize and repair DNA damage frequently provides a key step in tumor progression. p53 is well-known to function as an activator of the G1 checkpoint which is believed to provide cells with time for DNA repair following DNA damage. However, there is evidence that links p53 with the DNA repair process.

It has been found that p53 can bind to the regions of single stranded DNA which

are generated by DNA damage (Oberosler et al., 1993). p53 also can recognize primary DNA damage in the form of insertion/deletion mismatches and form stable complexes at the lesions (Lee et al., 1995). Furthermore, it has been demonstrated that p53 could associate with a nucleotide excision repair protein ERCC3 (Wang et al., 1994). These data suggest that p53 may directly participate in repair related processes.

p53 is a transcription factor. It has been also shown that p53 regulates the gene GADD45 which has a growth suppressive role following DNA damage and can stimulate nucleotide excision repair in vitro (Smith, et al., 1994). Studies showed that blockage of GADD45 gene expression by constitutive expression of GADD45 antisense RNA significantly reduced cell survival following UV irradiation (Smith et al., 1994). Thus p53 may be involve in the repair process by regulating the GADD45 gene.

The clearest data have come from comparison studies between cells with wild type p53, cells with wild type p53 but carrying the HPV E6 oncogene, and cells carrying a dominant-negative mutant p53. This study showed that disruption of normal p53 function in human colon carcinoma cells with either HPV E6 oncoprotein or a dominant-negative mutant p53 transgene results in reduced repair of UV-induced DNA damage (Smith et al., 1995).

Taken together, these studies suggest that p53 plays a positive role in DNA repair processes. It can bind to damaged regions in DNA and regulate genes involved in the repair process. The significance of the binding to damaged regions may be to provide signals for repair related molecules.

3.2.3. *Apoptosis-the p53 response to DNA damage*

Apoptosis or the process of programmed cell death is a fundamentally important part of normal development which preserves homeostasis by controlling the rate of cell death to balance cell proliferation (Raff, 1992). Apoptosis is an active and energy-dependent process, and requires specific gene expression (Martin et al., 1988; Schwartz et al., 1990). It was subsequently shown that the requirement of specific gene expression for the induction of apoptosis may reflect the need to synthesize molecules that activate or derepress the existing cell death machinery, rather than making components required for the basic cell death program itself (Raff et al., 1993; Jacobson et al., 1994). However, the regulation of apoptosis remains poorly understood.

Apoptosis is initiated by a variety of stimuli including physiologic activators, damage-related inducers, therapy-associated agents, and toxins (for review see Steller, 1995; Thompson, 1995). p53 appears to be predominantly required for mediating the apoptotic response to DNA damage (Lowe et al., 1993). This view is supported by following observations: 1) that p53 is required for the efficient activation of apoptosis following irradiation or treatment with chemotherapeutic compounds (for review see Fisher, 1994; Reed, 1994); 2) that significantly higher levels of p53 protein is observed following DNA damage. It is however not fully understood how p53 induces apoptosis.

It was recently shown that the relative ratio of Bax and Bcl-2 proteins is a major determinant of cellular susceptibility to apoptosis (Oltvai et al., 1993). Bax and Bcl-2 are homologous proteins that have oppositive effects on cell survival and death (for review see Reed, 1994). Bax acts as an accelerator of apoptosis, while Bcl-2 inhibits apoptosis

in response to DNA damage (Sentman et al., 1991). Bax and Bcl-2 can form heterodimers in cells, maintaining the balance between survival and apoptosis (Oltvai et al., 1993). p53 may feed into this decision step through activation of the bax gene (Miyashita and Reed, 1995) and down-regulation of the bcl-2 gene (Miyashita et al., 1994).

Therefore, taken together, a model for p53-induced apoptosis in response to DNA damage may be the following. Increased p53 activity in response to DNA damage or other means can reduce the resistance of cells to apoptotic stimuli through p53-mediated effects on Bax and Bcl-2 gene expression. Alternatively, in response to DNA damage the p53-induced changes in ratio of Bax and Bcl-2 proteins would result in cells with enhanced susceptibility to apoptotic death (Cox and Lane, 1995; Miyashita and Reed, 1995). However, a contradictory observation has been reported showing that transcriptional activation of p53-target genes is not required for p53-dependent apoptosis (Caelles et al., 1994).

In summary, p53 plays an important role in maintaining the integrity of genome. It provides a DNA damage G1 checkpoint control allowing cells sufficient time for DNA repair. p53 may also exert direct control of DNA repair and induce apoptosis following DNA damage. The mechanism by which p53 acts as tumor suppressor is believed to reside primarily in its ability to act as a transcriptional transactivator.

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4. MAP kinase signal transduction

4.1. *MAP kinase signal transduction pathway*

The mitogen-activated protein (MAP) kinase (MAPK) pathway is a conserved eukaryotic signaling module that links the signal from cell surface into a variety of outputs (for review see Thomas, 1992; Ruderman, 1993; Herskowitz, 1995; Hill and Treisman, 1995; Hunter, 1995; Marshall, 1994 and 1995). It can be activated by different receptors expressed at the cell surface including transmembrane domain-like receptors, cytokine- and lymphokine-like receptors, or growth factor-like receptors depending on the cell type (for review see Mordret, 1993). In addition to growth factors or cytokines, stimuli for this pathway can also include heat shock (Dubois and Bensaude, 1993) or mechanical (Yamazaki et al., 1993). The MAPK pathway is associated with cell proliferation and differentiation as well as stress responses (Ahn et al., 1992; Han et al., 1994; Rouse et al., 1994).

Three distinct MAPK pathways have been identified in vertebrates so far. The best-understood MAPK pathway leading to activation of MAPKs or ERKs is the Ras/Raf/MEK/ERK pathway as illustrated in Figure 4, where MEK refers to MAP/ERK kinase, and ERK refers to extracellular-signal regulated protein kinase and has a general name of MAPK (for review see Hunter, 1995). The Ras/Raf/MEK/ERK pathway is extensively used for transcytoplasmic signaling to the nucleus, where transcription of specific genes is induced through phosphorylation and activation of transcription factors. Both receptors for protein-tyrosine kinases and G protein-coupled serpentine receptors

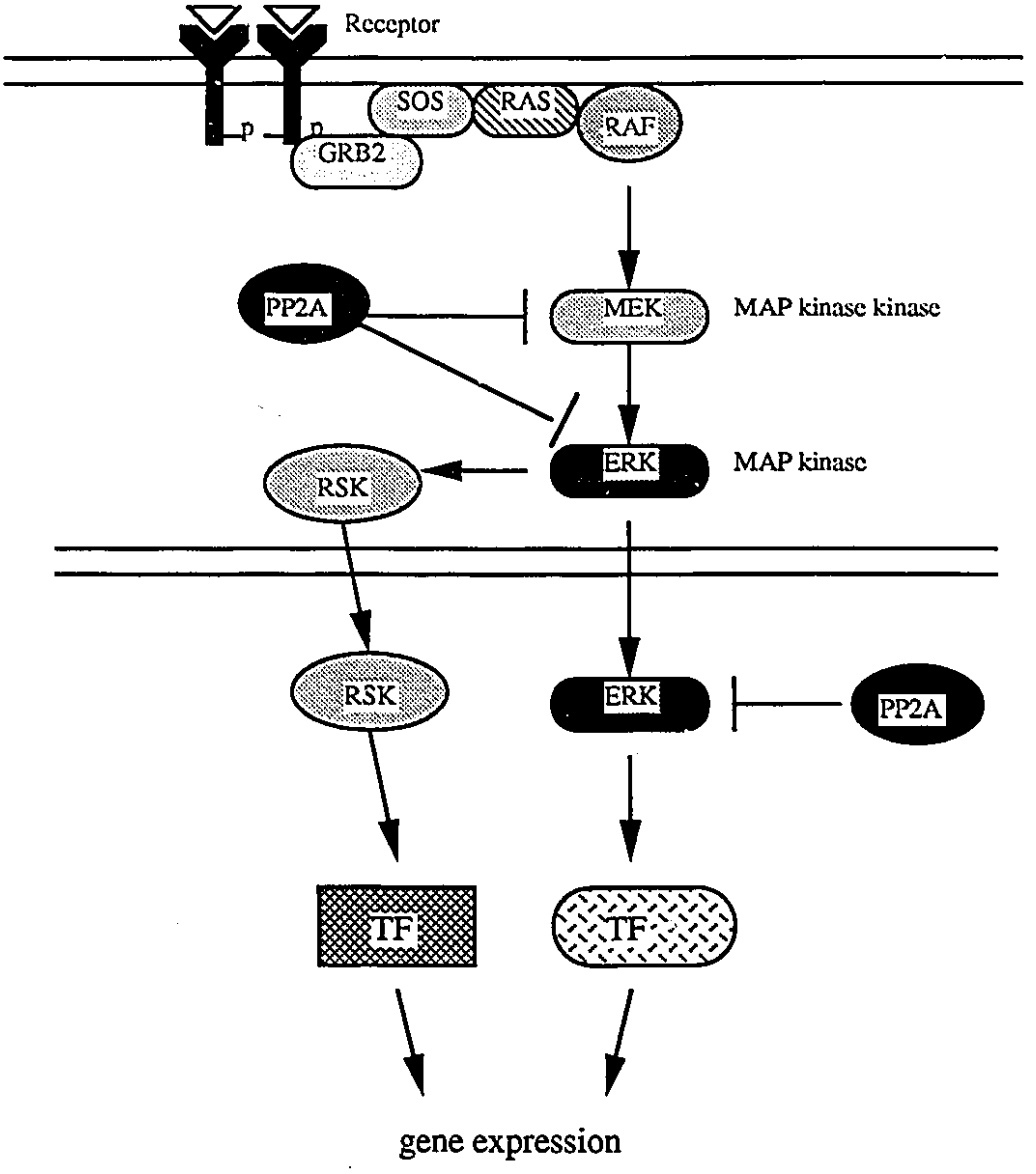

Figure 4. MAP kinase pathway (Adapted from Hunter, 1995). A schematic illustration of the Ras/Raf/MEK/ERK pathway and PP2A that negatively regulates activation of the pathway is presented. Abbreviations: GRB2, growth factor receptor-bound protein 2; SOS, son of seven less; RSK, ribosomal S6 kinase; TF, transcription factor; PP2A, protein phosphatase 2A.

link into this pathway (for review see Johnson and Vaillincourt, 1994).

In response to growth factor receptor binding, controllers of Ras exchange factors, such Grb2/Sem5, bind to phosphate groups which tyrosine kinase receptors attach to their tails when they are active. They recruit exchange factors to interact with Ras, and then the inactive form of Ras-GDP is converted to the active form of Ras-GTP. Downstream of Ras, a principal target is the Raf protein kinase. Once activated, Raf phosphorylates MAPK kinases which activate MAPKs. Activated MAPKs are translocated into the nucleus upon activation, where they can potentially phosphorylate a broad range of substrates which are involved in different cellular functions (for review see Hunter, 1995).

The MAPK pathway regulates translation through ribosomal S6 kinase (rsk) (Blenis, 1993) and protamine kinase (Reddy et al., 1993) which phosphorylates the eukaryotic protein synthesis initiation factor eIF4-E. MAPKs regulate transcription by phosphorylating nuclear transcription factors, such as c-Jun, c-Myc, and c-Tal1 (Cheng et al., 1993; Pulverer et al., 1991 and 1993), or through regulating transcription of genes encoding transcription factors (for review see Hill and Treisman, 1995). For instance, at the c-Fos serum response element (SRE), activated ternary complex factor (TCF) and activated SRE cooperate for maximal gene expression, and the signals from growth factors to TCF is mediated through MAPK pathway (Treisman, 1994). Activation of the MAPK pathway is rapidly down-regulated by protein phosphatases (PPs), removal of the activating phosphates from MAPK kinases and MAPKs, and possibly Raf, by PP2A, results in inactivation of these kinases (Anderson et al., 1990; Johnson and Vaillincourt,

1994).

In summary of this part, the MAPK signal pathway is composed of a cascade of protein kinases. The pathway mediates the transcytoplasmic signaling to the nucleus, where transcription factors are phophorylated and regulated. The pathway is also associated with cell proliferation and differentiation. It is controlled in a rapid switch on and off manner.

4.2. *MAP kinase signal transduction and cell transformation*

One of the hallmarks of the MAPK signaling pathway is its rapid transience (Nebreda, 1994; Johnson and Vaillincourt, 1994), and inappropriate activation or inhibition of its down-regulation can contribute to cell transformation. Indeed, the components of this pathway, such as Ras and Raf, have been identified as the products of transforming oncogenes (Feramisco et al., 1984; Heidecker et al., 1992). The current hypothesis is that oncogenes such as Ras, Raf, Src and Mos transform cells by prolonging the activated stage of MAPK kinases and of components downstream in the signaling pathway (Mansour et al., 1994).

The sustained activation of MAPKs can lead to a different cellular response because nuclear accumulation of active MAPKs will result in phosphorylation of transcription factors which alter gene expression (Dikic et al., 1994; Traverse et al., 1994). It has been demonstrated that sustained MAPKs activation could result in cell proliferation, differentiation, or transformation (Sontag et al., 1993; Dikic et al., 1994; Traverse et al., 1994). Constitutive activation of MAPK kinase can transform mammalian

cells (Mansour et al., 1994). The rate of internalization of receptors and whether they are down-regulated as a result of activation of the MAPK pathway may also affect the duration of signaling. One example is a study (Chapter IV) presented in this thesis. It is demonstrated within that E5 expression results in an increase in MAPK activity (Gu and Matlashewski, 1995) and this may be due to E5's ability to reduce growth factor receptor internalisation and degradation (Straight et al., 1993). It has also been shown that the constitutive activation of the MAPK pathway by SV40 small T could induce monkey kidney CV-1 cell proliferation (Sontag et al., 1993). SV40 small T inhibits the inactivation of MAPK kinases by PP2A (Sontag et al., 1993), thus together with the study on HPV E5 demonstrating that DNA tumour virus proteins could potentiate MAP kinase activity, although in a different manner. The increase in MAPK activity or sustained MAPK activation would alter cellular gene expression required for cell proliferation enhancing DNA replication of infected cells, thus ensuring replication of the viral genome in these cells.

Taken together, the MAPK pathways are important intracellular signaling pathways which serve to link signals from the cell surface to cytoplasmic and nuclear events. Inappropriate activation or the inhibition of down-regulation of this pathway can contribute to cell transformation and may also be the target of DNA tumour viruses.

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RATIONALE FOR THE STUDY

Chapter II:

Previous studies have suggested that the p53 tumor suppressor is the 'guardian of the genome' and maintains the integrity of the genome through arresting cells in G1 following DNA damage (Lane, 1992). This presumably allows cells sufficient time to repair the damage before entering replicative DNA synthesis (Kastan et al., 1991; 1992; Kuerbitz et al., 1992). It has been demonstrated that p53 is biologically activated as a transcriptional transactivator following DNA damage (Lu and Lane, 1993). Furthermore, it has been shown that the E6 proteins from the 'high-risk' HPVs can bind to and promote the degradation of the p53 protein (Scheffner et al., 1990; Crook et al., 1991). However, it was unclear whether E6 can abrogate induced p53-mediated transcription following a genotoxic insult. Thus it was proposed to examine the effect that HPV type 18 E6 has on p53-mediated transcription activity following UV irradiation. This work was published in the *Oncogene* in September 1994 (vol. 9:629-633).

Chapter III:

The p53 gene is only rarely mutated in cervical cancer and more than 90% cervical carcinomas contain and express HPV DNA sequences of 'high-risk' types including types 16, 18 and others. The E6 protein from 'high-risk' HPV types can complex with and promote p53 degradation and this is likely to be the major reason why the p53 gene is only rarely mutated in these neoplasias. Previous studies on the self-

association and transactivation activity of p53 revealed that the p53 monomeric variants retained transactivation activity (Tarunina and Jenkins, 1993). Furthermore, it has been shown that a p53 monomeric mutant 338 is only poorly targeted by E6 for degradation (Thomas et al., 1995; Kalita and Matlashewski, unpublished result). Based on this information, I have investigated the biological consequences of introducing into HPV-positive cervical cancer cells a transcriptionally functional p53 monomeric mutant which is resistant to E6 mediated degradation.

Chapter IV:

It has been proposed that oncogenes such as ras, src, raf, and mos transform cells in part by prolonging the activated stage of components within the mitogen-activated protein (MAP) kinase signal transduction pathway (Mansour et al., 1994). This signal transduction pathway mediates cellular responses to growth factors and differentiation factors. The HPV oncogenes E6 and E7 encode the major transforming proteins of the virus. The HPV type 16 E5 gene has also been shown to have weak transforming activity and cooperate with the epidermal growth factor receptor to amplify the signal transduction to the nuclear expression of c-fos (Leechanachai et al., 1992, Pim et al., 1992). Moreover, it has been revealed that the MAP kinase pathway is involved in the growth factor mediated signals for maximally expressing the c-fos (Treisman, 1994). It was therefore a relevant question to ask whether HPV type 16 oncogenes and in particular E5 could affect the MAP kinase signal transduction pathway. This work was published in the *Journal of Virology* in December 1995 (vol. 69, 12:8051-8056).

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CHAPTER II

DNA DAMAGE INDUCED p53 MEDIATED TRANSCRIPTION IS INHIBITED BY HUMAN PAPILLOMAVIRUS TYPE 18 E6

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Abstract

Cervical cancer is similar to other human cancers in that it develops through a multistep process. However, infection with oncogenic human papillomaviruses (HPVs) is believed to be essential for the initiation of this disease. Although HPV may play a central role in the early stages of neoplasia, the accumulation of mutations in an assortment of genes precedes the development of malignant cervical carcinoma. The mechanisms by which abnormalities accumulate are various, but it is possible that viral proteins are involved. In particular, the viral E6 oncoprotein has been show to interact with the cellular tumour suppressor protein p53, which is involved in DNA damage repair pathway. Hence, E6 may contribute to the genomic instability through this interaction with p53. We have tested this hypothesis by monitoring the effects of E6 upon DNA damage induced p53 transcriptional activity. This study shows that HPV-18 E6 inhibits p53 transcriptional activity following genotoxic stress with UV radiation. No effect was observed when a mutant E6 unable to direct the degradation of p53 was included in this assay. These results suggest that continued E6 expression may contribute to the accumulation of DNA damage associated with the progression of cervical cancer.

Introduction

On a worldwide basis, cervical cancer is one of the leading forms of cancer in women (Parkin et al., 1988) and epidemiologic studies have demonstrated that this cancer is associated with human papillomavirus (HPV) infection (zur Hausen, 1991; Lorincz et al., 1992). Laboratory studies have demonstrated that the HPV types associated with cervical cancer such as HPV-16 and HPV-18 contain oncogenes capable of transformating a variety of normal cells into tumour cells (Matlashewski et al., 1987a; Phelps et al., 1988; Munger et al., 1989; Storey et al., 1988). These studies have collectively established that the main transforming genes of oncogenic HPV types are E6 and E7. Additional evidence for the involvement of the E6 and E7 genes in the development of cervical cancer comes from the observations that these genes are selectively and consistently expressed in cervical tumours and cell lines derived from these tumours (Boshart et al., 1984; Schwarz et al., 1985; Smotkin & Wettstein, 1986; Banks et al., 1987). More recently, the E5 gene from various HPV types has been shown to be capable of stimulating anchorage independent growth of established rodent cells and thus establishing this as a third potential transforming region within the HPV genome (Chen & Mounts, 1990; Leechanachai et al., 1992; Pim et al., 1992; Straight et al., 1993).

The mechanism(s) in which HPV oncogenes participate in the development of cervical cancer is not fully understood. However, major advances have come from the demonstration that the E6 and E7 gene products interact with cellular proteins involved

in control of cell proliferation. The ability of E7 to induce proliferation and immortalisation of cells appears to be directly related to the ability of E7 protein to form complexes with cell cycle control proteins such as pRB, p107, p130, and cyclin A (Dyson et al., 1989; 1992; Banks et al., 1990; Tommasino et al., 1993. Likewise, the E6 protein is capable of binding to and promoting the degradation of the cellular tumour suppressor protein p53 (Scheffner et al., 1990; Crook et al., 1991). The degradation of the p53 protein is mediated through a cellular protein termed the E6 associated protein (E6-Ap) and this activity is ATP dependent (Huibrgtes et al., 1993). However, unlike E7, it remains to be established whether E6 modulation of p53 is important for the oncogenic properties of the E6 gene product and whether E6 can associate with additional cellular proteins.

Deletions or mutations in the p53 gene are the most common genetic lesion identified to date in human cancer cells (reviewed by Vogelstein & Kinzler, 1992). Recent observations have led to the conclusion that wild-type p53 is a tumour suppressor protein. This is consistent with the observation that mice which are p53 negative as a result of gene targeting, develop normally, but also develop spontaneous neoplasia within 6 months of age (Donehower et al., 1992). Moreover, the incidence of cancer is markedly increased in Li-Fraumeni individuals which are heterozygous for inherited germline mutations of p53 genes (Malkin et al., 1990). An emerging consensus is that normal p53 maintains the integrity of the genome through arresting cells in G1 following exposure to DNA damaging genotoxic agents. This presumably allows cells time to repair the damage before initiating replicative DNA synthesis (Kastan et al., 1991; 1992;

Kuerbitz et al., 1992). In contrast, cells expressing mutant p53 fail to exhibit G1 arrest following DNA damage. Taken together, these observations argue that p53 acts in a damage control manner to maintain the integrity of the genome and loss of p53 activity could result in the accumulation of genetic lesions which are the hallmark of tumour progression.

p53 has been shown to be capable of repressing the activity of promoters whose expression are TATA box-dependent (Seto et al., 1992; Mack et al., 1993). The shutdown of these genes probably contributes to the regulation of cell proliferation. p53 has also been shown to bind specifically to a DNA sequence motif and induce the expression of reported genes which contain this motif linked to a basal promoter (Kern et al., 1992). Thus, p53 can also act as a transacting transcription factor possibly activating genes involved in growth arrest and DNA damage repair.

Chromosomal abnormalities are observed in virtually every form of cancer and this likely contributes to the tumour cell phenotype. There is evidence for chromosomal instability in HPV-immortalised cells (Kaur & McDougall, 1988) and this may contribute to the progression of cervical lesions. Loss of wild-type p53 activity due to the action of HPV E6 gene could contribute to chromosomal instability. In the present study, we have begun to explore this possibility by examining the effect that HPV-18 E6 has on DNA damage induced p53 transcriptional activity. We demonstrate within that UV treatment of cells could induce p53 mediated transcriptional transactivation and that wild-type HPV-18 E6 could impair UV induced p53 activity. In this manner we demonstrate that E6 impairs p53 function under conditions where the normal p53 pathway is active in cells

under genotoxic stress. These data are consistent with the hypothesis that E6 may contribute to genomic instability and tumour progression.

Materials and methods

Plasmids The wild-type p53 cDNA used in this study was as previously described (Matlashewski et al., 1987b). This cDNA was cloned into the BamHI site of the pBluescript SK vector (Strategene) for the in vitro synthesis of labelled p53 protein. For expression of p53 in cells, cDNA was cloned into the BamHI site of pJ4 vector (Storey et al., 1988). The HPV-18 E6 wild-type and E6-δF mutant sequences extending from nucleotides 70 through 650 were cloned into the HindIII and EcoRI sites of the pBluescript SK and pJ4 vectors for expression in vitro and in cells respectively.

The mutant E6 sequence was generated using oligonucleotide-directed mutagenesis (Amersham). For generation of single stranded template DNA for mutagenesis, E6 sequence was cloned into M13 MP.19 and single stranded phage DNA purified Mutagenesis was performed using the Amersham system according to manufacturer's protocols. The E6-δF mutant coding for HPV-18 E6 with a deletion in amino acids 113 through 117 (Figure 1) was confirmed by dideoxy DNA sequencing.

E6 directed degradation of p53 For synthesis of E6 and p53 proteins in vitro the TNT system (Promega) was used according to manufacturer's instructions. Reactions were carried out in the presence of [^{35}S]-cysteine using E6 and p53 plasmids described above as templates for transcription/translation reaction. Assays for the E6 directed degradation of p53 was carried out essentially as described (Crook et al., 1991) using equal amounts of ^{35}S-labelled E6 and p53 proteins. Labelled proteins were incubated at 4°C or 22°C for

2 h in the TNT reaction mixture. The extent of p53 degradation following incubation was determined by immunoprecipitation of p53 with monoclonal antibody PAb1801 and SDS-PAGE as previously described (Matlashewski et al., 1986).

Transfections and CAT assays Transfections of cervical carcinoma derived C33I cells (Auersperg, 1964) which contain mutant p53 and U2-OS osteosarcoma cells (Ponten and Sakesela, 1967) which contain wild-type p53 were carried out using the standard calcium phosphate precipitation procedure (Matlashewski et al., 1987a). Cells were transfected with 5μg reporter plasmid PG13-CAT (Kern et al., 1992) and varying amounts of p53 and E6 expressing plasmids as indicated in figure legends. A total of 20 μg of DNA was present in each transfection. Forty-eight hours after transfection, cells were harvested in CAT buffer (40 mM TrisHCl pH7.5, 150 mM NaCl and 1 mM EDTA) and subjected to three cycles of freeze/thawing, followed by incubation at 65°C for 10 min. Samples were clarified by centrifugation at 14 000 r.p.m. for 5 min. and protein concentration determined by Bio-Rad protein assay. All transfections contained 1 μg of a RSV-beta-galactosidase plasmid and protein concentrations used per CAT assay were normalized against the beta-galactosidase activity measured in cell extracts from the transfected cells. CAT assays were carried out in the presence of 5 μl Acetyl CoA (33.3 mg mg^{-1} and 5 μl [^{14}C]-chloramphenicol (50 Ci $mmol^{-1}$; ICN) in a final volume of 100 μl at 37°C for 1.5 h. Following extraction with ethyl acetate, samples were analyzed by thin layer chromatography and autoradiography.

In some instances, U2-OS cells were treated with UV radiation. Media was

removed and the cells wash with PBS prior to UV treatment. Cells were treated with UV for 0, 1, 2, 3, and 4s (corresponding to 0, 35, 70, 105, 140, 175 mJm^2) and fresh media added back to the cells. Cell extracts were prepared 24 h after treatment with UV.

Results

Previous studies have shown that HPV-16 E6 amino acid residues 7 through 9 (Mietz et al., 1992), 45 through 49 and 106 through 115 (Crook et al., 1991) are involved in directing the degradation of p53. Since HPV-18 E6 has also been shown to be capable of binding and degrading p53, albeit at a lower rate than HPV-16 E6 (Scheffner et al., 1990; Werness et al., 1990), we were interested in determining whether a domain identified for binding p53 on HPV-16 E6 was conserved in HPV-18 E6. In addition, such a mutant would form a valuable control for further studies. Hence a mutant HPV-18 E6 was constructed (termed δF) lacking residues 113-117 (Figure 1). This region is well conserved between the oncogenic HPV E6 proteins and partially overlaps the sequence 106-115 previously identified as being necessary for p53 binding on the HPV-16 E6 protein (Crook et al., 1991). We then investigated whether this mutant E6 could promote the degradation of p53 in vitro. Wild-type and mutant HPV-18 E6 and p53 were synthesised in vitro in reticulocyte lysates in the presence of [^{35}S]-cysteine. The E6 and p53 protein containing lysates were then mixed together and incubated at 4°C or 22°C for 2 h to determine whether the E6 protein could direct the degradation of p53. The remaining p53 was then quantitated by immunoprecipitation followed by SDS-PAGE and autoradiography. As shown in Figure 2, p53 was efficiently degraded by wild-type HPV-18 E6. In contrast, mutant δF failed to direct the degradation of p53. Extending the degradation time to 4 h with δF still failed to mediate p53 degradation (data not shown). These results demonstrate that the wild-type HPV-18

E6 protein could direct the degradation of p53 and that the conserved region comprising amino acids 113-117 of HPV-18 E6 is necessary for this activity.

The wild-type and mutant HPV-18 E6 were then tested for their ability to impair p53 mediated transcription in the HPV negative human cervical carcinoma derived cell line C33I. A plasmid (PG13-CAT) containing the p53 responsive enhancer (Kern et al., 1992) was cotransfected with a plasmid expressing wild-type p53 (Matlashewski et al., 1987b) either alone or together with plasmids expressing wild-type or mutant E6. As shown in Figure 3, the PG13-CAT plasmid was transcriptionally active in those cells cotransfected with the p53 plasmid but was not active in the transfection which contained no p53 plasmid. This is consistent with C33I cells containing mutant p53 which does not stimulate transactivation of the PG13-CAT plasmid (Kern et al., 1992). Cotransfection of the wild-type HPV-18 E6 expressing plasmid impaired p53 mediated transcription in a dose dependent manner, whereas the plasmid expressing mutant HPV-18 E6 did not impair p53 mediated transcription (Figure 3). These data show that the wild-type but not the mutant HPV-18 E6 could abrogate p53 mediated transcription. These results are consistent with the data presented in Figure 2 which demonstrated that the E6-δF mutant could not direct the degradation of the p53 protein *in vitro*.

We were interested to determine whether HPV-18 E6 could likewise impair p53 mediated transcription in a cell line expressing endogenous wild-type p53. It has been previously established that U2-OS osteosarcoma cells contain wild-type p53 and arrest following exposure to DNA damaging agents (Kastan et al., 1992). Transfection of the PG13-CAT plasmid alone into these cells resulted in CAT activity confirming that these

cells do contain wild-type p53 (Figure 4). Cotransfection of the HPV-18 E6 expressing plasmid resulted in a dramatic decrease in p53 mediated transcription. In comparison, the mutant E6 expressing plasmid did not impair p53 mediated transcription (Figure 4). These data demonstrate that HPV-18 E6 could impair endogenous p53 transcriptional activity.

p53 is believed to be biologically active in cells exposed to genotoxic agents which induce DNA damage. It was a central objective of this study to determine whether E6 could impair p53 under biologically relevant conditions. We therefore determined whether HPV-18 E6 could impair p53 mediated transcription in cells where endogenous p53 activity was induced with a DNA damaging agent. It has been previously established that UV-radiation could induce high levels of endogenous p53 (Hall et al., 1993) and therefore we determined whether UV treatment could induce p53 mediated transcription in U2-OS cells. As demonstrated in Figure 5, p53 mediated transcription was induced in U2-OS cells with UV-radiation in a dose dependent manner. This allowed us to investigate whether HPV-18 E6 could impair UV induced p53 mediated transcription. As demonstrated in Figure 5, wild-type HPV-18 E6 efficiently impairing UV induced p53 mediated transcription. In this experiment, p53 mediated transcription at 4 s time interval was also lower in the presence of the mutant, δF, however this is not a consistent observation and we are investigating this further. Taken together, these data demonstrate that HPV-18 E6 could impair p53 under biologically relevant conditions where endogenous p53 activity was induced by genotoxic stress.

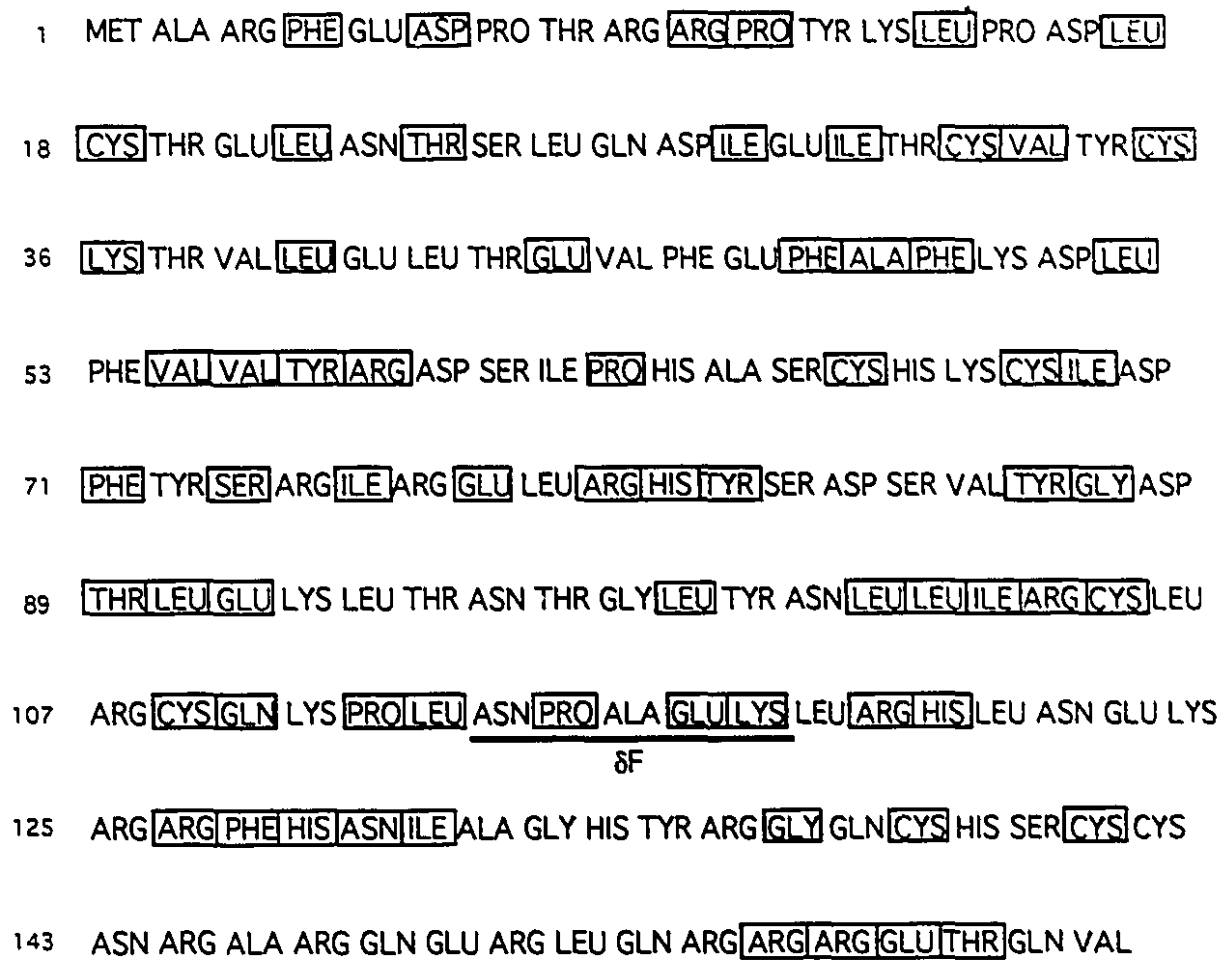

Figure 1. Primary structure of the HPV-18 E6 protein and the E6-δF deletion mutant used in this study. Mutant E6-δF has amino acids 113 through 117 deleted and this sequence is underlined. The boxed residues indicate those amino acids conserved between tumour-associated HPV types. The wild-type and mutant E6 sequences were cloned into the expression vectors pJ4 for expression in in vivo and pBluescript IIKS for expression in *in vitro*.

Figure 2. p53 degradation directed by wild type and mutant HPV-18 E6 protein. Labelled p53 and E6 proteins were synthesized in an in vitro transcription/translation system, mixed together at 4°C or 22°C and the p53 immunoprecipitated with anti-p53 Mab PAb1801 followed by SDS-PAGE. Immunoprecipitation were carried out on: lane 1; p53 incubated in the absence of E6 at 22°C; lanes 2 and 3; p53 incubated with wild type E6 at 4°C and 22°C respectively; lanes 4 and 5, p53 incubated with mutant E6-δF at 4°C and 22°C respectively. Note that only incubation with wild type E6 at 22°C resulted in degradation of p53.

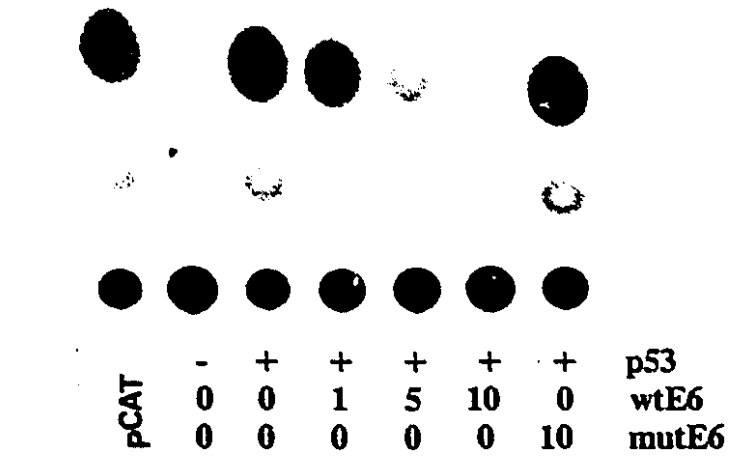

Figure 3. Repression of p53-mediated transcription in C33I cells by wild type but not mutant HPV-18 E6. The p53 responsive PG13-CAT plasmid (5 μg) was transfected into cells alone (lane labelled -) or together with 2 μg of the p53 expression plasmid (lanes labelled +) and 0 g, 1 μg, 5 μg or 10 μg of the wild type E6 expressing plasmid (lanes wtE6) or 10 μg of the mutant E6-δF expression plasmid (lanes mutE6) as indicated. pCAT represents a control transfection with a plasmid containing the CAT gene under the control of the SV40 promoter/enhancer.

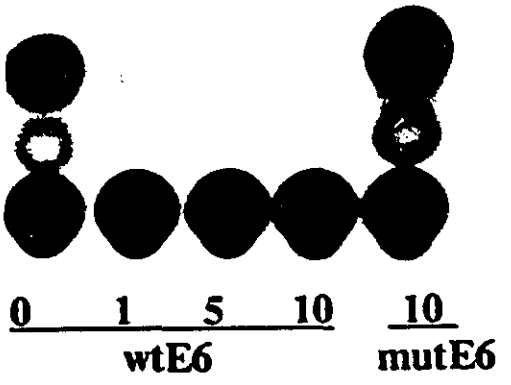

Figure 4. Repression of endogenous p53 mediated transcription in U2-OS cells by wild type but not mutant HPV-18 E6. The p53 responsive PG13-CAT plasmid (5 μg) was transfected alone (lane 0) or together with 1 μg, 5 μg or 10 μg of plasmid expressing wild type E6 (lanes labelled wtE6) or 10 μg of plasmid expressing mutant E6-δF (lane labelled mutE6) as indicated for each sample. Note that in the PG13-CAT plasmid alone was transcriptionally active in these cells without the co-transfection of wild type p53 expressing plasmid.

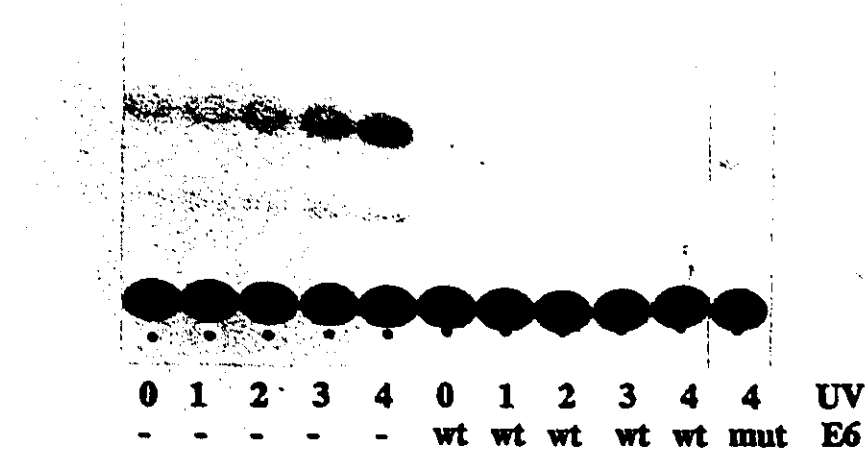

Figure 5. Repression of UV induced p53 mediated transcription in U2-OS cells by wild type but not mutant HPV-18E6. The p53 responsive PG13-CAT plasmid (2 μg) was transfected alone (lanes labelled -) or together with a 10 μg of plasmid expressing wild type E6 (lanes labelled wt). Transfected cells were treated with UV for 0, 1, 2, 3, or 4 s (represents 0, 35, 70, 105, 140, 175 mJm^2 respectively) as indicated. Cells cotransfected with the mutant E6-δF expression plasmid (lane labelled mut) were treated with UV for 4 s.

Discussion

The induction of p53 mediated transcription with a DNA damaging agent has allowed us to investigate whether E6 from oncogenic HPV-18 could impair p53 activity under biologically relevant conditions. The major conditions from this study was the demonstration that HPV-18 E6 was a potent inhibitor of p53 mediated transcription under conditions where p53 transcriptional activity was induced. Since recent studies suggest that p53 activity is important for maintaining the integrity of the genome, then these data are consistent with the view that E6 may contribute to the accumulation of genetic lesions in tumour progression.

Previous studies have revealed that oncogenic HPV E6 could inhibit p53 mediated transcription (Mietz et al., 1992; Hoppe-Seyler and Butz, 1993). This is however, the first example of E6 impairing p53 mediated transcription under biologically relevant conditions where p53 activity was induced with a DNA damaging agent. In addition, we have established that deletion of amino acid residues 113 through 117 of HPV-18 E6 abolished the ability of this mutant to direct the degradation of p53. This together with previous observations on HPV-16 E6 (Crook et al., 1991) demonstrate that this conserved region of the E6 molecule from different oncogenic HPV types is involved in directing the degradation of p53. We have not established whether this region of the HPV-18 E6 molecule is involved in binding to p53 or degrading p53 subsequent to binding. However, based on the observations that this region of HPV-16 E6 is required for binding p53 (Crook et al., 1991), it is likely that this region on HPV-18 E6 is also

involved in p53 binding. It is also possible that the removal of this region from the E6 molecule has induced a conformational change in the protein making it unable to bind or degrade p53.

Recent studies have demonstrated a central role for p53 in inhibiting replicative DNA synthesis after DNA damage and in maintaining the integrity of the genome (Kastan et al., 1991; 1992; Kuerbitz et al., 1992). The ability of p53 to induce transcription transactivation of target genes may be central to p53 control role. For example, ionising radiation induction of GADD45 gene expression is dependent on wild-type p53 activity (Kastan et al., 1992). Our findings that HPV-18 E6 impairs p53 transactivating activity in cells treated with a DNA damaging agent suggests that E6 can disrupt the DNA damage control role for p53. This is also consistent with a recent report demonstrating that inhibition of DNA synthesis and increase in p53 protein levels did not occur in HPV-16 E6 expressing cells treated with a DNA damaging agent (Kessis et al., 1993). Taken together, these observations suggest that the oncogenic E6 abolition of p53 activity may disrupt an important cellular response to DNA damage. Loss of this response may cause cells to become susceptible to genetic lesions which could in turn drive tumour progression. This is consistent with the knowledge that tumours arise through a multistep process and that HPV infection is believed to serve as the initiating event for the development of cervical cancer (reviewed by Franco, 1993).

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CONNECTING STATEMENT TO CHAPTER III

In Chapter II, it was first demonstrated that the HPV E6 oncoprotein inhibits DNA damage induced p53 transactivation activity. This study, and previous studies indicate that loss of p53 activity due to the action of the HPV E6 gene may increase the susceptibility of host cells to genetic lesions thus contribute to the progression of cervical carcinomas. The HPV E6 gene has also been shown to be necessary for maintaining transformed phenotype of HPV-positive cervical cancer cells. However, there is no direct evidence to reveal the importance of the viral E6 mediated p53 inactivation in maintaining the transformed phenotype of cervical carcinoma derived cells. Therefore, in the next chapter, I have undertaken to examine the biological consequences of introducing transcriptionally functional p53 into HPV-positive cervical cancer cells.

CHAPTER III

A FUNCTIONAL p53 MUTANT SUPPRESSES PROLIFERATION OF HUMAN PAPILLOMAVIRUS POSITIVE SiHa CELLS

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FOOTNOTES:

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Abstract

The cellular p53 protein contributes to tumour suppression at least in part by transactivating specific genes which are involved in cell cycle control and apoptosis. The p53 gene is one of the most frequently mutated genes found in human cancers but is only rarely mutated in cervical cancer. More than 90% of cervical carcinomas contain and express human papillomavirus (HPV) DNA sequences of the 'high risk' types including types 16, 18 and others. The E6 oncoprotein from 'high risk' HPV types complex with and promote p53 degradation through the ubiquitin proteolysis pathway and this is likely to be the major reason why the p53 gene is only rarely mutated in these neoplasias. In this study, we have investigated the biological consequences of introducing into HPV-positive cervical cancer cells a transcriptionally functional p53 monomeric mutant which is resistant to E6-mediated degradation. Stable transfection of HPV-16 positive SiHa cells with this functional p53 monomeric mutant resulted in a suppression of proliferation and induced a more differentiated cellular morphology. These data demonstrate that E6 impairment of p53 function contributes significantly to the transformed phenotype and that this may represent a target for the treatment of these tumours. These data also reveal that the C-terminal deletion mutant of p53 is functional as a tumour suppressor in transformed cells suggesting that oligomerisation is not needed for tumour suppressor activity. The implication of these results with respect to the treatment of cancer in general is discussed within.

Introduction

The pathologies associated with HPV infections include skin warts, respiratory papillomas, benign genital lesions, and cervical cancer (for review see Shah and Howley, 1990). Cervical cancer is similar to other human cancers in that it develops through a multistep process and infection with high risk HPVs is believed to be essential for the initiation and progression of this disease. The E6 and E7 genes from high risk HPVs are the major transforming genes within the viral genome and are selectively retained and expressed in HPV positive cervical cancers (for review see Matlashewski, 1989; DiMaio, 1991; Mansur and Androphy, 1993; Tommasino and Crawford, 1995). The biological activity of the E6 and E7 oncoproteins is widely accepted as contributing significantly to the transformed phenotype associated with cervical tumour cells.

The viral E6 and E7 proteins function at least in part by binding to and inactivating cellular tumour suppressor proteins, p53 (Werness et al., 1990; Crook et al., 1991) and Rb (Dyson et al., 1989) respectively. The interaction of E6 with p53 leads to the degradation of p53 through the ubiquitin proteolysis pathway (Scheffner et al., 1990) and this process requires a third cellular protein component termed E6-AP (Huibregtse et al., 1991, 1993). These observations suggest a mechanism for inactivation of p53 in HPV-positive cervical carcinomas.

The p53 tumour suppressor gene encodes a transcriptional regulator which is a sequence specific DNA binding protein (Kern et al., 1991; El-deiry et al., 1992; reviewed by Lane, 1994; Cox and Lane, 1995). p53 is believed to regulate various cell

growth regulatory processes, including cell cycle progression (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993), DNA damage repair (Smith et al., 1994) and apoptosis (Lowe et al., 1993; Caelles et al., 1994; Miyashita et al., 1994). Several nucleotide sequences specifically recognized by p53 protein have been identified (Bargonetti et al., 1991; Kern et al., 1991; Funk et al., 1992; Foord et al., 1993).

p53 is composed of several functional domains (for review see Donehower and Bredley, 1993; Haffner and Oren, 1995) involved in DNA binding (Bargonetti et al., 1993; Pavletich et al., 1993), transcriptional transactivation (Shaulian et al., 1992), oligomerisation (Sturtzbecher et al., 1992), and allosteric regulation (Hupp et al., 1995). Recent studies on the self-association and transcription transactivation function of p53 revealed the p53 monomeric variants retained transcription transactivation activity (Tarunina and Jenkins, 1993, Thomas et al., 1995b). It has also been established that the p53 monomeric mutant 338 (contains a C-terminal region truncation from amino acids 338 through 393) which retains transcriptional transactivation activity is only poorly targeted by E6 for degradation (Thomas et al, 1995a; Kalita and Matlashewski, unpublished data). Based on these observations, it was of interest to determine whether the p53 monomeric mutant 338 which was resistant to E6 mediated degradation could function as a tumour suppressor in HPV-16 positive cervical cancer cells. In this study it was observed that the p53 monomeric mutant 338 could suppress the proliferation of the SiHa cervical cancer cell line. Moreover, the p53 monomeric mutant was able to alter the morphology of the SiHa cells, making them larger, less refractive and more differentiated with respect to morphology. These results suggest that monomeric p53 is

functional and that oligomerisation is not necessary for tumour suppressor activity. These data also reveal in a cervical cancer derived cell line that E6 mediated p53 inactivation contributes significantly to the neoplastic phenotype.

Materials and Methods

Plasmids. The wild type p53 cDNA used in this study was previously cloned and described in detail (Matlashewski et al., 1987b). The p53 monomeric mutant 338 which is truncated from amino acids 338-393 lacks the oligomerisation domain and thus is monomeric (Tarunina and Jenkins, 1993). The resulting plasmid was termed pJ4-p53. The nonfunctional structural p53 mutant 175 has a histidine substitution at amino acid 175 and is unable to specifically bind DNA (Crook and Vousden, 1992). The above described p53 cDNAs were cloned into the BamHI/EcoRI site of the pJ4 plasmid expression vector (Storey et al., 1988) which contains the Moloney Murine Leukaemia Virus Long Terminal Repeat (MMLV-LTR) promoter and enhancer. The resulting plasmids were termed pJ4-wtp53, pJ4-338, pJ4-175, and pJ4 (no insert). Figure 1 is a representation of the p53 protein, showing the evolutionarily conserved regions (solid boxed) and regions of the protein important for oligomerisation, DNA binding, and transactivation (Crook et al., 1994) and the p53 mutants used in this study.

Cells and Transfections. The SiHa cells used in this study were initially derived from a human cervical squamous carcinoma and are epithelial-like and form poorly differentiated epidermoid carcinoma in Nude mice (Friedl et al., 1970). These cells contain wild-type p53 alleles (Wrede et al., 1991; von Knebel Doeberitz et al., 1994) and express the viral E6 and E7 genes from integrated HPV-16 specific DNA sequences (Yee et al., 1985). Cells were maintained in Dulbecco Modified Eagle (DME) Medium supplemented with

10% fetal calf serum (FCS).

The various p53 expression plasmids were stably transfected into SiHa cell lines using the standard calcium phosphate precipitation procedure (Matlashewski et al., 1987a, Leechanachai et al., 1992) and selected with G418. Briefly, the cells were cotransfected with 10 μg of plasmid pJ4, pJ4-p53, pJ4-338, or pJ4-175 together with 1 μg of pWL-Neo plasmid and cells taking up the transfected plasmids were selected in 500 μg of G418 per ml. Two weeks following the transfection, G418-resistant colonies were either counted for the colony formation assays or pooled and expanded for further studies. Colonies were counted in the following manner. G418-resistant colonies were fixed with methanol for 5 min. and stained with Giemsa solution for 15 min. Cells were then rinsed with PBS and colonies counted.

Growth curves for the various p53 transfected cells were performed in the following manner. Cells were grown in 60-mm tissue culture dishes in DME-medium supplemented with 10% of FCS and counted at 24-hour intervals using a Haemocytometer.

Morphology of various p53 expressing SiHa cell lines. A total of 4 x 10^5 pooled SiHa cells which were stably transfected with the various p53 expressing plasmids were seeded in 60-mm tissue culture dishes in DME-medium supplemented with 10% FCS and grown for 24 hours. Cells were examined with an OLYMPUS-CK2 inverted microscope and photographed at 200 X magnification.

Results

Growth suppression of HPV-positive cervical cancer cell SiHa by a functional p53 mutant 338. It has been previously demonstrated that p53 transcriptional activation was essential for its growth suppressive activities (Crook et al., 1994). In HPV-positive cervical cancer cell SiHa the endogenous p53-mediated transcriptional activation is dramatically impaired because of the presence of E6 in these cells (Butz et al., 1995). In the light of the functional properties of a p53 monomeric mutant 338 which retains its transcriptional transactivating activity (Tarunina and Jenkins, 1993) but is resistant to E6-mediated degradation (Thomas et al., 1995a; Kalita and Matlashewski, unpublished data), we were interested to determine whether this p53 mutant is capable of suppressing proliferation in cells containing high risk HPV E6.

In this study, the human cervical squamous carcinoma derived cell line SiHa was used. This cell line contains wildtype p53 alleles (Wrede et al., 1991; von Knebel Doeberitz et al., 1994) and expresses HPV type 16 oncogenic sequences (Yee et al., 1985). SiHa cells were transfected with plasmids expressing one of the following; wildtype p53 (pJ4-p53), the monomeric p53 mutant 338 (pJ4-338), the nonfunctional structural p53 mutant 175 (pJ4-175) or control plasmid with no insert (pJ4). Each of the above p53 expressing plasmids were cotransfected with the pWL-Neo plasmid and cells taking up the transfected plasmids were selected with G418 as described in the methods section. The resulting colonies were either pooled and expanded for further study or were fixed and stained to perform a colony forming assay.

The colony forming assay demonstrates whether any of the various p53s could suppress the growth of the recipient cells resulting in fewer colonies (Crook et al., 1994). The results from several colonies formation assays are summarized in Table 1. The monomeric p53 mutant 338 demonstrated a marked suppressive activity on SiHa cell proliferation by reducing the number of colonies when compared with the control and the structural p53 mutant 175 transfected cells. Wildtype p53 also resulted in a suppression of cell proliferation when compared with control or p53 mutant 175 transfected cells. These data demonstrate that both the wildtype p53 and the monomeric p53 mutant 338 suppressed the proliferation of SiHa cells whereas p53 mutant 175 had no effect on cell proliferation. Interestingly, the wildtype p53 was growth suppressive suggesting that its overexpression from the viral LTR could overcome the ability of E6 to effectively mediate its degradation. These data also suggest that the monomeric p53 mutant 338 was biologically active in these cells with respect to suppressing the proliferation of SiHa cells.

To further study the growth suppression by wildtype and monomeric p53 mutant 338, the proliferation rates of the pooled colonies of transfected Siha cells containing various p53 expressing plasmids were examined. The pooled colonies were used in this study to eliminate the variation of the expression of introduced gene between individual colonies. The rate of cell proliferation was determined by counting the cells at 24 hr intervals and the results from these experiments are shown in Figure 2. Monomeric p53 mutant 338 and wild type p53 transfected cells proliferated slower than did the control transfected or the cells transfected with the structural p53 mutant 175. These data suggest

that the surviving colonies expressed relatively low levels of the transfected plasmid DNA encoding wildtype p53 or monomeric p53 mutant 338 which reduced the rate of cell proliferation but did not result in complete cell cycle arrest or apoptosis. In comparison, transfected cells expressing higher levels of wildtype p53 or monomeric p53 mutant 338 would not have survived and thus resulted in fewer colonies as was observed in Table 1.

Morphology of p53 transfected SiHa cells. During the culturing of the resulting pooled colonies derived from the transfected cells, we observed interesting morphological differences in the different cells. The morphology of these transfected cells are shown in Figure 3. SiHa cells transfected with control pJ4 vector demonstrate the same morphology as untransfected SiHa cells or cells transfected with the structural p53 mutant 175, indicating that the simultaneous expression of exogenous proteins from the both vectors (pWL-Neo and pJ4) had not induced the morphological changes of SiHa cells. These cells are smaller, actively dividing, and are poorly differentiated. In contrast, cells transfected with monomeric p53 mutant 338 display a flattened morphology, an increase in size, and a more differentiated appearance when compared with that of the normal SiHa cells. Cells transfected with the wildtype p53 demonstrated a somewhat intermediate morphology between that of the control cells and the cells transfected with monomeric p53 mutant 338. These results demonstrate that monomeric p53 mutant 338 could not only reduce the proliferation of SiHa cells, but also morphologically altered them to a more differentiated type morphology.

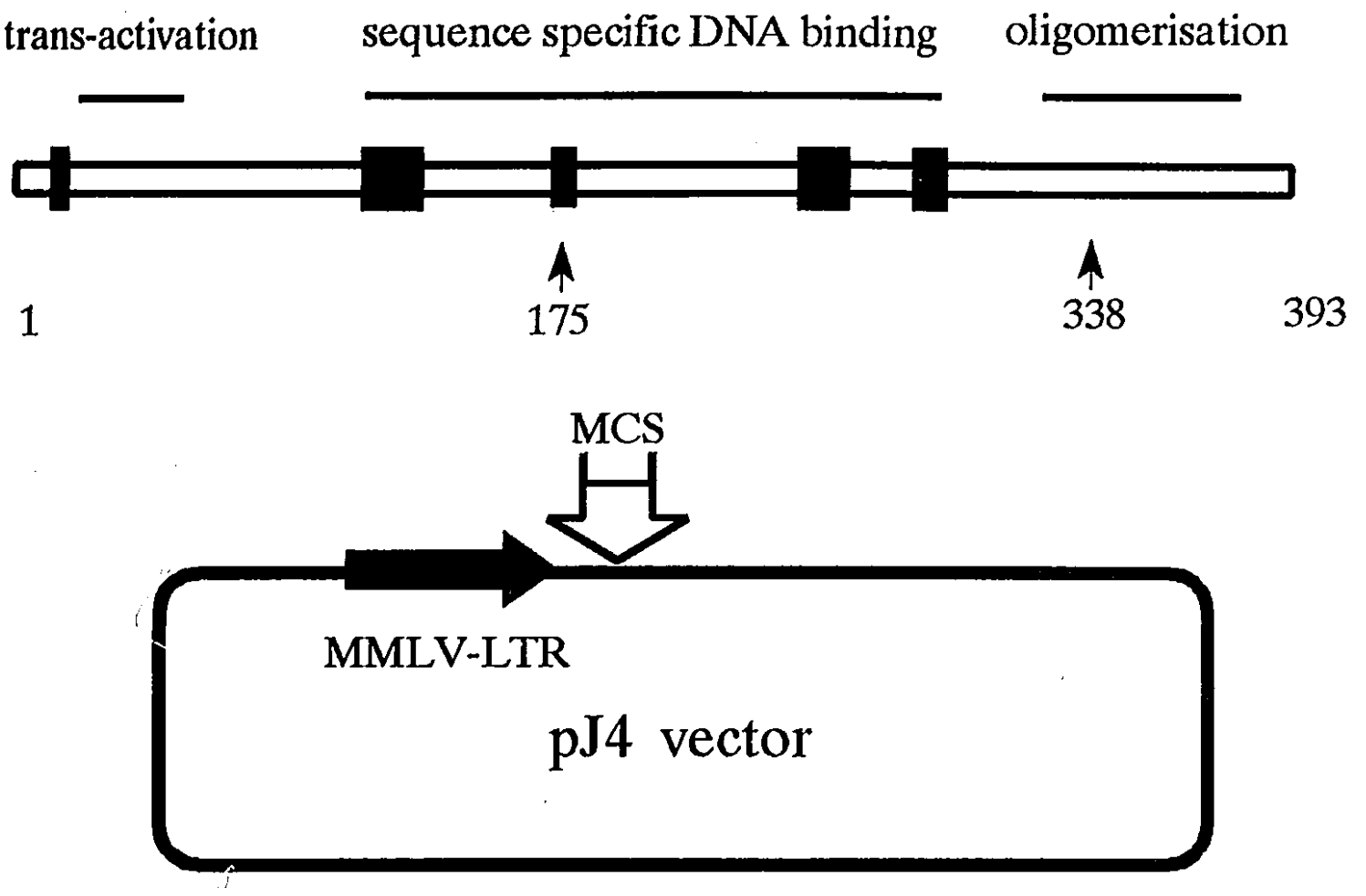

Figure 1. Representation of the p53 protein, showing the evolutionarily conserved domains (solid boxes), and the regions of p53 involved in oligomerisation, DNA binding, and transactivation. The region of the 175 mutation and the 338 truncation are also indicated with arrows.

Table 1. Suppression of SiHa Cell Growth by Functional p53 Mutant 338.

Transfected plasmid*	Number of colonies Experiment number 1	2	3	4
Vector	32	38	30	28
wtp53	17	26	22	11
338	13	20	17	16
175	31	29	25	28

*The SiHa cells were transfected with the indicated p53 expressing plasmids together with pWLNeo and selected in 500μg/ml G418 for two weeks. After this time the cells were fixed and stained (giemsa) and colonies counted.

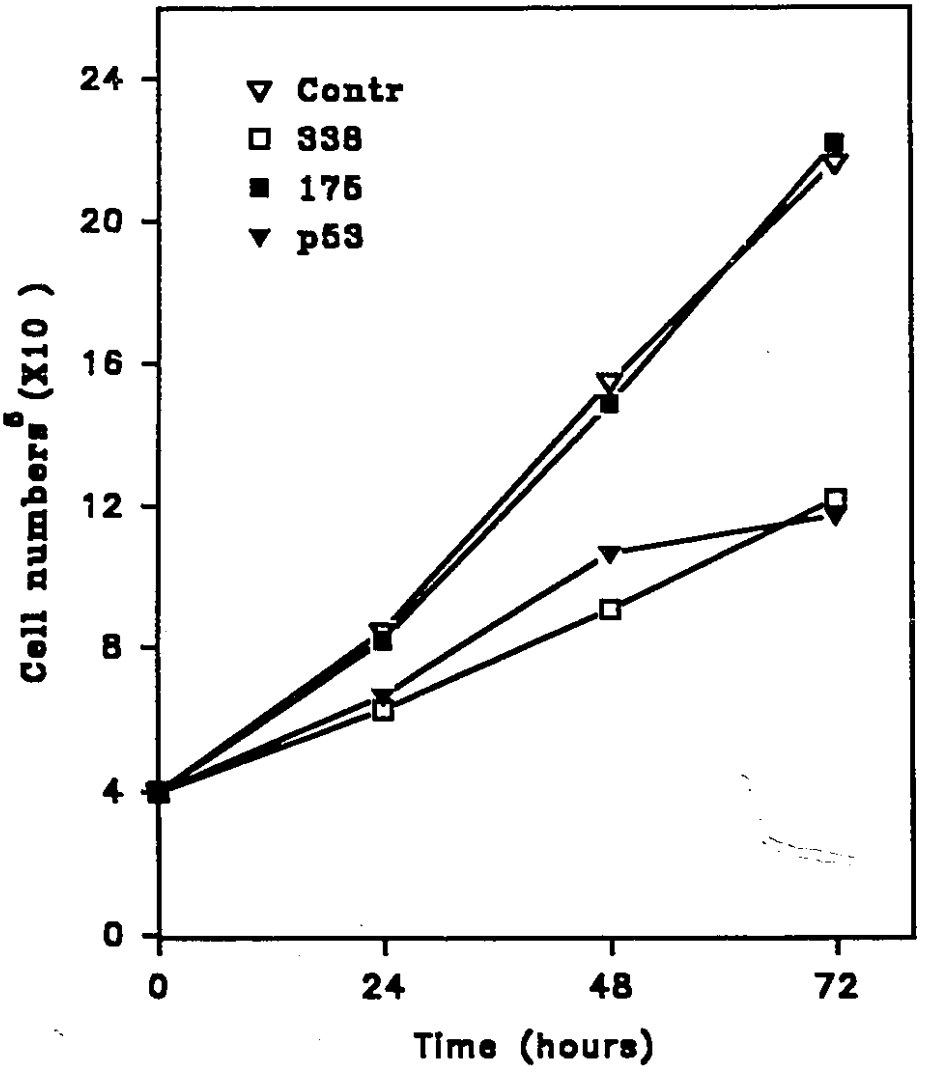

Figure 2. Growth curve of SiHa cells stably transfected with different p53 expressing plasmids. Cells stably transfected with the control vector (▽), the nonfunctional structural p53 mutant 175 (■), the monomeric p53 mutant 338 (□), or the wild type p53 expressing plasmid (▾) were cultured and the cell numbers were determined at the indicated time points.

Figure 3. Morphology of p53 transfected SiHa cells. Cells stably transfected with control vector (panel C) represent the normal morphology of SiHa cells. Panel 175 represents cells stably transfected with a nonfunctional structural p53 mutant 175 expressing plasmid; Panel 338, represents cells stably transfected with the monomeric p53 mutant 338 expressing plasmid; Panel p53, represents cells stably transfected with the wildtype p53 expressing plasmid. Phase contrast photomicrographs were obtained at a magnification of 200 X.

Discussion

Two significant observations have been made in the present study. First, it was revealed that the introduction of transcriptionally functional p53 into HPV-16 positive SiHa cells reduced the ability of these cells to proliferate and induced these cells to display a more differentiated morphology. This demonstrates that the viral E6 mediated p53 inactivation plays a major role in maintaining the transformed phenotype of cervical carcinoma derived cells. Second, the monomeric p53 mutant 338 was as efficient as wildtype p53 in suppressing the proliferation of SiHa cells. This demonstrates that p53 in its monomeric form with its C-terminal regulatory region removed retains biological activity with respect to impairing cell proliferation. These observations are unique in demonstrating the importance of the E6 mediated p53 inactivation in cervical cancer derived cells and also in defining the biological activity of monomeric p53 without its C-terminal regulatory region.

Initial studies have established that the E6 and E7 oncogenes participate in the transformation of primary cells in vitro (Matlashewski et al., 1987a, Pirisi et al., 1987, Phelps et al., 1988, and reviewed by Mansur and Androphy, 1993; Tommasino and Crawford, 1995). It has also been established that a major cellular target of high risk HPV E6 is the p53 tumour suppressor protein (Scheffner et al., 1990; Crook et al., 1991, and reviewed by Tommasino and Crawford, 1995). Finally, genetic mutations in the p53 gene are rare in cervical cancer cells (Park et al., 1994), and overexpression of wildtype p53 could restore the differentiation of human squamous carcinoma cells but not HPV-expressing cell lines (Brenner et al., 1993; Woodworth et al., 1993). Taken

together, these observations provided compelling evidence that the E6-p53 interaction plays a major role in the development of cervical cancer. The present study directly examined the biological importance of reduced p53 activity in cancer cells derived from a HPV-16 positive cervical tumour. The results demonstrated that restoring p53 activity to cervical cancer cells impaired the proliferation of these cells and morphologically altered them to a more differentiated appearance. These results have practical implications since they suggest that targeting the E6-p53 interaction may be a viable option for treating cervical neoplasia.

The activity of p53 most closely associated with its tumour suppressor function is its ability to act as a sequence specific transcriptional transactivator (Kern et al. 1992; El-Deiry et al., 1992). Several recent reports have shown that the C-terminal region of p53 contains a regulatory element which can interact with single stranded RNA or DNA and that this may regulate the ability of p53 to function as a transcriptional transactivator (Oberosler et al. 1993; Bakalkin et al., 1994; Jayaraman and Prives, 1995). It has also been established that phosphorylation of the C-terminal regulatory domain of p53 by either protein kinase C or casein kinase II activates sequence specific DNA binding (Hupp and Lane, 1994; Takenaka et al., 1995). In addition, a monoclonal antibody which binds to the C-terminal regulatory element is a potent activator of sequence specific DNA binding (Hupp and Lane, 1994). More recently, an allosteric model has been proposed for the regulation of p53 activity which suggests that posttranslational modification of the C-terminal regulatory region of p53 in vivo is a rate limiting step in the activation of sequence specific DNA binding by p53 (Hupp et al., 1995). Finally, it has been

demonstrated that the monomeric p53 deletion mutant used in this study (mutant 338) which has the C-terminal regulatory element deleted retained sequence-specific transcriptional activator activity in vivo (Tarunina and Jenkins, 1993). Based on these and other biochemical studies involving the C-terminal regulatory domain of p53, a major unresolved question was whether p53 without its C-terminal region regulatory element was biologically functional in cells with respect to suppressing cell proliferation. The present study addressed this issue by demonstrating that the monomeric p53 mutant 338 with the C-terminal 55 amino acids removed was functional with respect to impairing cell proliferation and inducing morphological changes consistent with a more differentiated state in HPV-16 positive SiHa cells. Although this C-terminal deletion mutant was chosen in this study because it is resistant to HPV E6 mediated p53 degradation (Thomas et al., 1995a), it would be interesting to further study the biology of this p53 mutant in cancer derived cell lines which do not contain HPV to determine whether it can also suppress proliferation in these cell types. Future studies must also examine whether the monomeric p53 mutant 338 can induce apoptosis and or expression of the p21 cell cycle regulatory gene. These studies are currently underway.

In conclusion, these results have several practical implications. First, these data suggest that targeting the interaction between E6 and p53 in advanced HPV containing cervical cancer cells is a viable option for causing tumour regression. Second, monomeric p53 molecules missing the C-terminal regulatory region may be effective tumour suppressors. Because such p53 mutants have lost the oligomerisation and regulatory domains, they may not be inactivated by the dominant negative properties of

naturally occurring mutant p53s, yet have retained sequence specific transcriptional transactivation activity.

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CONNECTING STATEMENT TO CHAPTER IV

The work presented in Chapter II and III demonstrated that HPV E6 impairs p53-mediated transcriptional activity induced by DNA damaging agents and that the loss of p53 contributes to the neoplastic phenotype of cervical cancer derived cells.

To further define the mechanisms in which HPV contributes to neoplasm, I have examined the effect of HPV oncogenes on the MAPK pathway. This pathway has been examined because of its involvement in transformation and its link to growth factor receptors which is a likely target of the E5 gene from HPV. This study has been published in the Journal of Virology.

CHAPTER IV

EFFECT OF HUMAN PAPILLOMAVIRUS TYPE 16 ONCOGENES ON MAP-KINASE ACTIVITY

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Abstract

The Mitogen-Activated Protein (MAP) kinase signal transduction pathway is an intracellular signaling cascade which mediates cellular responses to growth and differentiation factors. The MAP kinase pathway can be activated by a wide range of stimuli dependent on the cell types and this is normally a transient response. Oncogenes such as ras, src, raf and mos have been proposed to transform cells in part by prolonging the activated stage of components within this signaling pathway. The human papillomavirus (HPV) oncogenes E6 and E7 play an essential role in the in vitro transformation of primary human keratinocytes and rodent cells. The HPV type 16 E5 gene has also been shown to have weak transforming activity and may enhance the epidermal growth factor (EGF)-mediated signal transduction to the nucleus. In the present study, we have investigated the effects of the oncogenic HPV type E5, E6 and E7 genes on the induction of the MAP kinase signaling pathway. The E5 gene induced an increase in the MAP kinase activity both in the absence and in the presence of EGF. In comparison, the E6 and E7 oncoproteins do not alter the MAP kinase activity or prolong the MAP kinase activity induced with EGF. These findings suggest that E5 may function, at least in part, to enhance the cell response through the MAP kinase pathway. However, the transforming activity of E6 and E7 are not associated with alterations in the MAP kinase pathway. These findings are consistent with E5 enhancing the response to growth factor stimulation.

Introduction

More than 60 human papillomavirus (HPV) genotypes have been identified of which a subset including HPV types 6 (HPV-6), -11, -16 and -18, is associated with genital infections (56). HPV-16 and -18 are commonly associated with cervical cancers, while HPV-6 and -11 are commonly associated with benign condylomas (55,57). The association of high-risk HPV-16 and -18 with cancers is consistent with their ability to transform primary rodent cells (31,36,51) and human keratinocytes (17) in culture. In most of the advanced cervical neoplasias, the HPV DNA is integrated into the host chromosomal DNA, whereby only the E6 and E7 genes are retained and expressed (2,45,46,48). These observations suggest that these viral oncogenes are involved in the progression and maintenance of transformation. Significant advances in our understanding of E6- and E7-associated cell transformation have come from the observations that these viral oncoproteins target cellular tumour suppressor proteins. For example, E7 binds to and inactivates the cellular tumor suppressor Rb (14) and related proteins such as p107 and p130 (13), whereas E6 targets the cellular tumor suppressor p53 (54) and promotes its degradation through the ubiqutin proteolysis pathway (43). These observations provide an understanding of the molecular basis on which E6 and E7 manipulate the cell towards the transformed phenotype.

In contrast to the E6 and E7 genes, the E5 gene is often deleted from the viral genome during viral DNA integration in the more advanced cervical neoplastic lesions (2,46). However, in low grade lesions prior to integration, among the most abundant

mRNA transcripts are those which could potentially encode E5 and E4 (50). Therefore, if E5 does participate in the transformation process, this would be at an early stage when the HPV DNA is episomal. Several reports have described HPV-16 E5 as a potential third oncogene which can transform established rodent cells to anchorage-independent growth (25,26,37,53). The transforming activity of E5 may be due in part to its ability to inhibit downregulation of the epidermal growth factor (EGF) receptor (EGF-R). Evidence to support this view came from the observation that expressing HPV-16 E5 in human keratinocytes resulted in an increase in the number of EGF-Rs at the cell surface and that there was an inhibition of receptor degradation (53). Consistent with this observation, it was revealed that the inhibition of the downregulation of the EGF-R by E5 may be associated with the ability of E5 to bind a 16-kDa protein, a component of the vacuolar proton-ATPase pump complex (H^+-ATPase) involved in receptor protein degradation (10). Finally, it has recently been demonstrated that HPV-16 E5 expression results in an impairment of acidification of endosomes (52). The acidification of endosomes is essential for their proteolysis function, including those involved in the degradation of the EGF-R (32,44). The H^+-ATPase pump is responsible for the acidification, and E5 interaction with 16-kDa component may impair this process in HPV-infected human keratinocytes. Moreover, HPV 16 E5 has been demonstrated to cooperate with E7 to potentiate a mitogenic response which is enhanced in the presence of the EGF (5). The close association of E5 biological activities with growth factor receptors would suggest that E5 may contribute to the normal viral life cycle and the early stage of viral infection, by increasing cell responsiveness to growth factors such as

EGF. One way to further investigate this possibility would be to examine signal transduction mediators such as MAP-kinase which is downstream from the EGF-R and this is the objective of the present study.

The EGF-R is present on all epithelial cells, including cervical mucosal cells (7), and is a transmembrane receptor protein with ligand-activated tyrosine kinase activity (6). Stimulation of the receptor with EGF activates a number of signal transduction pathways including the MAP kinase pathway (4,12,29,30). The MAP kinase pathway is associated with both cell proliferation and differentiation (1,41).

In response to growth factor receptor binding, a number of intermediates have been identified upstream of MAP kinase, including the activation of ras GTPase activity followed by the stimulation of c-Raf protein kinase, MAP kinase kinase, and MAP kinase (21,29,30). Activated MAP kinase is translocated into the nucleus upon activation, where it can potentially phosphorylate transcription factors, such as c-Jun, c-Myc, c-Tal1, (8,20,28,38,39).

One of the hallmarks of this signaling pathway is its rapid transience (23,33) and inappropriate activation or inhibition of downregulation of the pathway can contribute to cell transformation. Indeed the components in the pathway, such as Ras and Raf, have been identified as the products of transforming oncogenes (15,19). The current hypothesis is that oncogenes such as ras, raf, src and mos transform cells by prolonging the activated stage of MAP kinase kinase and of components downstream in the signaling pathway (27). It has also been shown that the constitutive activation of the MAP kinase pathway by simian virus 40 small T could induce monkey kidney CV-1 cell proliferation

(49), thus demonstrating for the first time that a DNA tumour virus protein could potentiate MAP kinase activity.

On the basis of these observations, we have examined whether any of the HPV-16 oncogene products could alter cellular MAP kinase activity. In this report, we show that the E5 gene was able to induce an increase in the MAP kinase activity. In comparison, the E6 and E7 genes did not stimulate or prolong MAP kinase activity.

Materials and methods

Plasmids The HPV oncogenes expressing plasmids were constructed by inserting the open reading frames into the pJ4 vector (51), which placed the HPV oncogenes under the transcriptional control of the Moloney murine leukaemia virus long terminal repeat (MLV-LTR). The plasmids were designated pJ4-16E5, pJ4-16E6, pJ4-16E7, and pJ4-16M5. The plasmids, pJ4-16E5 and pJ4-16M5, were generated as follows. A BamHI/EcoRI restriction sites flanked E5 ORF (nts. 3849-4098) DNA fragment was synthesised by PCR and inserted into the pJ4 vector to generate pJ4-16E5. pJ4-16M5, a mutant in which the Kozak's sequence was introduced to increase the potential translation level (24), was created by inserting a HindIII/EcoRI DNA fragment into pJ4 vector, which was synthesised by PCR using a pair of primers (5'-AGCAAGCTTAAAATGGATCCGAATCTT and 5'-ATCGGAATTCTTATGTAATAAA-AA, Kozak's consensus sequence underlined). This generated a mutant E5 with the two amino acids aspartic acid and proline instead of threonine following the initiation methionine.

Transfections HPV oncogene-expressing HT1080 cell lines were developed by using the standard calcium phosphate transfection procedure and selected with G418 (25). Briefly, the cells were cotransfected with 10 μg of plasmid pJ4, pJ4-16E5, or pJ4-16M5, pJ4-16E6, pJ4-16E7 together with 1 μg of pWLNeo plasmid and selected in medium containing G418 (150 μg G418/ml). Two weeks after transfection, G418-resistant

colonies were pooled and expanded. Northern blot analysis was performed to verify that transfected cells expressed the individual HPV oncogenes.

Northern blot analysis Northern blot analysis was carried out as previously described (25). Briefly, total RNA was extracted with Trizol (Gibco/BRL). RNA samples (10 μg) were denatured for 1 hour at 50°C in the presence of 0.01M NaH_2PO_4 and 1M glyoxal, and resolved in 1.2% agarose gel. Following electrophoresis, RNA was transferred to Hybond-N membrane (Amersham, Canada), and prehybridized and hybridized at 42°C in 50% formamide with probes nick-translated in the presence of [α-^{32}p]dCTP (ICN, Canada).

MAP kinase activity assays HT1080 human fibrosarcoma cells were obtained from ATCC and maintained in DMEM supplemented with 10% fetal calf serum. For mitogen stimulation experiments, cells were starved with same medium containing 0.1% fetal calf serum for 6 hours and then treated with either EGF, PMA, or Okadaic acid. The cell lysates were prepared as previously described (49). Briefly, cells were washed three times with ice-cold buffer containing 0.15 M sodium chloride and 25 mM sodium phosphate (pH7.2) and lysed in 150 μl of ice-cold homogenization buffer containing 20 mM Tris (pH7.5), 50 mM p-glycerophosphate, 50 mM sodium fluoride, 2 mM DTT, 100 μM sodium orthovanadate, 5 mM benzamidine, 20 mM p-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cell lysates were cleared by centrifugation at 100,000 x g at 4°C for 30 min. The

supernatants were assayed for protein by Bio-Rad protein assay reagent and kinase activity was determined immediately or the samples were frozen at -70°C.

Two different MAP-kinase assays were performed in order to have independent confirmation of the activities measured. The first MAP-kinase assay was adapted from previously described methods (9,49) using Myelin Basic Protein (MBP) as the substrate. Briefly, cell lysates (10 μg) were assayed in a final volume of 25 μl containing 50 mM HEPES (pH8.0), 10 mM manganese chloride, 1 mM DTT, 1 mM benzamidine, 100 nM staurosporine, 50 μM [γ^{32}P]ATP (1 μCi) and 0.4 mg/ml MBP (Sigma, USA) at 30°C for 30 min. Assays were terminated with 2x SDS sample buffer and phosphorylated MBP was analyzed by a SDS polyacrylamide gel (15% acrylamide) electrophoresis and autoradiography.

In the second assay, the phosphorylation of MAP kinase target synthetic peptide (UBI, USA) which has the amino acid sequence of APRTPGGRR containing the amino acids 95-98 (underlined) of bovine myelin basic protein (42) was measured as described method (9). The phosphorylation reaction was similar to that for MBP phosphorylation. The reaction was stopped by spotting 20 μl aliquots onto a 1.5 cm^2 piece of Whatman P81 phosphocellulose paper and then the papers were dried briefly at room temperature, washed seven times by shaking for 10 min in phosphoric acid (1%, w/v), and radioactivity was then determined by scintillation counting.

Results

Activation of MAP kinases in HT1080 cells. It has been shown that the MAP kinase signaling pathway is involved in the cell growth, differentiation and transformation. It was therefore of interest to investigate whether the biological activities of HPV oncogenes could influence this signalling pathway. Since there is no one cell type which can be readily transformed by each of the HPV-16 oncogenes individually, we chose to use a cell type which was already fully transformed to provide a more uniform background to compare MAP kinase in cells expressing the different HPV oncogenes. HT1080 human fibrosarcoma cells were transfected with HPV oncogenes, and then the MAP kinase activity of the resulting cells was measured. Before transfecting these cells, it was, however, necessary to verify whether it was possible to stimulate and measure MAP kinase activity in these cells. Therefore, MAP kinase activity was determined in these cells following stimulation with EGF, phorbol myristate acetate (PMA), and okadaic acid (OA). MAP kinase activity is enhanced by OA because OA inhibits 2A protein phosphatase, which in turn dephosphorylates MAP-kinase (3,18). PMA may stimulate serine/threonine phosphorylation of MAP kinase kinase (16).

As shown in Figure 1, MAP kinase in HT1080 cells was stimulated with EGF in a dose-dependent manner and was maximally activated by eightfold with a concentration of 100 ng of EGF per ml. These data are consistent with a previous study demonstrating that HT1080 cells contain normal levels of EGF-Rs (47). Treatments with PMA or OA

increased MAP kinase activities by five- and threefold, respectively. EGF was used in subsequent assays because it induced the highest level of MAP-kinase activity. As also shown in Figure 1, two different MAP-kinase assays were performed. In Figure 1A, the phosphorylation of myelin basic protein (MBP) was determined by autoradiography. In Figure 1B, the phosphorylation of a MAP-kinase specific peptide was determined by liquid scintillation counting. The data obtained by the two different MAP-kinase assays was very similar, and therefore these two assays were used in subsequent analysis involving the HPV oncogene expressing cells.

Expression of HPV 16 E5, mutant E5, E6, and E7 in HT1080 cells. The preceding data showed that the MAP kinase activity could be determined in HT1080 cells following EGF stimulation. This allow us to use this cell line to examine the effects of expression of HPV oncogenes on EGF-activated MAP kinase signal transduction. HT1080 cells were cotransfected with HPV oncogene expressing plasmids pJ4-16E5, pJ4-16M5, pJ4-16E6, pJ4-16E7, or control plasmid pJ4 together with pWLNeo plasmid and selected in the medium containing G418. The resulting resistant colonies were pooled and expanded as polyclonal pools. The expression of the respective HPV oncogenes was verified by Northern (RNA) blot analysis. As shown in Figure 2, the respective HPV oncogene transcripts were present in the resulting pooled cells. There was no visible change in the morphology or growth characteristics of the HPV-oncogene expressing cells. Given that these cells are already fully transformed (40), demonstrate a loss contact growth inhibition in culture, form colonies in agar, and are tumorigenic in immunocompromised

mice, it was perhaps not surprising that the expression of HPV oncogenes did not further alter the growth characteristics of these cells.

MAP kinase activities in HPV oncogenes expressing HT1080 cells. The major purpose of this study was to determine whether expression of HPV type 16 oncogenes, E5, E6, and E7 could affect the EGF-activated MAP kinase signaling pathway. Therefore, the MAP kinase activity in the absence or presence of EGF was determined in the cells expressing the individual HPV oncogenes. Cells were serum starved for 6 h. stimulated with EGF (100 ng/ml) for 5 min, and then lysed. Lysates containing 10 μg total protein were assayed for MAP kinase activities with MBP and MAP kinase target synthetic peptide as substrates. The results from both these assay conditions are presented in Figure 3. These data demonstrate that expression of E6 or E7 in HT1080 cells did not affect the MAP kinase activity in these cells. In contrast, there was modest yet reproducible increase in MAP kinase activity in the E5- and mutant E5-expressing cells as determined by both assay conditions. The mutant E5 used here was altered in order to potentially increase the translation efficiency of the corresponding E5 mRNA by introducing the Kozak consensus sequence around the initiation ATG of the E5 gene. This resulted in two amino acid changes following the initiation methionine. In vitro transcription-translation analysis of the mutant E5 compared to the wild-type E5 showed that the mutant E5 was at least five times more efficiently translated in vitro (data not shown). However, in vivo the wild-type E5- and mutant E5-expressing cells both demonstrated a similar increase in the MAP-kinase activity, suggesting that this mutation

did not increase translation efficiency in vivo. There was approximately a twofold increase in MAP kinase activity in the E5 expressing cells which was observed both in the presence and in the absence of EGF stimulation.

Effects of E5 expression on the duration of MAP-kinase activity. The hallmark of the EGF-activated MAP kinase signaling pathway is its transience. Previous studies have shown that constitutive activation or prolongation of the activated stage of the MAP kinase pathway could lead to cell proliferation and transformation (11,27,49). Moreover, HPV type 16 E5 has been shown to inhibit degradation of EGF-R and cause a greater number of EGF-Rs to be recycled back to the cell surface after 2 h of stimulation (53), and this may therefore lead to a prolongation of the MAP kinase pathway by E5.

On the basis of this information, we examined the influence of E5 expression on the duration of EGF-activated MAP-kinase activity. As before, control cells and E5-expressing cells were serum starved for 6 h and then left untreated or treated with EGF (100 ng/ml) for various times up to 3 h. Because the previous assays showed that the MBP phosphorylation data and the peptide phosphorylation data yielded comparable results, only the MBP was used as the MAM kinase substrate in this experiment. The results of two similar such experiments are shown in Figure 4, in which the upper and lower panels represents the MAP kinase activity determined up to 2 h and 3 h respectively. These data show that the expression of E5 induced an increase in the MAP kinase activity by up to two- to threefold (as determined by densitometry of the X-ray

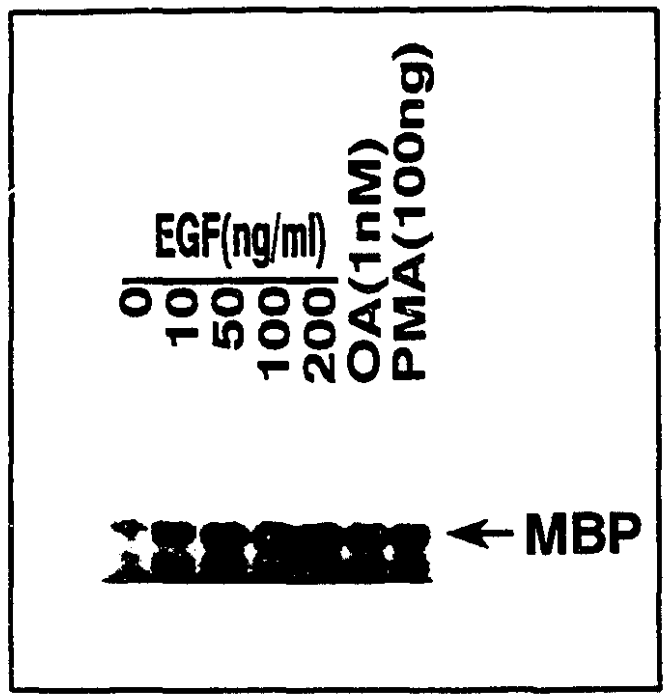
films [data not shown]) compared with the control cells at each time point analysed. These data also demonstrate that E5 could induce a sustained increase in MAP-kinase activity for up to at least 3 h. Therefore, although E5 had a modest effect on MAP-kinase activity, this effect was evident over an extended period. This is more significant than if the E5-mediated increase in MAP kinase activity was restricted only to the early 5 min interval.

MAP-kinase activity in transiently transfected COS-1 cells. Because of the modest increase in E5 mediated MAP kinase activity observed in the HT1080 cells, it was necessary to confirm this observation with an independent approach. Several previous reports have used COS-1 cells in transient transfection assays to characterise BPV E5 (10,34) and HPV-16 E5 (22). We therefore have used this approach to determine whether a transiently transfected HPV-16 E5 expressing plasmid in COS-1 cells would result in a detectable increase in MAP kinase activity. As shown in Figure 5, cells transiently transfected with the E5 expressing plasmid demonstrated more MAP-kinase activity than cells transfected with a control plasmid. As with the stably transfected HT1080 cells, the increase in MAP kinase activity in the E5 transfected cells was about twofold greater than in the control cells, and this was both in the presence and the absence of EGF stimulation. However, we estimated through the use of ß-galactosidase activity staining in the presence of X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) that about 20 to 30% of the cells were transfected in this manner. This argues that within the E5 plasmid transfected cells, the MAP-kinase as shown in Figure 5 is an underestimate.

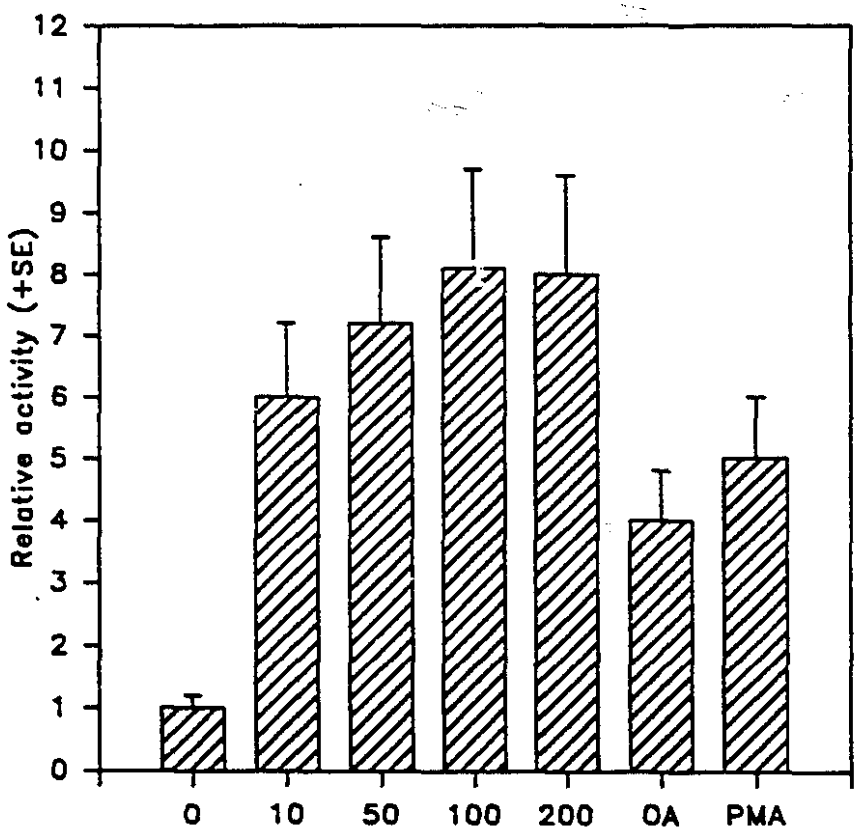
These data are consistent with the experiments carried out in the HT1080 cells and provide further support for the argument that HPV-16 E5 can enhance cellular MAP-kinase activity.

Figure 1: Activation of MAP-kinase in HT1080 cells. HT1080 cells were treated with 0, 10, 50, 100, 200 ng/ml EGF for 5 minutes, or 1 nM OA, or 100 ng/ml PMA for 10 minutes. The kinase activities were assayed as described below. In **Panel A,** myelin basic protein (MBP) was used as the substrate for MAP-kinase and the phosphorylation of MBP was determined by SDS-PAGE followed by autoradiography. In **Panel B,** a specific MAP-Kinase target peptide was used as the substrate for MAP-kinase activity. Phosphorylation of this peptide was measured by liquid scintillation counting. The relative levels of radioactivity are shown as a bar graph and represent the mean $\pm$ SD of determinations from three experiments. Note that the data obtained by the two assay conditions were very similar.

A


EGF(ng/ml)
0
10
50
100
200
OA(1nM)
PMA(100ng)
MBP

B


Relative activity (+SE)
0
1
2
3
4
5
6
7
8
9
10
11
12
0
10
50
100
200
OA
PMA

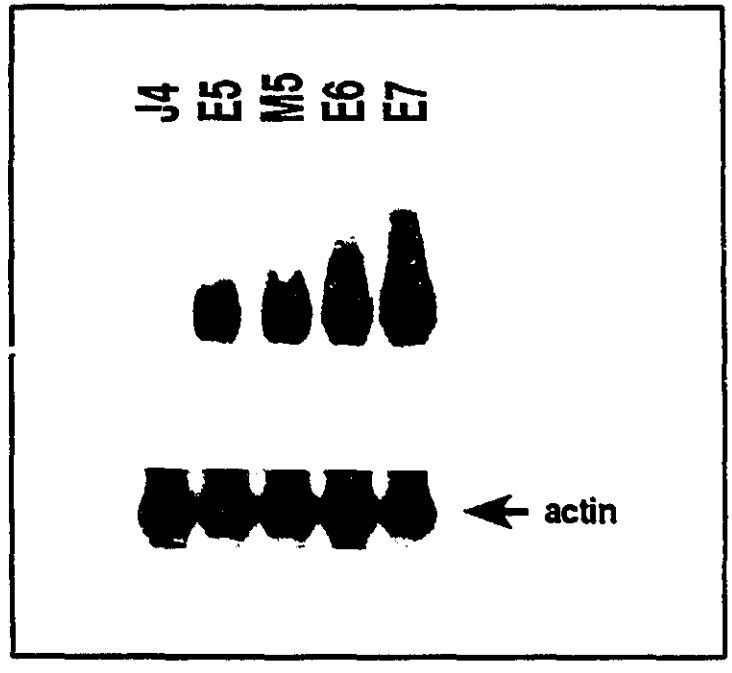
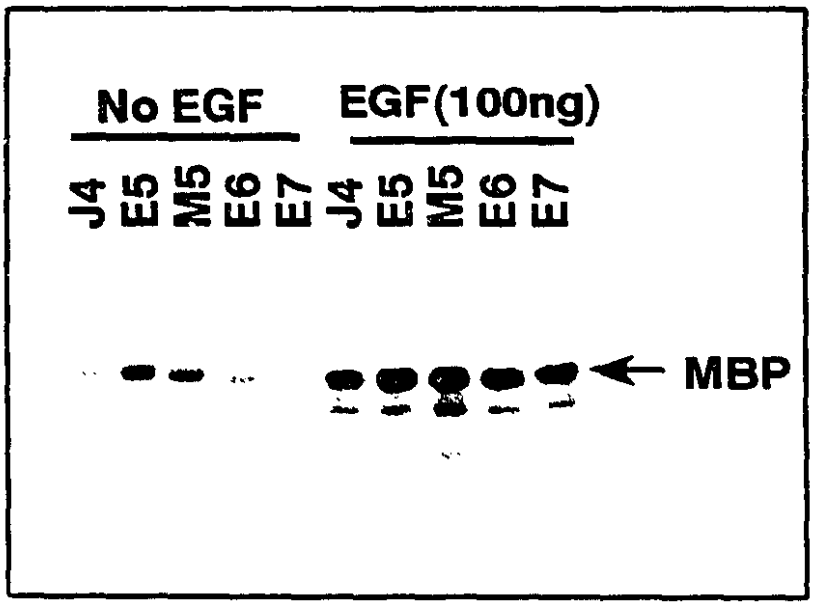


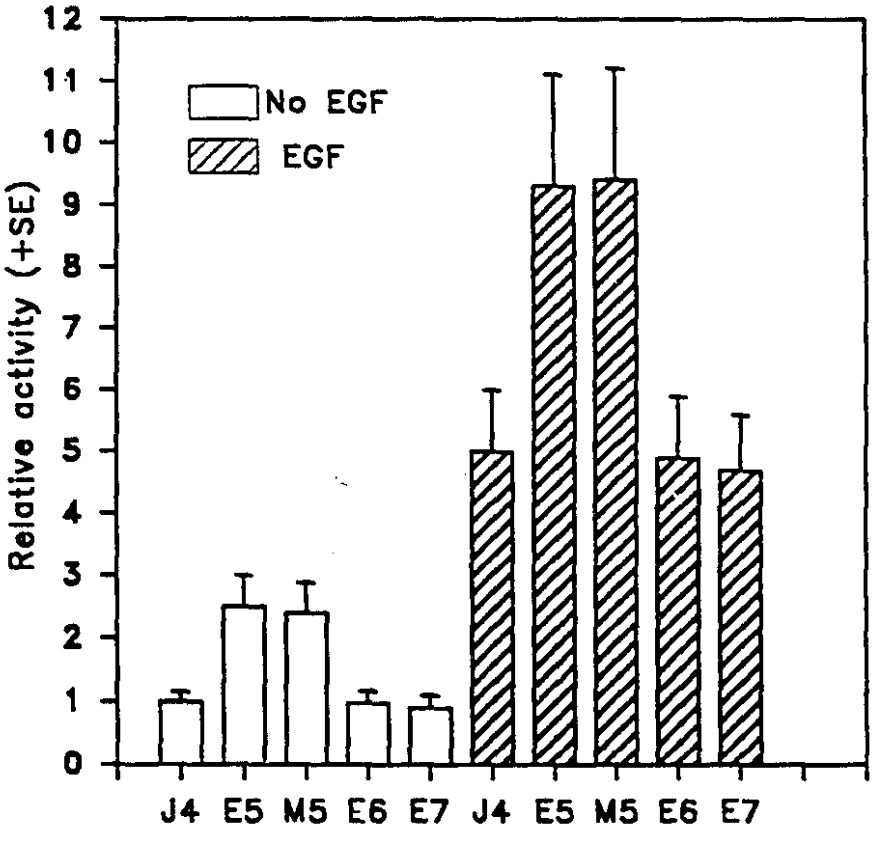
Figure 2: Northern blot analysis of cell lines expressing individual HPV oncogenes. Ten microgram of the total RNA from the individual cell lines stably transfected with control pJ4 vector and HPV oncogenes, E5, M5 (mutant E5), E6 and E7 were probed with [α^{32}P]dCTP labelled nick translated DNA fragments from the open reading frames of each viral gene. The blot was reprobed with an actin probe to verify equal loading of RNA in each lane.

Figure 3: MAP-kinase activity in the HPV oncogenes expressing HT1080 cell lines. Cells expressing individual HPV oncogenes were grown for 24 hours (80% confluence), serum starved for 6 hours, and then treated with 100 ng/ml EGF for 5 minutes or left untreated. **Panel A**, the MAP-kinase activities were assayed by the MBP phosphorylation assay. **Panel B**, MAP-kinase activity determined by the peptide phosphorylation assay where the numbers represent relative MAP-kinase activities and the mean ± SD of determinations from three experiments. The J4 represents the cell line stably transfected with control pJ4 vector.

A


No EGF
EGF(100ng)
J4 E5 M5 E6 E7 J4 E5 M5 E6 E7
MBP

B


No EGF
EGF
Relative activity (+SE)
0 1 2 3 4 5 6 7 8 9 10 11 12
J4 E5 M5 E6 E7 J4 E5 M5 E6 E7

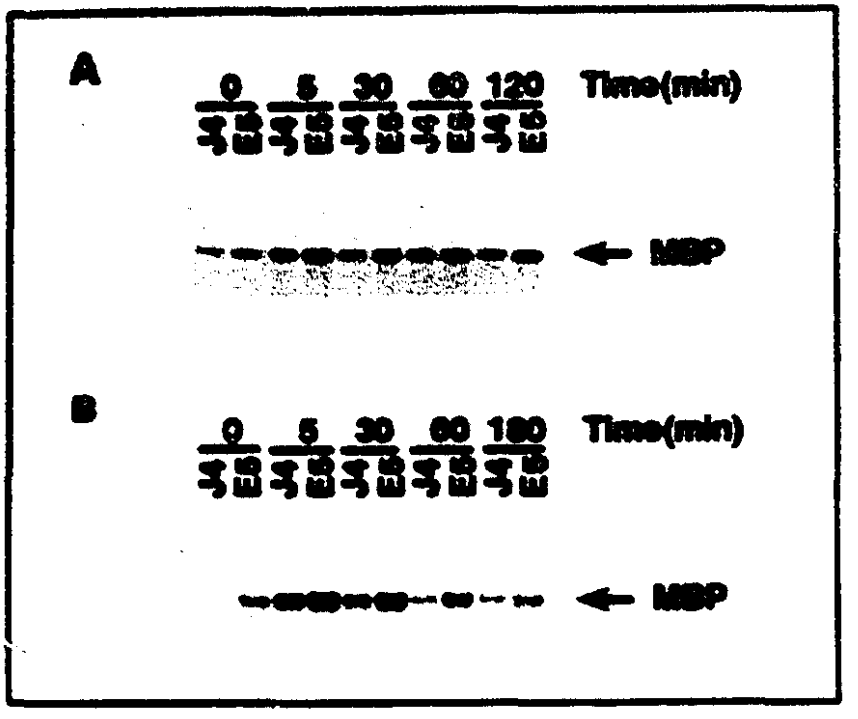


Figure 4: Duration of EGF-induced MAP-kinase activity in E5 expressing cells. Cells expressing E5 and control cells we assayed for MAP-kinase activity following treated with 100ng/ml EGF for various times. In the **Panel A**, data is shown for a two hour time course. In the **Panel B**, data is shown for a three hour time course. MAP-kinase activity was determined as described in materials and methods. Two such similar assays are shown here to demonstrate reproducibility in this assay. The J4 represents the cell line stably transfected with control pJ4 vector. These data demonstrate that the increased level of MAP-kinase activity in the E5 expressing cells was observed for at least three hours following EGF stimulation.

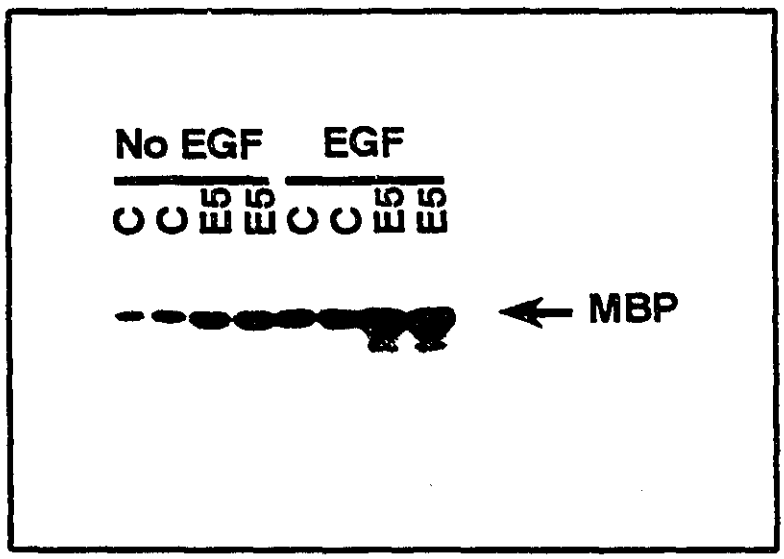


Figure 5: MAP kinase activity in COS-1 cells transiently transfected with the E5 gene. COS cells transiently transfected with control or E5 expressing plasmid were assayed for MAP-kinase activity 48 hours after transfection. Cells were either untreated or treated with 100 ng/ml EGF for 5 minutes as indicated. Each lane represents the MAP-kinase activity determined from a separate dish of transfected cells. Duplicate dishes of transfected cells were used in this assay. Note that the E5 transfected cells contained more MAP-kinase activity than the control cells.

Cells were transfected with the E5 expressing plasmid pMT2-H16E5KC by the Calcium phosphate procedure as previously described (25). MAP-kinase assays were performed as described in Figure 1.

Discussion

The stimulation of cell proliferation by growth factors is largely controlled by a series of specific kinases including MAP-kinase. Subtle differences in the concentration, activity, or period of activity of these regulators could have profound influences on cell replication. Viruses such as HPV may influence growth factor responses in subtle but specific ways to influence host cell behaviour to ensure virus replication and subsequent propagation. In the present study, we have demonstrated that expressions of HPV type 16 E5 resulted in a modest enhancement of MAP-kinase activity. In comparison, expression of E6 or E7 did not alter MAP kinase activity. The increase in MAP kinase activity in the E5 expressing cells was seen in both the presence and the absence of EGF and was observed for at least 3 h following EGF stimulation. This increase in MAP-kinase activity may enhance DNA replication of infected cervical cells, thus ensuring replication of the viral genome in these cells.

Activation of the EGF-R results in the stimulation of a number of signal transduction pathways including the downstream activation of MAP-kinase (4,21,29,30). Therefore, the observation reported within that MAP-kinase is more active in the E5 expressing cells is consistent with increased EGF-R activity in these cells. These data are in agreement with previous studies showing that expression of E5 inhibits degradation of EGF-R and causes EGF-R to be recycled back to cell surface (53) and that HPV type 16 E5 expression could cooperate with EGF to induce anchorage-independent growth and

proliferation of established and primary rodent cells (5,25,37).

It was also apparent that MAP kinase activity was increased in the E5-expressing cells in the absence of EGF stimulation. It is therefore possible that, like bovine papillomavirus E5 (34,35), HPV-16 E5 can associate directly with growth factor receptors and that this results in an increase in growth factor receptor activity. This would be consistent with the recent observation that HPV-16 E5 is capable of complexing with a variety of growth factor receptors including the EGF-R, the platelet-derived growth factor receptor, the colony stimulating factor-1 receptor, and p1850neu (22). Furthermore, HPV-16 E5 has transforming activity in the absence of EGF (25,26,53), and this may be due to in part to its association with and potential activation of growth factor receptors, thus resulting in the increased MAP kinase activity in the absence of growth factors. Another possibility is that E5 may inhibit the degradation of cellular growth factor receptors in addition to the EGF-R. These receptors may be able to respond to factors within the serum, and this may also contribute to increased MAP kinase activity in the absence of EGF.

The E5 gene is usually lost during viral DNA integration in the more advanced cancers, thus demonstrating that it is not required for disease progression to the later advanced stages. It is possible that the subtle activity which E5 has on the MAP-kinase activity becomes redundant in the more advanced transformed cells because of the more dominant activity provided by the major transforming genes, E6 and E7. For example,

loss of Rb regulatory activity due to E7 may override any requirement for increased MAP-kinase activity. Likewise, E6-mediated p53 degradation could result in unchecked cyclin-dependent kinase activity because of reduced p21 activity, and this could also override a requirement for increased MAP-kinase activity.

In summary, we have examined cellular MAP-kinase activity in HPV-16 oncogene expressing cells. Although E5 could enhance MAP-kinase activity, E6 and E7 had no effect on this activity. These data suggest that one of the cellular targets of HPV-16 E5 may be the MAP kinase associated signal transduction pathway.

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GENERAL CONCLUSION

Discussion

Cervical cancer develops through a multistep process. The preponderance of biochemical, biological, and epidemiological evidence all support the causal role of human papillomavirus in the development of this malignancy. The observation that the E5, E6, and E7 genes are oncogenic in a variety of transformation assays and that the E6 and E7 genes are selectively retained and expressed in HPV-associated malignancies supports the role for these proteins in both initiation and progression of the malignancies (Boshart et al., 1984; Smotkin and Wettstein, 1986; Banks et al., 1987). The fact that the majority of HPV-associated epithelia lesions are usually limited to benign lesions or warts and those infected with 'high-risk' HPVs progress toward a malignant phenotype after a long latency implies that viral proteins can modulate cellular functions. This modulation could be direct viral regulation, for instance, E6 and E7 have transactivation activity, or could be indirect through viral/cellular protein interaction.

Considering the biological functions of the cellular tumor suppressors, it has been speculated that by targeting the tumor suppressor gene products p53 and RB, HPV modulates cellular mechanisms to facilitate viral replication and this also can contribute to malignant progression. The E5 gene in comparison enhances the activity of growth factors receptors, and these activated receptors subsequently transduce a mitogenic signal to the nucleus that results in induction of cellular DNA synthesis. In addition, viral

oncogenes may act by indirectly inducing genomic lesions that accumulate and thereby drive malignant progression.

The main objective of the study presented within the thesis was to investigate the biological functions of E6-mediated inactivation of p53 and to explore the cellular signal transduction pathway which E5 may influence.

Previous studies have shown that E6 from 'high-risk' HPVs interacts with p53 and promote its degradation (Scheffner et al., 1990; Crook et al., 1991), and inhibits p53 mediated transcription (Mietz et al., 1992). However, it remained unclear whether E6 could impair p53 mediated transcription under biologically relevant conditions such as during the activation of p53 with DNA damaging agents. In Chapter II, the ability of HPV type 18 E6 to impair p53 mediated transcription activity in UV irradiation cells was investigated. One of the other objectives of this study was also to examine whether deletion of a region from amino acid residues 113 through 117 of HPV type 18 E6 could abolish the ability of the protein to promote the degradation of p53.

The principle conclusions were i) that HPV type 18 E6 inhibits p53 mediated transcription activity under biologically relevant conditions where p53 activity was induced with UV irradiation, ii) that the region from amino acid residues 113 through 117 is necessary for the function of HPV type 18 E6 to direct the degradation of p53. This study was the first demonstration that E6 targets p53 under conditions where p53 activity was induced. These data provided evidence for the involvement of E6 in the development of cervical carcinomas by potentially destabilising the genome by inactivating p53 under conditions where DNA damage has occurred.

To further evaluate the significance of E6/p53 interaction, a functional p53 monomeric mutant which is resistant to E6 mediated degradation was transfected into HPV-16 positive cervical carcinoma derived cells, and the biological characteristics of the resulting cells were examined. The results were presented in Chapter III. The main observations made from this study were i) that the proliferation of HPV-16 positive SiHa cells was inhibited and these transfected cells displayed a more differentiated phenotype, ii) that the monomeric p53 mutant 338 was as efficient as wild-type p53 in suppressing the proliferation of SiHa cells. These findings demonstrate that the viral E6 mediated p53 inactivation plays a role in maintaining the transformed phenotype of cervical carcinoma derived cells, and that p53 in its monomeric form with its C-terminal regulatory region removed retains biological activity with respect to impairing cell proliferation.

Finally, the effect of HPV type 16 oncogene E5, E6 and E7 on the MAPK signal transduction was evaluated and the results were presented in Chapter IV. This study revealed that expression of E5, but not E6 and E7, induced an increase in MAPK activity both in the presence and absence of EGF. This is consistent with the previous finding that E5 can inhibit down-regulation of EGF-R (Straight et al., 1993; 1995) and cooperate with EGF to enhance the signal transduction to the nucleus (Leechanachai et al., 1992; Pim et al., 1992).

Infection with human papillomavirus, for instance HPV type 16 or 18, and the concomitant expression of the three viral oncogenes E5, E6 and E7 in vivo does not immediately nor necessarily lead to cancer. Nevertheless, at least in the case of E6 and E7, these genes do play significant roles in the development of cervical cancer. The fact

that more than 90% of cervical carcinomas contain HPVs and the E6 and E7 genes are continuously expressed suggest that the E6 and E7 genes are important for HPV to induce cervical carcinomas and modulate cellular control mechanisms.

It has been established by a variety of in vitro biochemical and in vivo biological experiments that HPV oncoproteins modulate cellular proteins which are critical for regulating the cell cycle. For example, E7 forms complexes with cell cycle control proteins such as RB and related proteins (Dyson et al., 1989; Tommasino et al., 1993) and E6 interacts with the tumor suppressor p53 and promotes its degradation (Scheffner et al., 1990; Crook et al., 1991). Recent studies showing that E5 interacts with growth factors receptors and enhance mitogenic signal transduction credit HPV with targeting another important cellular control mechanism (Leechanachai et al., 1992; Hwang et al., 1995, Gu and Matlashewski, 1995).

Cell cycle studies have shown that RB plays a key role in regulating cell proliferation (Buchkovich et al., 1989). Rb interacts with several cellular proteins, such as E2F and Myc, which themselves function as transcription factors, and these interactions result in the impairment of cell cycle progression (Hamel et al., 1992). The interaction of E7 with RB inhibits binding of E2F to RB thus leading to the inappropriate transactivation of E2F-regulated genes which impairs the normal control of the cell cycle (Chellappan et al., 1992). The interactions of E7 with several other cell cycle regulators has been found, such as p130, p107 and cyclin A/cdk2 (Davies et al., 1993; Tommasino et al., 1993). This implies that interaction with cell cycle control molecules could be one of the mechanisms by which E7 advances cells to malignant transformation.

Wild type p53 protein is most commonly described as a tumor suppressor. Alteration or loss of p53 is associated with a wide variety of human cancers including cervical cancer. The protein has been postulated to act as a 'guardian of the genome' (Lane, 1992). The transcriptional activation activity of wild-type p53 is induced following DNA damage (Hall et al., 1993; Lu and Lane, 1993). p53 activates transcription of genes including those which suppress the G1/S transition (Kastan et al., 1992; Kuerbitz et al., 1992), thus providing cells with a G1 arrest checkpoint following DNA damage. In this manner, p53 protects the integrity of the genome by not allowing cells with damaged DNA to replicate. There is also evidence that p53 is directly involved in the DNA damage repair (Obercosler et al., 1993; Wang et al., 1994; Lee et al., 1995) and apoptosis (Oltvai et al., 1993; Miyashita et al., 1994; Miyashita and Reed, 1995). Therefore, blocking p53 activity promotes accumulation of cellular mutations which in turn contribute to tumour progression (Donehower et al., 1992).

The E6 mediated degradation of p53 plays a major role in inactivation of p53 activity in cervical carcinomas. The study presented in Chapter II demonstrates the ability of oncogenic E6 to abolish p53 activity following DNA damage, and provides evidence that E6 may disrupt p53-mediated cellular responses to DNA damage. This is consistent with previous studies demonstrating that E6 causes p53 degradation and p53 protein levels did not increase in HPV-16 E6 expressing cells treated with a DNA damaging agent (Kessis et al., 1993). The data in Chapter II suggests that E6 may induce cells to become susceptible to genomic lesions following DNA damage thus contributing to the accumulation of genetic mutations and the development of neoplasia.

Previous studies have shown that E6 and E7 are necessary to maintain the neoplastic phenotype of cervical cancer cells (Boshart et al., 1984; Smotkin and Wettstein, 1986; Banks et al., 1987). The biological importance of the E6-p53 interaction was directly examined in the study presented in Chapter III. The results demonstrated that restoring p53 activity in HPV positive cervical carcinoma cells results in impairing proliferation of these cancer cells and produced a more differentiated cellular morphology. This provided evidence that E6 mediated p53 degradation contributes to maintaining the neoplastic phenotype of HPV positive cervical carcinoma cells. The results from this study also demonstrated that p53 in its monomeric form without its C-terminal regulatory region retains biological function with respect to impairing cell proliferation.

The monomeric p53 mutant 338 used in the study is resistant to HPV E6 mediated p53 degradation (Thomas et al., 1995). The endogenous p53 activity is impaired by HPV E6 in HPV positive cervical cancer cells (Butz et al., 1995). Introducing this unique p53 form into HPV containing cervical cancer cells allowed us to directly examine the role of the E6/p53 interaction in inducing cancerous growth and maintaining the neoplastic phenotype of the cells. The cells containing this monomeric p53 mutant 338 are less actively proliferating and display a more differentiated phenotype. The results demonstrated the importance of the E6 mediated p53 inactivation in HPV positive cervical cancer derived cells.

An important genetic characteristic of cervical cancer is that the p53 protein activity is lost due to E6 from high risk HPV sequences or that the p53 gene is mutated

(Scheffner et al., 1991; Crook et al., 1992; Park et al., 1994). This loss of p53 activity may account partly for uncontrolled cell growth, disrupted differentiation, and maintenance of the malignant phenotype of cervical cancer cells (zur Hausen, 1991). It has been shown that p53 transactivation activity is required for its growth suppressive function (Crook et al., 1994; Pietenpol et al., 1994). p53 has also been shown to be involved in the regulation of differentiation. For example, introduction of wild type p53 leads to partial differentiation of a pre B-cell (Shaulsky et al., 1991), and overexpression of p53 induces signs of differentiation in a number of cells, including leukaemic K562 cells, HL-60 cells, and Friend virus-transformed erythroleukemic cells (Feinstein et al., 1992; Johnson et al., 1993; Soddu et al., 1994; Ehinger et al., 1995). Similarly, overexpression of p53 induces expression of differentiation markers in normal human keratinocytes and squamous carcinoma cells (Brenner et al., 1993; Woodworth et al., 1993), and leads to reversion of the malignant phenotype of various cell lines (Mercer et al., 1990; Baker et al., 1990; Diller et al., 1990). The results presented in Chapter III are consistent with previous studies showing that p53 plays a role in growth suppression (Crook et al., 1994; Pietenpol et al., 1994) and preventing neoplastic phenotype (Brenner et al., 1993; Woodworth et al., 1993).

Studies on the structure and function of the p53 protein have revealed that p53 contains separated domains which are important for transactivation, sequence-specific DNA binding, and oligomerization (see Chapter I, section 3). Several recent studies have shown that the C-terminal region of p53 contains a regulatory element which can interact with RNA and single stranded DNA, suggesting that this may regulate the ability of p53

to function as a transcriptional transactivator (Oberosler et al. 1993; Bakalkin et al., 1994; Jayaraman and Prives, 1995). This view was further supported by the observations that phosphorylation of the C-terminal regulatory domain of p53 by cellular protein kinases or by targeting this region with a monoclonal antibody in vitro activates sequence specific DNA binding (Hupp and Lane, 1994; Takenaka et al., 1995). More recently, an allosteric model has been proposed for the regulation of p53 activity which suggests that post-translational modification of the C-terminal regulatory region of p53 in vivo is a rate limiting step in the activation of sequence specific DNA binding by p53 (Hupp et al., 1995). It has also been demonstrated that the monomeric p53 mutant which has the C-terminal regulatory element deleted retained sequence-specific transcriptionally activating activity in vivo (Tarunina and Jenkins, 1993). It still remained unclear whether the monomeric form of p53 without its C-terminal region regulatory element was biologically functional, eg., capable of suppressing growth and inducing apoptosis.

The results presented in Chapter III argued that a monomeric form of p53 mutant 338 lacking C-terminal region was biologically functional with respect to suppressing cell growth. This monomeric p53 mutant was capable of inhibiting proliferation of HPV positive cervical cancer cells and inducing a more differentiated morphology in the cells. These results defined the growth suppression function for the monomeric p53 without its C-terminal regulatory region.

It would be interesting to further study the biology of this p53 mutant. For instance, can this monomeric p53 mutant induce apoptosis? What biological function does this p53 mutant have in cancer cells which have mutated p53 because the monomeric p53

mutant would not form complexes with dominant negative p53 mutants?

The HPV E5 gene is not required for the maintenance of advanced neoplastic lesions because it is usually lost during the viral DNA integration into host cellular genome (Schwartz et al., 1985; Baker et al., 1987). Therefore, if it is involved in transformation, this would be at an early stage of disease progression.

Recent studies have demonstrated that the E5 gene is a transforming gene and can enhance the signal transduction from growth factor receptors to the nuclear expression of c-fos gene (Chen and Mounts, 1990; Leechanachai et al., 1992; Pim et al., 1992). E5 has been shown to be capable of inhibiting growth factor receptor internalisation and degradation (Straight et al., 1993; 1995), and directly interacting with several growth factors receptors (Hwang et al., 1995). Thus it seems likely that the biological activity of E5 is associated with its ability to increase growth factor receptor activity.

The study presented in Chapter IV revealed that E5 could enhance the MAPK signal transduction pathway both in the presence and absence of EGF. This is consistent with previous studies showing enhanced EGF-R activity in E5 expressing cells and that over-expression of c-Raf, which is a mediator of the MAPK pathway, was capable of functionally substituting for E5 in a co-mitogen assay (Valle and Banks, 1995). It has been established that E5 enhances c-fos expression in response to growth factor (Leechanachai et al., 1992) and that the MAPK pathway mediates the maximal expression of c-fos (Treisman, 1994). These observations are also consistent with the data presented in Chapter IV showing that E5 expression resulted in an increase in MAPK activity both in the presence and absence of EGF. These observations further

strengthen the argument that E5 functions at least in part to enhance growth factor mediated signal transduction in HPV infected cells.

In summary, the following observations have been made in the studies presented within this thesis. i) HPV-18 E6 inhibits p53 mediated transcription activity following DNA damage, suggesting that E6 could contribute to genomic instability and the accumulation of genetic mutations. ii) the loss of p53 activity in HPV transformed cells is required to maintain the transformed phenotype. iii) the monomeric p53 mutant without its C-terminal regulatory domain is biologically active with respect to impairing cell proliferation. iv) the MAPK signal pathway is more active in cells expressing HPV-16 E5 than control cells or cells expressing E6 and E7. These observations contribute to our understanding of how HPV proteins interact with key cellular control proteins and how these interactions contribute to the development of neoplasia.

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