Down-regulation of MHC class I by human papillomavirus type 16 E5 protein

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May 2006

This thesis is submitted to the University of Glasgow in accordance with the requirements for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine

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To my wife & children

Acknowledgements

I would like to thank the Mazandaran University of Medical Sciences and Education section of Ministry of Health and Medical Sciences of Iran for providing me with a PhD scholarship. My special thanks are due to my supervisors Prof M. Saveria Campo and Dr. G. Hossein Ashrafi for their general guidance, constant support, patients, and help in so many ways and so many times. It would not have been possible to complete this work without their encouragement and support. Many thanks to all members of papillomavirus lab for their support and help during my studies. I will never forget you.

Thanks to all friends and staffs of the Institute of Comparative Medicine for their kindly help.

Last, but certainly not least, I would like to acknowledge my wife, Fariba, and my lovely children, Mehdi, Rahim and Maryam I say thank you for putting up with me and supporting me during my PhD study. I would also like to express my gratitude to my parents and all members of the family.

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ABBREVIATIONS

ATP	Adenosine triphosphate
AIDS	acquired immunodeficiency syndrome
MAP	mitogen activated protein
ATL	adult T-cell leukemia
β_2M	β_2-microglobulin
bp	base pairs
BPV	bovine papilomavirus
BSA	Bovine serum albumin
CDK	cyclin-dependent kinase
CIAP	calf intestinal alkaline phosphatase
CIN	Cervical intraepithelial neoplasia
cm	Centimeters
Co-IP	Co-Immunoprecipitation
CR	conserved regions
CRPV	Cottontail rabbit papillomavirus
CTL	cytotoxic T lymphocyte
DAPI	4'-6'-Diamidino-2-phenylindole
DD	death domain
DEPC	Diethyl Pyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	3' deoxyribonucleoside 5' triphosphate

DR	death receptor
E.coli	Escherichia Coli
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
EGF-R	epidermal growth factor receptor
EtBr	Ethidium bromide
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal calf serum
FITC	Flurorescein-isothiocynate
G418	Geneticin, G418-sulphate
GFP	Green Fluorescent Protein
HaCaT	Human immortalised keratinocyte stable cell line
HBV	Hepatitis B virus
HCC	hepatocellular carcinoma
HCMV	Human cytomegalovirus
HEPES	N-[2-Hyroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HFF	human foreskin fibroblast
HFK	human foreskin keratinocyte
HHV8	human herpesvirus 8
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	Human papillomavirus
HSV	Herpes Simplex Virus
HTLV-1	Human T cell leukaemia virus-1
IFN	Interferon
Ig	immunoglobulin
IP	Immunoprecipitation
IRF	interferon regulatory factor
kbp	Kilo-base pairs
kDa	KiloDalton
KS	Kaposi's sarcoma

LCR	Long control region
mAb	Monoclonal antibody
MHC	major histocompatibility complex
ml	millilitre
mM	Millimolar
mRNA	messenger ribonucleic acid
NK	natural killer
°C	degree centigrade
OD	Optical density (light absorbance)
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline plus 0.1% Tween
PCR	Polymerase chain reaction
PDGF-R	platelet-derived growth factor receptor
PV	Papillomaviruse
Rb	retinoblastoma
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
RSV	Rous sarcoma virus
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulpate
SV40	Simian virus 40
TAP	transporter associated with antigen processing
TNF	tumour necrosis factor
TRAIL	TNF related apoptosis-inducing factor
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet
V	Volts
v/v	Volume per unit volume

wt	Wild type
µg	microgram
µl	microlitre
^{35}S	Sulfur isotope 35 atom

Single letter DNA nitrogenous base

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Single letter amino acid code

Alanine	Ala (A)
Arginine	Arg (R)
Asparagine	Asn (N)
Aspartic acid	Asp (D)
Cystein	Cyc (C)
Glutamic acid	Glu (E)
Glutamine	Gln (Q)
Glycine	Gly (G)
Hisitdine	His (H)
Isoleucine	Ile (I)
Leucine	Leu (L)
Lysine	Lys (K)
Methiionine	Met (M)
Phenylalanine	Phe (F)
Proline	Pro (P)
Serine	Ser (S)
Threonine	Thr (T)
Tryptophan	Trp (W)
Tyrosine	Tyr (Y)
Valine	Val (V)

Abstract

Like many viruses, papillomaviruses appear to have evolved mechanisms resulting in escape from host immune surveillance and delay of resolution of infection. The E5 protein is expressed early in papillomavirus infection in the deep layers of the infected epithelium. Human papillomavirus type 16 (HPV-16) E5 is a small protein, 83 amino acids long, and has three hydrophobic domains connected by less hydrophobic regions. It is located mainly at the endosomal membranes, Golgi apparatus (GA) and, to a lesser extent, the plasma membrane. The E5 protein of bovine papillomavirus (BPV) impairs the synthesis and stability of major histocompatibility (MHC) class I complex and prevents their transport to the cell surface due to retention in the GA.

The results in this thesis demonstrated that HPV-16 E5 wild type down-regulates surface expression of MHC class I, retains MHC class I complexes in the GA and impedes their transport to the cell surface, which is rescued by treatment with interferon. Unlike BPV E5, HPV-16 E5 does not affect the synthesis of MHC class I heavy chains, and the morphology of the GA. HPV-16 E5 physically interacts with MHC heavy chain in the GA and this interaction is associated with the down-regulation of surface MHC class I. Moreover, we determined that HPV-16 E5 down-regulates selectively HLA-A and B but does not inhibit HLA-C and non-classical HLA-E thus potentially escaping both cytotoxic T lymphocyte (CTL) and natural killer (NK) cells.

Deletion mutants of HPV-16 E5 lacking each one of the hydrophobic domains were generated to define its functional domains. HaCaT cells expressing either HPV-16 E5 wild type or its mutants were analysed for their ability to down-regulate surface MHC class I and determine the localization in the cells. All deletion mutants were expressed and localised in the GA. The deletion mutants containing the first hydrophobic domain were all able to down-regulate

surface MHC class I, to retain the complex in the GA and interact with MHC heavy chain to extent as HPV-16 E5 wild type. Only the deletion mutant which lacks the first hydrophobic domain was unable to down-regulate surface MHC class I, to retain the complex in the GA and to interact with MHC heavy chain. This study demonstrated that the separate domains of HPV-16 E5 did not have the same function. We found that the first hydrophobic domain of HPV-16 E5 is important for down-regulation of surface MHC class I.

Lack of surface MHC class I in infected epithelial cells expressing E5 would allow evasion of CTL killing and thus establishment of viral infection. However, MHC class I down-regulation by E5 is selective, as E5 does not down-regulate HLA-C and non-classical HLA-E. It remains to be seen if E5 expressing cells evade CTL killing, through down-regulation of classical MHC I, and NK killing, through lack of down-regulation of non-classical MHC I.

Chapter One

Introduction

1 Introduction

1.1 Cancer

Uncontrolled cell proliferation is the hallmark of cancer. Cancer evolves from a single precursor cell acquiring a series of genetic and epigenetic changes conferring a growth advantage for the cell and leading to progressive conversion of normal cells into cancer cells. In cancer cells, various mutagens, oncogenic viruses and errors in replication trigger the genes regulation of their cell cycles. Genetic alterations and changes in DNA methylation may affect a variety of genes such as proto-oncogene and tumour suppressor genes (Lengauer *et al.,* 1998; Ponder, 2001). Normally, all cells reproduce by dividing in two, which each parental cell giving rise to two daughter cells on completion of each cycle of cell division. The division of all cells must be carefully regulated and coordinated with both cell growth and DNA replication in order to ensure the formation of progeny cells containing intact genomes. The cell cycle machinery is itself regulated by the growth factors allowing the division of individual cells to be coordinated. The growth factors control cell proliferation, differentiation, apoptosis and necrosis. When cell regulation is lost, cancer cells will grow and divide, and ultimately spread throughout the body by interfering with the function of tissues and organs. In a benign or malignant tumour, several genes regulate processes that are abnormal. During carcinogenesis, regulatory genes can be damaged, leading to production of more cells or reduction in normal cell death rate (Krontiris and Cooper 1981; Lengauer *et al.,* 1998). Tumours are classified according to the type of cell from which they arise, including carcinomas, tumours that begin in epithelial tissue (e.g. cervical carcinoma) sarcomas, tumours that originate in the connective tissues, predominantly the muscles, skeleton and cartilage, and leukaemia and lymphomas originating the tissue forming the blood cells and immune system. Cancer results from alterations in critical regulatory genes that control cell

proliferation, differentiation, and survival. It is believed that the development of cancer involves a complex series of interactions between several environmental factors and genetic factors. The environmental factors, which are exogenous factors, include physical agents or radiation agents (e. g. UV and X-ray), chemical agents (e.g. carcinogens and mutagens) and infectious agents (e.g. bacteria and viruses).

1.1.1 Oncogenes and tumour suppressor genes

Genetic changes in the progression of cancer typically affect two different types of genes, oncogenes (e.g., *src* and *ras*) and tumour suppressor genes (e.g., p53 and Rb). Both of the oncogenes and tumour suppressor genes encode many kinds of proteins that play a key role in cancer induction. These genes control cell growth and proliferation and mutations in these genes can contribute to the development of cancer (Yokota, 2000; Balmain *et al.,* 2003; Yokota and Kohno, 2004).

1.1.1.1 Oncogenes

Oncogenes are activated cellular proto-oncogenes, since these genes code for proteins that are directly involved in regulation of cell growth and proliferation. The oncogenes are abnormally expressed or mutated forms of the corresponding proto-oncogenes. As a consequence of such alterations, the oncogenes induce abnormal cell proliferation and tumour development. The viral and cellular oncogenes have defined a large group of genes that can contribute to the abnormal behaviour of malignant cells. The majority of oncoproteins function as elements of the signalling pathways that regulate cell proliferation and survival in response to growth factor stimulation. They include polypeptide growth factors, growth factor receptors, elements of intracellular signalling pathways, and transcription factors (Lengauer *et al.*, 1998; Todd and

Wong, 1999). The first was isolated in Rous sarcoma virus (RSV), which transforms chicken embryo fibroblasts in culture and induces large sarcomas rapidly after inoculation into chickens. The malignant changes are the consequence of the expression of a single viral gene termed *src* for sarcoma. The *src* gene is not required for virus replication; spontaneous deletions of the *src* gene can occur in the viral genome resulting in the generation of nonconditional transformation-defective viruses that replicate normally (Erikson *et al.,* 1980). Like *src*, many of these genes encode proteins that are now recognized as key components of signalling pathways that stimulate cell proliferation. Some of the oncogenes identified in human tumours are cellular homologs of oncogenes that were characterized in retroviruses. Subsequently, the activation of oncogene was definitely proven in human cancer. Activation is mostly induced by point mutation of oncogenes such as *ras* family. These genes are involved in approximately 20% of all human malignancies, including about 50% of colon and 25% of lung carcinomas (Salgia and Skarin, 1998; Downward, 2003; Soreide *et al.*, 2006). The *ras* genes, encode guanine-nucleotide binding proteins that function in transduction of mitogenic signals from a variety of growth factor receptors (Campbell *et al.*, 1998). The action of growth factors as oncoproteins results from their abnormal expression, leading to a situation in which a tumour cell produces a growth factor to which it also responds. The result is autocrine stimulation of the growth factor-producing cell, which drives abnormal cell proliferation and contributes to the development of a wide variety of human tumours. A large group of oncogenes encode growth factor receptors, most of which are protein-tyrosine kinases. These receptors are frequently converted to oncoproteins by alterations of their amino-terminal domains, which would normally bind extracellular growth factors (Nahta *et al.*, 2003).

1.1.1.2 Tumour suppressor genes

Tumour suppressor genes are a group of genes, which play an important role in regulating when cells are allowed to divide and increase in number. When DNA damage is detected in a cell, some tumour suppressor genes can stop the cell from multiplying until the damage is repaired. When tumour suppressor genes do not function correctly, the cells with DNA damage continue to divide and can accumulate further DNA damage that can eventually lead to the formation of a cancer cell. In that way, a tumour suppressor gene is similar to an oncogene. Most cancers have inactivating mutations in one or more proteins that normally function to restrict progression through the gap 1 (G_1) stage of the cell cycle such as retinoblastoma protein. Virtually all of human tumours have inactivating mutations in proteins such as p53 that normally function at crucial cell cycle checkpoints, stopping the cycle if a previous step has occurred incorrectly or if DNA has been damaged (Yokota, 2000; Sherr, 2004).

1.1.1.2.1 Retinoblastoma

The first tumour suppressor gene was identified as a genetic locus associated with the development of a retinoblastoma (Rb), a rare childhood eye tumour (Knudson, 1971; Friend *et al.*, 1986). Children with hereditary retinoblastoma inherit a defective copy of the Rb gene, sometimes seen as a small deletion on chromosome 13. They develop retinal tumours early in life and generally in both eyes. The realization that it was a loss of function of Rb that was associated with disease established the tumour suppressor paradigm (Cavenee *et al.*, 1983). The characterization of Rb as a tumour suppressor gene served as the prototype for the identification of additional tumour suppressor genes that contribute to the development of many different human cancers. Rb genes encode pocket proteins including pRb, p107 and

p130. Rb family negatively regulates progression from G_0 through to G_1 and into S-phase. These proteins bind to viral oncoproteins and cellular factors such as the E2F family of transcription factors. E2F family are functional transcriptional regulator of genes involved in cell cycle progression, DNA synthesis, apoptosis and other cellular processes (Dyson, 1998; Classon and Dyson, 2001). Rb binds E2F and inhibits transcription by blocking the E2F transcriptional activation domain. In cycling cells, Rb is inactivated in mid to late G_1 through phosphorylation by several cyclin-cyclin-dependent kinase (CDK) complexes. Hyperphosphorylated Rb no longer binds E2F; consequently, repression of E2F-dependent promoters is relieved. pRb, p107 and p130 also differ with regard to binding to E2F family members, with pRb binding to E2F1, E2F2 and E2F3 and p107/p130-binding E2F4 and E2F5. This binding represses E2F-dependent transcription. These E2F family members are generally thought of as transcriptional activators because they activate transcription when not bound by repressor molecules such as pRb (Dyson, 1998). Both p107 and p130 interact with E2F4 and E2F5, which are weak transcriptional activators when not bound by Rb family members. The expression patterns of Rb family members differ during the G_0/G_1/S-phase transition, with p130 highly expressed during G_0, p107 highly expressed in S-phase and pRb expressed at a fairly steady level throughout the cell cycle. This observation, together with the fact that different pocket proteins bind to distinct E2F family members, suggests the pocket proteins may regulate various E2F target genes at different times during the G_0/G_1/S-phase transition (Classon and Dyson, 2001). The pocket proteins are regulated in part via phosphorylation by CDKs. Hyperphosphorylation of pRb results in a loss of binding to both E2F and chromatin remodeling factors, and reverses pRb-mediated cell cycle arrest by pRb (Dyson, 1998). Similar to pRb, p107 and p130 regulate cell cycle progression via interactions with E2F and are CDK substrates (Classon and Dyson, 2001). Both Rb and the CDK inhibitor p21 prevent

S phase in differentiated cells (Woo *et al.*, 1997; Mal *et al.*, 2000). Rb binds to the E2F family of transcription factors in G_1 and represses transcription of E2F-controlled S-phase genes (Dyson, 1998), while p21 inhibits the activity of G_1 and S-phase cyclin/CDK complexes that is important for cell cycle progression (Sherr and Roberts, 1995). The Rb protein is a key target for the oncoproteins of several DNA tumour viruses, including polyomaviruses such as SV40 large T antigens, adenoviruses E1-A, human papillomaviruses E7 (Helt and Galloway, 2003), human T-cell leukemia virus type 1 Tax (Kehn *et al.*, 2005), Epstein–Barr nuclear antigen 3C (EBNA3C) (Knight *et al.*, 2006) and Hepatitis C virus NS5B polymerase (Munakata *et al.*, 2005). Inactivation of Rb family members by large T antigen, E1-A and E7 oncoproteins may serve mainly to induce transcription of E2F-controlled cell cycle and DNA synthesis genes, thereby establishing an environment permissive for viral replication (Nevins, 1995).

1.1.1.2.2 P53

The second tumour suppressor gene is p53 which is frequently inactivated in wide variety of human cancer. Mutations in the p53 gene or inactivation of its encoded protein by viral oncoproteins generally lead to a loss of these cellular defense functions. p53 mutations are common in human cancer and estimated to occur in up to 50% of all human cancers, making it the most common target of genetic alteration in human malignancies (Nigro *et al.*, 1989; Hollstein *et al.*, 1991; Olivier *et al.*, 2002). The p53 tumour suppressor gene encodes a nuclear phosphoprotein that functions as a key regulator of DNA repair, cell cycle progression and apoptosis. The p53 protein is upregulated in response to a diverse array of cellular stresses, including DNA damage, hypoxia, oxidative stress, ribonucleotide depletion and oncogene activation (Ko and Prives, 1996; Giaccia and Kastan, 1998). When activated, p53

can directly interact with DNA to yield transcription of a number of genes, including p21, and a temporary arrest of the cell cycle in the G_1 or G_2/M phase, prior to mitosis to allow for DNA repair. It is also capable of interacting with other cellular pathways to trigger apoptosis or differentiation (Lane *et al.,* 1994). After DNA damage, p53 holds the cell at a checkpoint until the damage is repaired. If the damage is irreversible, apoptosis is triggered. The mechanism by which p53 promotes apoptosis is still not fully understood (Benchimol, 2001). The normal p53 function can be inactivated by somatic and germ line mutations and by binding to different viral oncoproteins (human papillomavirus protein E6, SV40 large T antigen, hepatitis B viral X protein and adenovirus protein E1-B) (Harris, 1996).

1.1.2 Viruses and cancer

Many different agents have been implicated in various cancer including viruses, bacteria and parasites. Viruses have been the most studied in their relation to tumour formation. They are thought to be associated with 15-20 percent of all human cancers worldwide of which about 80 percent are cancers of the cervix and liver (zur Hausen, 1991; Parkin, 2006). Viruses have evolved multiple strategies to transform host cells. One common route is to alter the expression of cellular genes by integration of the viral genome into the cellular DNA. Viruses also help cause malignancy by introducing an oncogene into a cell to disrupt the regulation of cell division (DiMaio *et al.,* 1998; Murakami *et al.*, 2005; Kamihira *et al.*, 2005; Laurent-Puig *et al.,* 2006; Arias-Pulido *et al.*, 2006). Tumour viruses, which can cause human cancer, contain two groups: DNA tumour viruses and RNA tumour viruses. They include Hepatitis B virus, which is associated with hepatocellular carcinoma (Kao *et al.,* 2000; Cougot *et al.*, 2005); the human T cell leukaemia virus type 1, which is a retrovirus, is associated with adult T cell leukaemia and lymphomas; Epstein-Barr virus which is linked to several human

cancers including Burkitt's lymphoma, Hodgkin's lymphoma and nasopharyngeal carcinoma (zur Hausen, 2001; Kurokawa *et al.*, 2005); Kaposi's Sarcoma-Associated Herpesvirus etiologically linked to Kaposi's sarcoma (Moore *et al.*, 1996; Leao *et al.*, 2002) and human papillomavirus which is associated in carcinomas of the uterine cervix (Munoz *et al.*, 2003), cancer of the oropharynx (Herrero *et al.*, 2003), and head and neck cancers (zur Hausen, 2002; Ferris *et al.*, 2005).

1.1.2.1 Hepatitis B Virus

The hepatitis B virus (HBV) is one of the most common chronic pathogens in the world, which is the major cause of liver disease and hepatocellular carcinoma (HCC). Chronic infection with this virus often leads to chronic liver disease, including cirrhosis or primary HCC. Over 2 billion of the world's population have been exposed to this virus. About 350 million of these, making 5% of the world's population, are chronic carriers (Maynard, 1990). Annually up to 1 million of this population die due to the consequences of this infection such as cirrhosis and HCC (Mast *et al.*, 1999). The prevalence of hepatitis B carriers varies in different parts of the world, ranging from less than 1% to 15% (Maynard, 1990). Many cases of infection recover completely but in 5-10% of patients, failure of the immune system to clear the virus results in chronic carriers with HBV circulating in their blood for many years (Hollinger, 1996). These individuals usually develop persistent infections and an increased risk of liver cirrhosis and HCC. Integrated HBV genome is frequently detected in chronic virus infection and HCC patients. Epidemiology data have shown that HBV is one of the etiologic agents in HCC development. However, the molecular mechanism(s) of HBV leading to HCC remains unclear. A large number of cases are seen in eastern Asia and sub-Saharan Africa, where two of the most important health problems are chronic liver disease and liver

cancer. Up to 80% of liver cancers are believed to result from this viral infection, which is the most important cause of cancer mortality worldwide (Mast *et al.*, 1999).

1.1.2.2 Epstein-Barr virus

Epstein-Barr virus (EBV) has a large double stranded circular DNA genome. It belongs to the gamma-herpesvirus family that latently infects the majority of adults. EBV is the causative agent of infectious mononucleosis and is associated with malignancies that originate from lymphoid cells (e.g., Burkitt's lymphoma and Hodgkin's lymphoma) and epithelial cells (e.g., nasopharyngeal carcinoma) (Baumforth *et al.*, 1999; Hsu and Glaser, 2000). Over 95% of the adult population worldwide are infected with EBV. Primary infection usually occurs during childhood and results in a mild, self-limiting illness. When infection is delayed until adolescence, infectious mononucleosis, characterized by fever, lymphadenopathy and pharyngitis, occurs in around 50% of individuals (Cohen, 2000). EBV enters via the oropharyngeal route and infects resting B cells (Niedobitek *et al.*, 1997) and/or epithelial cells (Sixbey *et al.*, 1984). Virus released during this productive primary phase subsequently infects B cells circulating through the oropharynx, resulting in a latent infection. The infected B cells are highly immunogenic and therefore induce a massive expansion of virus-specific and nonspecific T cells that cause the clinical symptoms of infectious mononucleosis. A small number of B cells express only latent membrane protein-2 and small nonpolyadenylated viral RNAs (EBV-encoded RNA 1 and 2) out of the almost 100 viral proteins encoded by the viral genome. This limited viral antigen expression allows these EBV-infected B cells to evade the immune response (Baumforth *et al.*, 1999; Hsu and Glaser, 2000). The virus can then persist in its latent state for the life of the individual.

1.1.2.3 Human T-cell leukemia virus type 1

Human T-cell leukemia virus type-I (HTLV-1) is the first discovered human retroviral pathogen (Poiesz *et al.*, 1980). HTLV-1 causes adult T-cell leukemia (ATL), which is an aggressive lymphoproliferative disease, and tropical spastic paraparesis or HTLV-1 associated myelopathy, which is a neurological progressive inflammatory syndrome (Barmak *et al.*, 2003; Jeang *et al.*, 2004). In culture HTLV-1, which is an onco-retrovirus, can infect a wide variety of cell types from different species. However, in natural human infections this virus targets mainly mature $CD4^+$ helper T-cells (Richardson *et al.*, 1997), resulting in benign expansion the infected cells. HTLV-1-infected T-cells and deterioration of host immune activities are prerequisites for ATL development and 90–96% of the HTLV-I genome is found to segregate with CD4 cells in the peripheral blood of ATL patients (Richardson *et al.*, 1990). HTLV-2 is molecularly and biologically similar to HTLV-1 (Seiki *et al.*, 1983) but is not associated with ATL or any other leukaemia (Sugamura and Hinuma, 1993). HTLV-1 encodes several proteins (e.g. Tax) that are responsible for distinct pathogenic activities of virus. It originally interacts with transcription factors and activates or represses transcription (Chen *et al.*, 1985). Also the activation of Tax protein is essential for transforming primary human T-cells and T-cell immortalization. In addition, in transgenic animals Tax induces various malignancies such as fibrosarcoma and natural killer cell leukemia (Robek and Ratner, 1999).

1.1.2.4 Kaposi's sarcoma-associated herpesvirus

Kaposi's sarcoma-associated herpesvirus or human herpesvirus 8 (HHV8) is a member of the gamma-herpesviruses like EBV, etiologically linked to Kaposi's sarcoma (KS) (Moore *et al.*, 1996). In 1994, HHV-8 was first detected in KS tissues from a patient with acquired

immunodeficiency syndrome (AIDS) by representational difference analysis (Chang *et al.*, 1994). Since its initial discovery, HHV-8 has been found in all forms of KS: classical, endemic, and AIDS-associated acquired KS (Schulz, 2001). HHV-8 is detected in 95% of all KS. HHV-8 has been shown to infect a variety of cell types both in vivo and in vitro, including B cells, endothelial cells, keratinocytes, and macrophages (Moore and Chang, 2003). This broad cellular host range suggests that HHV-8 may interact with ubiquitous host cell molecules to gain access to target cells.

1.2 Papillomavirus

Papillomaviruses are classified in family of their own, papillomaviridae, unrelated to the polyomaviruses, which belongs to polyomaviridae (de Villiers *et al.*, 2004). PVs are non-enveloped, circular double stranded and relatively small DNA viruses with a genome of approximately 8 kilo-bas pairs (kbp). The genome of papillomavirus is divided into three regions: a long control region and regions containing open reading frames corresponding to early and late genes. They have been found in many different types that infect a wild range of animals (from birds to rabits, dogs, sheep and cattle) and several primates including humans (Campo, 1997). These viruses are a very heterogeneous group of viruses, and individual types are associated with specific lesions. They are spread by contact and enter the body through minute abrasion in the skin and induce hyperproliferation of epithelial cells of the skin or mucosa (papillomas). The papillomas are mostly benign but some tumours may eventually undergo malignant conversion in both humans and animals when genetic or environmental factors are involved. Papillomaviruses-associated neoplasias comprise anogenital carcinoma and skin squamous cell carcinoma in humans, skin cancer in rabbits and upper gastrointestinal cancer and urinary bladder cancer in cattle (Campo, 1997 and 2002). Most cases of infections

in both humans and animals are cleared following activation of the host immune system against viral antigen after several months (Frazer and Tindle, 1996). However, occasionally the lesions do not regress and can progress to cancer. The most commonly studied of these viruses are the cottontail rabbit papillomavirus (CRPV), bovine papillomavirus (BPV) and human papillomavirus (HPV). Animal papillomaviruses, particularly CRPV and BPV have been used as model systems in which to study virus biology, the interaction of the virus with its natural host and with environmental cofactors, and the host immune response to the virus.

1.2.1 Cottontail rabbit papillomavirus

CRPV was the first model of papillomavirus carcinogenesis, demonstrated in 1933 by Shope (Shope, 1933). CRPV induces papillomas in both cottontail and domestic rabbits infected under experimental conditions. These papillomas progress to carcinomas with high frequency (Breitburd *et al.*, 1997). A first link between papillomaviruses and skin cancer was obtained in 1935 from an animal model system, when Rous and Beard described the development of squamous cell carcinomas in rabbits after experimental infection with CRPV (Rous and Beard, 1935). Thus far, the only model system for studying the progression of papillomavirus-induced skin tumours is the domestic rabbit infected with CRPV. Infection of domestic rabbits with CRPV particles or viral DNA leads to the development of local tumours within 2 to 8 weeks post infection. These papillomas progress within 8 to 14 months in all of the cases into invasively growing and finally metastasizing carcinomas (Han *et al.*, 2000). These events parallel the outcome of HPV type16 infections in human populations (explained later) (Hildesheim, 1997).

1.2.2 Bovine papillomaviruses

BPVs induce papillomas of cutaneous or mucosal epithelia in cattle. Six different types of bovine papillomaviruses have so far been characterized, each one associated with a specific disease. These viruses were divided three subgroups Delta (δ)-papillomavirus, Xi (ξ)-papillomavirus and Epsilon (ε)-papillomavirus, on molecular, immunological, histological, pathological and clinical manifestation. δ-papillomavirus includes BPV type 1 and BPV type 2, which cause lesions of both dermal fibroblasts and keratinocytes, ε-papillomavirus includes BPV type 5 that like δ-papillomavirus cause lesions of both dermal fibroblasts and keratinocytes and ξ-papillomavirus comprises the epitheliotropic papillomaviruses BPV type 3, BPV type 4 and BPV type 6, which involve only keratinocytes (de Villiers *et al.*, 2004). The BPV genome, even though retaining the general organization common to all papillomaviruses, differs between δ-papillomavirus, ξ-papillomavirus and ε-papillomavirus. The δ– papillomavirus and ε-papillomavirus have a genome of approximately 7.9 kbp but the ξ-papillomavirus have a smaller genome of approximately 7.3 kbp (Campo, 1995). The BPV genomes are divided into a long control region, which includes responsive sites for viral transcription and replication, the early region, which encodes the early proteins (E1-E8) that are responsible for the establishment and maintenance of the viral infection and the late region that encodes structural proteins, L1 and L2. It has been demonstrated that the ξ-papillomaviruses lack the E6 oncogene, which is replaced by the E5 gene (Patel *et al.*, 1987; Jackson *et al.*, 1991). The E5 gene is functionally homologous to the E5 gene of BPV-1 and 2 (Faccini *et al.*, 1996; Ashrafi *et al.*, 2000). Both BPV-1 and BPV-2 are capable of transforming primary bovine fibroblast or epithelial cells to complete oncogenicity (Campo, 1992), but BPV-4 DNA alone is not sufficient to induce transformation of primary bovine cells and needs the cooperation of an activated *ras* oncogene (Jaggar *et al.*, 1990). In BPV-1,

the E5 and E6 ORF encode functionally major transforming protein whereas E7 is a lesser extent to the transformation of bovine cells (Campo, 1992); while in BPV-4 the E5 and E7 ORF encode the major transforming protein. Both E5 and E7 cooperate. The three groups of BPVs are evolutionarily more closely related to each other within three subgroups then they are among subgroups and there is a little or no immune cross-reaction between the members of the subgroups (Campo, 1995).

Table 1. Bovine papillomaviruses and the tumours they cause

Genus	type	Tumour caused
Delta (δ)-PV	BPV-1	Fibropapillomas of paragenital areas
	BPV-2	Fibropapillomas of skin and alimentary canal
Epsilon (ε)-PV	BPV-5	Fibropapillomas of teats and udders
Xi (ξ)-PV	BPV-3	Papillomas of skin
	BPV-4	Papillomas of alimentary canal
	BPV-6	Papillomas of teats and udders

from de Villiers *et al.*, 2004

Some of the BPV types have been identified to be associated with cancer in cattle, BPV-1 and BPV-2 which cause skin warts, also cause cancer of the urinary bladder, BPV-4 has been found as the etiologically agent of upper alimentary canal cancer; BPV-1 is also involved in cancer of the penis (Campo, 1997 and 2002). Both urinary bladder cancer and upper alimentary canal cancer develop as a result of the interaction between the virus, environmental factors (bracken fern) and host cell factors activation. BPV-1, BPV-5 and BPV-6 can cause economic consequence, as cows with papillomatosis of teats and udders cannot be milked, young calves cannot suckle (Campo, 1997).

1.2.3 Human papillomaviruses

HPVs are small double-stranded DNA tumour viruses of approximately 8 kbp. HPVs are ubiquitous in the human population, and occasionally infection leads to cervical cancer. They infect cutaneous and mucosal epithelial tissues of the anogenital tract, the hands, or the feet, which cause a wide variety of disease from skin and anogenital warts to laryngeal papillomas and anogenital interaepithelial neoplasias that often progress to malignancy (Walboomers *et al.*, 1999). To date, over 100 different types of HPV have been identified, and about one-third of these infect epithelial cells in the genital tract. The HPV types that infect the genital tract are divided into two categories: high-risk and low-risk (Table 2) (Munoz *et al.*, 2003). The low-risk HPV types such as HPV-6 and HPV-11, commonly cause benign warts and low-grade premalignant lesion that regress and do not progress to cancer while the high-risk types of HPV which include HPV-16, HPV-18, HPV-31, HPV-33, and HPV-45 are associated with the development of anogenital cancers and found in more than 99% of all cervical carcinomas (Walboomers *et al.*, 1999; Munoz *et al.*, 2003), with HPV-16 occurring most frequently (Munoz and Bosch, 1997; Munoz *et al.*, 2003). Infection by high-risk HPVs is not limited to the genital tract, since approximately 20% of cancers of the oropharynx contain DNA from these HPV types (Herrero *et al.*, 2003).

Table 2. Genital HPV and their associated risk of cancer

Low risk	6, 11, 40, 42–45, 53–55, 57, 59, 61, 67, 69, 71, 74, 82
High risk	16, 18, 31–35, 51–52, 56, 58, 61, 66, 68, 70, 73

1.2.3.1 HPV and Cervical cancer

Cervical cancer is the most common cause of cancer death in women worldwide. Both incidence and mortality from cervical cancer are second only to breast cancer and in parts of the developing countries, cervical cancer is the major cause of death in women of reproductive age (Boffetta and Parkin, 1994). Cervical intraepithelial neoplasia (CIN), which is a precursor of cervical cancer, is classified histologically into three classes (CIN I, CIN II and CIN III), based on its severity. The level of cellular changes is determined by histopathology and microscopy of Pap smears. Infection of the genital tract by HPVs can initially result in low-grade lesions termed dysplasias or CIN I (Bosch *et al.,* 2002). These lesions exhibit only mildly altered patterns of differentiation, and many of them are cleared by the immune system in less than a year (Jenson *et al.,* 1991; Hopfl *et al.,* 2000). The mechanisms by which the cellular immune response clears HPV infections are still not clearly understood. Some of these lesions, however, are not cleared by the immune system and can persist for periods as long as several decades. Persistence of infection by high-risk HPV types is the greatest risk factor for development of genital malignancies (zur Hausen, 1996 and 2000). Certain strains of HPV are associated with different risk levels of the transformation into cervical tumours. High-risk HPV-16 and 18 are found with the highest frequencies in cervical cancer and account for approximately two thirds of all cervical carcinomas worldwide (Munoz, 2000; Munoz *et al.,* 2003). HPV-16, 18, and 31 are constantly associated with moderate to severe cases of cervical dysplasia and less associated with invasive cancers of the vulva, penis, and anus. It has been demonstrated that the presence of even minimal amounts of HPV DNA is associated with an increased risk in the development of cervical cancer (Munoz *et al.,* 1992). HPV DNA is found in up to 100% of cervical tumours, while in cervical tissue of healthy women the figure is typically below 10% (Wallin *et al.,* 1999). It has

been reported that the DNA of HPV-16, 18 can be found in about 70% of cervical cancer biopsies and HPV-16 is present in about 50% of these case (Figure 1) (Bosch *et al.*, 1995). The majority of the remaining positive biopsies contain several additional types of HPV.

Figure 1. Worldwide prevalence of HPV DNA in cervical tumors

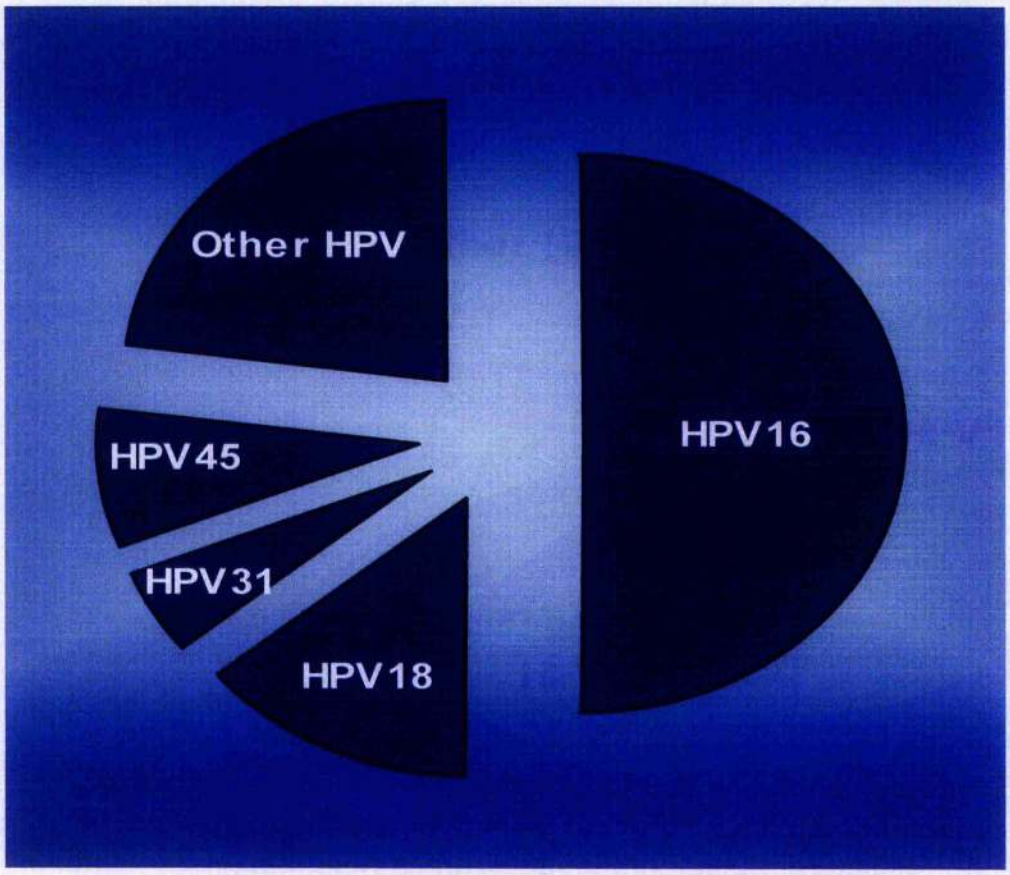


Figure 1: The prevalence of HPV DNA in cervical tumours in worldwide have been shown that HPV 16 50%, HPV 18 14%, HPV 31 5%, HPV 45 8% and other HPV types 23%, (Bosch *et al.*, 1995).

1.2.3.2 HPV type 16

HPV-16 is the most prevalent HPV subtype detected in CIN (Clifford *et al.,* 2003). It is also found in cancer of the tonsils, anus, penis and cancer of neck (Klussmann *et al.,* 2001 and 2003). The HPV-16 genome consists of a single molecule of double-stranded, circular DNA containing approximately 7,900 bp associated with histones (Favre, 1975). The HPV-16 genome is functionally divided into three regions of which at the end of each coding region there are polyadenylation sites (Figure 2). The first is a non-coding region, which has been referred to as the long control region (LCR), or the upstream regulatory region (URR). This region contains the p97 main early promoter, and sequences that regulate DNA replication. This region also contains the highest degree of variation in the viral genome. The second is an early region, consisting of ORFs E1, E2, E4, E5, E6, and E7, which are involved in viral replication and oncogenesis. The third is a late region, which encodes the L1 and L2 structural proteins for the viral capsid. HPVs express many different polycystronic transcripts throughout the viral life cycle thus co-ordinating the expression of the viral gene products with the differentiation of the host cell. Additionally transcription initiates from several different promoters during keratinocyte differentiation (Doorbar *et al.,* 1990). Viral transcription is controlled by cellular transcription factors binding the LCR and by the virally encoded E2 protein (Bouvard *et al.,* 1994b; Steger and Corbach, 1997). The epithelial specificity of HPV infection is thought to be due in part to specific control elements in the enhancer and promoter sequences in the viral LCR (Vance *et al.,* 1999). Viral replication is initiated within the viral origin of replication (Ori) and is dependent on the virally encoded replication factors E1 and E2 (Desaintes and Demeret, 1996). HPV-16 encodes two major oncoproteins: E6 and E7, which together modify the expression patterns and activities of many cellular gene and proteins. They can be retained and expressed in cervical cancer and

cancer-derived cell lines and are enough for host-cell immortalization. The virus also encodes E5 protein, the minor transforming protein in HPV-16 but the major transforming protein in BPV.

Figure 2. Diagrammatic structure of HPV-16 gene

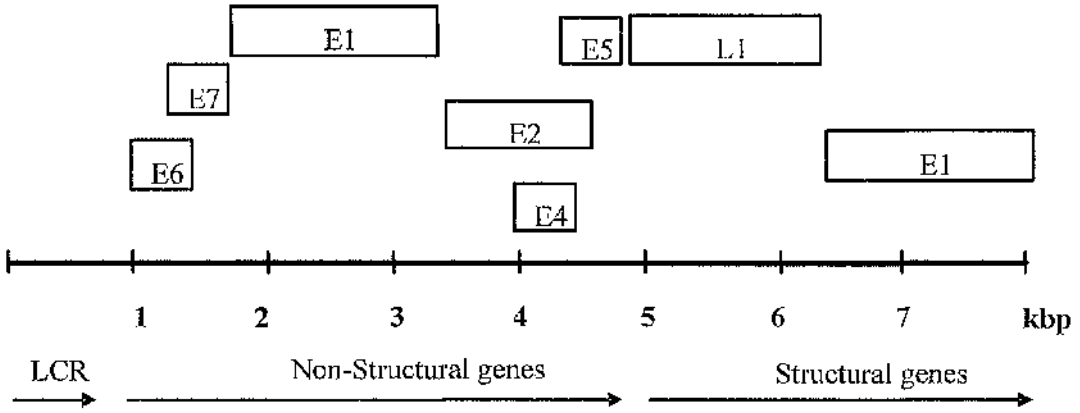


Figure 2: Schematic of genome organization of HPV-16. ORFs are labeled E or L depending on their position in the early or late region of the genome. The early region contains the early genes E1, E2, E4, E5, E6 and E7 and the late region contains the late genes L1 and L2. The LCR, which is a non-coding sequence, has the position of the E1 and E2 binding sites. Also LCR has an origin of replication site that is required for efficient viral DNA replication.

1.2.3.3 The HPV life cycle

In the case of infection by low-risk HPV types (HPV-6 and 11), which cause papillomas or warts, the viral genome appears to remain episomal, and this extrachromosomal DNA maintains gene expression. This is in contrast to high-risk HPV types (HPV-16 and 18), which are associated with anogenital dysplasia and cancer. HPV infects the squamous epithelium through micro-traumas and its life cycle is closely linked to the differentiation

status of host epithelial tissue (Laimins, 1993). Infection is thought to occur in cells where viral genomes are established as episomes in the nucleus at approximately 50-100 copies per cell in the basal layers of the epithelium and are replicated synchronously with chromosomal DNA and following cell division (LaPorta and Taichman 1982; Stubenrauch and Laimins, 1999). The establishment and maintenance of HPV genomes are associated with the expression of the HPV early proteins, E1, E2, E4, E5, E6 and E7, involved in DNA replication and transcription in host cell. Coincident with the distal movement of infected cells, late gene expression occurs, giving rise to L1 and L2 capsid proteins and virion formation in the upper-layers (LaPorta and Taichman 1982). The HPV genome replication is dependent on E1 and E2 and the cellular replication apparatus and E2 also controls viral transcription (Fehrmann and Laimins, 2003). Additionally, it has been demonstrated that E6 and E7 play important roles in the HPV life cycle (Thomas *et al.,* 1999; Flores *et al.,* 2000). This results in the proliferation of infected cells and a drive into S-phase that allows the replication of the viral genome and its segregation into the daughter cells (Lambert, 1991). The presence of E7 protein leads to the characteristic retention of nuclei throughout all layers of infected epithelial cells (Cheng *et al.,* 1995). Both E6 and E7 have also been shown to be necessary for the maintance of extrachromosomal forms. Following infected cell division, viral DNA is distributed between both daughter cells. One of the infected daughter cells migrates away from the basal layer and begins to differentiate and the other daughter cell continues to divide in the basal layer and provides a reservoir of viral DNA for further cell division. In contrast to uninfected keratinocytes, which exit the cell cycle as soon as they detach from the basement membrane, HPV-infected cells do not exit the cell cycle. Amplification of the genome to high levels prior to encapsidation is thought to occur with HPV-16 in cells of the suprabasal layer. This is followed by the synthesis of the E1^E4

proteins and both of the L1 and L2 capsid proteins, resulting in the assembly of infectious virions. Infectious virions are released into the environment as the upper layer of the epithelium is shed to infect new individuals or new sites in the same host (Frattini *et al.*, 1996). The E1^E4 protein is suggested to help counteract the effects of the E7 protein, facilitating a productive life cycle (Flores *et al.*, 2000).

Figure 3. Schematic of HPV-16 life cycle

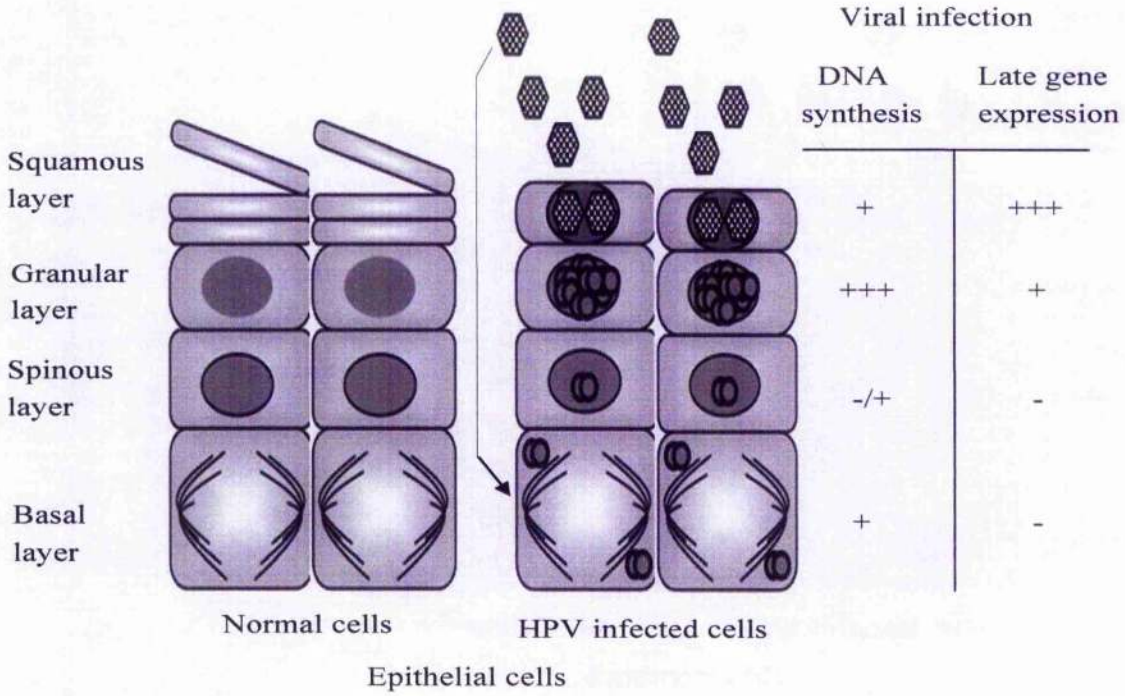


Figure 3: Schematic normal and HPV-infected epithelia are compared, and differentiation-dependent viral functions are shown. When a basal cell divides, a daughter cell will migrate upwards in the cell layers and start to differentiation. The migration of cells will continue to the upper layer of the epithelium until it is terminally differentiated whereby it is sloughed off. Papillomavirus enters the epithelium through micro-traumas and infects the basal layer cells. In the early stage, virus infection will be established but no viruses will be produced.

Late in cell differentiation, the viral structural genes will be expressed and infectious virus assembled. Viruses are released as cells are sloughed off (Fehrmann and Laimins, 2003).

1.2.3.4 Integration of the HPV-16 genome in the host cell

HPV types are associated with the development of malignant lesion such as squamous cell carcinoma (zur Hausen, 1996). Initial infection with the HPV-16 causes low-grade disease and the viral DNA exists in episomal form in the cell nucleus. In the majority of the HPV-associated cancers, the viral DNA has been integrated into the genome of the host cell by accidental disruption of the viral genome from circular to linear (Schwarz *et al.*, 1985). The integration of HPV-16 genome is apparently non-specific with respect to the site in the host cell genome, although in some cases integration has occurred in the vicinity of cellular oncogenes (Lazo *et al.*, 1992). HPV DNA integration has been suggested to be an important event in the development of cervical cancer, since this often results in a disruption of the E2 region (Figure 4) (Schwarz *et al.*, 1985; Baker *et al.*, 1987; Fehrmann and Laimins, 2003), which controls viral replication and transcription. This not only allows the viral oncoproteins E6 and E7 to remain expressed but also up-regulates their expression due to loss of the viral transcriptional regulatory gene E2 (Fehrmann and Laimins, 2003).

Figure 4. Schematic of HPV-16 genome integration in the host cell

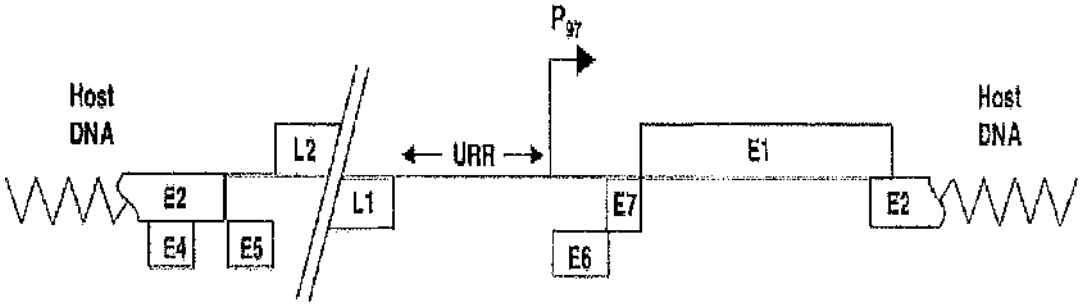


Figure 4: Papillomavirus DNA integration into host cells may accelerate progression to malignancy, since this often results in a disruption of the E2 region. The loss of the viral E2 genome allows the E6 and E7 to express. The early and late ORFs of an integrated form of HPV are indicated (Fehrmann and Laimins, 2003).

1.2.3.5 HPV-16 proteins

1.2.3.5.1 Viral DNA replication proteins

Viral DNA replication occurs in the infected cell by using the cellular DNA replication machinery. HPV encode two non-structural viral proteins E1 and E2, which are efficient HPV DNA replication factors. Both of the E1 and E2 proteins together recruit the cellular proteins required for the viral genome replication. E1 and E2 proteins bind the viral DNA, which is necessary to initiate replication from ori, the viral origin of replication. Also it has been found out that the E1^E4 protein has a significant role in the vegetative replication of the viral genome (Longworth *et al.*, 2004).

1.2.3.5.1.1 HPV-16 E1 protein

The HPV-16 E1 protein is a nuclear phosphoprotein of 649 amino acids that contains blocks of homology in the C-terminal amino acids with the SV40 replication protein large T antigen and other pollyomaviruses (Clertant and Seif, 1984; Fouts *et al.*, 1999). The E1 protein of papillomavirus has ATPase functions and ATP dependent helicase activity, which are essential for both initiation of viral replication and elongation of viral DNA (Hughes and Romanos, 1993). It is capable both of binding to the origin of replication and interacting with the E2 protein (Liu *et al.*, 1995; Benson and Howley, 1995) as a hexamer, where it hydrolyses ATP to unwind the DNA (Sedman and Stenlund, 1998). The E1 amino terminus binds to the

p68 of the polymerase α-primers, which is the only polymerase able to initiate DNA synthesis, and it is essential for the replication of DNA fork (Masterson *et al.,* 1998; Conger *et al.,* 1999). The p68-binding domain is in a region of the E1 C-terminus where E2 binds; therefore it is possible that the initiation of DNA synthesis by E1 is after the function of E2 in HPV replication has been fulfilled. Both E1 and E2 have been shown to be essential for the replication of the DNA of various HPV types (Ustav and Stenlund, 1991). As well as binding to specific sequences at the origin of replication, both E1 and E2 proteins bind each other. E1 binds the origin with weak affinity but the additional binding of E2 increases this affinity. The E1 binding site is AT rich region with more variability than the E2 binding site and lies between E2 sites (Frattini and Laimins, 1994; Sun *et al.,* 1996). As mentioned above, both proteins are required for episomal replication of the genome in human keratinocytes and when either ORF is disrupted the genome integrates into the host chromosome.

1.2.3.5.1.2 HPV-16 E2 protein

The E2 ORF encodes a 42-48 kilo dalton (KDa) protein that contributes to multiple biological processes including viral transcription, viral DNA replication, and is essential for completion of the viral life cycle. HPV E2 protein can be divided into three functional domains; an N-terminal acidic transactivation domain, a C-terminal DNA binding domain and a central flexible hinge domain. E2 binds to 12 base pairs (bp) palindromic sequences (5'-ACCGN_4CGGT-3') in the viral LCR as a homodimer to the C-terminus domain (Hegde, 2002). The central flexible hinge domain is rich in potential phosphorylation sites and is involved in tethering the HPV episomal circular DNA to the chromosomal DNA (Gauthier *et al.,* 1991). The amino terminal transactivation domain is involved in transcriptional regulation, replication and direct association with the E1 protein (Ferguson and Botchan,

1996; Sakai *et al.*, 1996). Interaction between E1 and E2 is essential for replication of the viral genome, E2 binds to DNA binding sites that flank the E1 binding site and the amino-terminal of E2 interacts with E1 and recruits E1 to the origin of replication (Chao *et al.*, 1999; Titolo *et al.*, 1999). The activation of E2 protein usually occurs when the full-length molecules are presented but acts as a repressor of the early promoter in high-risk HPV (e.g. HPV-16). The HPV genome is frequently integrated in the host cell genome by accidental disruption of the viral genome from circular to linear. In cell lines derived from HPV associated cervical cancers with an integrated high-risk. In HPV associated cancer cell lines a common event is the deletion of E2 coding sequences during viral integration. This results in very high levels of expression of the viral oncogenes E6 and E7. Expression of either HPV-16 or 18 E2 in these cells represses the expression of E6 and E7 resulting in the induction of apoptosis (Dowhanick *et al.*, 1995; Desaintes *et al.*, 1999).

1.2.3.5.1.3 HPV-16 E4 protein

The HPV E4 protein is translated from spliced transcripts as a fusion with the first 5 amino acids of E1 to the protein encoded by the E4 ORF hence is often referred to as E1^E4. The E1^E4 protein can be easily detected in papilloma HPV-16 positive lesions. In genital lesion, E1^E4 expression occurs in the supra-basal layers (Doorbar *et al.*, 1997), whilst in HPV-1 positive cutaneous warts, it occurs much deeper in the epithelial layers and observed as early as the supra-basal layers (Chow *et al.*, 1987). In some HPV infection the E1^E4 proteins are expressed in the late phase of the viral life cycle and expression correlates with the onset of vegetative viral DNA amplification, prior to the expression of the viral structural proteins (Doorbar *et al.*, 1997; Middleton *et al.*, 2003). The E1^E4 protein is also present in superficial cells that express the structural proteins L1 and L2 (Crum *et al.*, 1990; Brown *et al.*, 1994).

The expression of the HPV-16 E1^E4 protein in cultured epithelial cells can cause reorganization of the keratin intermediate-filament network by interaction with the keratins which are major structural protein in epithelial cells. It has been determined that transient expression of the HPV-16 E1^E4 protein in human keratinocytes (the natural host cell for HPV infection) associates with intermediate filaments resulting in the total collapse of the cytokeratin matrix within the epithelial cell. It was proposed a role for E1^E4 in facilitating viral egression by destroying the keratin matrix and compromising the strength of the keratinized squama in infected tissue. However, in natural infections of high-risk types only a limited amount of collapse has been observed. It has also been demonstrated that the HPV-16 E1^E4 also binds to a DEAD-box RNA helicase protein (a tetra-peptides Asp-Gla-Ala-Asp) (Doorbar *et al.,* 1991), which regulates gene expression at various levels. Hence, E1^E4 may regulate the expression of either cellular or viral gene or both of them by binding to DEAD-box proteins (Doorbar *et al.,* 2000). However a direct role for E1^E4 protein in the viral life cycle has not yet been demonstrated but several studies suggest how it may be able to significantly enhance viral replication. It has been shown that many HPV E1^E4 proteins, including HPV-16 E1^E4, induce a cell cycle arrest at the G2 (Davy *et al.,* 2002; Nakahara *et al.,* 2002). It is suggested that the role of E1^E4 protein in the productive life cycle may be to counteract the effects of E7, which acts to push cells into the S phase (Chang and Laimins, 2000). It has been suggested that HPV-1 E4^E1 protein may be involved in the replication of the virus in the differentiated cells (Davy *et al.,* 2002; Roberts *et al.,* 2003).

1.2.3.5.2 Viral transforming proteins

HPVs of the high-risk type such as HPV-16 encode three transforming proteins E5, E6 and E7. While E6 and E7 are mainly responsible for their transforming properties (Munger and

Howley, 2002) E5 has weak transforming activity but increases greatly the oncogenic properties of E7 (the E5 protein will be discussed later) (Valle and Banks, 1995; DiMaio and Mattoon, 2001).

1.2.3.5.2.1 HPV-16 E6 protein

HPV-16 E6 ORF encodes a small nuclear protein, 151 amino acids, containing two zinc finger domains. E6 protein induces several important changes in the host cell that impact both are the normal viral life cycle and the process of immortalization. E6 protein interacts with several cell proteins and this function can alter the cell, making it more amenable to production of new viral particles (Li *et al.,* 2005). The main activity of the high-risk HPV E6 proteins is the stimulation of the ubiquitin mediated degradation of p53 tumour suppressor protein. Both the binding of E6 to p53 and the ubiquitination are dependent on the cellular E6-associated protein (E6-AP) (Li and Coffino, 1996; Scheffner and Whitaker, 2003). The low-risk E6 proteins are able to interact with p53 "*in vitro*" but do not target p53 for proteolysis "*in vivo*" (Elbel *et al.,* 1997). p53 is a transcription factor that stimulates the expression of genes involved in cell cycle arrest and apoptosis, such as the cyclin dependent kinase inhibitor, p21. The prevention of translocation and degradation of p53 by E6 inhibit the ability of p53 to activate or repress transcription of target genes (Mietz *et al.,* 1992). Pan and Griep showed (Pan and Griep, 1994 and 1995) that using transgenic mice with targeted expression of E6 in the lens of the eye, E6 could block apoptosis. Another study (Jackson and Storey, 2000) showed that using E6 genes from mucosal and cutaneous subtypes of HPV all E6 proteins was capable of blocking apoptosis induced by ultraviolet (UV) radiation. This interaction probably contributes to the p53 independent inhibition of apoptosis. If left unchecked, the increase in p53 and induction of p53 dependent apoptosis would most likely

kill an infected cell before viral replication could take place. Thus, the ability of E6 to modulate p53 levels may be integral during productive infection. It has been shown that E6 can up-regulate the activity of the cellular telomerase complex, which is an enzyme that maintains the telomeric DNA at the ends of chromosomes (Gerider and Blackburn, 1985). Without telomerase, telomeres gradually shorten with each cell division, until they reach a critically short length. It has been suggested that telomere shortening functions as a sort of molecular clock, controlling the number of cell divisions. Additionally, cells that normally proliferate in vivo have detectable telomerase activity, as do most tumour cells, while cells that normally have a limited lifespan in vivo generally do not show telomerase activity (Harle-Bachor and Boukamp, 1996). It has been shown, in vitro, that the telomerase activity is increased in HFK (human foreskin keratinocyte), HFF (human foreskin fibroblast) and HMEC (human mammary epithelial cell) expressing HPV-16 E6 (Klingelhutz *et al.,* 1996; Stoppler *et al.,* 1997). E6 expression was shown to transactivate the HPV early promoter in the URR (Gius *et al.,* 1988). The mechanism for this transcriptional alteration was not dependent on p53. HPV-16 E6 proteins are capable of interacting with p300 and CBP to inhibit p300/CBP mediated transcriptional activation. Both p300 and CBP are transcriptional co-activators and bring together transcription factors and the basal transcription machinery at gene promoters. They also have intrinsic histone acetyltransferase activity, allowing them to acetylate histone core proteins and alter chromatin structure (Patel *et al.,* 1999; Zimmermann *et al.,* 1999). Therefore, an explanation of the earlier work on the transcriptional repression by E6 may be the interaction of E6 with the coactivators CBP and p300. E6-CBP/p300 binding probably impacts transcription of other genes.

1.2.3.5.2.2 HPV-16 E7 protein

The HPV-16 E7 ORF is predicted to encode a 98-amino-acids protein, which is divided into three conserved regions (CR1, CR2, and CR3), which share significant homology with conserved domains 1 and 2 of adenovirus oncoprotein E1A. The homology with conserved domain 2 is also found in the SV40 large T proteins. E7 induces significant biological changes, particularly in regard to cellular growth (Barbosa *et al.*, 1990; Dyson *et al.*, 1992). E7 binds and inactivates pRb and two pRb related proteins p107 and p130 (Munger *et al.*, 2001). The retinoblastoma protein family members play a central role in the regulation of the eukaryotic cell cycle. More specifically, in its hypophosphorylated state, Rb can bind to transcription factors such as the E2F family members and repress the transcription of particular genes. As cells progress from G_0 through G_1 and into S phase, Rb family members become progressively hyperphosphorylated by G_1 cyclins- CDKs, consequently releasing the transcription factor E2F, which in turn activates genes involved in DNA synthesis and cell cycle progression (Dyson, 1998). Since E7 is able to bind to unphosphorylated Rb, it is believed that E7 can prematurely induce cells into S phase by disrupting Rb-E2F complexes (Huang *et al.*, 1993; Patrick *et al.*, 1994). Additionally, E7 is able to bind transcription factor AP-1 and activate its activity and therefore hypothetically after the differentiation of the cells (Antinore *et al.*, 1996).

1.2.3.6 Papillomavirus E5

The analysis of the DNA sequence of the papillomavirus genome revealed that E5 ORFs commonly is located at the 3' end of the early region before the L2 ORF and overlaps the E2 ORF in the early region. However, in the ξ-papillomavirus epitheliotropic BPVs the E5 ORF (previous named E8) is located 5' to the E7 ORF and replaced the E6 ORF (Jackson *et al.*,

1991). The CRPV genome has two E5-like (E8 and E5) ORFs in the early region (Han *et al.*, 1998), suggesting either gene duplication or translocation without loss of the E6 ORF. The E5 genes of PVs encode small hydrophobic proteins, many of which have been shown to have transforming activity, from 42 amino acids residues in BPV-4 to 83 amino acids residues in HPV-16. The most studied PV E5 proteins in animal and human are BPV-1 and HPV-16 respectively. BPV-1 E5 protein can be divided into two domains, N-terminus and C-terminus. The N-terminus, which is a hydrophobic domain, includes 30 amino acids and is thought to exist in an α-helical conformation important for membrane anchoring and cell transformation. The C-terminal amino acids are generally hydrophilic, 14 amino acids in BPV-1 including two cysteine residues which are important for homodimerization and transforming activity (Burkhardt *et al.*, 1987; Horwitz *et al.*, 1988). It has been suggested that E5 protein is a type II transmembrane protein that is localized asymmetrically in Golgi membranes with its C-terminus being oriented toward the Golgi lumen (Burkhardt *et al.*, 1989). HPV-16 E5 is a hydrophobic membrane protein, 83 amino acids long, located mainly at the endosomal membranes, Golgi apparatus and, to a lesser extent, the plasma membranes (Conrad *et al.*, 1993). It has been determined by hydropatic analysis that HPV-16 E5 protein has three hydrophobic domains connected by less hydrophobic regions (Bubb *et al.*, 1988), and in vitro transcription-translation experiments show the protein is rapidly incorporated into microsomal membranes. In contrast to BPV-1 where E5 is the main oncoprotein, little is known about the biological activity of HPV-16 E5. However, the amino acid analysis of the HPV E5 proteins and BPV E5 has revealed that they are very different (Table 3).

Table 3. Amino acid sequences of BPV-1 E5 and HPV-16 E5 proteins

BPV-1 E5	1 MPNLWFLLFL GLVAAMQLLL LLFLLLFFLV YWDHFECSCT GLPF 44 1 atgccaaatc tatggtttct attgttcttg ggactagttg ctgcaatgca 51 actgctgcta ttactgttct tactcttgtt tttcttgtta tactgggatc 101 attttgagtg ctcctgtaca ggtctgccct tttaa
HPV-16 E5	1 MTNRDTASTT LLACFLLCFC VLLCVCLLIR PLLLSVSTYT SLIILVLLLW ITAASAFRCF IVYIIFVYIP LFLIHTHARF LIT 83 1 atgacaaatc gtgatactgc atccacaaca ttactggcgt gctttttgct 51 ttgcttttgt gtgcttttgt gtgtctgcct attaatacgt ccgctgcttt 101 tgtctgtgtc tacatacaca tcattaataa tattggtatt actattgtgg 151 ataacagcag cctctgcgtt taggtgtttt attgtatata ttatatttgt 201 ttatatacca ttatttttaa tacatacaca tgcacgcttt ttaattacat 251 aa

But some limited sequence similarities have been suggested between the HPV-16 E5 and BPV-1 E5 proteins, such as a few hydrophobic residues (Bubb *et al.,* 1988). The HPV-16 E5 protein is nearly twice as long as BPV-1 E5, and is likely to form three transmembrane segments. The C-terminal portion of BPV-1 E5, which appears to be important for biological function, does not have an identifiably similar region in the HPV-16 E5. It has been reported that in BPV-1 and HPV-16-transfected cells, most of the synthesized E5 protein localizes to the Golgi apparatus but a small proportion can be found at the plasma membrane (Burkhardt *et al.,* 1989; Conrad *et al.,* 1993). The expression of the PV E5 occurs during the early stages of infection in the deep layers of the infected epithelial cells like as E6 and E7, and its expression is extinguished as the lesion progresses (Burnett *et al.,* 1992; Anderson *et al.,* 1997). Initial infection with the high-risk HPV-16 can cause low-grade disease and the viral DNA exists in episomal form in the cell nucleus. During the progression to malignant disease, HPV DNA frequently integrates into the host cell genome (Choo *et al.,* 1987; Cullen *et al.,* 1991). The integration of viral DNA allows for persistent expression of the E6 and E7 oncogenes. In the episomal form of the viral DNA present in the initial stages of HPV-infection, the E5 ORF is present and transcribed in the major abundant viral transcript, E1^E4 mRNA (Zur Husen and de Villiers, 1994). However, the E5 gene is frequently deleted when

the HPV genome is integrated (Zur Husen and de Villiers, 1994; DiMaio and Mattoon, 2001). Therefore, E5 gene expression is often extinguished after integration of the viral genome during the progression from low-grade to malignant disease. For E5 protein to exert effects on carcinogenesis, it should therefore act at any early stage. In addition, in well-developed carcinomas, E5 has been found to be frequently deleted upon incorporation of the viral DNA into the host genome, suggesting that E5 is not necessary to maintain the malignant state (Bauer-Hofmann *et al.*, 1996). It was suggested that HPV E5 plays a role in the first steps of cellular transformation. This is supported by the fact that in cervical intraepithelial neoplasia (low-grade intraepithelial neoplasia) lesions large amounts of E5 mRNA as well as of E5 protein can be detected (Stoler *et al.*, 1992). The E5 protein is able to interact with several different cellular proteins, including the epidermal growth factor receptor and the 16 k subunit c of the vacuolar H^+-ATPase. In addition, BPV-1 E5 protein interacts with platelet-derived growth factor receptor (Petti and DiMaio, 1994).

1.2.3.6.1 BPV-1 E5 and platelet-derived growth factor receptor

The BPV E5 protein induces tumorigenic transformation of rodent fibroblasts and is the major transforming protein of this virus (Bergman *et al.*, 1988). This small, 44-amino-acid transmembrane protein exists as a dimer with a subunit size of 7 kDa and localizes primarily to the endoplasmic reticulum and Golgi membranes of transformed cells (Burkhardt *et al.*, 1989). It has been suggested that the E5 protein transforms cells by modulating the activity of a cellular membrane-associated growth-regulatory protein. Several studies have demonstrated that the BPV E5 protein is critical for binding and activation of the platelet-derived growth factor receptor (PDGF) β receptor and for cell transformation (Sparkowski *et al.*, 1996). It has also been shown that the interaction between BPV E5 oncoprotein and the activated PDGF β-

receptor takes place in naturally occurring urinary bladder tumours in cattle (Borzacchiello *et al.*, 2006). It has been demonstrated that the E5 protein forms a stable complex with and activates the PDGF β-receptor in E5-transformed fibroblasts (Petti *et al.*, 1991; Petti and DiMaio, 1992). The PDGF β-receptor is a cell surface receptor tyrosine kinase, which stimulates cell proliferation and has been associated with some cancers. The binding of PDGF to its receptor causes activation of the tyrosine kinase receptors and receptor autophosphorylation, followed by activation, leading to cell transformation (Claesson-Welsh, 1994). The binding of the E5 protein to the PDGF receptor (PDGF-R) results in PDGF-R activation and this activated PDGF-R transmits a sustained growth-stimulatory signal, resulting in cell transformation in the absence of ligand (Petti and DiMaio, 1992 and 1994). Complex formation between the E5 protein and the PDGF β-receptor is mediated by interactions between the transmembrane and juxtamembrane regions of the two proteins, and removal of the ligand-binding domain of the receptor does not disrupt interaction with the E5 protein. A positive charge at position lysine 499 in the extracellular juxtamembrane region of the receptor and threonine 513 in the transmembrane domain are required for interaction with the E5 protein and for E5-induced receptor activation but not for activation by PDGF. The E5 protein does not interact with the related PDGF α-receptor, a difference that map to the transmembrane/juxtamembrane region of the receptor (Staebler *et al.*, 1995): both a positively charged juxtamembrane residue and the transmembrane threonine are absent from the PDGF α-receptor. The E5 protein is thought to be a type II membrane protein and consequently would be inserted into membranes in an orientation opposite that of the PDGF β-receptor (Claesson-Welsh, 1994). This places aspartate 33 of the E5 protein and lysine 499 of the receptor on the extracytoplasmic side of the membrane at the membrane surface, with glutamine 17 and threonine 513 buried in the membrane at roughly the same distance relative

to the membrane surface. These considerations have led to the proposal that two pairs of interacting residues, aspartate 33-lysine 499 and glutamine 17-threonine 513, are essential for complex formation between the E5 protein and the PDGF β-receptor. It has been shown that the dimeric E5 protein in lipid bilayers is largely α-helical and has a transmembrane orientation. Computational searches generated two related structural models for the E5 dimer that account for the role of glutamine 17 and aspartate 33 in complex formation and are consistent with the position of conserved and transformation-sensitive residues (Surti *et al.*, 1998).

1.2.3.6.2 HPV-16 E5 and epidermal growth factor receptor

The transforming activity of E5 may be due in part to its ability to inhibit down-regulation of the epidermal growth factor (EGF) receptor (EGF-R). It has been suggested that HPV E5 associates with the EGF-R that the function of this protein is in a manner similar to BPV E5 and the PDGF-R (Hwang *et al.*, 1995). In human keratinocytes, the major growth factor receptor is the EGF-R, so multiple studies have suggested a link between the HPV-16 E5 gene and EGF-R signaling. In addition to the interaction between BPV E5 with PDGF-R, it has been reported that BPV-1 E5 cooperates with EGF-R in the transformation of NIH 3T3 cells and induces receptor hyperphosphorylation as well as altered down-regulation (Martin *et al.*, 1989). The EGF-R that is a transmembrane receptor protein with ligand-activated tyrosine kinase activity is present on all epithelial cells, including cervical mucosal cells (Carpenter and Cohen, 1990). The expression of HPV-16 E5 in human keratinocytes results in an increase of the number of EGF-Rs at the cell surface and in inhibition of receptor degradation (Straight *et al.*, 1993). In other studies, E5 has been shown to increase cellular proliferation in the presence of EGF in the mouse fibroblasts transfected with HPV-16 E5 (Leechanachai *et*

al., 1992). The molecular basis for this effect is not clear, although it has been suggested that E5 associates with the EGF-R (Hwang *et al.*, 1995). Stimulation of the receptor with EGF activates a number of signal transduction pathways including the mitogen activated protein (MAP) kinase pathway that is associated with both cell proliferation and differentiation (Sumbayev and Yasinska, 2005). It has been reported that E5 gene was able to induce an increase in the MAP kinase activity (Gu and Matlashewski, 1995). In comparison, the E6 and E7 proteins did not stimulate or prolong MAP kinase activity. Additionally, it was revealed that the inhibition of the down-regulation of the EGF-R by E5 may be associated with the ability of E5 to bind to a 16 kDa proteolipid, which is a component of the vacuolar proton-ATPase pump (H^+-ATPase) (Conrad *et al.*, 1993) and to inhibit the acidification of endosomes (Straight *et al.*, 1995). This binding has been suggested to result in a delay in EGF-R degradation and an increased recycling of EGF-R to the cell surface (Straight *et al.*, 1993). Moreover, HPV-16 E5 has been also demonstrated to cooperate with E7 to potentiate a mitogenic response, which is enhanced in the presence of the EGF (Bouvard *et al.*, 1994a). The close association of E5 biological activities with growth factor receptors would suggest that E5 might contribute to the normal viral life cycle and the early stage of viral infection, by increasing cell responsiveness to growth factors such as EGF.

1.2.3.6.3 E5 and 16 K subunit c/ductin

Several viral oncoproteins such as BPV-1 E5 protein (Goldstein and Schlegel, 1990), HPV-16 E5 protein (Conrad *et al.*, 1993) and T-cell leukaemia virus type 1 p12 (Franchini et al., 1993) bind to 16 K subunit c/ductin, which is a hydrophobic polypeptide. It is found in two distinct membrane complexes, subunit c is a component of the vacuolar H+ ATPase (Mandel *et al.*, 1988) and as a ductin in a component of gap junctions (Finbow and Pitts, 1993). The binding

of E5 to ductin has led to the suggestion that the function of the ductin's transport might be impaired. The V-ATPase is a universal transmembrane proton pump of eukaryotic cells and is responsible for acidification of cytoplasmic organelles (e.g. Golgi, endosomes, lysosomes, and certain secretory vesicles) (Finbow and Harrison, 1997). Thus, it has been postulated that binding of E5 to the 16K proteolipid would inhibit endosomal acidification leading to an increase in receptor recyling to the plasma membrane (Straight *et al.*, 1993 and 1995). The effect of this mechanism would be to increase the number of receptors at the cell surface signalling in E5-expressing cells (Leechanachai *et al.*, 1992; Pim *et al.*, 1992). It has been determined that E5 binds to 16K subunit c which is thought to increase the pH of Golgi (Schapiro *et al.*, 2000). Since growth factor receptors are processed in the Golgi apparatus and are internalized via clathrin-coated vesicles, E5 binding to the 16 K subunit c within these components may be an intermediate step to receptor activation and signal transduction.

1.2.3.6.4 E5 and gap junction

Gap junction channels span the plasma membrane of two adjacent cells and allow cell–cell communication via diffusion of small molecules. This channel is called connexon (Simon and Goodenough, 1998). Each hemi-channel consists of the oligomers of six protein subunits. Most cells such as epithelial cells form gap junctions. They contribute to the maintenance of homeostasis between cells in a tissue or in a proliferative unit (Kam and Hodgins, 1992). Numerous reports have shown that gap junction communication can be regulated at various cellular levels, including mRNA transcription, mRNA stability and channel gating (Bruzzone *et al.*, 1996). Often, gap junctions are not functional in transformed cells and the isolation of transformed cells from their normal neighbours is thought to contribute to the neoplastic process (Budunova *et al.*, 1995). 16 K subunit c is also the 'ductin' component of a type of

gap junction, an intercellular complex that provides sites of cell-to-cell movement (Finbow and Pitts, 1993; Finbow *et al.,* 1995). Connexins also form gap junction channels, and mutant forms of ductin that have transforming ability disturb connexin localization and down-regulate gap junction communication (Saito et al., 1998). It has been reported that several E5 proteins (e.g. BPV-1, 4 and HPV-6, 16) bind to ductin and this interaction is deemed responsible for the observed down-regulation of gap junctions (Goldstein *et al.,* 1991; Conrad *et al.,* 1993; Faccini *et al.,* 1996; Ashrafi *et al.,* 2000). In addition, HPV-16 E5 reduces gap junction-mediated intercellular communication via dephosphorylation of connexin 43 (Oelze *et al.,* 1995). This results in the inhibition of tissue homeostatic feedback, which has also been described as an early event in carcinogenesis progression (King *et al.,* 2000).

1.2.3.6.5 E5 and Apoptosis

Cell growth normally reflects a dynamic balance of cell proliferation, differentiation, and apoptosis. Apoptosis is a morphologically and biochemically distinct form of eukaryotic cell death that occurs under a variety of physiological and pathological conditions (Arends *et al.,* 1991). Two main apoptotic routes have been identified that include the death receptor (DR) pathway and the mitochondrial pathway (Danial and Korsmeyer, 2004). The activation of the DR pathway takes place by ligation of members of the tumour necrosis factor (TNF) family, such as Fas ligand (FasL; also called Apo1) and Apo2 ligand (Apo2L; also called TNF-related apoptosis-inducing factor [TRAIL]), to DR on the plasma membrane. This family consists of trimeric ligands that induce defined cellular responses upon binding to their respective receptors. They include the adaptor protein FADD (Fas-associated death domain) and subsequent recruitment of procaspase-8 and this protein complex is called death-inducing signaling complex (DISC). DISC formation provides the necessary environment for activation

of procaspase-8 by autocatalytic cleavage (Scaffidi *et al.*, 1999; Krueger *et al.*, 2001). FasL, which is a 40-kDa, binds to the Fas (CD95) receptor leading to activation and clustering of the death receptors (Schneider *et al.*, 1997; Walczak *et al.*, 2000). TRAIL interacts with several cellular soluble receptors (Emery *et al.*, 1998), the TRAIL receptors 1 and 2 that contain intracellular death domains (DDs) to transmit the apoptotic signal (Pan *et al.*, 1997; Kischkel *et al.*, 2000; Sprick *et al.*, 2000). Fas and TRAIL receptors are expressed by a broad panel of normal epithelial cells (Leithäuser *et al.*, 1993; Wiley *et al.*, 1995). Down-regulation of Fas expression is a common abnormality in gynecological cancers (Das *et al.*, 2000), whereas the expression of TRAIL receptors is not reduced in cervical cancer (Ryu *et al.*, 2000) compared to normal tissue. Therefore, it is plausible that HPV-16, like many other viruses (Teodoro and Branton, 1997; O'Brien, 1998), has developed mechanisms to delay apoptosis of the infected cell and one of its early proteins, namely, E6, has been identified to prevent intrinsic, p53-dependent apoptosis (Scheffner *et al.*, 1990). It has been reported that the HPV-16 E5 protein protects the human keratinocyte cell line from TRAIL and FasL-mediated apoptosis and that this effect correlates with the level of E5 expression. It proposed that this is a primary function of the viral protein (Kabsch and Alonso, 2002).

1.2.3.7 HPV-16 capsid proteins

HPVs encode two capsid proteins L1 and L2. The L1 protein is the major capsid protein and the L2 protein is the minor capsid protein in the virion. Both of them are expressed in the terminally differentiated layers of the epithelium and form a protective shell around the viral genome (Okun *et al.*, 2001). The L2 protein is found mainly on the inside of the virion and is required for infectious virions to form and has been shown to interact with a cell surface receptor (Kawana *et al.*, 2001). The HPV L2 protein may play a major role in papillomavirus

capsid assembly by introducing HPV DNA into the virus particles formed by the self-assembly of the L1 major structural protein (Zhou *et al.*, 1994). It has been shown that the antibody against L2 protein neutralises papillomavirus without preventing virion attaching to the cell surface (Roden *et al.*, 1994). The L1 protein is the major capsid protein and when expressed in various eukaryotic an expression system is able to self-assemble into virus-like particles (Kirnbauer *et al.*, 1992). On encapsidation of the double stranded circular viral genome the virion conformationally changes into an icosahedral structure with 72 pentamers of L1.

1.3 The human immune system

Two main arms of the immune response play an important role in the natural clearance of viral infection, innate and adaptive immunity. Innate immunity consists of a quickly induced, non-specific response, which does not result in immunological memory. The innate immune system is localized at epithelial borders and is stimulated via several immunomodulatory cytokines and cellular effectors including monocytes, macrophages, natural killer and antigen presenting cells (APC). The innate immune system is likely to play an important first line of defence in the control of viral infections (Uthaisangsook *et al.*, 2002). The adaptive immune response arises from a breach of the innate immune response resulting in the generation of antigen specific effectors cells and their products that specifically target the pathogen or pathogen infected cell, as well as memory cells that will prevent or limit subsequent infection with the same organism (Sigal, 2005). It is now presumed that activation of the innate immune system stimulates the adaptive immune responses. Different pathogens are handled by specific immune responses. At the same time, unwanted immune responses against self-proteins are avoided. However due to the polymorphism of the vertebrate major

histocompatibility complex (MHC) genes, different individuals in a population respond differently to identical antigens (Ploegh, 1998; Sigal, 2005).

1.3.1 The human leukocyte antigen system

The human MHC is known as human leukocyte antigen (HLA) system, which is located on the short arm of chromosome 6, encodes class I proteins (presenters to cytotoxic T cells) and class II proteins (presenters to helper T cells), as well as class III proteins (components of the complement subunits). The MHC locus consists of a linked set of genetic loci encoding many of the proteins involved in antigen presentation to T cells, most notably the MHC class I and class II glycoproteins that present peptides to the T-cell receptor (Sigal, 2005). The outstanding feature of the MHC molecules is their extensive polymorphism. This polymorphism is of critical importance in antigen recognition by T cells. A T cell recognizes antigen as a peptide bound by a particular allelic variant of an MHC molecule, and will not recognize the same peptide bound to other MHC molecules. This behaviour of T cells is called MHC restriction (Dunbar and Ogg, 2002). The main function of the MHC class I molecules is to present antigenic peptides to cytotoxic T cells (CTLs). When a pathogen infects a host cell, the proteins of the pathogen are degraded intracellularly, and a subset of different peptides are loaded onto the groove of class I MHC molecules that are transported to the cell surface. Once the MHC-peptide complex is present on the cell surface, CTLs can mount an immune response against pathogens. The MHC class II genes are very polymorphic in human population. They encode proteins that are found on antigen processing/presenting cells such as B-lymphocytes and macrophages. The MHC Class II proteins present 13-18 amino acid peptides derived from extracellular pathogens to T helper lymphocytes and are important in the priming and maintenance of the humoral immune response (Dunbar and Ogg,

2002). MHC class III genes are located between the class I and class II genes and contained a variety of genes with known and unknown immune mechanisms. These genes encode for some components of the complement system, TNF-α and TNF-β (Ryder and Svejgaard, 1981; Beck and Trowsdale, 2000).

1.3.2 MHC class I

The MHC class I molecules are subdivided into two families, classical MHC I or MHC class Ia or and non-classical MHC I or MHC class Ib.

1.3.2.1 Classical MHC class I

The classical MHC I molecules are a family of exceedingly polymorphic cell surface glycoproteins found on almost all nucleated somatic cells. These proteins play an important role in alerting the immune system to virally infected cells. In human, the classical MHC class I glycoproteins, HLA-A, B and C, are expressed on the cell surface of all nucleated cells and present peptide fragments derived from intracellular proteins (Hewitt, 2003). These peptides are normally derived from the cell's own 'house-keeping' proteins but in a virally infected cell, peptides derived from viral proteins are also presented. MHC class I molecules are heterodimers of a heavy chain and β_2-microglobulin (β_2M). The extracellular region of the heavy chain includes α1, α2 and α3 domains with β_2M contributing a fourth domain. The α1 and α2 domains form the peptide-binding site that is a groove on the upper surface of the MHC class I molecule and binds antigenic peptides of 8–10 amino acids in length (Hewitt, 2003; Princiotta *et al.,* 2003). These proteins are cotranslationally inserted into the lumen of the endoplasmic reticulum (ER). In the ER, the MHC/β_2M heterodimer binds peptide that is generated by proteasomal protein degradation in the cytosol and translocated into the ER by

the transporter associated with antigen processing (TAP) (Pamer and Cresswell, 1998). The loading of peptides into MHC class I molecules occurs in an assembly complex which includes TAP. Upon peptide binding, MHC class I molecules leave the ER and traverse to the cell surface via Golgi and vesicular transport (Reits *et al.*, 2000). At the surface, the peptides are exposed for recognition by T lymphocytes able to lyse infected cells, an outcome that pressures viruses to take defensive measures (Figure 5) (Hewitt, 2003). Given the role that the MHC class I antigen presentation pathway plays in the detection of virally infected cells by CTLs, it is not surprising that many viruses have evolved proteins that interfere with this pathway (Tortorella *et al.*, 2000).

Figure 5. The processing of viral proteins for presentation of peptides to cytotoxic T cells

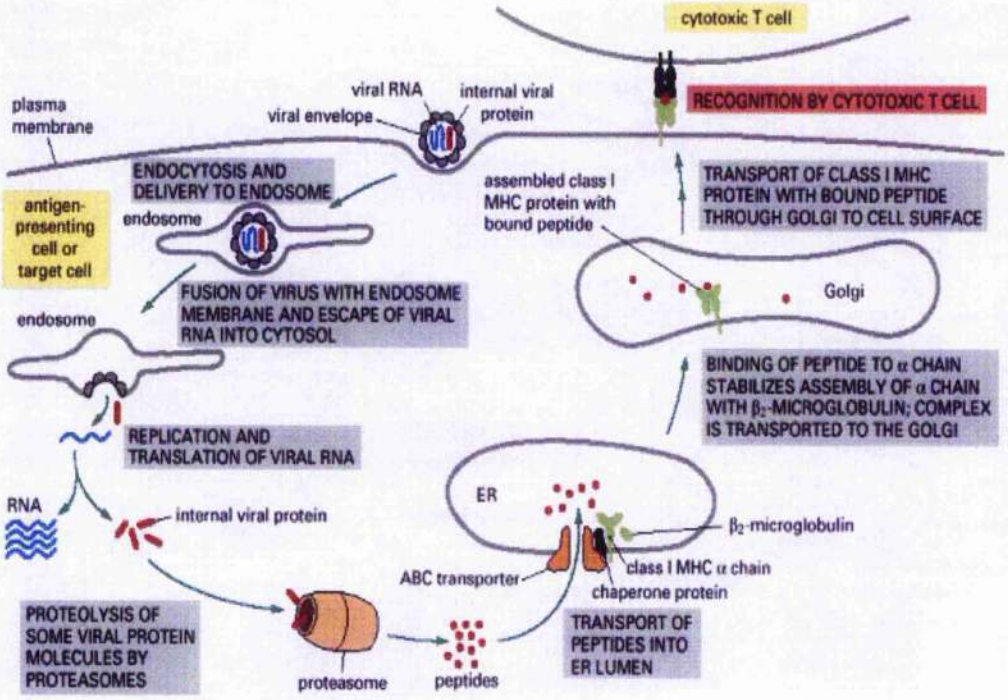


Figure 5: An effector cytotoxic T cell kills a virus-infected cell when it recognizes fragments of viral protein bound to class I MHC proteins on the surface of the infected cell. Not all

viruses enter the cell in the way that this enveloped RNA virus does, but fragments of internal viral proteins always follow the pathway shown. Some of the viral proteins synthesized in the cytosol are degraded, and this is a sufficient amount to attract an attack by a cytotoxic T cell. The folding and assembly of a class I MHC protein is aided by several chaperone proteins in the ER lumen, only one of which is shown. The chaperones bind to the class I MHCα chain and act sequentially. The last one binds the MHC protein to the ABC transporter, as shown (Molecular Biology of the cell)

1.3.2.2 Non-classical MHC class I

The human non-classical MHC I includes HLA-E, HLA-F, and HLA-G molecules. Like the classical MHC I loci, the non-classical MHC I genes are highly transcribed in many tissues, but they display only limited genetic variation. These molecules show homology to classical MHC I molecules but generally have limited polymorphism, low cell surface expression, and more restricted tissue distribution (Shawar *et al.*, 1994). The function of the non-classical MHC I molecules remains unclear, but some of them may have more specialized antigen presentation activities. In humans, the non-classical HLA-G molecule binds a wide range of peptides derived from cellular proteins and has been suggested to play an important role in the maintenance of maternal tolerance to the fetus by interacting with inhibitory receptors on natural killer (NK) cells (Lee *et al.*, 1995). HLA-E is another non-classical MHC I molecule and, like classical MHC I loci, the HLA-E gene is highly transcribed in many tissues (Koller *et al.*, 1988). HLA-E preferably binds to a peptide derived from amino-acid residues 3–11 of the signal sequences of most HLA-A, B, C, and G molecules, but cannot bind its own leader peptide. Cell-surface expression of HLA-E depends on a functional TAP, and is up-regulated by the binding of the peptides derived from MHC molecule signal sequences (Braud *et al.*,

1998). The close correlation between the surface expression of HLA-E and other classical MHC I molecules (HLA/A and B) suggests a possible role for HLA-E in NK cell-mediated recognition of target cells.

1.3.3 Natural killer Cells

While classical MIIC I molecules (HLA-A/B) are the main presenters of antigenic peptides to CTL, HLA-C and non-classical HLA-E inhibit NK cell-mediated lysis by interacting with inhibitory receptors. NK cells play an important role in the non-adaptive immune response, which is related to their ability to kill target cells lacking MHC class I without prior instruction (Ploegh, 1998; Natarajan *et al.,* 2002). NK cells express receptors that interact with MHC class I serving to inhibit or activate their effector function. Two types of receptors are specific for self-HLA class I molecules to inhibit NK cells. They include the killer Ig-like receptors (KIR) and the lectin-like heterodimer CD94/NKG2 receptors. The KIRs recognize classical MHC I to inhibit the activation of NK cells (Colonna and Samaridis, 1995; Wagtmann *et al.,* 1995), whereas the CD94/NKG2 receptors recognize HLA-E which is a non-classical MHC I molecule (Lopez-Botet *et al.,* 1997). The inhibitory receptors provide the mechanism by which NK cell effector function is prevented against cells expressing normal levels of class 1 HLA molecules but is permitted against cells expressing reduced levels of class I HLA molecules, such as on virally infected or tumour cells (Ploegh, 1998). The activating receptors provide a mechanism by which NK cell function is stimulated. Thus, NK cells would kill classical MHC I negative virus-infected cells, unless non-classical MHC-E molecules are present.

1.3.4 MHC and virus infection:

The MHC class I molecules, which are essential for presentation of foreign peptides to the host CTL, are targets of many pathogens, including viruses (Ploegh, 1998; Tortorella *et al.*, 2000). CTLs recognize virus-infected cells through the specific interaction of their T-cell receptor with an MHC class I molecule presenting a viral peptide. The MHC class I complex consists of a heavy chain containing the peptide binding site and β_2M, which assemble very rapidly in the lumen of the ER. Peptides, generated by the proteasome in the cytoplasm, are translocated by TAP into the ER where they assemble in ternary complexes and are transported to the cell surface for presentation to CTL (York and Rock, 1996). Interference with the assembly and/or trafficking of the MHC class I complex can contribute to the persistence of a virus, although NK cells can recognize and lyse cells that lack MHC class I antigens (Karre, 1991).

Several viruses that induce chronic infections encode proteins that target or modulate the host's immune system. Adenovirus was the first virus demonstrated to express E3/19K adenovirus protein that binds to MHC class I in the ER and prevents its transport to the cell surface. Human cytomegalovirus (HCMV) encodes multiple proteins that target MHC class I synthesis, peptide loading, and transport. Murine CMV glycoprotein, gp34, also interacts with the heavy chain β_2M complex in the ER and has been recently shown to target MHC class I for degradation in the lysosomes. Infected cell protein (ICP) 47, a protein encoded by herpes simplex virus, inhibits the TAP transporter (Tortorella *et al.*, 2000). More recently, it was demonstrated that the K3 and K5 proteins encoded by Kaposi's sarcoma-associated herpesvirus down-regulate MHC class I from the cell surface (Ishido et al., 2000). It has been demonstrated that the human immunodeficiency virus (HIV) Nef and HCMV US10/UL40 proteins down-regulate HLA-A/B but does not down-regulate HLA-C/E, which are mostly

involved in positive selection of inhibitory receptors on NK cells (Cohen *et al.*, 1999: Tomasec *et al.*, 2000; Furman *et al.*, 2002).

In HPV infections, several lines of evidence suggest that the immune responses play an important role in the control of HPV infection. Generally, HPV infection is characterized by a very high rate of spontaneous clearance suggesting that immune responses play the essential role in the control of infection (O'Brien and Campo, 2003). The first line of defence would be at the mucosal surface via the innate immune system. It has been reported that the BPV E5 protein down-regulates the expression of MHC class I molecules on the cell surface (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). Cells that express E5 have reduced levels of MHC class I mRNA and protein and surface MHC class I (Ashrafi *et al.*, 2002). E5 can retain MHC class I interacellularly in different cell types, and the site of blockade has been shown to be the Golgi (Marchetti *et al.*, 2002). It has also been shown, *in vivo*, that BPV-4 E5 protein down-regulates MHC class I in BPV-4 papilloma samples (Araibi *et al.*, 2004).

1.3.5 HPV-16 and immune evasion

Interferons (IFNs), which consist of type I (α and β) and type II (γ) IFNs, play an important role in host defence, having both anti-viral and anti-tumour effects, and may influence expression of a number of genes associated with both apoptosis and cell cycle progression (Sangfelt *et al.*, 1997). One of the functions of IFNs is to up-regulate the expression of MHC class I. The role of MHC class I is to present antigen peptides to CTL. Many viruses down-regulate MHC class I either directly or indirectly to establish infection. It has been demonstrated that both E6 and E7 proteins of the high-risk HPV (types 16 and 18) inhibit IFN α and β pathway. It has been reported that HPV 16 E6 protein binds to interferon regulatory factor-3 (IRF-3) and can inhibit its trans-activation function. IRF family members like as IRF-

3 are believed to play a critical role in the regulated expression of the IFN α and β genes (Ronco *et al.,* 1998). It has been demonstrated that HPV E7 protein is functionally and physically associated with IRF-1, *in vivo*, and *in vitro*. The interaction between the IRF-1 and the HPV E7 proteins hinder the interferon response (Park *et al.,* 2000). Additionally, HPV 16 and HPV 18 E7 protein repress the MHC class I heavy chain gene promoter. Expression of the HPV 18 E7 oncoprotein represses MHC class I heavy chain promoter, as well as repression of a bidirectional promoter that regulates the expression of the genes encoding TAP-1 and a proteasome subunit, low molecular weight protein (LMP2) (which enhances degradation of cytoplasmic proteins) (Georgopoulos *et al.,* 2000). E7 down-regulates MHC class I by interacting with and inhibiting TAP-1 (Vambutas *et al.,* 2001). HPV-16 E7 also interacts with the human subunit 4 (S4) ATPase of the 26 S proteasome and degrades Rb through the 26 S proteasome pathway (Berezutskaya and Bagchi, 1997).

1.3.6 E5 and MHC class I

MHC class I molecule is essential for presentation of foreign peptides to the host CTL and plays a critical role in immune surveillance to target many pathogens (Ploegh, 1998; Tortorella *et al.,* 2000). The BPV-1 and 4 E5 proteins which are a major transforming protein causes a down-regulation of surface MHC class I molecules in transformed cells by reducing transcription of the MHC class I heavy chain gene. It also causes a lower level of the MHC class I molecules by prevention of transport of the MHC class I complex to the cell surface (Ashrafi *et al.,* 2002; O'Brien and Campo 2003). This is achieved by the trapping of MHC class I in the Golgi apparatus and prevention of its transport to the cell surface (Marchetti *et al.,* 2002). It has been also reported that BPV E5 proteins causes a down-regulation of surface MHC class I molecules in transformed cells (Ashrafi *et al.,* 2002) and IFN treatment increases

transcription of the MHC class I heavy chain gene, but can not overcome the block in exocytic transport (Marchetti *et al.*, 2002). It has been investigated that the expression of BPV-4 E5 protein down-regulates MHC classes I in clinical samples of BPV-4 papillomas (Araibi *et al.*, 2004). Down-regulation of MHC class I in the cell expressing BPV-4 E5 is caused by physical interaction between the C-terminus of E5 and MHC class I heavy chain (Marchetti *et al.*, 2006).

1.4 Aim of Ph.D. project

Previous studies have shown that cells transformed by BPV E5 do not express MHC class I molecules on their surface, and that E5 expression also impairs both transport of MHC class I to the cell surface and production/stability of the MHC class I complex (Ashrafi *et al.*, 2002, Marchetti *et al.*, 2002 and O'Brien and Campo, 2003). Also BPV E5 down-regulates MHC class I in clinical samples of BPV-4 papillomas (Araibi *et al.*, 2004). It has been shown that the C-terminus of BPV-4 E5 is important to down-regulate MHC class I by interaction to heavy chain (Marchetti *et al.*, 2006). To this end, E5 protein escapes immune systems by reducing the level of MHC class I on the cell surface.

The overall aim of project was to investigate the relationship between HPV-16 E5 and MHC class I. The objectives are:

-To determine whether HPV-16 E5 down-regulates classical MHC I on the cell surface and localization of MHC class I.

-To determine whether down-regulation of MHC class I by E5 is reversible.

-To determine whether the domain of HPV-16 E5 responsible for down-regulation of classical MHC I on the cell surface and the mechanism of HPV-16 E5 down-regulates classical MHC I on the cell surface.

-To determine whether there is a physical interaction between HPV-16 E5 and MHC heavy chain.

-To determine whether HPV-16 E5 does not down-regulate non-classical MHC I on the cell surface.

Chapter Two

Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Antibodies

-Sigma

Monoclonal antibody (mAb) W6/32 and FITC conjugated against human HLA/A, B & C

mAb 4A3 raised against the Golgi protein GM130

DAPI (4'-6'-Diamidino-2-phenylindole)

Anti mouse IgG FITC conjugated antibody

Anti mouse IgG TRITC conjugated antibody

Horse Radish Proxidase

mAb HA against HA-Tag

mAb AB-1 (anti-actin)

-Serotec

mAb W6/32 raised against HLA/A,B and C

mAb MEM-E/02 raised against HLA-E

-kind gifts from

mAb DT9 anti human HLA-C/E (for FACS & IF) (A kind gifts from Dr. Veronique Braud, Centre National de la Recherrche Scientifique, Sophia Antipolis, France)

Anti HPV-16 E2 (A kind gifts from Dr.Merlin Hibma, Tumour Virus Group (TVG), Department of Pathology, University of Cambridge, England)

E5 N-terminus and E5 C-terminus antisera against E5 (kind gifts from Dr. DiMaio, Department of Genetics, Yale University School of Medicine, New Haven, USA).

mAb HC10 against HLA-A2 heavy chain (kind gifts from Dr. Stephen Man, University of Cardiff)

2.1.2 Antibiotics

-Sigma

Ampicillin

Kanamycine

-Invitrogen

Geneticine (G-418)

2.1.3 Bacteriology

-Institute of Comparative Medicine Central Services

L-broth

LB-agar

-Becton Dickinson Labware (Oxford, U.K.)

Falcon 1059 polypropylene tubes

Falcon 2059 polypropylene tubes

-Bibby Sterilin Ltd (Staffordshire, U.K.)

90mm bacteriological petri dish

-Invitrogen

E.coli DH5-α competent cells with SOC medium

-Nunc (Hereford, U.K.)

Sterile disposable inoculating loops

2.1.4 Cell line

Immortalised Human Keratinocyte (HaCaT)

2.1.5 Cell Culture materials

-Institute of Comparative Medicine

Sterile phosphate buffered saline (PBS)

-Becton Dickinson Labware (Oxford, U.K.)

90 mm tissue culture dishes

Falcon 2097 polypropylene tubes

Falcon 2098 polypropylene tubes

Serological plastic pipettes

24 well cell culture

Cover glass 22x22mm

Cover glass 13mm

Sterile plastipak syringes

Sterile syringe needles

-Bibby Sterilin Ltd (Staffordshire, U.K.)

Sterile plastic bijoux

Sterile plastic universal containers

-Costar Corporation (Bucks, U.K)

Disposable cell scrapers

-Invitrogen

Dulbecco's Modified Eagle Medium (DMEM), without $CaCl_2$ supplemented

2.5% Trypsin-EDTA (1X)

Foetal Calf Serum (FCS)

L-Glutamine (100X)

Sodium pyruvate MEM 100MM

-Nunc (Hereford, U.K.)

Cryotubes

T25, T75 and T175 cm^2 tissue culture flasks

-Gelman Science, Northampton, England

Sterile 0.2 μm acrodisc filters

-Sigma

Interferon-β Human

2.1.6 Chemicals and Reagents

-Amersham International plc (Buckinghamshire, U.K.)

Enhanced Chemiluminescence (ECL-plus) Western detection agent

-Molecular Probes Europe BP, The Netherland

Bodipy® TR Ceramide

-Invitrogen

Agarose (ultrapure electrophoresis grade)

Tablet of Phosphate Buffered Saline (PBS)

20 X MES SDS running buffer

20 X MOPS SDS running buffer

20 X MOPS SDS transfer buffer

NUPAGE® Antioxidant

NUPAGE® Sample Reducing Agent (10X)

NUPAGE® LDS Sample Buffer (4X)

Tricine Glycin SDS Sample Buffer

Tricine SDS Runing Buffer (10X)

Nu PAGE™ 4-12% Bis-Tris gel

Nu PAGE™ 16% Tricine gel

-Institute of Comparative Medicine

1X TBE buffer

10 X loading buffer (65% Sucrose, 10mM Tris-HCl, 10mM EDTA, 0.3% BPB, Bromophenol Blue up to 10ml dH_2O)

-Gibco

HEPES

-Sigma

β-mercaptoethanol

Bicinchonoinic Acid (BCA) solution

Bovine Serum Albumen (BSA)

Copper (II) sulphate (pentahydrate 4% (w/v) solution)

Ethidium bromide

Nonidet P-40 (NP-40)

Tween-20 (Polyoxyethylene sorbitan nonolaurate)

-Roche (Lewes, U.K.)

dATP

dCTP

dGTP

dTTP

dUTP

-University of Glasgow (Glasgow, U.K.)

Crude Ethanol

-VWR International (Dorset, U.K.)

Acetic acid

Dimethyl sulfoxide (DMSO)

100% pure ethanol

Ethylene diamine tetraacetate (EDTA) disodium salt

Hydrochloric acid

Magnesium chloride

Methanol

Propan-2-ol

Sodium acetate

Sodium chloride

Sodium dihydrogen orthophosphate

Sodium dodecyl sulphate (SDS)

Tris base

2.1.7 Enzymes and Kits

-Applied Biosystems (Warrington, U.K.)

Amplitaq® DNA polymerase with Geneamp® buffer

Bigdye v3.1 DNA sequencing kit

Hi-Di formamide

Taq-Man® EZ RT-PCR® kit

Taq-Man® β-actin Detection Reagents

-Invitrogen

Superscript one-step RT-PCR with platinum Taq (100 reaction)

The restriction enzymes *EcoR-I*, *Nhe-I*, *Begl-II* and their respective reaction buffers.

Calf Intestinal Alkaline Phosphatase (CIAP)

T4 DNA ligase

5X ligase buffer

-Promega

Transcription and Translation assay (T/T)

Canine Pancreatic Microsomal Membranes

-Qiagen Ltd (Crawley, U.K.)

QIAquick PCR purification kit

QIAprep plasmid maxiprep kit

QIAprep Spin plasmid miniprep kit

QIAquick Gel extraction kit

RNease mini kit

-Gentra Systems, USA

Generation Capture column kits

2.1.8 Molecular Weight Markers

-Invitrogen

1kb ladder

100bp ladder

See-Blue 2 protein markers

2.1.9 Plasmids

pcDNA empty vector carrying the universal immediate early (IE) promoter of cytomegalovirus (CMV) (Invitrogen, Glasgow, UK).

pL2 empty vector carrying the ED-L2 promoter of Epstein-Barr Virus (EBV) active only in basal epithelial cells (A kind gifts from Dr. J. Wilson, Division of Genetics, University of Glasgow).

pL2-16E5 carrying the E5 ORF of HPV-16 E5 under the transcriptional control of the EBV ED-L2 promoter, active only in epithelial cells (A kind gifts from Dr. H. Ashrafi, Institute of Comparative Medicine, University of Glasgow, Glasgow).

pc16E5 expresses the wild type HPV-16 E5 protein under the control of the CMV IE promoter.

pc16E5-R79 expresses a mutant of HPV-16 E5 protein, which lacks the last 5 amino acids, under the control of the CMV IE promoter.

pc16E5-A54 expresses a mutant of HPV-16 E5 protein, which lacks the third hydrophobic domain, under the control of the CMV IE promoter.

pc16E5-V36 expresses a mutant of HPV-16 E5 protein, which contains the first hydrophobic domain plus the 6 amino acids of the hydrophylic region between the firs hydrophobic domain and second hydrophilic region, under the control of the CMV IE promoter.

pc16E5-R30 expresses the mutant of HPV-16 E5 protein, which contains the first hydrophobic domain, under the control of the CMV IE promoter. (pc16E5 and the above mutants were kind gifts from Professor Alonso, German Cancer Research Centre, Heidelberg, Germany).

pc16E5-Del-1 expresses a mutant of HPV-16 E5 protein, which lacks the first hydrophobic domain, under the control of the CMV IE promoter.

pHA-1 (pcDNAHA-1) vector carrying three HA tag, a kind gifts from Dr.Winnie Boner, Institute of Comparative Medicine, University of Glasgow.

pEGFP-C1 is a eukaryotic expression plasmid for the Green Flourescent Protein (GFP). Expression is driven by the CMV promoter (BD Biosciences Clontech).

pEGFP-C1-16 E5 is a pEGFP plasmid expressing the GFP protein fused to the amino terminus of the full-length HPV-16 E5 protein.

pEGFP-C1-16 E5Del-1 expresses a mutant of HPV-16 E5 protein, which lacks the first hydrophobic domain.

pEGFP-C1-16 E5R79 expresses a mutant of E5 protein, which lacks the last 5 amino acids.

pEGFP-C1-16 E5A54 expresses a mutant of HPV-16 E5 protein, which lacks the third hydrophobic domain.

pEGFP-C1-16 E5V36 expresses a mutant of HPV-16 E5 protein, which contains the first hydrophobic domain plus the 6 amino acids of the hydrophylic region between the firs hydrophobic domain and second hydrophobic domain.

pEGFP-C1-16 E5R30 expresses a mutant of HPV-16 E5 protein, which contains the first hydrophobic domain.

pAL356HLA-A2 was a kind gifts from Dr Stephen Man from University of Cardiff, Cardiff, and HLA-A2 was cut from pAL356HLA-A2 and put in pBS/A2 between EcoRV/SpeI sites, which is a plasmid [pBlueScriptII SK(+)] carrying HLA-A2, a kind gifts from Barbara Marchetti, Institute of Comparative Medicine, University of Glasgow.

2.1.10 Radiochemical

-Promega

REDIVUE [^{35}S] Methionine

2.1.11 Miscellaneous

-Amersham International plc (Buckinghamshire, U.K.)

Hyperfilm ECL

-Eastman Kodak Co., Rochester, New York, USA

X-ray film

-Whatman International Ltd (Maidstone, U.K.)

Whatman 3MM filter paper

Whatman 1 filter paper

-Applied Biosystems Ltd (Warrington, U.K.)

Real-time PCR 96-well plates and their caps

-Costar Corporation (Bucks, UK)

96-well plates

-Elkay International (Basingstoke, U.K.)

Microcentrifuge tubes

Pastettes

-Equibio (Kent, U.K.)

2mm electroporation cuvettes

-Sigma

Protein G immobilised on agarose beads

Formaldehyde

Saponin

-Institute of Comparative Medicine

Diethyl Pyrocarbonate (DEPC)

-Invitrogen

Lipofectamin Reagent

Plus Reagent

NuPAGE 4x LDS sample buffer

NuPAGE 10x sample reducing agent

NuPAGE Antioxidant

-Roche Diagnostics, Mannheim, Germani

Protease inhibitor cocktail tablets

-Camlab, Cambridge, England

1.5 ml Screw Top Microtubes

-Amersham Biosciences

Amplify solution

-Merck, England

Steel and plastic cloning rings

Silicon Grease

-Safeway Supermarkets (Glasgow, UK)

Marvel (dried skimmed milk)

2.1.12 Water

Distilled water (dH_2O) for the preparation of buffer stocks was obtained from a Millipore MilliRo 15 system, and when used for protein, enzyme, RNA or recombinant DNA procedure was further purified on a Millipore MilliQ system to 18MΩ/cm. Sterile distilled water for making up tissue culture media was supplied by the Institute of Comparative Medicine Technical Service.

2.2 Methods

2.2.1 Transformation of Competent Bacterial Cells

E. coli DH5-α competent cells were used for the propagation of plasmid DNA unless stated otherwise. Stocks of competent cells were stored at –70°C until use when they were thawed slowly on ice. 20 µl of competent cells were aliquoted to a 1.5 ml micro-centrifuge tube and then 4 ng of plasmid DNA was added into each tube and left on ice for 30 minutes. Cells were heat shocked at 42°C for from 45 second to 1 minute and then returned to ice for 2-5 minutes. 180 µl sterile SOC medium (25 bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to each sample and the tubes were incubated at 37°C for 1 hour with shaking at 225 revolution per minute (rpm). 50 µl of the transformation mixes were spread on LB-agar plates containing the appropriate antibiotic, and then plates were inverted and incubated overnight at 37°C to allow colony formation.

2.2.2 Small Scale Preparation of Plasmid DNA (Miniprep)

Small amounts of plasmid DNA were obtained from transformed bacterial colonies to allow the identification of positive transformants. A single bacterial colony was used to inoculate 4-5 ml of L-broth [1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] medium in Bijoux tubes containing the appropriate antibiotic (100 µg/ml Ampicillin or 50 µg/ml Kanamicin unless stated otherwise) and was grown overnight at 37°C with shaking. The bacteria culture was transferred into 1.5 ml micro-centrifuge tube and pelleted by centrifugation at 14000 rpm for 1 minute. Plasmid DNA was prepared from the colony using the QIAprep Spin plasmid miniprep kit according to the manufacturer's instructions.

2.2.3 Large Scale Preparation of Plasmid DNA (Maxiprep)

One colony of the overnight bacterial culture was used to inoculate 200 ml of L-broth containing the appropriate antibiotic in a 0.5 litre glass conical flask. The culture was incubated overnight at 37°C with shaking at 225 rpm. Bacterial cells were transferred in a big centrifuge tube and pelleted by centrifugation 7000 rpm at 4°C for 15 minutes, using a Backman J2-21 centrifuge. The supernatant was removed and the bacterial pellet was resuspended in 10ml buffer P1 (50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100 μg/ml RNase A) and transferred to a medium centrifuge tube (JA-20), then gently mixed with 10ml buffer P2 (200 mM NaOH, 1% SDS (w/v)) and allowed to stand at room temperature for 5 minutes to ensure efficient lysis. Next, 10 ml of chilled buffer P3 (3M potassium acetate pH 5.5) was added and inverting the tube sharply several times mixed the contents. The solution was incubated on ice for 20 minutes and then centrifuged at 13000 rpm for 30 minutes at 4°C and the supernatant was removed in a fresh medium centrifuge tube then centrifuged at 13000 rpm for 15 minutes again. The bacterial debris forms a tight pellet on the bottom of the tube. The supernatant was filtered through nylon gauze and was loaded onto a QIAfilter Mega Cartridge. The column was washed twice with 30ml of QC (1M NaCl, 50 mM MOPS pH 7, 15% isopropanol (v/v), 0.15% Triton (v/v)). Plasmid DNA was eluted by 15 ml of QF (1.25 M NaCL, 50mM Tris-Cl pH 8.5, 15% isopropanol (v/v)). DNA was precipitated by the addition of 10.5 ml isopropanol. Nucleic acid was pelleted by centrifugation at 12000 rpm for 30 minutes, the supernatant was removed and the pellet was washed with 1 ml 70% ethanol (v/v). Next the nucleic acid was transferred to a micro-centrifuge tube and pelleted by centrifugation at 14000 rpm for 10 minutes, the supernatant was removed and the pellet was allowed to air dry. The pellet was re-suspended in 200 μl dH_2O and DNA quantification was determined as explained later (see section 2.2.21).

2.2.4 Cell culture

All cell culture work was performed following strict aseptic techniques inside a laminar flow hoods (Class-2 Microbiological Safety Cabinets, Gelaire BSB-4). Cells were incubated in a dry atmosphere at 37°C containing 5% (v/v) CO_2 (Nopco Scientific).

2.2.5 Maintenance of Human Keratinocyte cells

The human immortalised keratinocyte stable cell line (HaCaT) was grown in Dulbecco's Modified Eagle Medium (DMEM) without $CaCl_2$ supplemented with 1mM Sodium Pyruvate, 2mM L-Glutamine and 10% Fetal Calf Serum (FCS) at 37°C in a dry atmosphere containing 5% (v/v) CO_2. The cells were fed twice weekly, old medium was aspirated from sub-confluent tissue culture flask and fresh medium, which had been pre-warmed at 37°C was added. HaCaT cells were grown until 80% confluent and replating was performed as follows: the medium was aspirated off and the cells were washed twice with sterile phosphate-buffered saline (PBS). Then the PBS was removed and trypsin was added to cells (0.25% trypsin in 1x PE buffer; PBS with the addition of EDTA to 1 mM, pre-warmed to at 37°C). Flasks were incubated at 37°C until the cells had detached from the flasks (5-10 minutes). Complete medium was added to neutralised trypsin and the cells suspension transferred to a universal tube. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes at room temperature. The pellet was resuspended in fresh growth medium and the cells reseeded at an appropriate density.

2.2.6 HPV-16 E5 expression vectors

The HPV-16 E5 ORF from 3850 to 4101 of the HPV-16 was cloned in two different expression plasmids, either under the transcriptional control of the universal IE promoter of

CMV in pcDNA3 or pcI-neo (Invitrogen, UK) and called pc16 E5; or under the transcriptional control of the EBV ED-L2 promoter, active only in the basal epithelial cells, in pL2 and called pL2-16 E5 (A kind giftsss fro Dr. J. Wilson, Division of Genetics, University of Glasgow).

2.2.7 Generation of HPV-16 E5 Del-1

HPV-16 E5 Del-1 mutant protein was made by deletion of the first hydrophobic domain of the HPV-16 E5 ORF. The primers used to make deletion mutant in the HPV-16 E5 ORF were designed as follows: forward primer of the Del-1 was made from 91 to 105 of the HPV-16 E5 including *Nhe-I* restriction enzyme site and HA-tag and four non specific nucleotides; 5' CAT TGC TAG CAT GTA CCC ATA CGA TGT TCC AGA TTA CGC TCC GCT GCT TTT GTC T 3' and reverse primer of the HPV-16 E5 Del-1 was made from 252 to 231 of the HPV-16 E5 including *EcoR-I* restriction enzyme site and four non specific nucleotides; 5' TCG CGA ATT CTT ATG TAA TTA AAA AGC GTG 3'. All reagents were provided in the Genamp® PCR reagent Kit and core Reagents with AmpliTaq® DNA polymerase. For this mutation, 5 ng of pc16 E5 was mixed with 100 ng of each forward and reverse primers, with 1x PCR kit buffer, MgCL2, 2 units Taq DNA polymerase and dNTPs (32 μM of each dATP, dGTP, dCTP and dTTP) in a total volume of up to 50 μl dH_2O (control included distilled water) and then aliquoted into 0.5 ml GeneAmp PCR reaction microfuge tubes. The tubes were placed into the PCR machine (PTC-200 Peltier Thermal Cycle) to make PCR products. For PCR amplification, the sample was heated to 95°C for 3 minutes to inactivate DNase and ensure all DNA duplexes were melted, in addition to activating the Taq DNA polymerase. The DNA was then amplified for 28 times as follows: the sample was heated to denaturalise double stranded DNA at 94°C for 30 seconds, 57°C for 30 seconds for annealing and 72°C for

30 seconds to elongate DNA. After completion of the cycles, the reaction was incubated at 72°C for a further 7 minutes to ensure full extension and then got cold to 4°C. After PCR amplification, 5 µl of each sample was analysed by agarose gel electrophoresis using appropriate marker to check the correct product was amplified. The PCR products were digested by using appropriate restriction enzymes and reaction buffer and then purified by using PCR purification kit. The pcI-neo vector was digested (Figure 6) with *EcoR-I* and *Nhe-I* sites, which located multi-cloning site by using appropriate restriction enzymes and reaction buffer according to the manufacturer instructions.

Figure 6. pCI-neo vector multiple cloning region and circle map

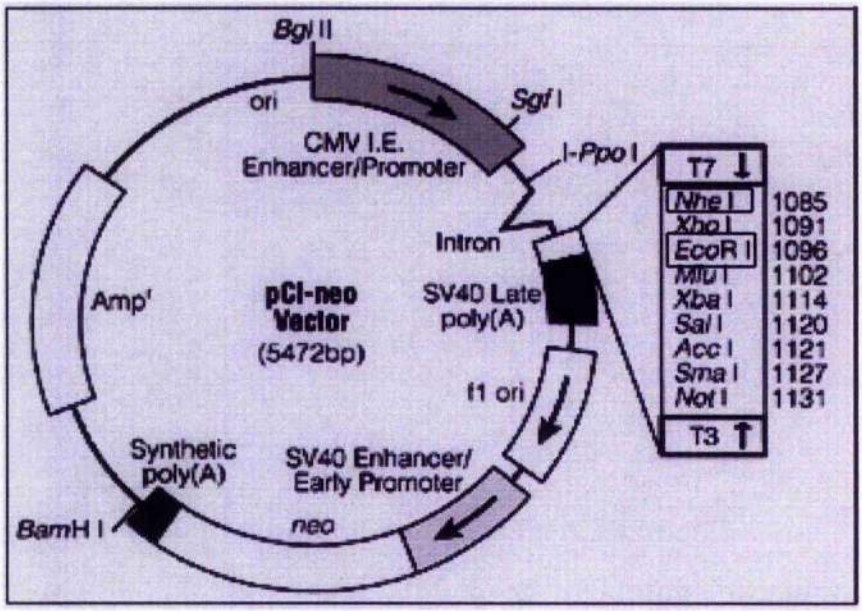


Figure 6: pCI-neo vector circle map and sequence reference points (Promega).

Neo = neomycin phosphotransferase; CMV I.E. = cytomegalovirus immediate-early.

The digestion of the pcI-neo vector was monitored by using agarose gel electrophoresis and the cut DNA was purified by using gel extraction kit. The DNA fragment was ligated into the

cut vector at different inserts:vectors ratio, 1:1, 3:1, 6:1 using 1 μl (4 Units) of T4 DNA ligase in 1x ligase buffer in a 20 μl reaction volume (see section 2.2.11). It was found that the best ratio of inserts:vectors was 6:1 for ligation. 5 μl of the ligated samples was transformed in *E.coli* DH5-α competent cells (see section 2.2.1). 50 μl of transformation mixes were spread on LB-agar plates containing the appropriate antibiotic (100 μg/ml Ampicilin) and incubated at 37°C overnight. A single bacterial colony was used to inoculate 5 ml L-broth medium containing the appropriate antibiotic in Bijoux tubes and grown overnight at 37°C (to select 10 colonies in 10 Bijoux tubes). Plasmid DNA was prepared from the colony using the QIAprep Spin plasmid miniprep kit according to the manufacturer's instructions (see section 2.2.2). The plasmid DNA was sequenced, confirmed and transfected into HaCaT cells.

2.2.8 Cloning of E5 and E5 mutants into a Green Fluorescent Protein plasmid

The pEGFP-C1 vector, which is a eukaryotic expression plasmid for the Green Fluorescent Protein (GFP) was digested with *EcoR-1* and *Bgl-II* sites, located in multi-cloning sites by using appropriate restriction enzymes and reaction buffer according to the manufacturer instructions. The primers were designed for making HPV-16 E5 and all mutants (R79, A54, V36, R30 and Del-1), with the oligonucleotides are listed as follows:

a) Forward primers of HPV-16 E5 and mutants (R79, A54, V36, R30) were from 1 to 22 of HPV-16 E5 sequences;

5' CAT TAG ATC TAT GAC AAA TCG TGA TAC TGC AT 3'

b) Forward primer of HPV-16 E5Del-1 was from 91 to 112 of HPV-16 E5 sequences;

5' CAT TAG ATC TCC GCT GCT TTT GTC TGT GTC TA 3'

c) Reverse primers HPV-16 E5 wild type and HPV-16 E5Del-1 were from 252 to 231 of HPV-16 E5 sequences;

5' TCG CGA ATT CTT ATG TAA TTA AAA AGC GTG CA 3'

d) Reverse primer HPV-16 E5R79 was from 237 to 216 of HPV-16 E5 sequences;

5' TCG CGA ATT CTT AGC GTG CAT GTG TAT GTA TTA AA 3'

e) Reverse primer HPV-16 E5 A54 was from 162 to 141 of HPV-16 E5 sequences;

5' TCG CGA ATT CTT AGG CTG CTG TTA TCC ACA ATA GT 3'

f) Reverse primer HPV-16 E5V36 was from 111 to 90 of HPV-16 E5 sequences;

5' TCG CGA ATT CTT AAG ACA CAG ACA AAA GCA GCG GA 3'

g) Reverse primer HPV-16 E5R30 was from 93 to 72 of HPV-16 E5 sequences;

5' TCG CGA ATT CTT AAC GTA TAA TAG GCA GAC A.

All forward and reverse primers were contained four non-specific nucleotides with restriction enzyme site (for forward primers *Bgl-II* and reverse primers *EcoR-I*) using for cloning. The DNA fragments were made by PCR and digested by using appropriate enzymes and reaction buffer. The DNA fragment was ligated into the cut vector with inserts: vectors ratio 6:1 and transformed in *E. coli* DH5-α competent cells. 50 µl of transformation mixes were spread on LB-agar plates containing 50 µg/ml kanamycin and incubated at 37°C for overnight. A single bacterial colony was used to inoculate 5 ml L-broth medium containing the appropriate antibiotic in Bijoux tubes and grown overnight at 37°C. Plasmid DNA was prepared from the colony using the QIAprep Spin plasmid miniprep kit according to the manufacturer's instructions (see section 2.2.2). The plasmid DNA was sequenced, confirmed and transfected into HaCaT cells.

2.2.9 DNA purification

DNA fragments for PCR used for cloning were routinely purified using the Qiagen QIAquick® PCR Purification Kit. DNA samples were added 5 volumes of buffer PB to 1 volume the PCR samples and mixed together. It was placed a QIAquick spin column in a provided 2 ml collection tube and centrifuged at 13000 rpm for 30-60 seconds. The DNA samples were washed with 0.75 ml buffer PE and centrifuged as before. The QIAquick column was placed in a clean 1.5 micro-centrifuge tube and added 50 µl or 30 µl H_2O to the center of the QIAquick membrane and then centrifuged the column at 13000 for 1 minute.

2.2.10 Digestion of DNA with restriction enzymes

Restriction digests were performed in small reaction volumes using the appropriate enzymes and reaction buffers according to the manufacturer instructions. Plasmid DNA was incubated with 5-10 units of restriction enzyme/µg DNA in a buffer solution. In general, small quantities of plasmid DNA (<5 µg) were digested in a 20 µl reaction volume for 2-3 hours at 37°C and large quantities of plasmid DNA were typically digested in a 50µl reaction volume overnight at 37°C. The digestion of the vector plasmid was monitored by using agarose gel electrophoresis and the cut DNA was purified by using a gel extraction kit. Digestion of HPV-16 E5 wild type and mutant fragments were carried out using the appropriate restriction enzymes at 37°C for overnight and purified by using DNA purification kit.

2.2.11 Ligation of DNA fragment

Both plasmid DNA and the DNA fragment to be inserted into the vector were separately digested using the appropriate restriction enzymes and isolated by gel electrophoresis and

then purified as described above (see section 2.2.10). The linearised vector DNA was dephosphorylated to prevent vector religation. 1 µl (1 unit) of calf intestinal alkaline phosphatase (CIAP) and dephosphorylation buffer were added at the end of a restriction digest reaction DNA vector and then the reaction mixture was incubated for 30 minutes at 37°C. The reaction was followed by a second incubation at 70°C for 10 minutes to stop all enzyme activity. The plasmid and DNA fragment were purified by using the PCR purification kit. The DNA fragment was ligated into the cut vector using 1 µl (4 units) of T4 DNA ligase in 1x ligase buffer in a 20 µl reaction volume. This was carried out according to the manufacturer's instruction. An excess of DNA fragment compared to vector was used for ligation reactions. Reactions were routinely incubated overnight at 16°C.

2.2.12 DNA sequencing

The fidelity of all plasmid constructions was verified using an Applied Biosystems 3100 automated DNA sequencer. The region to be sequenced was sequenced using a primer complementary to the appropriate region of the vector DNA (see table 1). Reactions containing 500 ng DNA, 3.2 pmol forward or reveres primer, 2 µl 5x buffer and 4 µl Big Dye Terminator Reaction premix were made up to 20 µl with dH_2O. 250 µl thin walled eppendorf tubes were used for all sequencing PCR reactions. Samples were heated to 95°C for 10 seconds, 50°C for 15 seconds and 60°C for 4 minutes. This cycle was repeated 25 times. PCR products were precipitated using Spin Column Purification. The purification columns were centrifuged at 3K for 2 minutes then transferred to sample collection tubes. Next, the PCR products were transferred into centre of the gel to spin at 3K for 2 minutes and dried under vacuum. The samples were resuspended in 25 µl Hi-Di formamide and transferred into 96-

wells plate. Subsequently the samples were analysed using an ABI3100 genetic analyser (sequencer) and confirmed by DNASTAR program.

Table 4. Oligonucleotides used for sequencing

Primer	Nucleotide Sequence	Plasmid Sequenced
Forward primer	5' CAT GGT CCT GCT GGA GTT CGT G 3'	pEGFP-C1
Reverse primer	5' CGT CGC CGT CCA GCT CGA CCA G 3	pEGFP-C1
T3 primer	5' T3 ATT AAC CCT CAC TAA ACG G 3'	pcI-neo
T7 primer	5' ATT AAT ACG ACT CAC TAT AGG GA 3'	pcI-neo

2.2.13 Seeding of cell lines

When seeding cells, the culture medium was removed and cells were washed with sterile PBS. 4 ml trypsin-EDTA was added into T175 cm^2 flasks, 2ml to T75 cm^2 and 1 ml to T25 cm^2 flasks. The cells were incubated at 37°C until the cells had detached from the flask bottom. Two volumes of appropriate medium were added to neutralise the trypsin and the cells were centrifuged at 1000 rpm for 5 minutes. The cells were resuspended in fresh medium at the required cell density and were maintained in new flasks as before.

2.2.14 Counting cells

Total live cell count was performed using trypan blue which stains dead cells dark blue. 50 µl of cell suspension was added to 50 µl of trypan blue (Gibco BRL, UK) and mixed. 10 µl of mixing sample was loaded onto a haemocytometer with a coverslip added. The number of

non-coloured cells in each of the 4 open squares was counted under 10x objective and a mean of these counts was taken. This mean multiplied by 1x 10^4 represented the number of cells per ml.

2.2.15 Transfection of HaCaT cells

HaCaT keratinocytes were stably transfected with a range of plasmid DNA; pcDNA, pL2, pHA-1, pc-16E5, pL2-16E5, pEGFP-C1, pEGFP-C1-16E5 and all mutants (R79, A54, V36, R30 and Del-1, see section 2.1.9) by using Lipofectamine Plus (Invitrogen). One day before transfection, 1x 10^6 HaCaT cells had been seeded in 80 mm petri dishes with 15 ml complete medium and incubated at 37°C for over night. The following day, the medium was removed and the cells were washed twice with sterile PBS, then 5 ml medium without FCS was added. 4 μg plasmid with 750 μl medium without FCS was mixed with 20 μl of Plus Reagent and incubated at room temperature for 15 minutes. 30 μl Lipofectamin Reagent was mixed with 750 μl medium without FCS in a separate reaction vial. Both solution were mixed together and incubated for 15 minutes at room temperature. This mixture was then slowly added to the petri dishes of cells and incubated at 37°C for 3 hours. After this time, cells were washed twice with PBS, fed with fresh medium with FCS and incubated at 37°C for overnight. Sixteen to eighteen hours later, the old medium was removed and the cells were re-fed with fresh growth medium.

2.2.16 Selection and Isolation of Transfected HaCaT cells

Following transfection, HaCaT cells were selected in medium containing 500 μg/ml G418 for 3-4 weeks. After this time, G418-resistant colonies were marked under the microscope using a microscope ring marker attachment. The cells were then washed with sterile PBS. A sterile 6

mm steel or plastic cloning rings coated with sterile silicon Grease (Merck, England) at the base was then placed over the identified colony thus providing a waterproof seal round each isolated colony. 100 µl of trypsin, which had been pre-warmed to 37°C was added within each cloning ring to cover the whole colony and incubated for 5-10 minutes at 37°C. An equal volume of complete medium which had been pre-warmed to 37°C was added within each cloning ring and the cell suspension transferred to a small flask (T25 cm^2) and incubated at 37°C. Where appropriate, several colonies were picked from each transfection class and expanded into cell lines for analysis.

2.2.17 Long-term cells storage

For long-term storage of cells, confluent cultures were trypsinised, and pelleted as described above. The pellet was then resuspended at a concentration of approximately $1x10^6$ cells/ml in growth medium containing 10% (v/v) DMSO and 40% (v/v) FCS. The DMSO in the medium acts as a cryoprotectant but all solution must be chilled as DMSO is toxic to cell at room temperature. Suspensions were aliquoted into 2 ml Nunc cryotubes and placed in a polystyrene box and frozen, well insulated, at -70°C for at least 24 hours to ensure a slow rate of cooling. The following day the tubes were placed in liquid nitrogen until further use. When needed, the ampoules were removed from liquid nitrogen and placed into a small, covered bucket of water at 37°C. Once thawed, the cells were added to 10 ml of the appropriate pre-warmed growth medium, centrifuged, re-suspended in fresh growth medium and transferred to a T75 cm^2 flask.

2.2.18 DNA preparation from HaCaT cells

For DNA extraction, HaCaT cells were grown until 80% confluent and washed with PBS and then tripsinized. The cells were washed twice with PBS and resuspended in 500 µl PBS and then applied into the Capture column by using Generation Capture column kits (Gentra Systems, USA). After quick centrifugation, the cells were washed twice with 500 µl purification solution and quickly centrifuged (2-3 seconds). Next, the cells were washed once with elution solution and pelleted by a quick centrifugation. 200 µl of elution solution was added again and wrapped with parafilm around the either top of the tube and then put them in the boiled water (99°C) for 10 minutes and then spin them down as above. The DNA was determined and the PCR product was performed using PCR purification kit.

2.2.19 RNA preparation from HaCaT cells

RNA extraction from cells was done using a Qiagen RNeasy kit with QIAshredder columns. Cells were grown until sub-confluent, washed twice with PBS and detached, then counted and lysed by addition of RTL buffer with *B*-mercaptoethanol (if the cells were less than $5x10^5$, it should be added 350 µl of RTL buffer with *B*-mercaptoethanol but if the cells were more than $5x10^5$ should be added 600 µl). Cells were then scraped into a QIAshredder spin column then centrifuged to homogenise the sample at 8-10x 10^3 rpm for 15 seconds. Next 350 µl or 600 µl of 70% ethanol was added to the lysate and the sample was added to an RNeasy mini column by centrifugation. Subsequently the column was washed once with 700 µl of RW1 and with 500 µl RPE by centrifugation as above. The sample was washed once again with 500 µl RPE by centrifugation at 8-10x 10^3 rpm for 2 minutes and then centrifugation at 14000 rpm for 1 minute to remove all RPE buffer. The purified RNA was eluted from the column in 30 µl or 50 µl of RNase-free water or dH_2O by centrifugation at 8-10x 10^3 rpm for 1 minute and then the concentration of nucleic acid was determined. Following preparation RNA sample prior to

RT-PCR, 1 μg of RNA sample, 1 μl of 10x DNase I reaction buffer, 1 μl of the DNase I Amp Grade 1U/μl, and up to 10 μl DEPC were mixed together and incubated 15 minutes at room temperature. For inactivation of the DNase I, 1 μl of 25 mM EDTA was added and mixed and then heated at 65°C for 10 minutes.

2.2.20 Concentration and purity determination of nucleic acids

The concentration of nucleic acid was determined by absorbance measurement at 260 nm and 280 nm using a Biotech spectrophotometer model UV1101. Samples were diluted in dH_2O and transferred to a quartz cuvette with a pathway of 1 cm. The spectrophotometer was initially calibrated using dH_2O only as a blank and the optical density (OD) reading were obtained at 260 nm and 280 nm. An OD_{260} reading of 1 corresponds approximately to 33 μg/ml single stranded DNA or 50 μg/ml double stranded DNA and for RNA an OD_{260} of 1 was taken to correspond to 40 μg/ml. The ratio, which is 1.8 for DNA and 2 for RNA between reading at OD_{260} and OD_{280} provided an estimate of the purity of the nucleic acid.

2.2.21 Protein extract preparation

The cells were washed twice with PBS, then trypsinised, and washed two times with 10 ml of ice cold PBS. The cell pellet was then resuspended in 1ml of ice cold PBS and transferred to a 1.5 ml microcentrifuge tube and pelleted. They were then resuspended in 100 μl of lysis buffer (0.5% NP40, 50mM Tris pH 7.8, 150 mM NaCl with a protease inhibitor cocktail (Roche, Lewes, UK) dissolved in the lysis buffer. The extracts were then incubated on ice for 30 minutes with occasional mixing. Following this they were centrifuged in a refrigerated microfuge for 10 minutes at maximum speed at 4°C. The supernatant was then removed to

another tube and the cell debris discarded. The protein concentration was then determined using the BCA/$CuSO_4$ assay.

2.2.22 Amplification of HPV-16 E5 wild type and all mutants DNA sequences from HaCaT cells

For PCR amplification of DNA from HaCaT cells to see if viral E5 sequences were in the cell transfected. HaCaT cells expression with HPV-16 E5 wild type and HPV-16 E5Del-1 mutant were grown until sub-confluent and tripsinized and then washed with 500 µl PBS (see section 2-2-18). All reagents were provided in the Genamp® PCR reagent Kit and core Reagents with AmpliTaq® DNA polymerase. Primer sequences are followed; forward primer of E5 was designed from 91 (5'CCG CTG CTT TTG TCT GTG TCT A 3') to 112 and reverse primer was designed from 249 (5'TGT AAT TAA AAA GCG TGC ATG TGT A 3') to 225. The reaction mixture comprised, see section, 32 µM of each dATP, dGTP, dCTP and dTTP, 1 × PCR kit buffer, 100 ng of each primer, 2 units Taq polymerase and 5 ng of DNA sample (controls included distilled water and 5 ng of pc16E5) up to 50 µl dH_2O and then aliquoted into 0.5 ml GeneAmp PCR reaction microfuge tubes. The tubes were placed into the PCR machine (PTC-200 Peltier Thermal Cycle) and heated to 95°C for 3 minutes to inactivate DNase and ensure all DNA duplexes were melted, in addition to activating the Taq DNA polymerase. The DNA was then amplified for 28 cycles at 94°C for 30 seconds, 57°C for 30 seconds, to allow the primers to anneal to the template DNA, followed by 72°C for 30 seconds to allow extension of the amplimer sequences. After completion of the cycles, the reaction was incubated at 72°C for a further 7 minutes to ensure full extension and then cooled to 4°C. 5 µl of each sample was analysed by agarose gel electrophoresis (as described before) to check the correct product was amplified.

2.2.23 Amplification of E5 RNA from HaCaT cells by RT-PCR

RNA was prepared (see 2.2.19) and used as the template for reverse transcription and PCR amplification by using Superscript one-step Reverse Transcriptase PCR (RT-PCR) with platinum Taq, 100 reaction (from Invitrogen Ltd.). Primer sequences are as follows: forward primer of HPV-16 E5 was designed from 91 (5'CCG CTG CTT TTG TCT GTG TCT A 3') to 112 and reverse primer was designed from 249 (5'TGT AAT TAA AAA GCG TGC ATG TGT A 3') to 225. For each sample were used 25 µl of 2x Reaction Mix, 1µl RT/Platinum® Taq Mix, 100 ng RNA sample and up to 50 µl dH_2O. Amplification proceeded at 50°C for 30 minutes and then 94°C for 2 minutes and then for 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 70°C for 45 seconds and extension at 72°C for 10 minutes. PCR was carried out in a PTC-200 Peltier Thermal Cycle. 5 µl of each sample containing 1x loading buffer was then analysed by agarose gel electrophoresis to ensure correct amplification. Primers sequences for β-actin quantitation were purchased from Applied Biosystems.

2.2.24 Real time RT-PCR

Total RNA was isolated from HaCaT cells using the RNeasy Mini kit (Qiagen, Sussex, UK), and residual DNA was removed by DNase I treatment according to the manufacturer's guidelines (Invitrogen, UK). Real-time RT-PCR for HPV-16 E5 wild type, all mutants and β-actin mRNA was carried out using the Taq-Man EZ RT-PCR kit (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in triplicate using 100 ng of RNA samples, 5 µl of 5x TaqMan E2 buffer, 6 µl of 25 mM $MgCl_2$, dNTPs (16 mM of each dATP, dGTP, dCTP and dUTP), 10 µM of each forward and reverse primer, 5µM of probe, 2.5 U/µl of the rTth DNA polymeras, 1U/µl of Amperase and up to 50 µl sterile dH_2O. Oligonucleotide primers for HPV-16 E5 wild type and all mutants (R79, A54, V36, R30 and Del-1) were

designed using Primer Express (v1.7, Perkin-Elmer, Oak Brook, IL) as follows in the table 5 (Forward=F, Reverse=R and probes).

For β-actin quantitation, the primers and probe sequences were purchased from Applied Biosystems. The samples were placed into the 96-well real time RT-PCR plate and amplified in triplicate. Amplification was proceeded at 60°C for 30 minutes, 95°C for 5 minutes and then for 40 cycles of 94°C for 20 seconds and 62°C for 1 minute. PCR reaction was performed using an ABI prism 7700 sequence detector. Standard carves were generated using 10-fold serial dilutions of each template DNA, which were used to quantitate the relative levels of E5 and β-actin mRNA. The Level of E5 mRNA was normalized according to the β-actin controls.

Table 5. Oligonucleotides used for Real Time RT-PCR

Primer	Nucleotide Sequence
F. primer E5,R79,A54,V36,R30	5' GAC AAA TCG TGA TAC TGC ATC CA 3'
R. primer E5,R79,A54	5'CTG CTG TTA TCC ACA ATA GTA ATA CCA ATA G3'
R. primer V36,R30	5' TAA TAG GCA GAC ACA CAA A 3'
Probe E5,R79,A54,V36,R30	5'AAC ATT ACT GGC GTG CTT TTT GCT TTG CT3'
F. primer Del-1	5' CTG CTT TTG TCT GTG TCT ACA TA 3'
R. primer Del-1	5' TAT AAT ATA TAC AAT AAA ACA CCT AA 3'
Probe Del-1	5' TTA ATA ATA TTG GTA TTA CTA TTG TGG ATA 3'

2.2.25 Detection of MHC class I by Fluorescence Activated Cell Sorting

Fluorescence Activated Cell Sorting (FACS) was used to analyse the level of surface and total MHC class I in the control cells and cells expressing HPV-16 E5 wild type and mutants. HaCaT cells were grown in big flasks (T175 cm^2) with complete DMEM until sub-confluent. After removal of the medium, the cells were washed once with PBS, then detached from the flask with trypsin/EDTA and pelleted at 1000 rpm for 5 minutes at room temperature. The cell pellet was re-suspended in complete medium for 1 hour at 37°C to allow surface antigens to be re-expressed. The cells were washed and re-suspended in PBS/1% BSA (Sigma) at $1x10^7$ cells/ml. Next, the cells were divided in four microcentrifuge tubes (for surface and total MHC class I) and incubated at room temperature for 30 minutes. For the detection of surface MHC class I, 100 µl of cells were aliquoted and incubated with an equal volume of either MHC class I monoclonal antibody (mAb) W6/32 (1/100; Serotec) or anti-HLA-C/E mAb DT9 (1:50; a kind giftss from Dr. Veronique Braud, Centre National de la Recherche Scientifique, Sophia Antipolis, France) at 4°C for 30 minutes. The cells were washed three times in PBS-1%BSA and incubated with anti-mouse IgG-FITC (1/100; Sigma) at 4°C for 30 minutes in the dark. The cells were washed as above and re-suspended in 500 µl PBS-1% BSA and analysed by FACS. If the FACS analysis was not performed immediately, the cells were re-suspended in 500 µl of 3.4% paraformaldehyde (fixing solution) in PBS and kept at 4°C in the dark. For the detection of intracellular MHC class I, the cells were treated as above and permeabilised with 0.5% Saponin (Sigma) in PBS-1% BSA for 30 minutes at room temperature. Following a wash in PBS 1% BSA solution, the permeabilised cells were incubated with primary antibody as described above. All samples were examined in a Beckman Coulter EPICS Elite analyser equipped with an ion argon laser with 15 mV of excitation at 488 nm. The data were analysed using Expo 2 software.

2.2.26 Detection of MHC class I by immunofluorescence microscopy

HaCaT cells were aliquoted into 24-well plates containing coverslips, at $1x10^4$ cells per well, approximately 25-50% confluent, and grown overnight (at least two coverslips cell line). After removal of the medium, cells were washed twice with PBS and fixed in fixing solution (19ml PBS, 1ml 37% formaldehyde and 0.4g sucrose) for 10 minutes at room temperature. After that, the cells were washed twice and incubated in permeabilising solution (19 ml PBS, 1 ml 10% NP40 and 0.4 g sucrose) at room temperature for 10 minutes and then washed twice with PBS+1% FCS, then incubated with mAb W6/32 (1/50) against MHC class I or DT9 (1/50) against HLA-C and E for 1 hour at room temperature and washed twice as above. The cells were then incubated with anti-mouse IgG-FITC (1/500; Sigma) at room temperature for 1 hour in the dark. Next, the cells were washed twice with PBS and incubated with DAPI (1/1000, Sigma), which stains DNA for 10 minutes at room temperature in the dark. Following two final washes with PBS and one with distilled water, the slides were mounted in Citifluor™ (glycerol/PBS solution, Sigma) and analysed with a Leica DM-IRE2 fluorescence confocal microscope at 488nm wavelength for FITC, 405 for DAPI and 543 for TRITC. Images were acquired using Leica confocal software.

2.2.27 Visualisation of Golgi apparatus by immunofluorescence microscopy

The Golgi apparatus was visualised by immunofluorescence detection using mAb 4A3 raised against golgin GM130, an integral GA protein (1/200; Sigma) or Bodipy®-TR Ceramide (Molecular Probes Europe BP, The Netherland). HaCaT cells were aliquoted, grown, fixed and permeabilised as described above (see section 2.2.26). The cells were incubated with mAb 4A3 for 1 hour at room temperature and washed twice with PBS+1% FCS and then incubated with anti-mouse IgG- TRITC (1/1000, Sigma) or FITC (1/500) at room temperature

for 1 hour in the dark. Following three final washes (twice with PBS and one with dH_2O), the slides were mounted in Citifluor™ and analysed with a Leica DM-IRE2 fluorescence confocal microscope. Images were acquired using Leica confocal software. For Bodipy®-TR staining (Golgi staining), the cells were washed twice with DMEM without FCS plus 25 mM Hepes (PH 7.5). 200 µl of 5 mM Bodipy®-TR in DMEM without serum plus 25 mM Hepes was added and incubated at 4°C for 30 minutes and then the cells were washed twice as above. Next, 200 µl of DMEM without FCS 25 mM Hepes was added and incubated at 37°C for 30 minutes. The cells were washed twice with PBS and fixed with fixing solution as above (see 2.2.26).

2.2.28 Co-localisation of Golgi apparatus and MHC class I

For co-localisation of Golgi apparatus and MHC class I in the control HaCaT cells (pcDNA and pL2) and cells expressing HPV-16 E5 (pc16E5 and pL2-16E5), the cells were aliquoted, grown, fixed and permeabilised as above (see section 2.2.26). The cells were washed twice with PBS+1% FCS and incubated with mAb 4A3 at room temperature for 1 hour. The cells were washed as above and incubated with anti-mouse IgG-TRITC (1/1000, Sigma) and with mAb W6/32 conjugated with FITC (1/10, Sigma) at room temperature for 1 hour in the dark. The cells were then washed twice as above and incubated with DAPI for 10 minutes at room temperature in the dark. Following three final washes (twice with PBS and once with dH_2O), the slides were mounted in Citifluor™ and analysed as above. The merge between FITC and TRITC fluorescent signals was achieved with the Leica confocal software.

2.2.29 SDS-Polyacrylamide Gel Electrophoresis

Protein samples were resolved according to the molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were electrophoresed on Nu PAGE™ 4-12% Bis-Tris gel (Invitrogen, UK) under denaturing conditions. Prior to loading, equivalent amount of each protein samples (10 µg) was mixed with 5µl of 4x SDS gel loading buffer (Invitrogen, UK) and 2 µl of 10x reducing agent (Invitrogen, UK) made up to 20 µl with dH_2O. Samples were then boiled at 75°C for 10minutes. The prepared protein samples were then loaded into consecutive wells and 10 µl See Blue® Plas2 (Invitrogen), which is a protein molecular weight marker, added to the first and/or last well on the gel. The gel was run by electrophoresis at a constant voltage of 200V for 45 minutes. The running buffer (Nu PAGE™ MES buffer, Invitrogen, UK) was used with 500 µl antioxidant (Invitrogen, UK) added to 200 ml of the running buffer in the inner tank. Once the dye front was approximately 1-3 cm from the bottom of the gel, the gel was removed and used for western blot.

2.2.30 Detection of MHC class I by Western Blotting

HaCaT cells were removed from the flasks by trypsinisation, washed with PBS, then lysed by syringe in lysis buffer (100mM Tris HCl, pH 7.5, 2% SDS, 20% glycerol) and insoluble material was removed by centrifugation at 20,000g. 10 µg of lysate were electrophoresed in 4-12% NuPAGE gels (Invitrogen), and proteins transferred to nitrocellulose membrane (Invitrogen) using a semidry blotting apparatus at 30V/150A for 1 hour. For this purpose the gel was removed from the electrophoresis tank and transferred onto a nitrocellulose membrane (Invitrogen, UK). Before transferring to the membrane, the Nu PAGE™ gel sandwich was pre-soaked with transfer buffer with 500 µl antioxidant added to 200 ml of

transfer buffer (with 10% or 20% methanol depending on the number of gels being transferred). The gel sandwich contains a pre-cut nitrocellulose membrane sandwiched between two filter papers. The gel tank was set up as follows: the first sheet of filter paper, soaked in transfer buffer was placed neatly on to the gel avoiding any air bubbles. Then the nitro-cellulose membrane was laid on the gel, and then a further sheet of filter paper, all soaked in transfer buffer was added to the top. Next, the sandwich was rolled with a glass pipette to eliminate any air bubbles and placed into the transfer tank and run at 30V for approximately 60 minutes, the time taken for the pre-stained marker proteins to be completely transferred. Once the transfer was completed, the membrane was blocked by shaking for a minimum of 1 hour in 50 ml of block buffer (5% Marvel [dried milk] in PBS-0.01% Tween) at room temperature. The nitro-cellulose filter was washed in washing buffer PBS-Tween (PBS-T) for 10 minutes. The filter was then placed in 7 ml blocking buffer containing mAb HC-10 (1/50; Kind giftss from Dr. S. Man) or mAb MEM-E2/02 against HLA/E (1/50; Serotec) and mAb AB-1 (anti-actin, 1/1000) and incubated at room temperature for 1 hour with gentle shaking. The primary antibody solution was removed and the filter rinsed in blocking buffer then washed 1x 15 minutes and 2x 5 minutes in 100 ml volumes of fresh PBS-T. The filter was then incubated in 20 ml blocking buffer containing anti-mouse Horse Radish proxidase (1/5000, Sigma) for 1 hour at room temperature with gentle shaking. The filter was washed 1x for 15 minutes and 2x for 5 minutes with PBS-T buffer. Excess surface liquid was removed from the filter by briefly blotting with a piece of Whatman 3MM paper. The detection consisted of incubating the filter in an equal volume of an Amersham Enhanced chemilluminescence (ECL) detection reagents 1 and 2 for 5 minutes at room temperature. The excess detection solution was drained off the nitro-cellulose filter and this was then wrapped

in Saran wrap and exposed to Amersham ECL film for 30 seconds and up to 30 minutes (depending on the strength of the signal).

2.2.31 Treatment of cells with interferon

One million parental HaCaT cells, control cells carrying empty vectors and cells expressing HPV-16 E5 were seeded in tissue culture dishes. The following day, the old medium was removed and cells were washed once with sterile PBS then the fresh medium was added with or without 500 U/ml β-IFN (Sigma). Next the cells were incubated in a dry atmosphere at 37°C containing 5% (v/v) CO_2 for 48 hours. After 48 hours, the cells were harvested for detection of MHC class I by immunofluorescence staining or FACS analysis as described before (see sections 2.2.25 and 2.2.26).

2.2.32 Immunoprecipitation and Co-Immunoprecipitation

2.2.32.1 *In vitro* transcription and translation assay

For translation of proteins labelled with [^{35}S]methionine (Amersham Pharmacia Biotech) HPV-16 E5 wild type and mutants; R79, A54, V36, R30 and Del-1 transcription and translation proteins synthesized *in vitro*, were preformed as follows: 20 µl of Transcription and Translation assay (T/T), 1 µl Canine Pancreatic Microsomal Membranes (from Promega), 2 µl of [^{35}S]methionine, 0.5-1 µg DNA plasmids and dH_2O up to 25 µl were added into 1.5 ml Screw Top Microtubes (Camlab, Cambridge, England) and incubated at 30°C for 90 minutes to translate protein. Prior to loading, 5µl of each protein samples was mixed with 5µl of 4x SDS gel loading buffer (Invitrogen, UK) and 2 µl of 10x reducing agent (Invitrogen, UK) made up to 20 µl with dH_2O. Samples were then boiled at 75°C for 10 minutes. Separated protein samples were transferred to the SDS-PAGE gel at a constant voltage of 200V for 45

minutes and fixed using fixing solution (50% Methanol, 10% Acetic Acid, 40% dH_2O) for 1 hour and then incubated in Amplify solution (Amersham Biosciences) for 20-30 minutes. The gel was dried and then visualized by autoradiography to check the size of protein.

2.2.32.2 *In vitro* immunoprecipitation and co-immunoprecipitation assays.

Immunoprecipitations and co-immunoprecipitation of HPV-16 E5 and all mutants were performed (see section 2.2.32.1) as follows: 25 µl of translated protein was added in the tube with 3 µl of HA antibody (1/10; Sigma) against HA-tag on the HPV-16 E5 and all mutants, 10 µl E5 N-terminus/ E5 C-terminus antisera (1/3; kind gifts from Dr.DiMaio) against E5, or 10 µl HC10 antibody against HLA-A2 (1/3) and then incubated at 4°C for overnight on the rotator. For co-immunoprecipitations, 25 µl of HLA-A2 translated samples with 25 µl of HPV-16 E5 or mutants were mixed in the tube and incubated at 4°C for 4-6 hours and then appropriate antibody (6 µl HA antibody, 20 µl E5 N-terminus/ E5 C-terminus antisera or 20 µl HC10 antibody) added and incubated at 4°C overnight on the rotator. Next 10 µl or 20 µl of Sepharose beads protein-G (Amersham Pharmacia) with protease inhibitor cocktail (Roche) was added to the mixture and incubated for 1 hour at 4°C. The beads were washed two times with washing buffer, once with washing buffer I or high salt (50 mM Tris HCL PH 7.5, 500 mM NaCl, 0.1% Nonidet-P 40, 0.05% NaDoc and up to 10 ml dH_2O) and once with washing buffer II or low salt (50 mM Tris HCL PH 7.5, 0.1% Nonidet-P 40, 0,05% NaDoc and up to 10 ml dH_2O). The immunoprecipitation samples were resuspended in the 5 µl of 4x SDS gel loading buffer with 2 µl of 10x reduce agent and boiled at 75°C for 15 minutes. The bound proteins were resolved by SDS-PAGE and dried and then visualized by autoradiography.

2.2.33 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using horizontal gel cast apparatus (Biorad). 1-1.5% agarose gels were routinely used. The appropriate amount of ultrapure electrophoretic grade agarose was dissolved in 1x TBE buffer (10x TBE: 900mM Tris base, 900 mM boric acid, 25 mM EDTA, pH 8.0) by heating the solution in a glass conical flask in a microwave. The gel cast apparatus with a comb containing the appropriate number and size of teeth to form the sample wells was assembled and the gel was poured. The solidified gel was placed in the gel tank and submerged in 1x TBE buffer containing 0.5 μg/ml ethidium bromide. Samples containing 1x loading buffer (10x loading buffer is 65% (w/v) sucrose, 10mM Tris-HCl pH7.5, 10 mM EDTA, 0.3% (w/v) Bromophenol Blue) were loaded into individual wells. An appropriate sized DNA ladder was loaded into the first and/or last well in the gel and the DNA was separated by running at 100-150 constant voltage until the dye front was 1-3 cm from the end of the gel. Separated DNA was visualised by illumination on a short wave (312 nm) UV light box and photographed using UVP Gel Documentation System. Agarose gel images were captured on a P.C.

Chapter Three

Results

3 Results

Introduction

The analysis of the HPV-16 DNA sequence revealed that E5 ORF is commonly located at the 3' end of the early region before the L2 ORF and overlaps the E2 ORF in the early region (Bravo *et al.*, 2004). E5 ORF encodes a small hydrophobic membrane protein, 83 amino acids long, located mainly at the endosomal membranes, GA and occasionally the plasma membranes (Burkhardt *et al.*, 1989 and Conrad *et al.*, 1993). By hydropatic analysis, it has been determined that HPV-16 E5 protein has three hydrophobic domains connected by less hydrophobic regions (Bubb *et al.*, 1988). In contrast to BPV-1, where E5 is a major oncoprotein, little is known about the biological activity of HPV-16 E5. However, the amino acid analysis of the HPV-16 E5 protein and BPV-1 E5 has revealed that they are very different. The HPV-16 E5 protein is nearly twice as long as BPV-1 E5, but some limited sequence similarities have been suggested between the HPV-16 E5 and BPV-1 E5 proteins such as a few hydrophobic residues (Bubb *et al.*, 1888).

Previous studies have shown that cells transformed by BPV E5 show retention of MHC class I molecules in the GA and inhibition of transport of the complex to the cell surface (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002; O'Brien and Campo, 2003). Also BPV E5 inhibits both transcription of the MHC class I heavy chain gene and affects the stability of the heavy chain protein (Ashrafi *et al.*, 2002). Furthermore, BPV E5 down-regulates MHC class I in clinical samples of BPV-4 papillomas (Araibi *et al.*, 2004). The MHC class I molecule plays an important role in the eradication of virally infected and transformed cells. The importance of MHC class I in virus clearance is highlighted by the acquisition of numerous mechanisms of interference with the MHC class I pathway by many viruses (Hewitt, 2003). The MHC class I molecules are subdivided into two families, MHC class Ia or classical, and MHC class Ib or

non-classical. The classical molecules are a family of exceedingly polymorphic cell surface glycoproteins found on almost all nucleated somatic cells. These proteins play an important role in alerting the immune system to virally infected cells. Independently of the molecular nature of these mechanisms, employed by viruses to avoid host immunosurveillance the outcome is failure of the infected cells to effectively present viral peptides to effector CTL, resulting in avoidance of detection and destruction. The function of the non-classical MHC I molecules may have more specialized antigen presentation activities. In humans, the non-classical MHC I molecule binds a wide range of peptides derived from cellular proteins and has been suggested to play an important role in the maintenance of maternal tolerance to the fetus by interacting with inhibitory receptors on NK cells (Lee *et al.*, 1995).

The aim of this study is to investigate the ability of HPV-16 E5 to down-regulate classical and non-classical MHC I, and to investigate the mechanisms by which HPV-16 E5 mediates this down-regulation.

3.1 HPV-16 E5 down-regulates MHC class I

3.1.1 HPV-16 E5 constructs

HPV-16 E5 ORF was cloned into two different vectors: pcDNA and pL2. pcDNA is a universal expression vector in which the inserted gene is under the control of the CMV early promoter, while pL2 is a plasmid in which expression is directed by the epithelial cell-specific promoter of EBV ED-L2. In both cases the E5 ORF was tagged at its 5' and with the sequence encoding the HA epitope, a small polypeptide of nine amino acids (YPYDVPDYA).

3.1.2 Transfection of HPV-16 E5 in human keratinocyte cells (by Dr.H.Ashrafi)

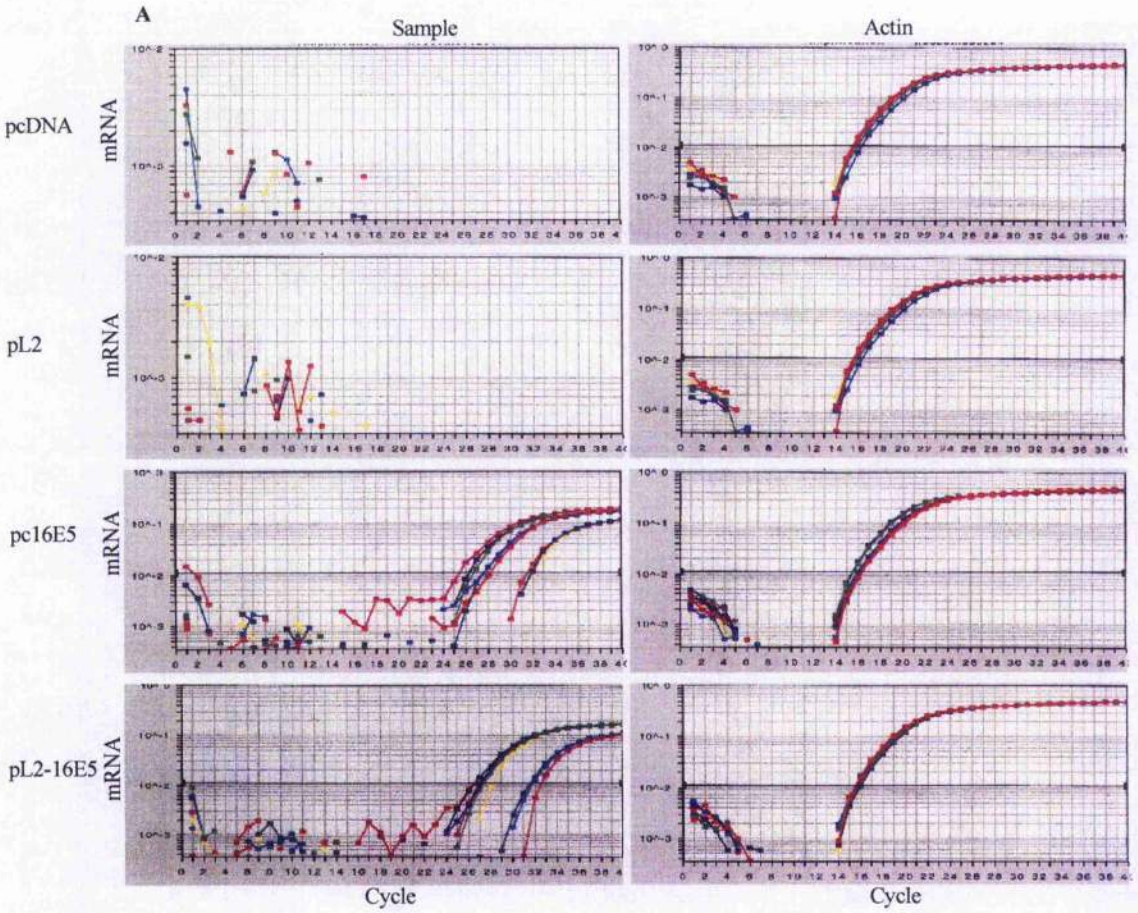
As E5 is expressed at very low levels in cells and there are no reliable antibodies against the protein, it is very difficult to detect its expression (Disbrow et al., 2003). Therefore, we used HA-(YPYDVPDYA, peptide from human influenza haemaglutinin protein) tag was incorporated at the N-terminus of HPV-16 E5 wild types and mutants. The human keratinocyte (HaCaT) cells were stably transfected with a range of plasmid DNA; pcDNA, pL2, pc16E5 and pL2-16E5 by using Lipofectamine Plus to investigate the role of HPV-16 E5 on MHC class I. One day before transfection, 1x 10^6 HaCaT cells had been plated in 80 mm petri dishes with appropriate medium and incubated at 37°C in 5% CO_2 overnight following the transfection protocol (see section 2.2.15). Following transfection, the cells were selected in medium containing 500μg/ml G418 for 21 days. After this time, G418 resistant clones were marked, individually picked from each transfection class and expanded into cell lines for analysis (see section 2.2.16).

3.1.3 E5 expression in HaCaT clones

It has been established that E5 is expressed at low levels in cells and there are no reliable antibodies against the E5 protein (Disbrow *et al.*, 2003). Therefore, we investigated that the E5 ORF was being transcribed in HaCaT cells using quantitative RT-PCR. RNA was isolated and the relative level of E5 mRNA expression was determined by comparison to β-actin mRNA using Real-time RT-PCR protocol (see section 2.2.21 and 2.2.24). We analysed three pc16E5 and three pL2-16E5 clones for the presence of E5 transcripts. In each experiment, additional reactions, with 10 fold serial dilutions of template DNA, were performed with each set of primers and probes on the same 96-well plates to generate standard curves. The quantitive RT-PCR was used to determine the relative amount of HPV-16 E5 and β-actin

mRNA in triplicate experiments of each E5 transfected cell line and from cells carrying empty vectors by using a standard curve (Figure 7A). The results show that all of the transfected cells (pc16E5 and pL2-16E5) express the E5 gene but at 10,000 fold less than β-actin (Figure 7B). As expected, no E5 RNA was amplified in the control cells (Figure 7A).

7. Expression of HPV-16 E5 RNA in transfected cell lines



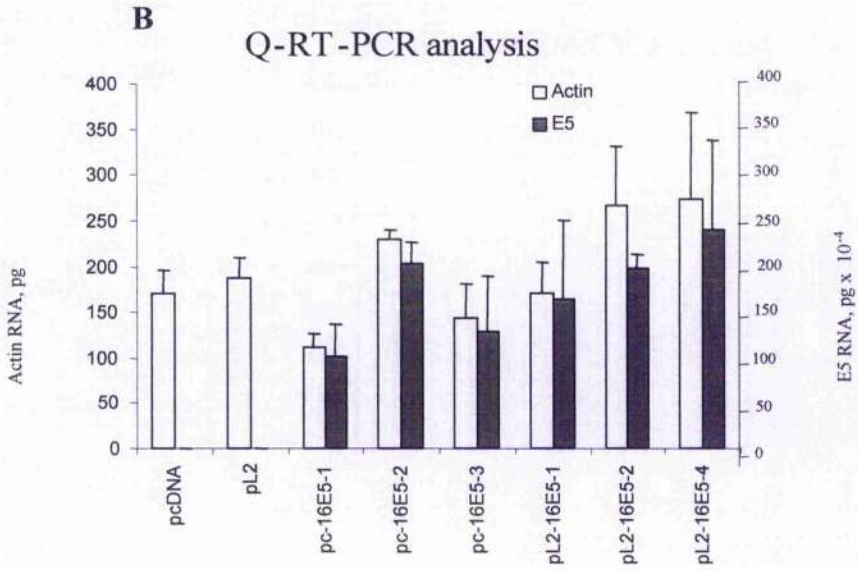


Figure 7: Taq-Man RT-PCR was used to determine the expression of E5 mRNA in the transfected HaCaT cells. **A)** The expression of HPV-16 E5 and actin mRNA was determined in HaCaT control cells (pcDNA and pL2) (one clone of each of pcDNA and pL2) or cells expressing HPV-16 E5 (pc16E5 and pL2-16E5) (three clones of each of pc16E5 and pL2-16E5) and each colour represents an RT-PCR experiment. **B)** Relative amount of HPV-16 E5 and actin mRNA in HaCaT control cells (pcDNA and pL2) or cells expressing HPV-16 E5 (pc16E5 and pL2-16E5). E5 is expressed at 10,000 fold less than β-actin.

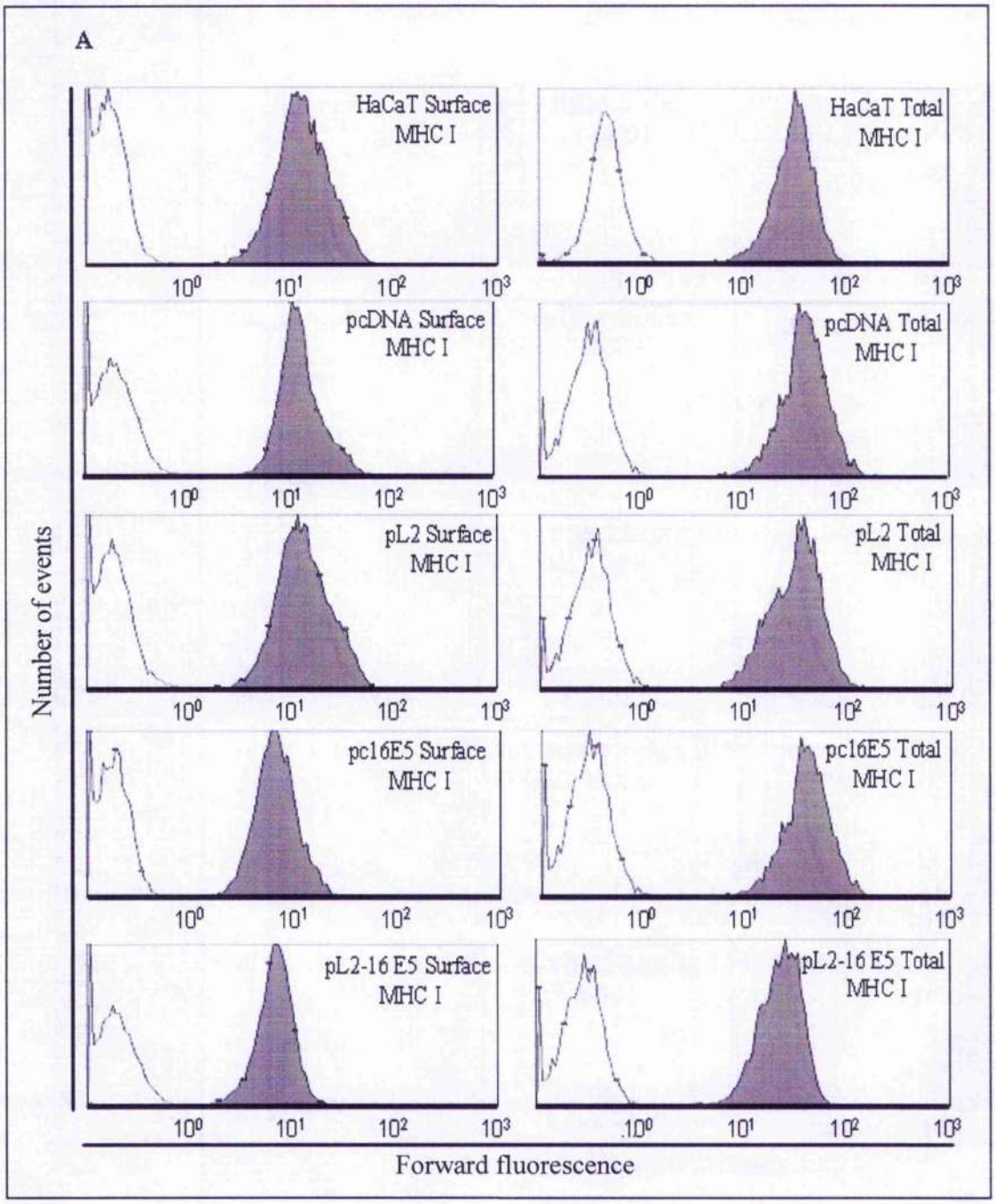
3.1.4 Down-regulation of MHC class I in cells expressing HPV-16 E5

The effect of HPV-16 E5 on MHC class I was investigated by FACS, immunofluorescence microscopy and western blots. FACS was used to investigate the level of MHC class I on the cell surface. For intracellular analysis; immunofluorescence microscopy was used to show the cellular localization of MHC class I, and western blots to show the expression of the MHC class I heavy chain in HaCaT parental cells, cells caring empty vectors or cells expressing HPV-16 E5.

3.1.4.1 Detection of MHC class I by FACS

It has been reported that BPV E5 can inhibit transport of MHC class I molecules to the cell surface (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). We investigated whether HPV-16 E5 would likewise down-regulate surface MHC class I. Three clones of HaCaT parental cells, control pcDNA and pL2 cells or pc16E5 and pL2-16E5 cells were incubated with mAb W6/32 (anti-MHC class I) and analysed for levels of both surface and total MHC class I. We determined that the clones harbouring empty vectors were no different from parental HaCaT cells, with approximately twice as much total MHC class I than surface MHC class I. On the contrary, all of the cells expressing HPV-16 E5 (pc16E5, pL2-16E5) the level of surface MHC class I reduced to approximately half that of control cells whereas the level of total MHC class I remained constant (Figures 8A and 8B).

8. Down-regulation of MHC class I by HPV-16 E5



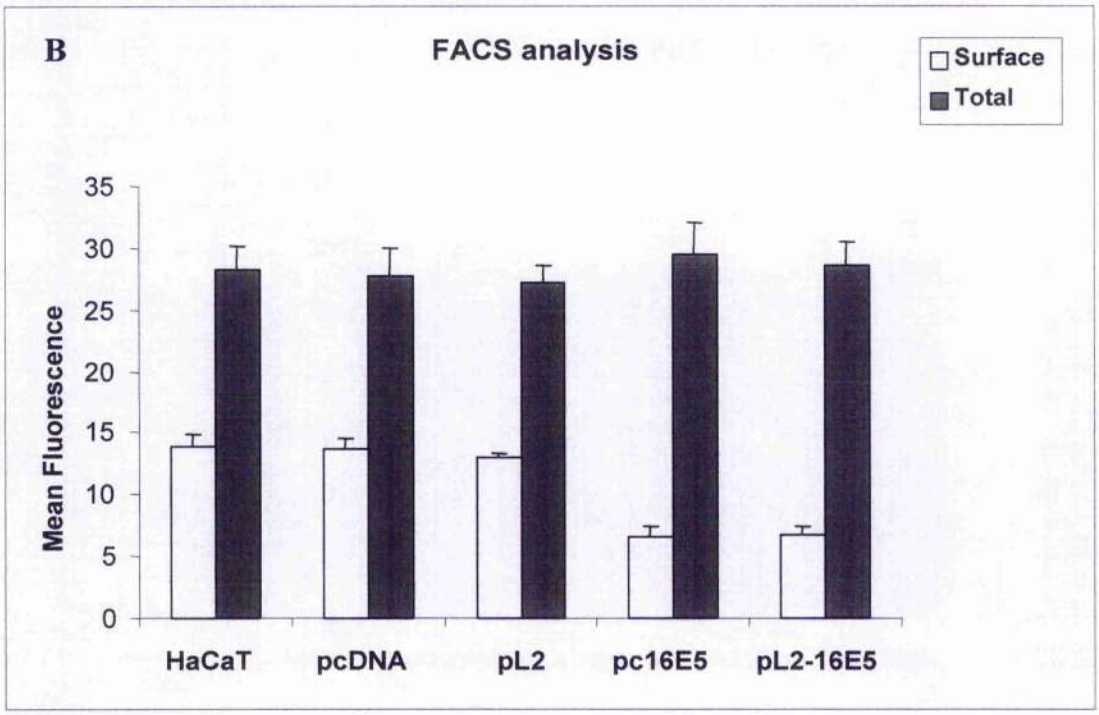


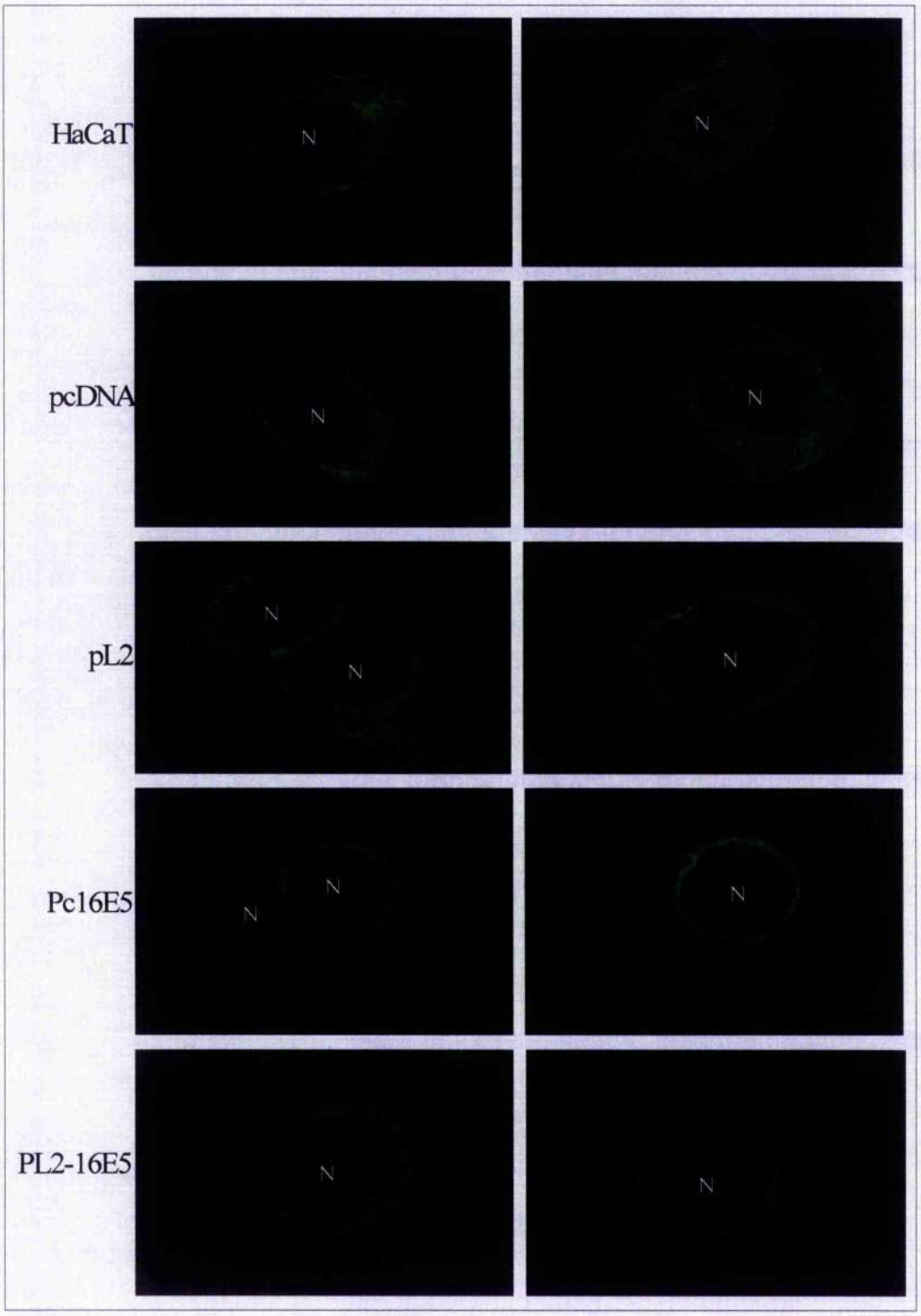
Figure 8: Down-regulation of MHC class I on the cell surface was investigated in the cells expressing HPV-16 E5. HaCaT parental cells, cells carrying empty vector (pcDNA, pL2) or cells expressing HPV-16 E5 (pc16E5, pL2-16E5) were stained with mAb W6/32 and analysed for expression of surface and total (surface plus intracytoplasmic) MHC class I by FACS. **A)** Representative FACS profiles of single cell lines. Dotted line and open histogram, forward fluorescence with secondary antibody only; solid histogram, forward fluorescence with primary and secondary antibody. **B)** The mean fluorescence from at least four experiments performed on three clones of each cell line was calculated from FACS analysis. A background of 0.4 (the reading of cells stained with only secondary antibody) was subtracted in all cell lines.

3.1.4.2 Detection of MHC class I by immunofluorescence microscopy

To investigate the cellular localization of MHC class I, the cells were aliquoted into 24-well plates containing coverslips at $1x10^4$ cells per well and grown overnight until 20%-50% confluent following the immunofluorescence staining protocol (see section 2.2.26). HaCaT parental cells, pcDNA, pL2, pc16E5 or pL2-16E5 cells were stained with mAb W6/32. In HaCaT parental cells and pcDNA or pL2 cells, MHC class I was detected both on the cell surface and in the cytoplasm, but it was detected only in the cytoplasm in the cells expressing HPV-16 E5 (Figure 9). These results confirmed that HPV-16 E5 down-regulates MHC class I on the cell surface.

Figure 9: HaCaT parental cells, cells carrying empty vector (pcDNA and pL2) or cells expressing HPV-16 E5 (pc16E5 and pL2-16E5) were stained with mAb W6/32 (anti MHC class I). The slides were mounted in Citifluor^{TM} and analysed with a Leica TCS SP2 fluorescence confocal microscope at 488nm wavelength. Images were acquired using Leica confocal software to show the localization of MHC class I. N=nucleus

9. Detection of MHC class I by immunofluorescence microscopy



3.1.4.3 Visualisation of the Golgi apparatus by immunofluorescence microscopy

It has been shown that BPV-4 E5 oncoprotein affects the structure of the Golgi Apparatus (GA), leading to GA swelling and fragmentation (Marchetti *et al.*, 2002). To see if the expression of HPV-16 E5 causes the same affect on the GA, the GA was visualised by immunofluorescence using mAb 4A3 raised against golgin GM130, an integral GA protein (Barr *et al.*, 1998). HaCaT cells were aliquoted, grown, fixed and permeabilised as described in section 2.2.27. The cells were incubated with mAb 4A3 for 1 hour at room temperature and washed twice with PBS+1% FCS. Cells were then incubated with anti-mouse IgG-TRITC at 4°C for 1 hour (in the dark). Following three final washes with PBS, the slides were mounted in Citifluor™ and analysed with a Leica TCS SP2 fluorescence confocal microscope at 543nm wavelength. Images were acquired using Leica confocal software. The results show that expression of HPV-16 E5 does not affect the morphology of the GA (see central panels of the Figure 10).

3.1.4.4 Localisation of MHC class I in the Golgi apparatus

It has been reported that BPV-1 and 4 E5 causes the retention of MHC class I in the GA and prevents its export to the plasma membrane (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). To see if HPV-16 E5 retains MHC class I in the GA in transformed cells, at least three of each control HaCaT clones carrying empty vectors, three pc16E5 and three pLJ2-16E5 clones were co-stained with mAb W6/32 and mAb 4A3. The cells were aliquoted, grown, fixed and permeabilised as described in Materials and Methods section 2.2.28. The merge between FITC and TRITC fluorescent signals was achieved with the Leica confocal software. In control cells, MHC class I was detected both on the cell surface and in the GA. In contrast, in E5 cells MHC class I was detected almost exclusively in the GA (Figure 10, shown only for

one of each clone of HaCaT control cells and E5 cells), confirming that E5 prevents the MHC class I complex from reaching the cell surface, and retains it in the GA, as already observed for BPV E5 (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002).

10. Immunofluorescence detection of MHC class I and Golgi apparatus

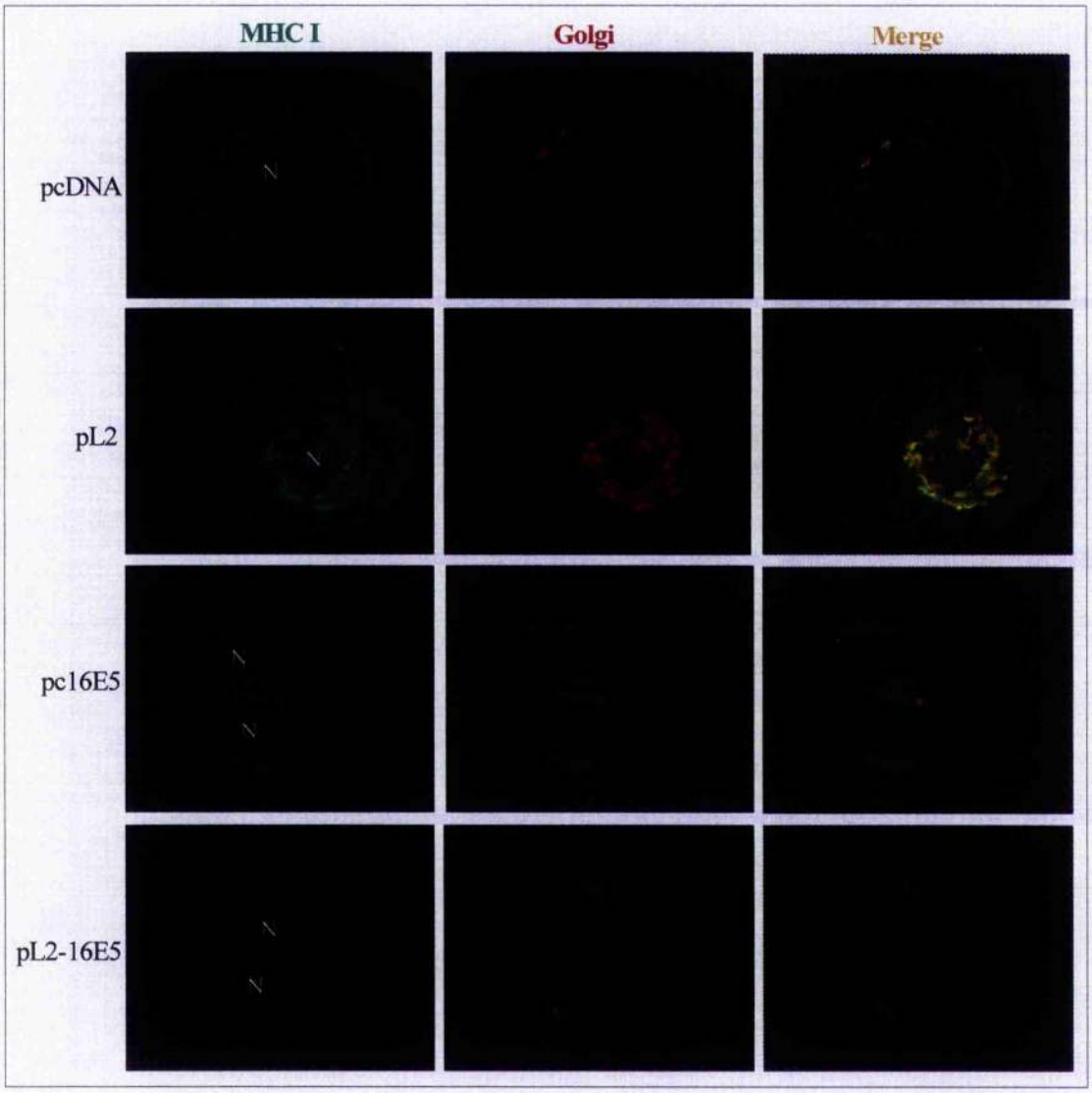


Figure 10: HaCaT cells carrying empty vectors or cells expressing HPV-16 E5 were co-stained with mAb W6/32 (anti MHC class I) and mAb 4A3 (anti golgin, GM130) and analysed with a Leica TCS SP2 fluorescence confocal microscope. Images were acquired using Leica confocal software to show visualisation of the GA and MHC class I. N=nucleus

3.1.4.5 Expression of MHC class I heavy chain is not inhibited by HPV-16 E5

The results above suggested that, in contrast to BPV, HPV-16 E5 did not have any effect on the overall levels of the MHC class I heavy chain. To confirm this observation, MHC class I was investigated by immunoblotting in HaCaT parental cells, pcDNA and pL2 cells or pc16E5 and pL2-16E5 cells using mAb HC10, specific for human MHC class I heavy chain, as described in section 2.2.30. We determined that HPV-16 E5 has no effect on the overall levels of the MHC class I heavy chain, which is in similar amounts in control cells and in cells expressing E5 (Figure 11). Although the levels of MHC class I heavy chain are slightly lower in the pL2 and pL2-16E5 cell lines than in the pcDNA and pc16E5 cell lines, there is no significant difference between them. The results confirm that HPV-16 E5 does not down-regulate the expression of total classical MHC I heavy chain.

11. Detection of MHC class I in the HPV-16 E5 cells by immunobloting

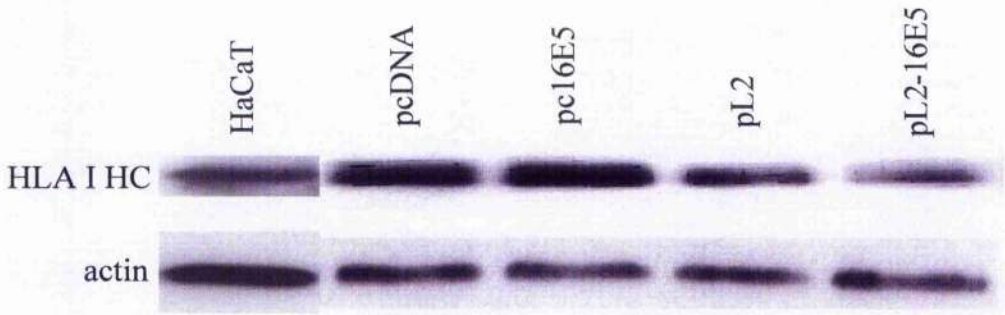


Figure 9: For detection of MHC class I, the membranes were incubated with mAb HC10 specific for MHC class I heavy chain and mAb AB-1, specific for β-actin.

3.1.5 Conclusion

The FACS results show that the levels of surface MHC class I are approximately half of the total in HaCaT parental cells and control cells but that in E5 cells, the levels of surface MHC class I is reduced to approximately half that of control cells whereas the level of total MHC class I remains constant (Figure 8). Furthermore, in control cells, MHC class I was detected both on the cell surface and in the GA. In contrast, in E5 cells MHC class I was detected almost exclusively in the GA (Figure 10), confirming that E5 prevents the MHC class I complex from reaching the cell surface, and retains it in the GA. However, despite the down-regulation of surface MHC class I, the overall expression of MHC class I heavy chain as measured by intracellular immunofluorescence is not inhibited by E5 (Figure 11).

3.1.6 Down-regulation of MHC class I by E5 is reversible

3.1.6.1 Introduction

IFNs, which consist of type I (IFN α and β) and type II (IFN γ) are a large family of multifunctional secreted proteins that regulate cellular antiviral, anti-tumour and immunological responses through IFN-stimulated gene expression. The IFN α and β are produced in virally infected cells but IFN γ is not virus inducible and is restricted to mitogen or cytokine-activated lymphoid cells such as T lymphocytes and natural killer cells. One of the functions of IFNs is to up-regulate the expression of MHC class I, which is able to present antigen peptides to CTLs. It has been reported that BPV E5 proteins causes a down-regulation of surface MHC class I molecules in transformed cells (Ashrafi *et al.*, 2002) and IFN treatment increases the transcription of the MHC class I heavy chain gene, but cannot overcome the block in exocytic transport (Marchetti *et al.*, 2002 and 2005). To see whether down-regulation of MHC class I by HPV-16 E5 is reversible, we treated cells with IFN to determine if IFN treatment up-regulated MHC class I expression. Therefore, HaCaT parental cells, pcDNA, pL2, pc16E6 and pL2-16E5 cells were treated with 500U/ml β-IFN for 48 hours and stained with mAb W6/32 then analysed for expression of total and surface MHC class I both by FACS and immunofluorescence.

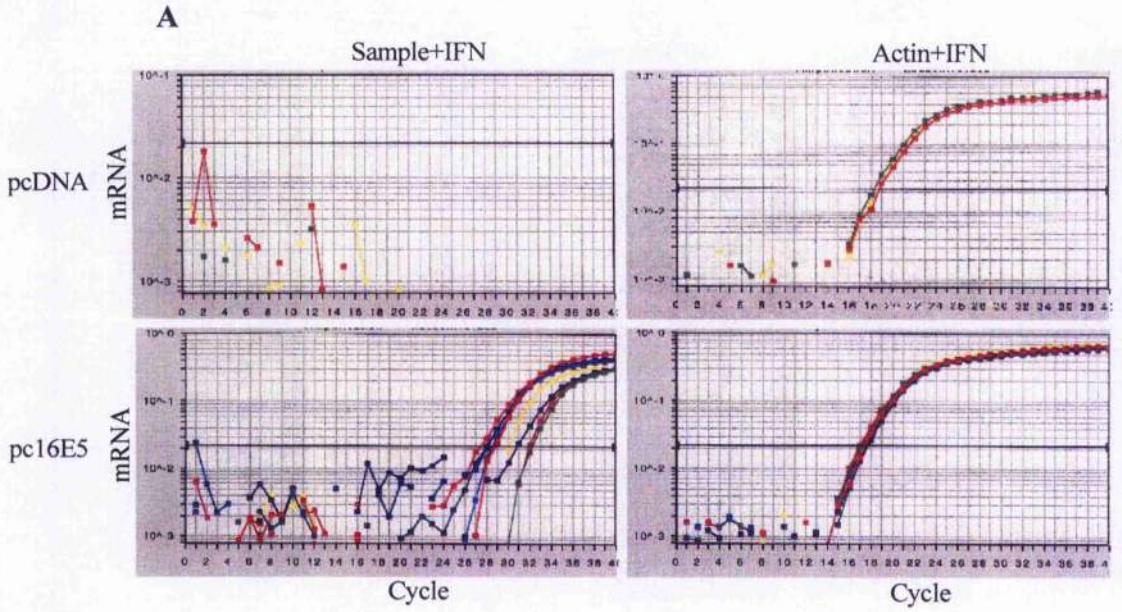
3.1.6.2 E5 expression in IFN treated HaCaT cells

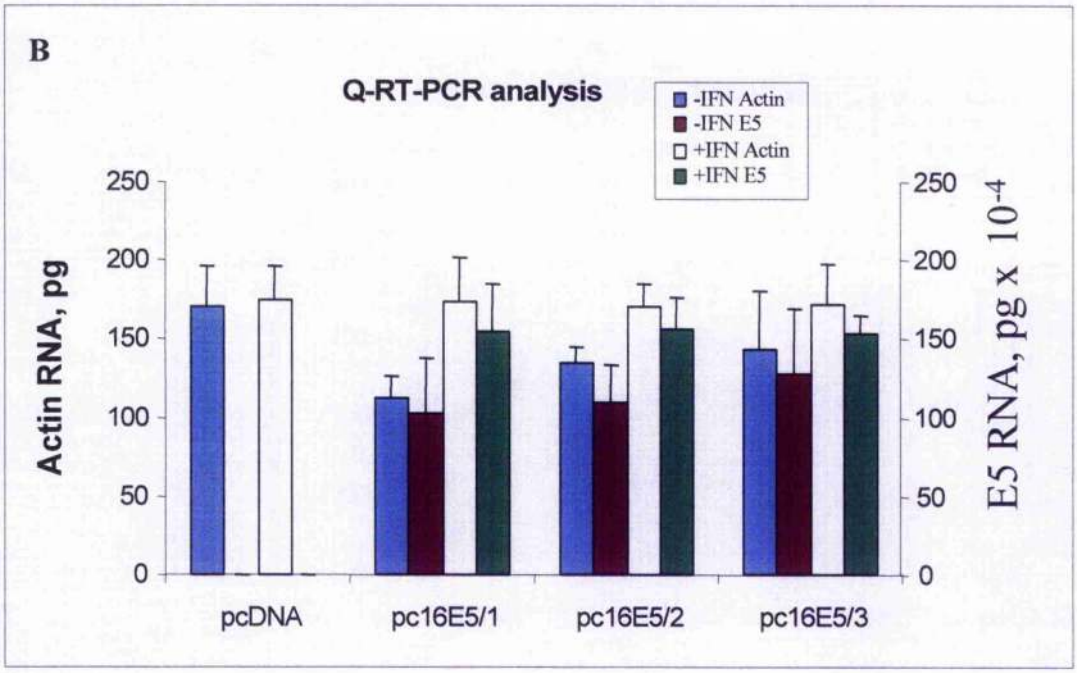
To see whether IFN treatment affected expression of E5, we measured level of E5 mRNA in IFN-treated and untreated cells by quantitative RT-PCR. RNA was isolated and the relative level of E5 mRNA expression was determined by comparison to β-actin mRNA (see section 2.2.21 and 2.2.24). We analysed three pc16E5 clones. In each experiment, additional reactions of 10 fold serial dilutions of template DNA were performed with each set of primers and

probes on the same 96-well plates to generate standard curves. The quantitive RT-PCR was used to determine the relative amount of HPV-16 E5 and β-actin mRNA in triplicate experiments of each transfected E5 cells and cells carrying empty vector by using a standard curve. The Taq-Man RT-PCR results show that IFN treatment has no major effect on E5 expression at the dose used (Figures 12A and 12B).

Figure 12: Taq-Man RT-PCR was used to determine the expression of E5 mRNA in the transfected HaCaT cells treated or not treated with 500U/ml β-IFN for 48 hours. **A)** The expression of HPV-16 E5 and actin mRNA was determined in HaCaT control cells (one clone of pcDNA) or cells expressing HPV-16 E5 (three clones of pc16E5) treated with β-IFN and each colour represents an RT-PCR experiment. **B)** Relative amount of HPV-16 E5 and actin mRNA in HaCaT control cells (pcDNA) or cells expressing HPV-16 E5 (pc16E5) treated or untreated with β-IFN. E5 is expresed at 10,000 fold less than β-actin.

12. Expression of HPV-16 E5 RNA in transfected cell lines treated with β-IFN

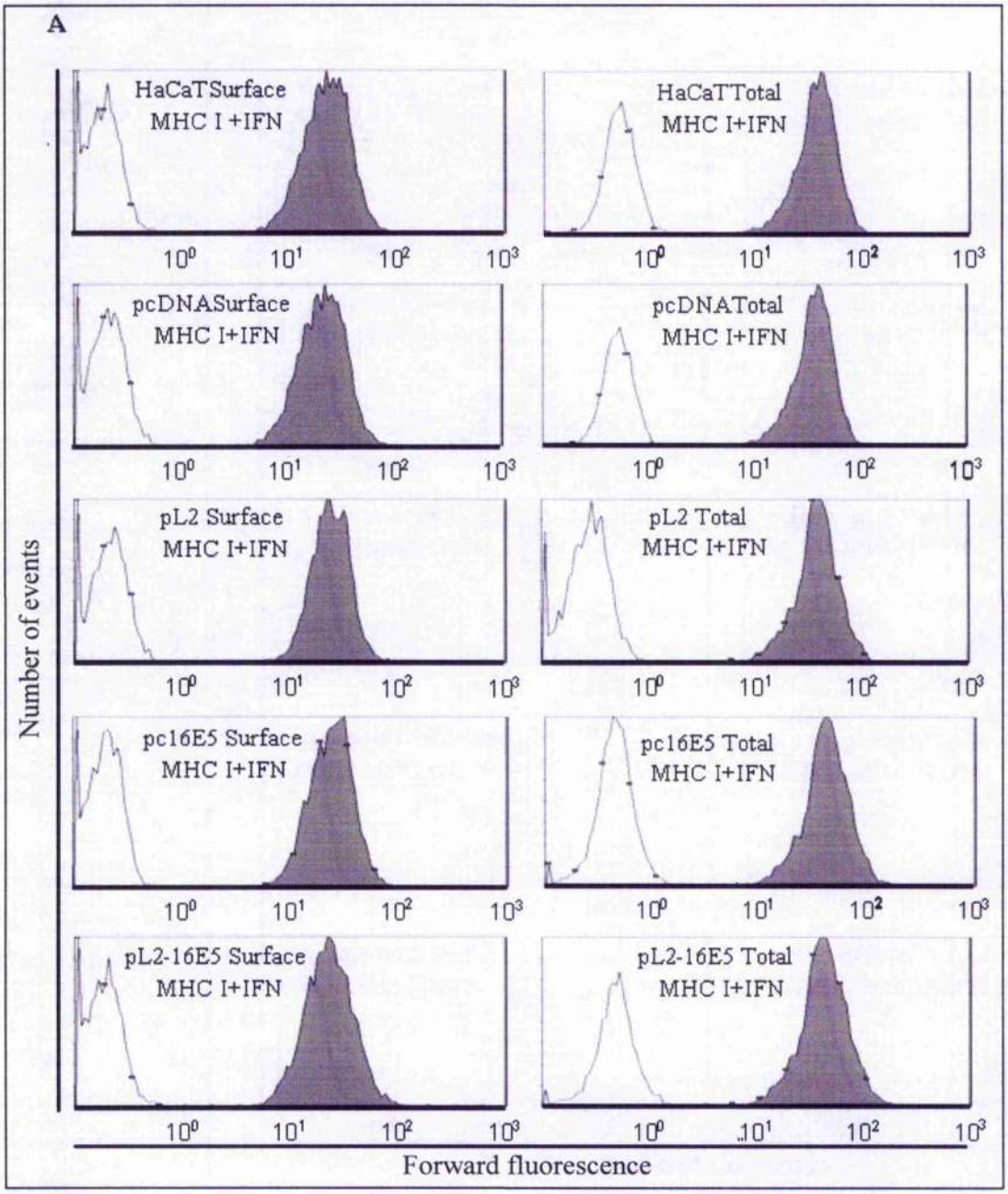


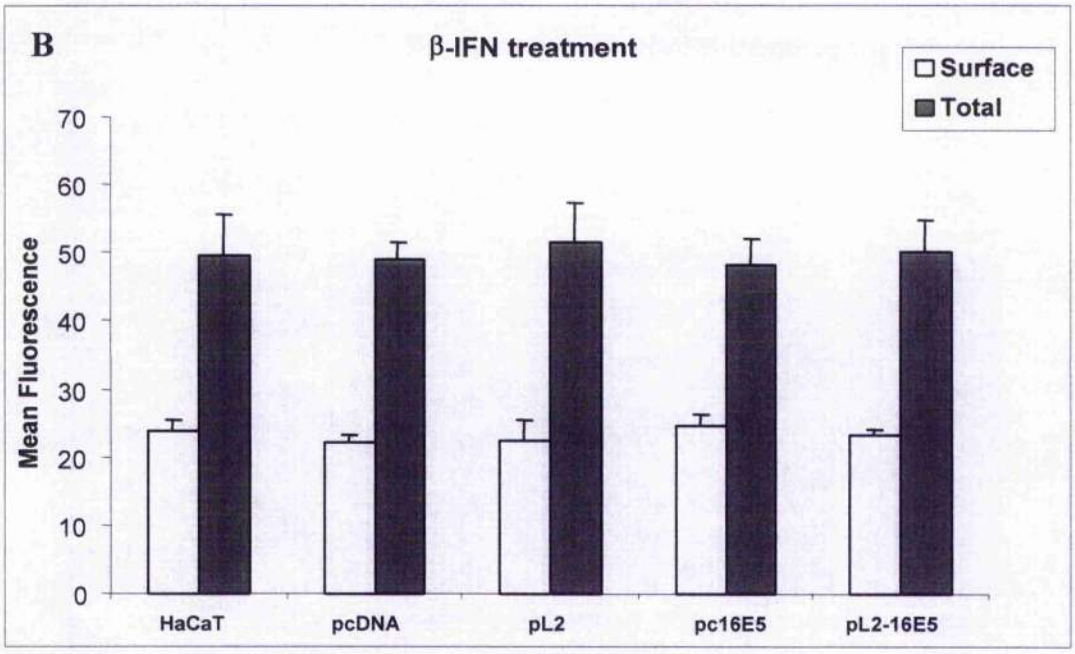


3.1.6.3 Treatment of HPV-16 E5 cells with IFN rescues MHC class I traffic to the cell surface

β-IFN increases the transcription activity of the MHC class I heavy chain gene promoter (Agrawal and Kishore, 2000) leading to higher expression levels of heavy chain. We have shown above that HPV-16 E5 retains MHC class I in the GA and impedes its transport to the cell surface (Figures 8 and 10). To see if increased synthesis of heavy chain led to an increase in transport of MHC class I complex to the cell surface, HaCaT parental cells, pcDNA and pL2 cells or pc16E5 and pL2-16E5 were treated with 500U/ml β-IFN for 48 hours, and analysed for MHC class I expression by FACS and localisation by immunofluorescence, as described in section 2.2.31. We determined that treatment with β-IFN increased the level of total MHC class I both in HaCaT control cells and in E5 cells to approximately the same level in all cell lines. Treatment with β-IFN increased total MHC class I approximately two folds in all cell lines. At the same time β-IFN treatment increased surface MHC class I approximately two folds in HaCaT parental cells, pcDNA and pL2 cells, and approximately four folds in E5 expressing cells (Figure 13). This resulted in all cell lines, including those expressing E5, having similar levels of surface MHC class I. These results indicate that β-IFN treatment can overcome the block exerted by E5 on MHC class I transport.

13. Detection of MHC class I by HPV-16 E5 is reversible by β-IFN




B
β-IFN treatment
Surface
Total
Mean Fluorescence
70
60
50
40
30
20
10
0
HaCaT
pcDNA
pL2
pc16E5
pL2-16E5

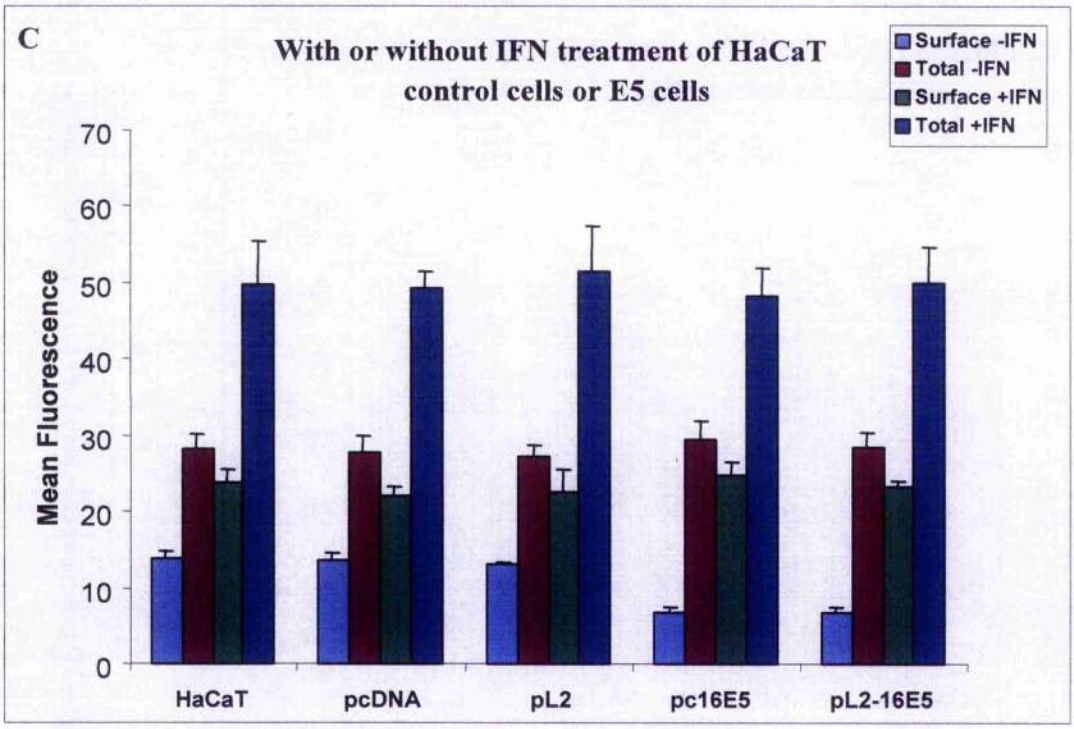

C
With or without IFN treatment of HaCaT control cells or E5 cells
Surface -IFN
Total -IFN
Surface +IFN
Total +IFN
Mean Fluorescence
70
60
50
40
30
20
10
0
HaCaT
pcDNA
pL2
pc16E5
pL2-16E5

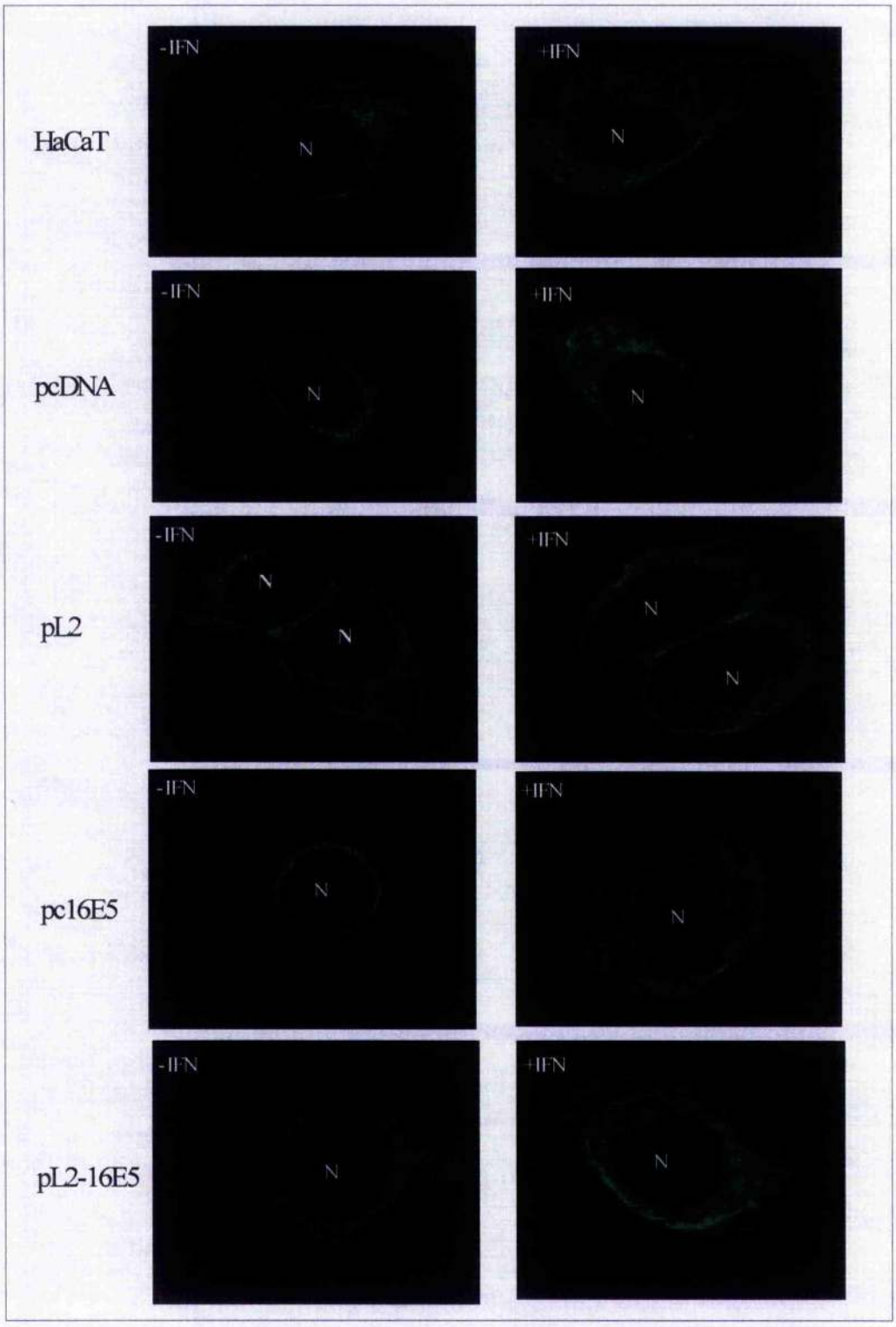
Figure 13: The level of MHC class I was investigated in HaCaT parental cells, cells carrying empty vectors (pcDNA, pL2) or cells expressing HPV-16 E5 (pc16E5 and pL2-16E5) with 500U/ml β-IFN for 48 hours. The cells were stained with mAb W6/32 and analysed for expression of surface and total MHC class I by FACS. **A)** Representative FACS profiles of single cell lines. Dotted line and open histogram, forward fluorescence with secondary antibody only; solid histogram, forward fluorescence with primary and secondary antibody. **B)** The mean fluorescence for each of the HaCaT control cells and E5 cells was calculated from three experiments and three clones from each cell line by FACS analysis. A background of 0.4 (the reading of cells stained with only secondary antibody) was subtracted in all cell lines. **C)** Average mean fluorescence for each of the cell lines treated with or without 500U/ml β-IFN for 48 hours and analysed for expression of total and surface MHC class I by FACS analysis with mAb W6/32.

3.1.6.4 Distribution of MHC class I in HPV-16 E5 cells after IFN-β treatment

To confirm this observation, HaCaT parental cells, cells carrying empty vectors or cells expressing HPV-16 E5, treated with β-IFN, were stained with mAb W6/32 and analysed for MHC class I localisation by immunofluorescence staining as described in section 2.2.26. We determined that MHC class I was mostly detected in the GA in untreated HPV-16 E5 cells, as before. In HPV-16 E5 cells treated with β-IFN, MHC class I was detected also on the cell surface (Figure 14). This result is in agreement with the FACS analysis, shows that E5 cells are responsive to β-IFN and that the E5-induced MHC class I transport inhibition is reversible by β-IFN.

Figure 14: HaCaT parental cells, cells carrying empty vector (pcDNA and pL2) or cells expressing HPV-16 E5 (pc16E5 and pL2-16E5) were treated with 500U/ml β-IFN for 48 hours and stained with mAb W6/32. The slides were mounted in Citifluor^{TM} and analysed with a Leica TCS SP2 fluorescence confocal microscope at 488nm wavelength. Images were acquired using Leica confocal software to show the localization of MHC class I. N=nucleus

14. Detection of MHC class I using β-IFN by immunofluorescence microscopy



3.1.6.5 Conclusion

The FACS results show that treatment of the HaCaT control cells and E5 cells with β-IFN increased the amount of total MHC class I approximately two-fold in all of the cell lines. Moreover, we found an approximately two-fold increase in surface MHC class I in the parental HaCaT cells and control cells (no E5 cells), and an approximately four-fold increase in the E5-expressing cells (pc16E5, pL2-16E5). We show that treatments with β-IFN increases the syntheses of MHC class I in the cells and rescue the transport of MHC class I to the cell surface. IFN has little effect on E5 expression so the abrogation of the retention of MHC class I in the GA is not due to inhibition of E5 expression. In fact the expression of E5 is slightly increased by IFN treatment, but this does not prevent transport of MHC class I to the cell surface. These results reveal that β-IFN treatment of the HPV-16 E5 cells did not affect the expression of E5. Therefore, the increase of the levels of MHC class I cannot be attributed to changes in E5 expression.

3.2 Which domain of HPV-16 E5 is responsible for down-regulation of MHC I?

3.2.1 Introduction

The E5 protein is a small hydrophobic protein from 42 amino acids residues in BPV-4 to 83 amino acids residues in HPV-16. BPV E5 protein can be divided into two domains, N-terminus, which is a hydrophobic domain and C-terminus, which is a generally hydrophilic domain. The C-terminus of BPV-1 E5 includes two cysteine residues, which are important for homodimerization and transforming activity (Burkhardt *et al.*, 1987; Horwitz *et al.*, 1988). It has been determined by hydropatic analysis that HPV-16 E5 protein has three hydrophobic domains connected by less hydrophobic regions (Bubb *et al.*, 1988). HPV-16 E5 protein is 83 amino acids long, located mainly at the endosomal membranes, GA and, to a lesser extent, the plasma membranes (Burkhardt *et al.*, 1989; Conrad *et al.*, 1993). The C-terminal portion of BPV-1 E5, which appears to be important for biological function, does not have an identifiable similar region in HPV-16 E5.

3.2.2 Generation of HPV-16 E5 Del-1

Like BPV E5 (Ashrafi *et al.*, 2002, Marchetti *et al.*, 2002), HPV-16 E5 can inhibit transport of MHC class I molecules to the cell surface (Figure 8). It is important to determine the region of the E5 protein responsible for down-regulation of MHC class I. To this end, deletion mutants of E5 (Figure 16) were used. HPV-16 E5 mutants (R79, A54, V36 and R30), in which double stop codons were introduced at these positions, were kind gifts from Professor Alonso. In addition, a mutant was constructed by PCR, which lacked the first hydrophobic domain, called HPV-16 E5 Del-1.

15. Sequence of HPV-16 E5 wild type and HPV-16 E5 mutants

HPV-16 E5 wild type

```
  1 atgacaaatc gtgatactgc atccacaaca ttactggcgt gctttttgct ttgcttttgt
 61 gtgcttttgt gtgtctgcct attaatacgt ccgctgcttt tgtctgtgtc tacatacaca
121 tcattaataa tattggtatt actattgtgg ataacagcag cctctgcgtt taggtgtttt
181 attgtatata ttatatttgt ttatatacca ttatttttaa tacatacaca tgcacgcttt
241 ttaattacat aa
```

1 MTNRDTASTT LLACFLLCFC VLLCVCLLIR PLLLSVSTYT SLIILVLLLW
51 ITAASAFRCF IVYIIFVYIP LFLIHTHARF LIT

HPV-16 E5 R79

```
  1 atgacaaatc gtgatactgc atccacaaca ttactggcgt gctttttgct ttgcttttgt
 61 gtgcttttgt gtgtctgcct attaatacgt ccgctgcttt tgtctgtgtc tacatacaca
121 tcattaataa tattggtatt actattgtgg ataacagcag cctctgcgtt taggtgtttt
181 attgtatata ttatatttgt ttatatacca ttatttttaa tacatacaca tgcataa
```

1 MTNRDTASTT LLACFLLCFC VLLCVCLLIR PLLLSVSTYT SLIILVLLLW
51 ITAASAFRCF IVYIIFVYIP LFLIHTIIA

HPV-16 E5 A54

```
  1 atgacaaatc gtgatactgc atccacaaca ttactggcgt gctttttgct ttgcttttgt
 61 gtgcttttgt gtgtctgcct attaatacgt ccgctgcttt tgtctgtgtc tacatacaca
121 tcattaataa tattggtatt actattgtgg ataacagcat aa
```

1 MTNRDTASTT LLACFLLCFC VLLCVCLLIR PLLLSVSTYT SLIILVLLLW
51 ITA

HPV-16 E5 V36

```
  1 atgacaaatc gtgatactgc atccacaaca ttactggcgt gctttttgct ttgcttttgt
 61 gtgcttttgt gtgtctgcct attaatacgt ccgctgcttt tgtcttaa
```

1 MTNRDTASTT LLACFLLCFC VLLCVCLLIR PLLLS

HPV-16 E5 R30

```
  1 atgacaaatc gtgatactgc atccacaaca ttactggcgt gctttttgct ttgcttttgt
 61 gtgcttttgt gtgtctgcct attaatataa
```

1 MTNRDTASTT LLACFLLCFC VLLCVCLLI

HPV-16 E5 Del-1

```
 91 ccgctgcttt tgtctgtgtc tacatacaca tcattaataa tattggtatt actattgtgg
151 ataacagcag cctctgcgtt taggtgtttt attgtatata ttatatttgt ttatatacca
211 ttatttttaa tacatacaca tgcacgcttt ttaattacat aa
```

31 PLLLSVSTYT SLIILVLLLW ITAASAFRCF IVYIIFVYIPLFLIHTHARF LIT

Figure 15: HPV-16 E5 wild type ORF has 249 nucleotides (83 amino acids), R79 lacks the last C-terminus 5 amino acids, A54 lacks the third hydrophobic domain, V36 contains the first hydrophobic domain plus amino acids 31-36, R30 contains only the first hydrophobic domain and Del-1 lacks the first hydrophobic domain.

16. Schematic diagram of the HPV-16 E5 wild type and HPV-16 E5 mutants.

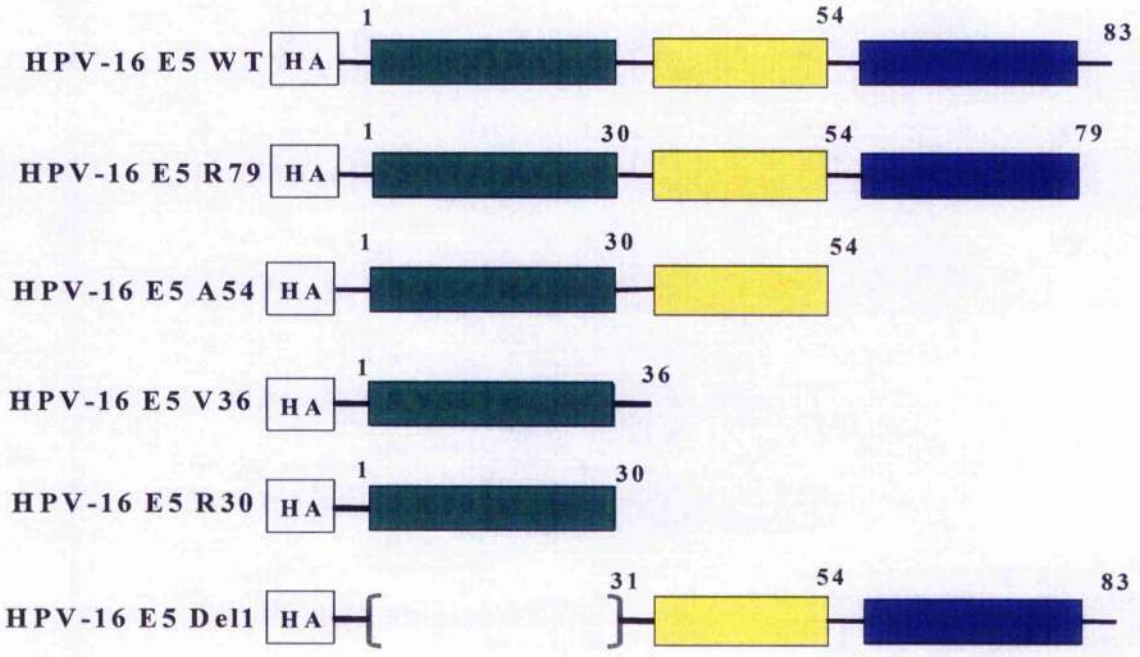


Figure 16: HPV-16 E5 mutants: HPV-16 E5 R30 contains only the first hydrophobic domain, V36 contains the first hydrophobic domain plus amino acids 31-36, A54 lacks the third hydrophobic domain, R79 lacks the last C-terminus 5 amino acids and Del-1 lacks the first hydrophobic domain. HA- (YPYDVPDYA, peptide from human influenza heamaglutinin protein) tag was incorporated at the N-terminus of all of E5 mutants. Each box represents a hydrophobic domain of the HPV-16 E5 protein, bound by less hydrophobic regions (lines).

3.2.3 Domain-directed deletion of HPV-16 E5

HPV-16 E5 Del-1 mutant was made by deletion of the first hydrophobic domain (from 1 to 30 first amino acids) of the HPV-16 E5 ORF. The primers used to make deletion mutant in the HPV-16 E5 ORF incorporated two restriction enzyme sites: *Nhe-I* and *EcoR-I*, for cloning in the multi cloning sites of pcI-neo vector, and the forward primer incorporated the HA-tag. DNA was constructed as described in section 2.2.7.

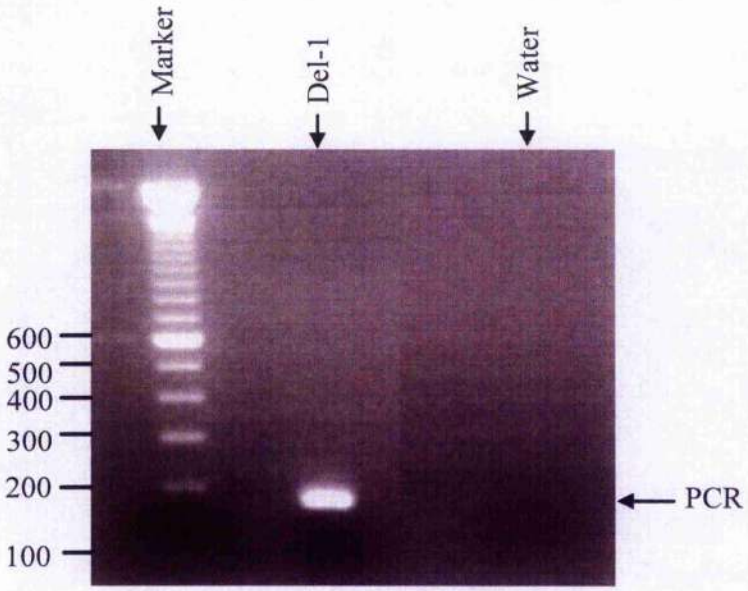
3.2.4 Generation of HPV-16 E5 Del-1

The oligonucleotide sequence containing the HPV-16 E5 Del-1 mutation was generated following DNA PCR protocol as described in section 2.2.7. The correct product was amplified by PCR and purified by using a PCR purification kit (see section 2.2.9). The DNA fragment was ligated into the cut vector at an insert:vector ratio of 6:1 using appropriate enzyme and buffer. The ligated samples were transformed in *E.coli* DH5-α competent cells and the bacterial culture grown on medium containing the appropriate antibiotic (see section 2.2.1). A single bacterial clone was used to inoculate 5 ml L-broth medium and grown overnight at 37°C (picked at least 10 clones). Plasmid DNA was prepared from the clone using the QIAprep Spin plasmid miniprep kit according to the manufacturer's instructions (see section 2.2.2). The plasmid DNA was sequenced and transfected into HaCaT cells.

3.2.5 Agarose gel electrophoresis

The correct size of this mutant was analysed by agarose gel electrophoresis. Samples were loaded into individual wells with an appropriate sized DNA ladder. Separated DNA was visualised by illumination on a short wave (312 nm) UV light box and photographed using UVP Gel Documentation System. We determined the size of HPV-16 E5 Del-1 mutant, which is 189 base pairs, encoding a protein of 62 amino acids long including the HA tag, of approximately 6 KDa (Figure 17).

17. DNA PCR product of HPV-16 E5 Del-1



Marker: 100 base pair DNA ladder

Figure 17: HPV-16 E5 Del-1 mutant which is 189 base pairs including HA-tag (162 bp for E5Del-1 plus 27 bp of HA tag).

3.2.6 DNA sequencing

The deletion of the first hydrophobic domain was verified using an Applied Biosystems 3100 automated DNA sequencer. Subsequently the samples were analysed using an ABI3100 genetic analyser (see section 2.2.12). The sequence of the HPV-16 E5 Del-1 construct was aligned and compared to HPV-16 E5 wild type sequence and confirmed by using the DNASTAR program.

3.2.7 Transfection of HPV16 E5 mutants in HaCaT clones

The HaCaT cells were stably transfected with a range of plasmid DNA; HPV-16 E5 wild type (pc16E5) and HPV-16 E5 mutants (pc16E5 R79, pc16E5 A54, pc16E5 V36, pc16E5 R30 and pc16E5 Del-1) by using Lipofectamine Plus to investigate the role of HPV-16 E5 mutants (see section 2.2.16).

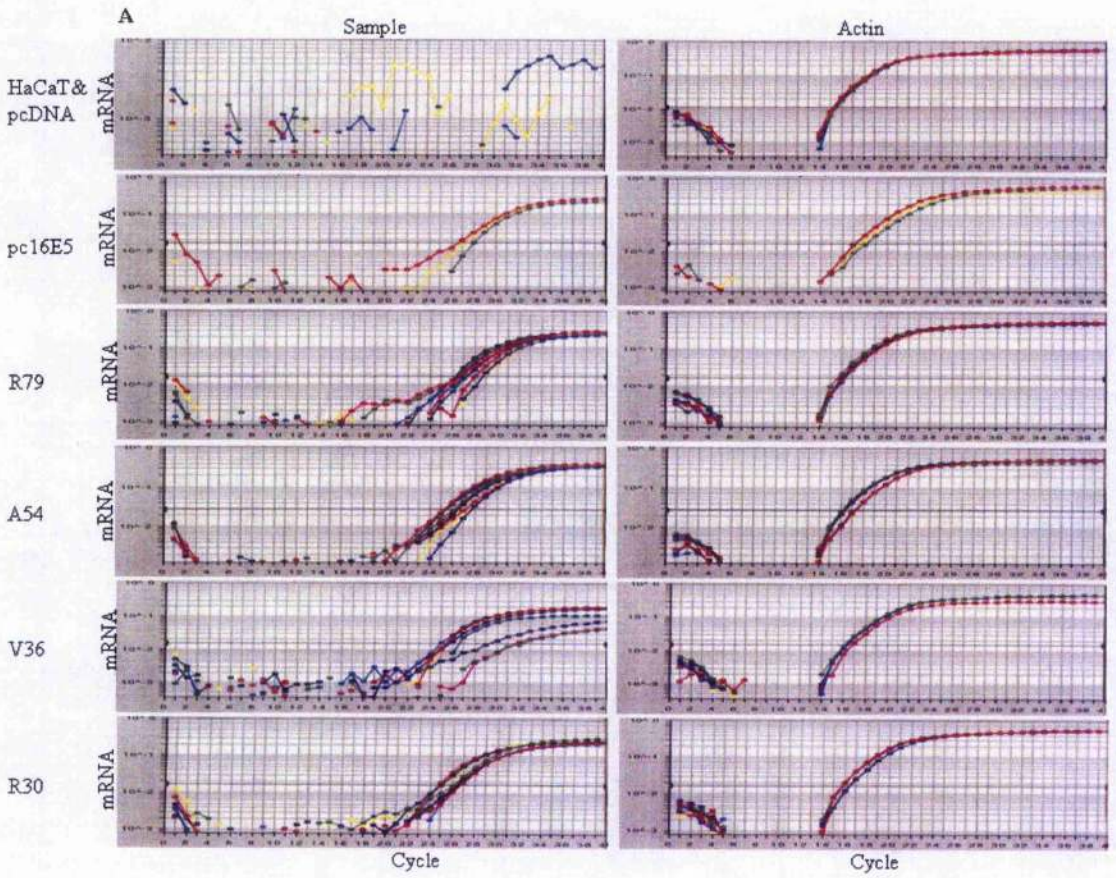
3.2.8 The expression of HPV-16 E5 mutants in HaCaT clones

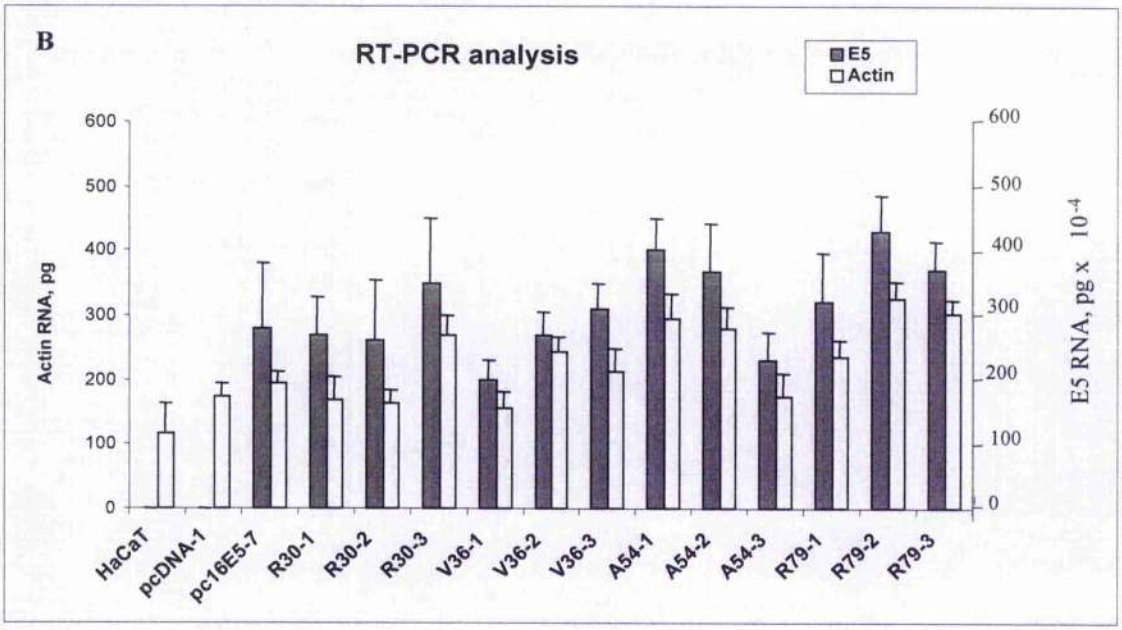
We investigated that the HPV-16 E5 wild type (pc16E5) and HPV-16 E5 mutants (pc16E5 R79, pc16E5 A54, pc16E5 V36, pc16E5 R30 and pc16E5 Del-1) were being transcribed using quantitative RT-PCR. RNA was isolated and the relative level of E5 mRNA expression was determined by comparison to β-actin mRNA using Real-time RT-PCR protocol (see sections 2.2.21 and 2.2.24). We analysed three clones of each HPV-16 E5 mutants for the presence of E5 transcripts. In each experiment, additional reactions, with 10 fold serial dilutions of template DNA, were performed with each set of primers and probes on the same 96-well plates to generate standard curves. The quantitive RT-PCR was used to determine the relative amount of HPV-16 E5 mutants and β-actin mRNA in triplicate experiments of each transfected cell line and cells carrying empty vectors by using a standard curve (Figure 18A).

The Taq-Man RT-PCR results show that the transformed cells (pc16E5 R79, pc16E5 A54, pc16E5 V36 and pc16E5 R30) express E5 mRNA but at 10,000 fold less than β-actin sample, similar to HPV-16 E5 wild type (Figure 18B). However, expression of HPV-16 E5 Del-1 could not be detected by real-time RT-PCR.

Figure 18: Expression of HPV-16 E5 wild type and HPV-16 E5 mutants (R79, A54, V36 and R30) mRNA in HaCaT cells. **A)** The expression of HPV-16 E5 wild type, HPV-16 E5 mutants and actin mRNA was determined in HaCaT parental cells (one clone), cells carrying empty vector (one clone of pcDNA) or cells expressing HPV-16 E5 (one clone of pc16E5) and HPV-16 E5 mutants (three clones of each of the pc16E5 mutants) and each colour represents an RT-PCR experiment. **B)** Relative amount of HPV-16 E5 wild type, HPV-16 E5 mutants and actin mRNA in HaCaT control cells (one clone), pcDNA (one clone), pc16E5 wild type (one clone) and pc16E5 mutants (three clones each of the mutants: R79, A54, V36 and R30). HPV-16 E5 wild type and HPV-16 E5 mutants are expressed at comparable levels approximately 10,000 fold less than β-actin.

18. Expression of HPV-16 E5 wild type and mutants RNA in transfected cell lines

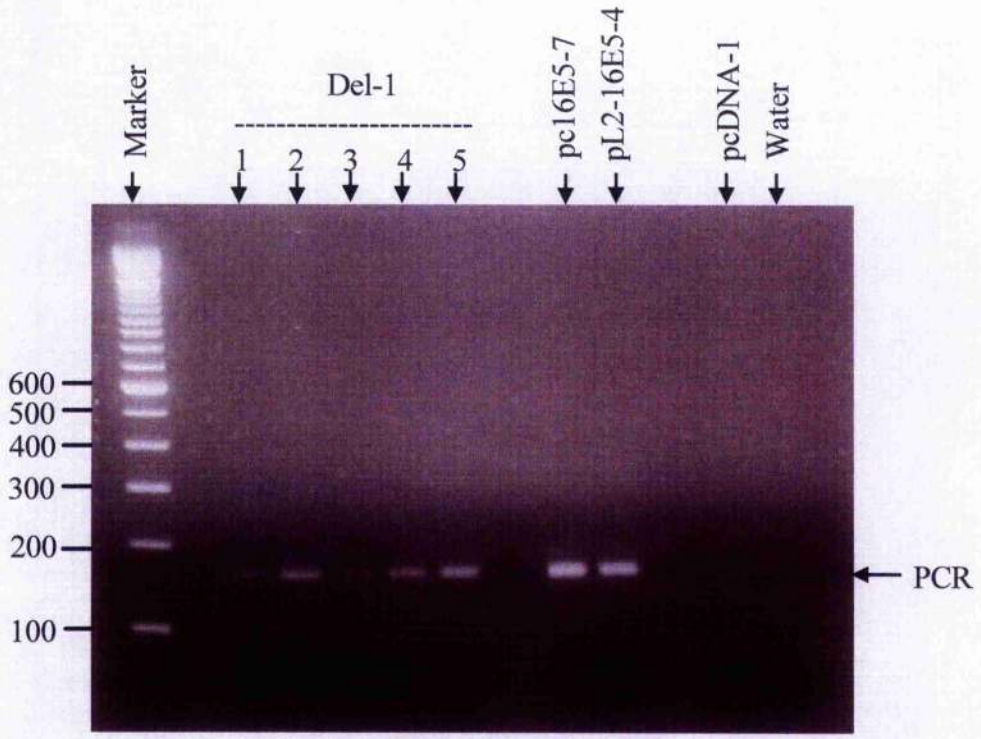




3.2.9 Detection of HPV-16 E5 Del-1 RNA from HaCaT cells

We investigated the expression of HPV-16 E5 mutants (R79, A54, V36 and R30) gene by Taq-Man RT-PCR as HPV-16 E5 wild type but we could not detect HPV-16 E5 Del-1 mutant. This mutant has the first hydrophobic domain deleted, which is the best target for the probe. Therefore, DNA PCR was used to investigate the presence of HPV-16 E5 Del-1 gene in the transfected HaCaT cells. The DNA was extracted from each clone of G418 resistant cells as described in section 2.2.18. All reagents were provided in the Genamp® PCR Reagent Kit and core Reagents with AmpliTaq® DNA polymerase and appropriated primers as in section 2.2.22. Thereafter, 5 µl of each sample was analysed by agarose gel electrophoresis to check the correct product had been amplified. The analysis of DNA PCR showed that there were detectable products in HaCaT cells with E5 wild type and in HaCaT cells transfected with E5Del-1, but not in the negative controls. These results confirm that the HPV-16 E5 Del-1 DNA was present in the transfected cells (Figure 19).

19. DNA PCR analysis of HaCaT cells transfected with HPV-16 E5 wild type and HPV-16 E5 Del-1 mutant



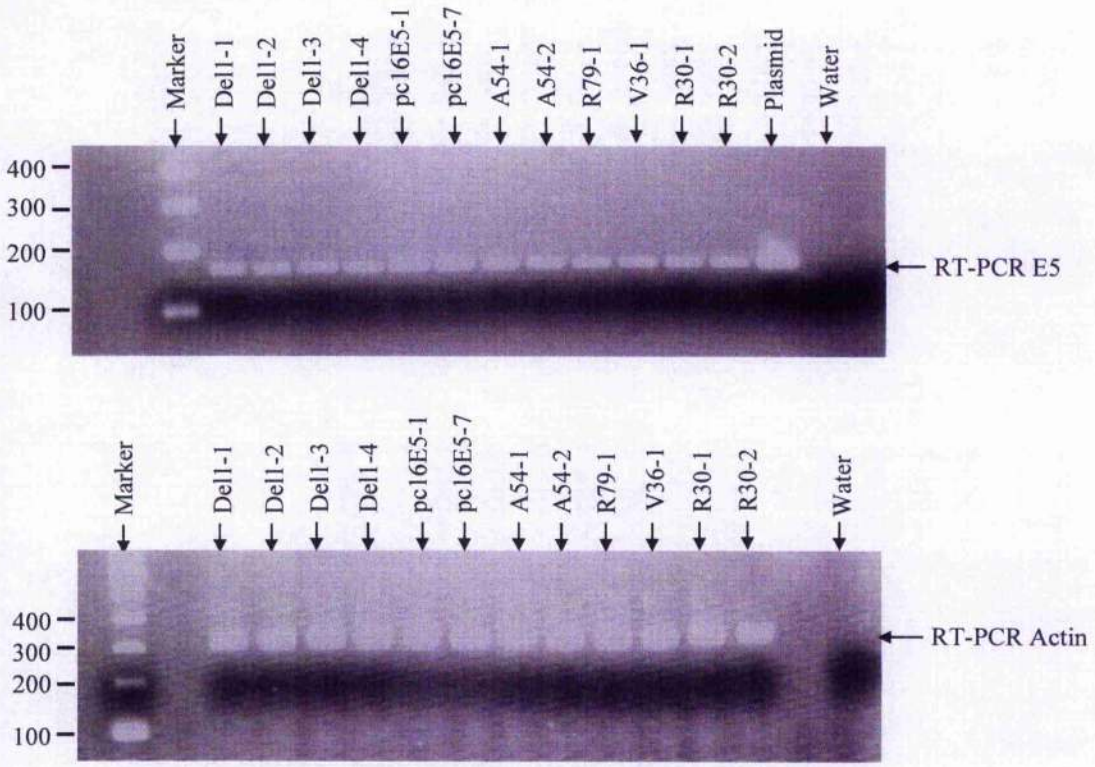
Marker: 100 base pair DNA ladder

Figure 19: Presence of the gene for HPV-16 E5 wild type (one clone each of pc16E5 and pL2-16E5) or five clones of HPV-16 E5 Del-1 mutant was analysed by DNA PCR.

3.2.10 Amplification of E5 RNA from HaCaT cells by RT-PCR

To investigate that the HPV-16 E5 Del-1 gene was transcribed in the transfected HaCaT cells, to the same extent of the other E5 mutants, we analysed each clone of HaCaT-E5Del-1 for the presence of mRNA by using RT-PCR. Total cell RNA was prepared as in section 2.2.19 and used as the template for reverse transcription and PCR amplification by using Superscript one-step RT-PCR with platinum Taq, 100 reaction and appropriate primers (see section 2.2.23). RNA was isolated and the relative level of E5 mRNA expression was determined by comparison to β-actin mRNA. We analysed the presence of E5 transcripts in HaCaT cells containing HPV-16 E5 wild type and HPV-16 E5 mutants including Del-1, along with positive and negative controls. The results show that the levels of expression of E5 wild type and mutants including Del-1 are approximately the same. The RT-PCR results show that all of the transformed cells express E5 but in lower amount than β-actin (Figure 20).

20. RT-PCR analysis of HaCaT cells with HPV-16 E5 wild type and mutants



Marker: 100 base pair DNA ladder

Figure 20: Expression of HPV-16 E5 wild type, HPV-16 E5 mutants (R79, A54, V36, R30 and Del-1) and actin mRNA in the transfected HaCaT cells were analysed by RT-PCR at 35 cycles. Two clones of HPV-16 E5 wild type (pc16E5), four clones of HPV-16 E5 Del-1, one clone of HPV-16 E5 R79, two clones of HPV-16 E5 A54, one clone of HPV-16 E5 V36 and two clones of HPV-16 E5 R30 were used, plus positive and negative controls.

3.2.11 RT-PCR analysis of HaCaT cells transfected with HPV-16 E5 wild type and HPV-16 E5 mutants

HPV-16 E5 wild type, HPV-16 E5 mutants (Del-1, R79, A54, V36 and R30) and actin mRNA were analysed by using RT-PCR protocol and run on the gel. The bands of the E5 and actin were analysed using Image-Quantification 5.2 P.C. program (Figure 21). The results show that cells carrying HPV-16 E5 wild type and all of the HPV-16 E5 mutants including Del-1, express E5 at approximately the same level.

21. RT-PCR analysis of HPV-16 E5 wild type and HPV-16 E5 mutants

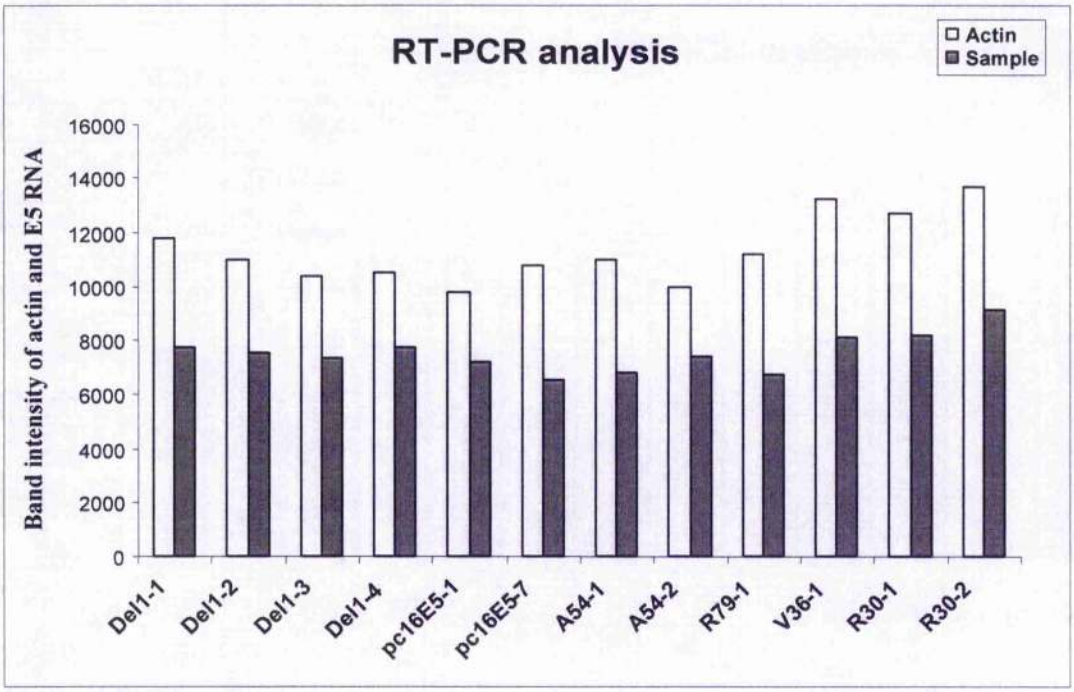
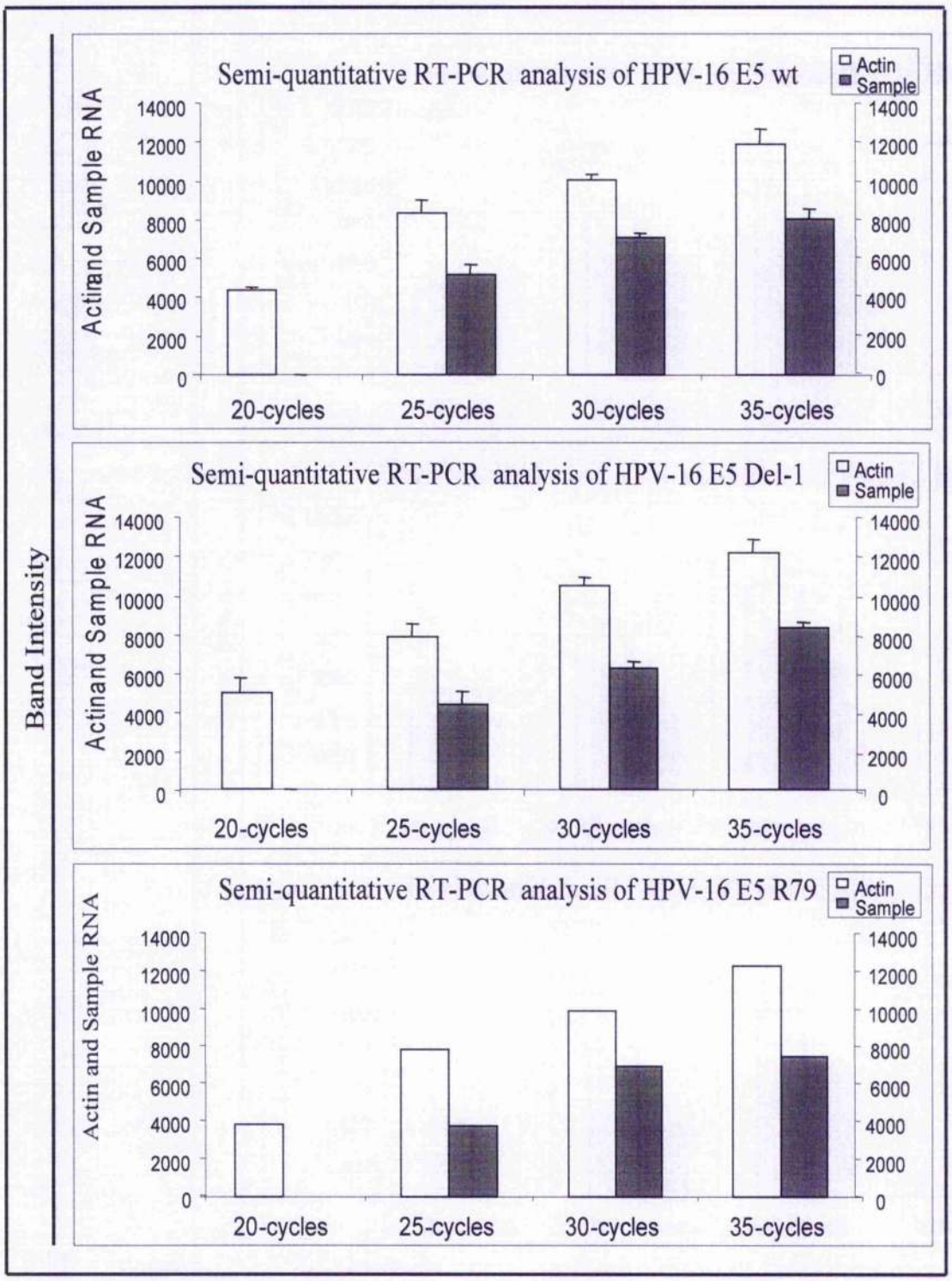


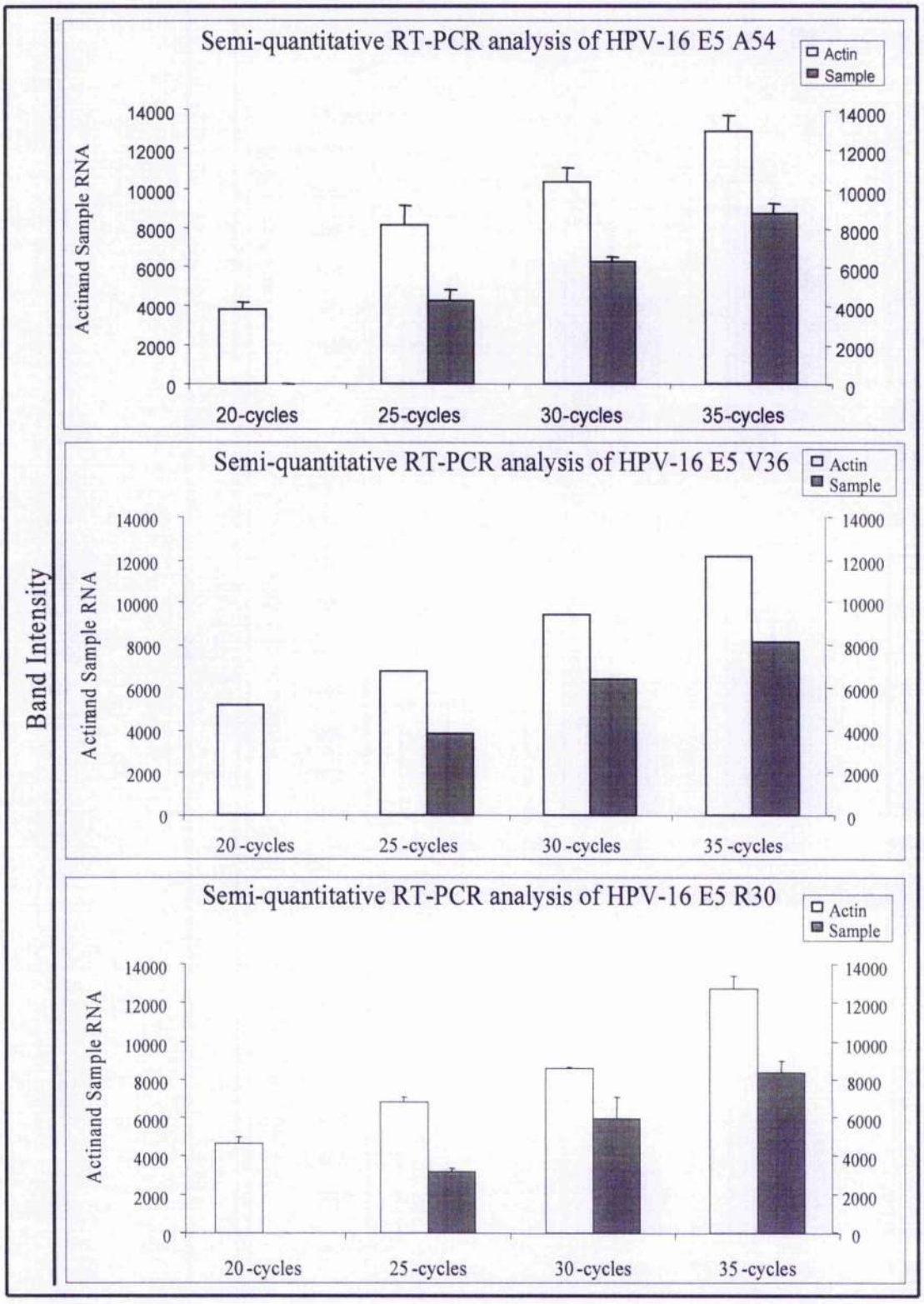
Figure 21: RT-PCR analysis shows that the level of expression of HPV-16 E5 wild type and HPV-16 E5 mutants (R79, A54, V36, R30 and Del-1) at 35 cycles was comparable. Two clones of HPV-16 E5 wild type (pc16E5), four clones of E5Del-1, one clone of E5R79, two clones of E5A54, one clone of E5V36 and two clones of E5R30 were used (the level of E5 and actin mRNA were analysed using Image-Quantification 5.2 P.C. program).

3.2.12 Semi-quantitative RT-PCR analysis using different numbers of cycles

The quantitation of RNA was done by RT-PCR at different cycles (15, 20, 25, 30 and 35 cycles) to determine the quantity of E5 RNA. Total cell RNA was prepared as in section 2.2.20 and used as the template for reverse transcription and PCR amplification by using Superscript one-step RT-PCR with platinum as previously described. 5 µl of each sample was run on the gel containing 1x loading buffer and carried out Image-Quantification 5.2 P.C. programme. No RNA could be detected at 15 cycles. Actin RNA could be detected at 20 cycles, whereas E5 RNA could be detected only at 25 cycles (Figure 22), confirming these is less E5 RNA than actin RNA. However, RNA from E5 wild type and E5 mutants, including E5Del-1, were detected at approximately the same levels (Figure 22).

22. Semi-quantitative RT-PCR analysis of HPV-16 E5 wild type and HPV-16 E5 mutants using different cycles (15, 20, 25, 30 and 35 cycles).




Band Intensity
Semi-quantitative RT-PCR analysis of HPV-16 E5 A54
Actin
Sample
Actinand Sample RNA
14000
12000
10000
8000
6000
4000
2000
0
20-cycles
25-cycles
30-cycles
35-cycles
Semi-quantitative RT-PCR analysis of HPV-16 E5 V36
Actin
Sample
Actinand Sample RNA
20 -cycles
25 -cycles
30 -cycles
35 -cycles
Semi-quantitative RT-PCR analysis of HPV-16 E5 R30
Actin
Sample
Actinand Sample RNA
20 -cycles
25 -cycles
30 -cycles
35 -cycles

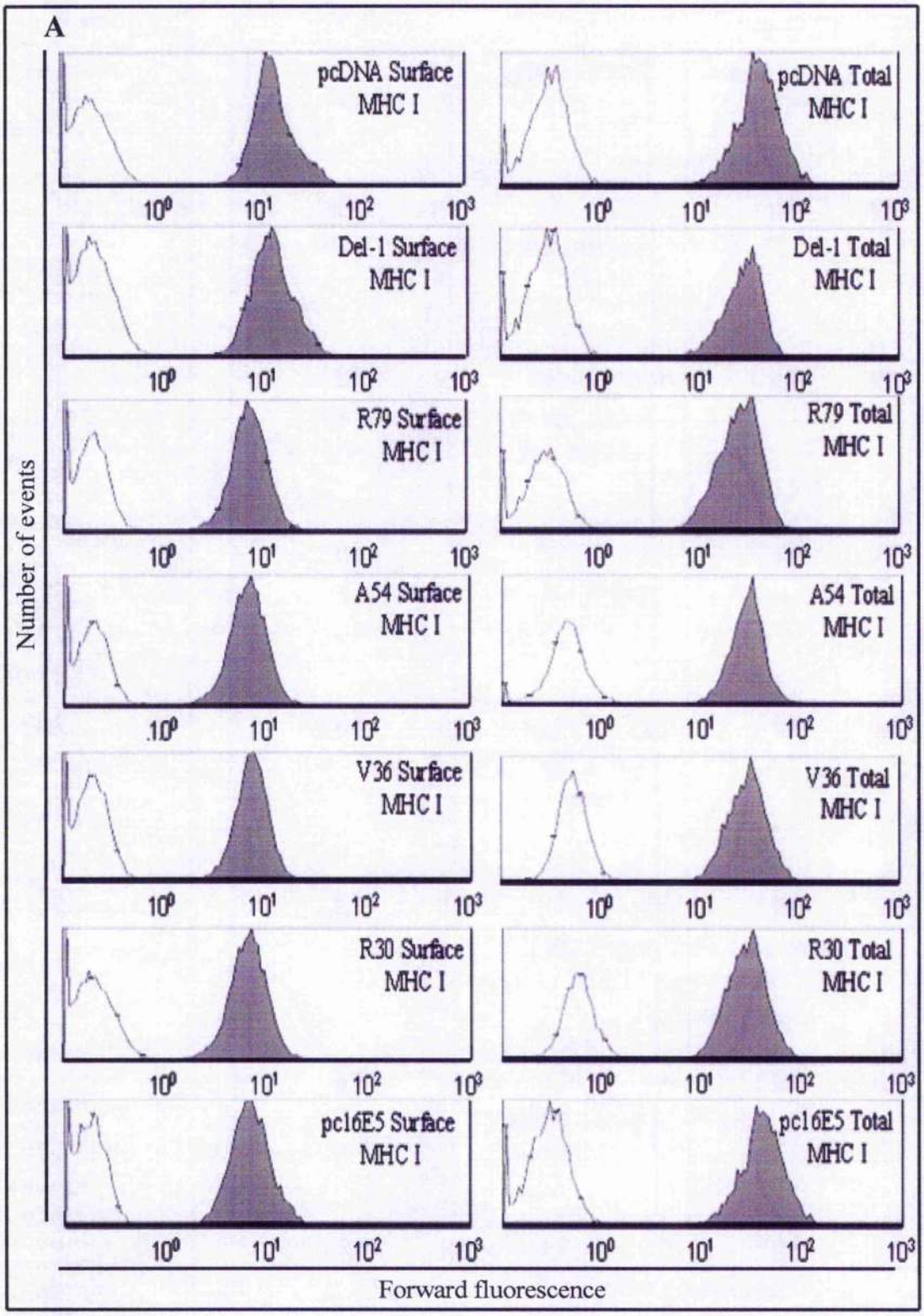
3.2.13 Down-regulation of MHC class I by HPV-16 E5 mutants

The effect of HPV-16 E5 mutants on MHC class I was investigated by FACS for the level of surface and total MHC class I and by immunofluorescence for the cellular localization of MHC class I.

3.2.13.1 Down-regulation of MHC class I by the first hydrophobic domain of HPV-16 E5

The aim was to investigate which domain of HPV-16 E5 protein is important for down-regulation of MHC class I. To this end we have shown that HPV-16 E5 wild type down-regulates surface MHC class I. Three clones of HaCaT parental cells, cells caring empty vectors (pcDNA), cells expressing HPV-16 E5 (pc16E5) or cells expressing HPV-16 E5 mutants (R79, A54, V36, R30 and Del-1) were incubated with mAb W6/32, and analysed by FACS for levels of both surface and total MHC class I. We determined that the cells expressing HPV-16 E5 Del-1 mutant were no different from parental or control HaCaT cells, with approximately twice as much total MHC class I than surface MHC class I. In contrast, in cells expressing HPV-16 E5 mutants (R79, A54, V36 and R30) the levels of surface MHC class I was reduced to approximately half of the HaCaT parental or control cells and indistinguishable from that of cells expressing E5 wild type. However, the level of total MHC class I was constant in the all cell lines (Figure 23). These results suggest that the firs hydrophobic domain of HPV-16 E5 is important to reduce surface MHC class I.

23. Detection of MHC class I by FACS



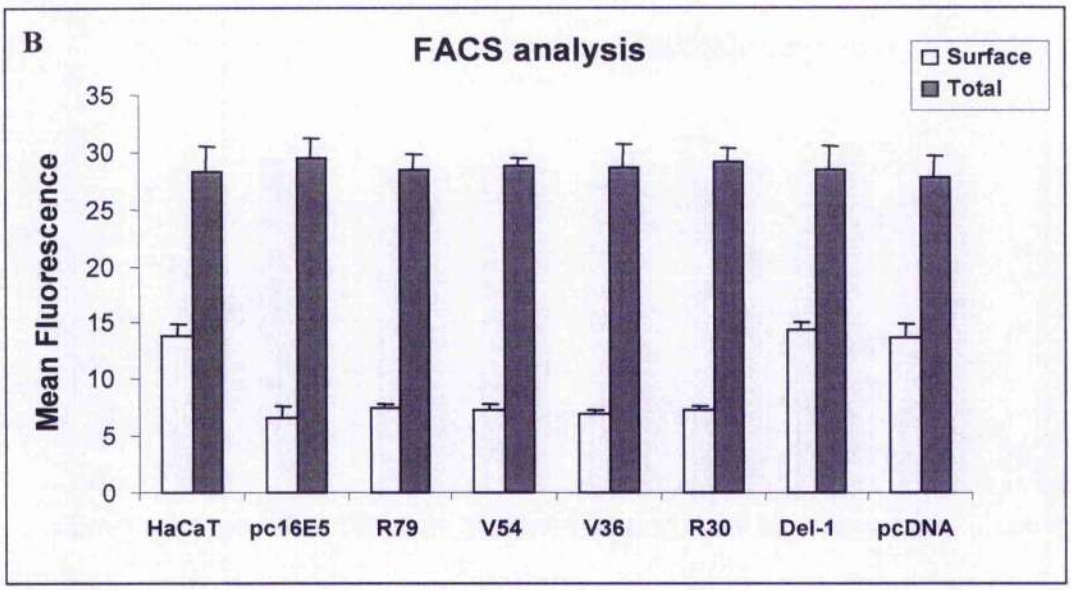


Figure 23: Down-regulation of surface MHC class I in the cells expressing HPV-16 E5 wild type (pc16E5) and HPV-16 E5 mutants (R79, A54, V36, R30 and Del-1). HaCaT parental cells, pcDNA, pc16E5 or E5 mutants (three cell lines of each) were stained with mAb W6/32 and analysed for expression of surface and total MHC class I by FACS. **A)** Representative FACS profiles of one clone of each cell line. Dotted line and open histogram forward fluorescence with secondary antibody only; solid histogram forward fluorescence with primary and secondary antibody. **B)** The average fluorescence for the different cell lines was calculated from the FACS analysis. A background of 0.4 (the reading of cells stained with only secondary antibody) was subtracted in all cell lines.

3.2.13.2 Detection of MHC class I by immunofluorescence microscopy

To confirm our observation with the FACS analysis we used immunofluorescence staining, as described in section 2.2.26, to detect the presence of MHC class I on the cell surface and in the cytoplasm of HaCaT carrying HPV-16 E5 wild type and mutants. We determined that MHC class I is present on the cell surface and in the cytoplasm in HaCaT E5Del-1 cells and control cells, but is present only in the cytoplasm of the cells expressing HPV-16 E5 wild type (pc16E5) and HPV-16 E5 mutants: R79, A54, V36 and R30 (Figure 24).

24. Detection of MHC class I by immunofluorescence microscopy

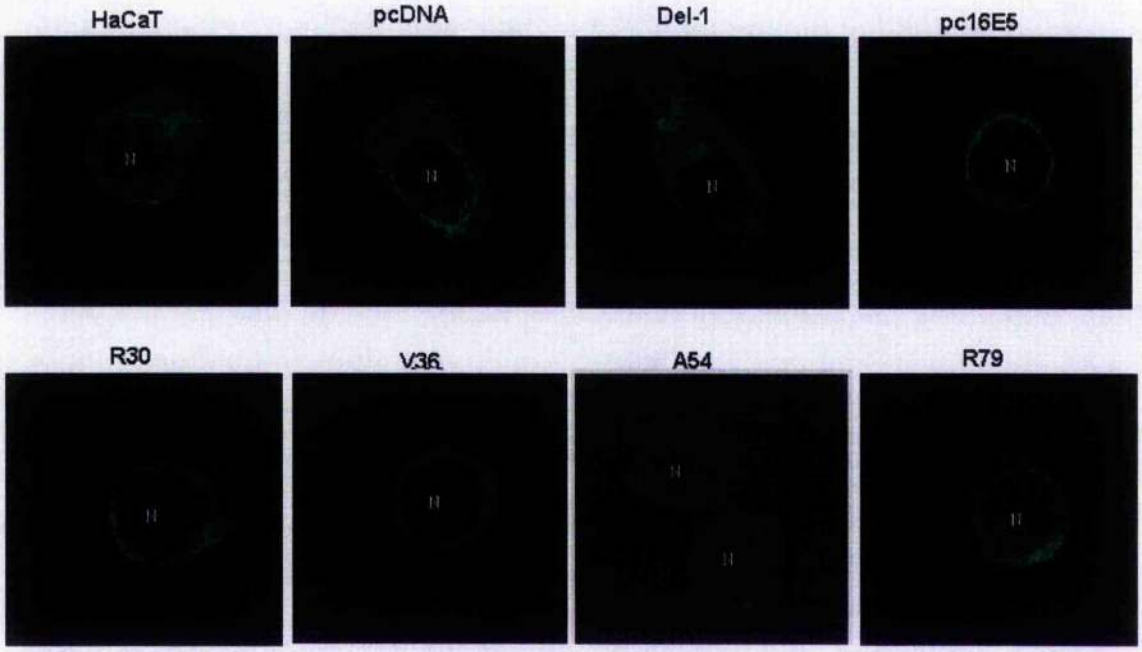


Figure 24: HaCaT parental cells, cells carrying empty vector (pcDNA), cells expressing HPV-16 E5 wild type and HPV-16 E5 mutants (R79, A54, V36, R30 and Del-1) were stained with mAb W6/32. The slides were mounted in Citifluor^{TM} and analysed with a Leica TCS SP2 fluorescence confocal microscope at 488nm wavelength. Images were acquired using Leica confocal software to show the localization of MHC class I. N=nucleus.

3.2.14 Cloning of HPV-16 E5 wild type and mutants into pEGFP-CI vector

We have established that HPV-16 E5 down-regulates MHC class I on the cell surface and the first hydrophobic domain is necessary for down-regulation (Figures 8 and 23). We have shown that MHC class I was detected only in the GA in the cells expressing HPV-16 E5, in contrast in HaCaT control cells or no E5-cells, MHC class I was detected both on the cell surface and the cytoplasm (Figure 10). It has been shown that BPV-4 E5 and MHC class I co-localizes in the GA (Marchetti *et al.,* 2002). To investigate the localization of HPV-16 E5 wild type and mutants we constructed fusion forms of HPV-16 E5 wild type and HPV-16 E5 mutants with the GFP as described in section 2.2.8. The plasmids pEGFP-C1 HPV-16 E5 wild type and the pEGFP-C1 HPV-16 E5 mutants were transfected in HaCaT cells by using Lipofectamine Plus. Following transfection, the cells were selected in medium containing 500 µg/ml G418. Cells were analysed by confocal microscope to ascertain where the HPV-16 E5 mutants localized. We determined that GFP alone is expressed throughout the cell in HaCaT cells but HPV-16 E5 wild type and all HPV-16 E5 mutants including Del-1 localized to the cytoplasm (Figure 25).

25. Localisation of the HPV-16 E5 wild type and mutants using GFP vector.

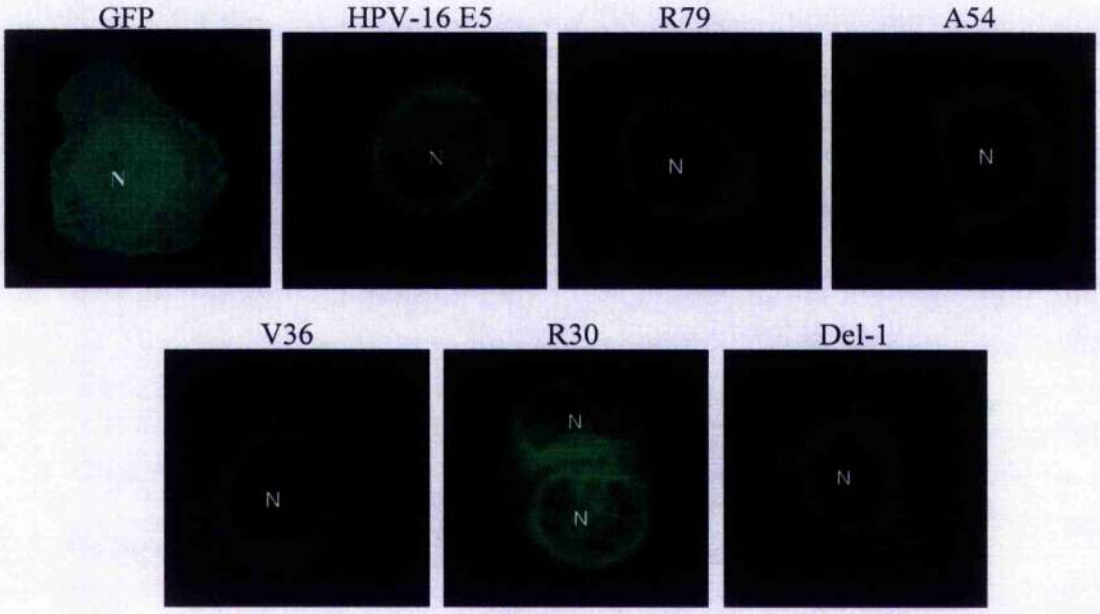


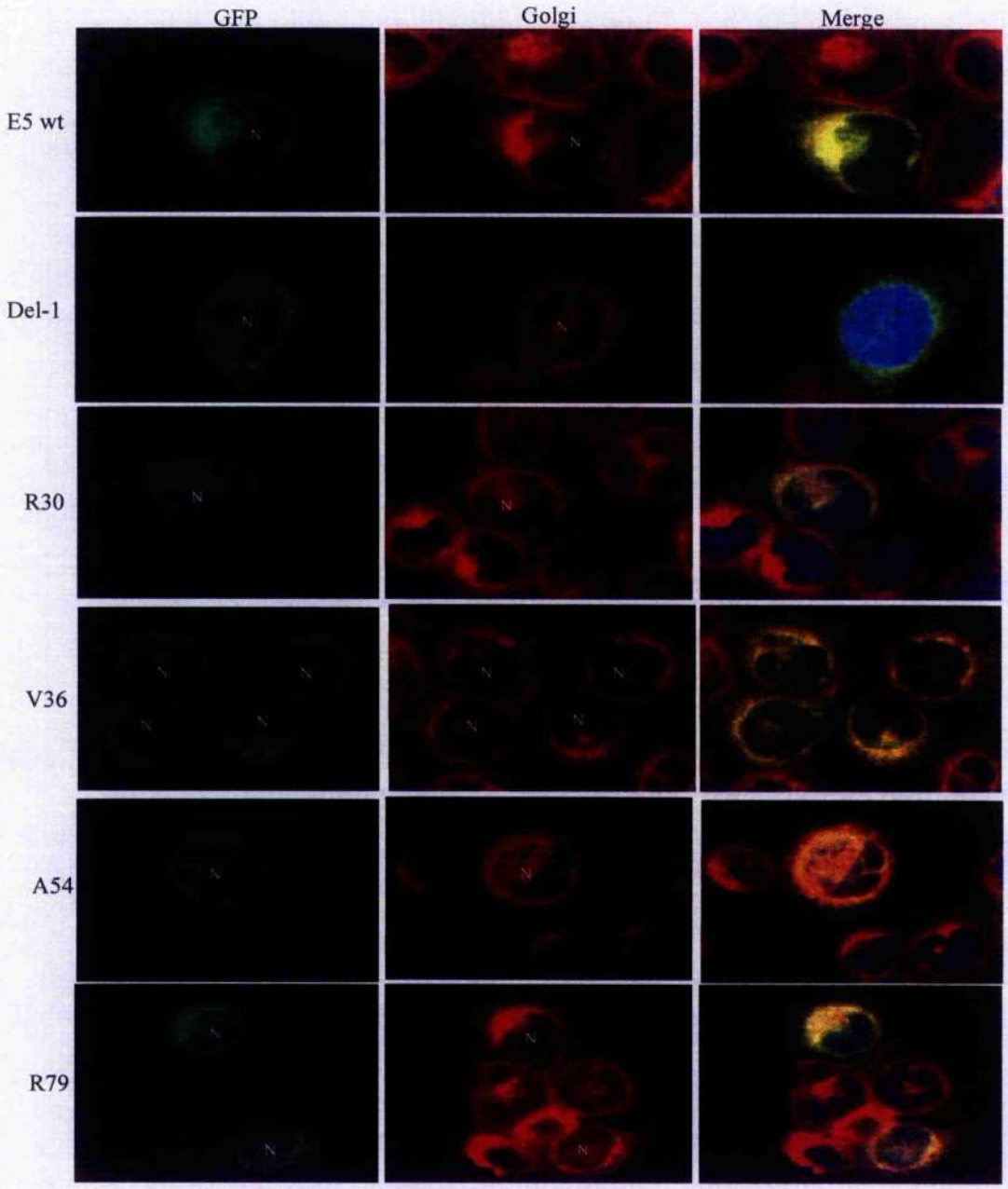
Figure 25: HaCaT cells expressing pEGFP-C1, pEGFP-C1 HPV-16 E5 wild type and pEGFP-C1 HPV-16 E5 mutants (R79, A54, V36, R30 and Del-1) were aliquoted into 24-well plates containing coverslips at $1x10^4$ cells per well. The cells were fixed using fixing solution at room temperature for 10 minutes, mounted in Citifluor™ and analysed with a Leica TCS SP2 fluorescence confocal microscope at 488nm wavelength. Images were acquired using Leica confocal software. N=nucleus

3.2.15 Localisation of pEGFP-E5 and visualisation of Golgi apparatus

We established that HPV-16 E5 wild type and HPV-16 E5 mutants including Del-1 localized to the cytoplasm but GFP is expressed throughout the cell. To see if HPV-16 E5 mutants (R79, A54, V36, R30 and Del-1) localize in the GA like HPV-16 E5 wild type, the GA was visualised by immunofluorescence detection using mAb 4A3. HaCaT cells were aliquoted into 24-well plates containing coverslips at $1x10^4$ cells per well and grown overnight till 20%-50% confluent. The cells were fixed and incubated in permeabilization solution at room temperature for 10 minutes and then stained with mAb 4A3 (for Golgi) and DAPI (for nucleus). Cells were analysed by two colours fluorescence to ascertain where the HPV-16 E5 mutants localized. We determined that HPV-16 E5 wild type and all HPV-16 E5 mutants including Del-1 are localized in the GA (Figure 26). The same results were obtained when the GA was stained with BODIPY-Ceramide (not shown).

Figure 26: HaCaT cells expressing pEGFP-C1 HPV-16 E5 wild type and pEGFP-C1 HPV-16 E5 mutants (R79, A54, V36, R30 and Del-1) were aliquoted, grown, fixed and permeabilised as previously described. The cells were stained with mAb 4A3 (for Golgi staining) and DAPI (for nucleus staining) and then analysed with the Leica TCS SP2 fluorescence confocal microscope. Images were acquired using Leica confocal software to show the localization of GFP-E5 and visualisation of the GA. The left panels are GFP-E5 wild type and GFP-E5 mutants, the middle panels are the GA and the right panels are merge between GFP-E5 wild type or GFP-E5 mutants and the GA with nucleus. N=nucleus

26. Localisation of the pEGFP-HPV-16 E5 wild type and mutants in HaCaT cells



3.2.16 Conclusion

We determined that the cells expressing HPV-16 E5 Del-1 mutant were no different from parental or control HaCaT cells, with approximately twice as much total MHC class I than surface MHC class I. In contrast, in cells expressing the other HPV-16 E5 mutants (R79, A54, V36 and R30) the levels of surface MHC class I was reduced to approximately half of the HaCaT parental or control cells and indistinguishable from that of cells expressing E5 wild type. However, the level of total MHC class I was constant in all the cell lines (Figure 23). To confirm this, we did immunofluorescence microscopy analysis of the same cell lines. The results show that MHC class I was detected on the cell surface and the cytoplasm in HaCaT Del-1 cells (Figure 24). These results suggest that down-regulation of MHC class I is due to the first hydrophobic domain of HPV-16 E5. To investigate the localization of HPV-16 E5 wild type and HPV-16 E5 mutants in the transfected HaCaT cells, we determined that HPV-16 E5 wild type and HPV-16 E5 mutants including Del-1 localized to the GA, whereas GFP is expressed throughout the cell (Figure 25 and 26). These results show that the localization of the E5 in the GA is important but not sufficient for the down-regulation of MHC class I as E5Del-1 fails to down-regulate of surface MHC class I despite its localisation in the GA; and that the first hydrophobic domain of HPV-16 E5 is necessary for down-regulation of MHC class I.

3.3 HPV-16 E5 protein physically interacts with MHC class I

3.3.1 Introduction

The E5 protein is a small hydrophobic protein that can interact with cellular proteins, including EGF-R, 16K subunit c/ductin and PDGE-R. It has been reported that BPV E5 protein (Goldstein *et al.,* 1990) and HPV E5 protein (Conrad *et al.,* 1993) bind to 16K subunit c/ductin, which is found in two distinct membrane complexes, V0 sector of the vacuolar H+ ATPase (Mandel *et al.,* 1988) and gap junctions (Finbow and Pitts 1993). The binding of the E5 to 16K subunit c/ductin has led to the suggestion that the function of both the gap junction and V-ATPase might be impaired. Previous studies (Rodriguez *et al.,* 2000) have identified the second domain of HPV-16 E5 as responsible for binding to the 16K subunit c. The V-ATPase is a universal transmembrane proton pump of eukaryotes and is responsible for acidification of the cytoplasmic organelles (e.g. Golgi, endosomes, lysosomes, and certain secretory vesicles) (Finbow *et al.,* 1997). Thus, complex formation between E5 and 16K subunit c would inhibit endosomal acidification leading to an increase in receptor recyling to the plasma membrane (Straight *et al.,* 1993 and 1995). It has been determined that the expression of E5 increases the pH of the GA (Schapiro *et al.,* 2000), which is thought to be through its binding to 16K subunit c. Since growth factor receptors are processed in the Golgi and are internalized via clathrin-coated vesicles, E5 binding to the 16K protein within these components may be an intermediate step to receptor activation and signal transduction.

The 'ductin' is a component of a type of gap junction, an intercellular complex that provides sites of cell-to-cell movement (Finbow and Pitts 1993). It has been reported that the interaction of HPV-16 E5 with ductin is responsible for the observed down-regulation of gap junctions (Conrad *et al.,* 1993). HPV-16 E5 reduces gap junction-mediated intercellular communication via dephosphorylation of connexin 43 (Oelze *et al.,* 1995). This results in the

cessation of tissue homeostatic feedback, which has also been described as an early event in carcinogenesis progression (King *et al.*, 2000).

It has been demonstrated that mouse fibroblasts transfected with HPV-16 E5, have increased cellular growth and this growth is accelerated by addition of epidermal growth factor (Pim *et al.*, 1992; Leechanachai *et al.*, 1992; Straight *et al.*, 1995) by binding to the EGF-R when both proteins are over-expressed in the transfected cells (Hwang *et al.*, 1995). In human keratinocyte cells, the target cells for the papillomavirus, long-term treatment with EGF results in an E5-mediated increase in the number of EGF-R at the cell surface (Straight *et al.*, 1993). Moreover, BPV-1 E5 may act in a similar manner as this transforming protein binds to the PDGF-R and BPV-1 E5 expressing cells respond to addition of PDGF (Petti *et al.*, 1991; Petti and DiMaio, 1992).

It has been shown (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002) that cells transformed by BPV E5 can inhibit transport of MHC class I molecules to the cell surface. Down-regulation of MHC class I in the cell expressing BPV-4 E5 is caused by physical interaction between MHC class I heavy chain protein and the C-terminus of the BPV-4 E5 protein (Marchetti *et al.*, 2006). We have found that HPV-16 E5 down-regulates MHC class I on the cell surface and the first hydrophobic domain of HPV-16 E5 is responsible for this down-regulation. It is important to determine how HPV-16 E5 protein down-regulates MHC class I on the cell surface. To this end, we have investigated the physical interaction of HPV-16 E5 and MHC heavy chain by Co-immunnoprecipitation.

3.3.2 *In vitro* immunoprecipitation and Co-immunoprecipitation assays

Immunoprecipitation (IP) and co-immunoprecipitation (Co-IP) are key techniques for studying protein-protein interactions. These methods utilize immobilized Sepharose beads

protein-G or protein A to isolate antibody-bound target antigens. IP is a powerful immunochemical technique that has been used to study antigen characteristics such as interactions with proteins. The IP procedure involves interacting antigens with antibody to allow formation of immune complexes, and precipitating those complexes with beads. Co-IP is a key technique used to study protein-protein interactions, such as interactions of subunits within a protein complex. Typically, an antibody specific for one protein is incubated with a protein mixture to form an immune complex with the target protein (antigen). The target protein may be interacting with one or other more proteins to form a protein complex. The entire protein complex is then precipitated with beads. We have done *in vitro* transcription and translation of HPV-16 E5 wild type, HPV-16 E5 mutants (R79, Δ54, V36, R30 and Del-1) and HLA-A2 were performed as described in section 2.2.32.1.

3.3.3 HPV-16 E5 wild type physically interacts with HLA heavy chain by Co-IP

Given that BPV E5 can inhibit transport of MHC class I molecules to the cell surface (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002 and 2006), we investigated that HPV-16 E5 inhibits the transport of MHC class I to the cell surface, reducing the level of surface MHC class I to approximately half of that in the control cells. It is necessary to determine how HPV-16 E5 protein down-regulates surface MHC class I on the cell surface. We have shown that both MHC class I and HPV-16 E5 co-localize in the GA by using GFP vector, as does BPV E5 (Marchetti *et al.*, 2002). The complete co-localization of HPV-16 E5 and the residual MHC class I in the GA would suggest an interaction between HPV-16 E5 and the MHC complex. To this end, we performed Co-IP experiments to investigate if any such interaction existed. HPV-16 E5 and HLA-A2 were separately transcribed/translated, *in vitro,* in the presence of ^{35}S-labelled methionine and were then kept separately or mixed together as described in

section 2.2.32.1. The protein samples were precipitated with four different antibodies: mAb HC10 against human MHC heavy chain, mAb HA against HA-tag of HPV-16 E5 and rabbit polyclonal antisera against synthetic peptides corresponding to the amino-terminal (E5N-terminus antiserum) or carboxy-terminal (E5C-terminus antiserum) sequences of the HPV-16 E5 protein. The samples were then loaded into consecutive wells in the SDS-PAGE gels. The results showed that there was no significant precipitation in the absence of antibody (Figure 27A, lanes 1, 4; Figure 27B, lanes 2, 4, 6; Figure 27C and 27D, lanes 3, 5, 7). The HC10 antibody precipitated HLA heavy chain but not E5 (Figure 27A, lane 3), and co-precipitated HLA heavy chain and E5 (Figures 27A, lane 2). The HA antibody precipitated E5 and co-precipitated E5 and HLA heavy chain (Figure 27B, lanes 5 and 7). The HLA heavy chain band was also visible in the lanes without the antibody (Figure 27B, lanes 2 and 6) or without E5 (Figure 27B, lane 3); this was often observed with the HA antibody (see Figure 30A and Figure 31A), but we consider this an artifact as the heavy chain band was stronger in the lanes with the co-precipitated.

The Co-IP experiment between HPV-16 E5 and HLA heavy chain was repeated using antisera against the N-terminus and the C-terminus of E5. Also in this case, E5 and heavy chain co-precipitated (Figures 27C and 27D, lanes 6), while there were no bands in the lanes without antibody (Figures 27C and 27D, lanes 7). However, HLA heavy chain was detected also in the lanes without E5 and without antibody (Figures 27C and 27D, lanes 2 and 3). The reason for this artifact is not known.

Taking all these results together (see also Figure 34, lane 4), we conclude that HPV-16 E5 is likely to interact physically with MHC class I heavy chain

Figure 27: For Co-IP, HPV-16 wild type and HLA-A2 were translated and labelled with [^{35}S]methionine and precipitated with four different antibodies: mAb HC10 against HLA heavy chain, mAb HA against E5HA tag, E5 N-terminus antiserum and E5 C-terminus antiserum against E5 N-terminus or E5 C-terminus.

A panel: immunoprecipitated with HC10 antibody against HLA-A2 heavy chain. Lanes 1 and 2 are Co-IP with HLA-A2+HPV-16 E5 with or without HC10 antibody. Lanes 3 and 4 are IP-A2 with or without HC10 antibody. Lane 5 is E5 input (5µl) and lane 6 is HLA-A2 input (5µl).

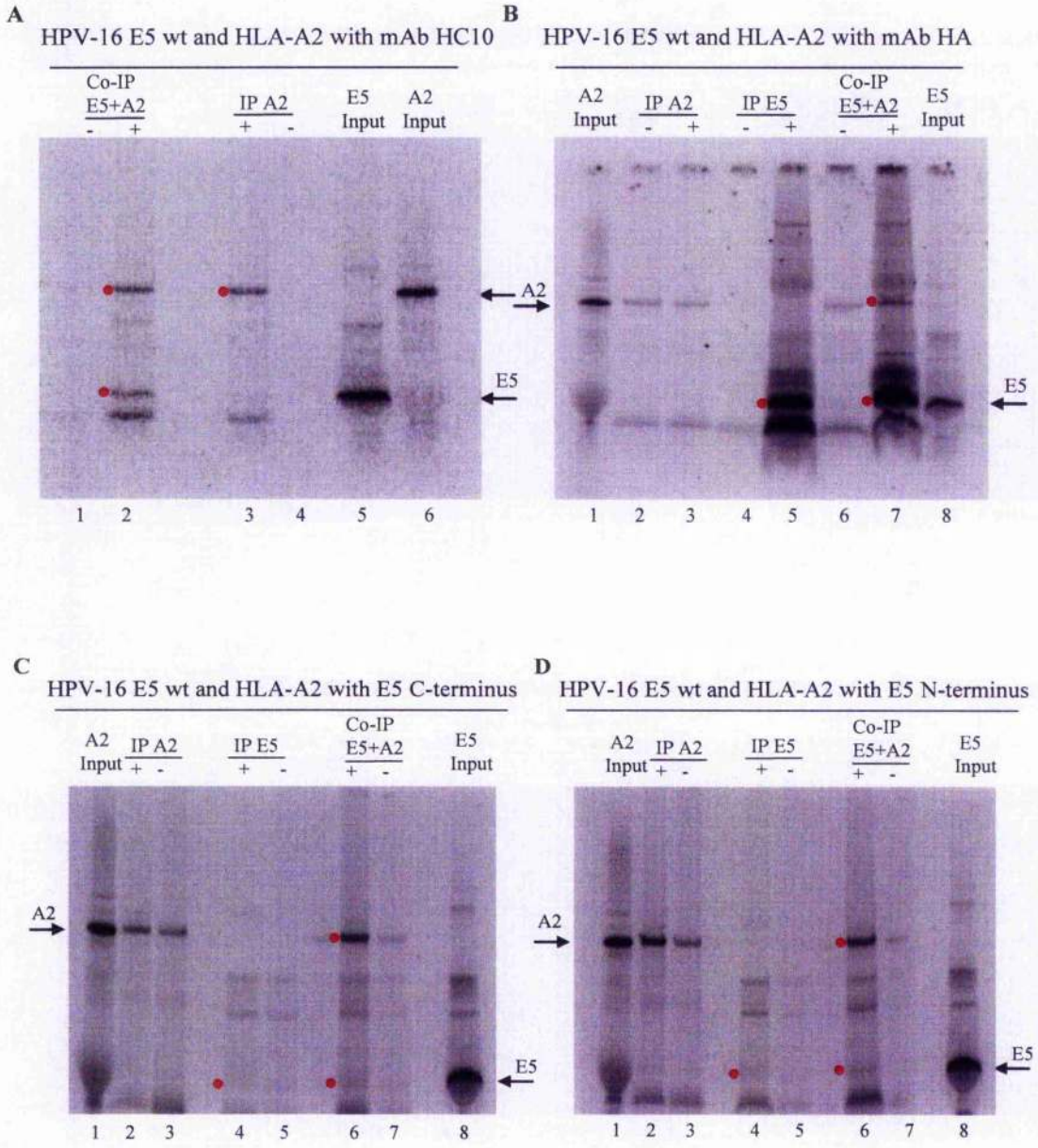
B panel: immunoprecipitated with HA antibody against E5HA-tag. Lane 1 is HLA-A2 input (5µl). Lanes 2 and 3 are IP-A2 with or without HA antibody. Lanes 4 and 5 are IP-E5 with or without HA antibody. Lanes 6 and 7 are Co-IP with HLA-A2+HPV-16 E5 with or without HA antibody and lane 8 is E5 input (5µl).

C panel: immunoprecipitated with E5 C-terminus antiserum against E5. Lane 1 is HLA-A2 input (5µl). Lanes 2 and 3 are IP-A2 with or without E5 C-terminus antiserum. Lanes 4 and 5 are IP-E5 with or without E5 C-terminus antiserum. Lanes 6 and 7 are Co-IP with HLA-A2+HPV-16 E5 with or without E5 C-terminus antiserum and lane 8 is E5 input (5µl).

D panel: immunoprecipitated with E5 N-terminus antiserum against E5. Lane 1 is HLA-A2 input (5µl). Lanes 2 and 3 are IP-A2 with or without E5 N-terminus antiserum. Lanes 4 and 5 are IP-E5 with or without E5 N-terminus antiserum. Lanes 6 and 7 are Co-IP with HLA-A2+HPV-16 E5 with or without E5 N-terminus antiserum and lane 8 is E5 input (5µl).

The E5 and MHC class I heavy chain bands are indicated by red dots and the other bands originate from the translated E5 protein (see input lanes) and may represent multimers of E5.

27. Interaction of HPV-16 E5 wild type and HLA heavy chain



3.3.4 IP and Co-IP of HPV-16 E5 mutants and HLA-A2

We have determined that the first hydrophobic domain of HPV-16 E5 is necessary for down-regulation of surface MHC class I. In contrast, in BPV E5, the C-terminus is important for down-regulation of MHC class I (Marchetti *et al.*, 2006). We have found that both MHC class I and HPV-16 GFP-E5 mutants co-localize in the GA, similar to HPV-16 E5 wild type. We have also found that HPV-16 E5 wild type physically interacts with HLA heavy chain. To determine the region of the HPV-16 E5 protein responsible for binding to HLA heavy chain, we used the previously described deletion mutants. E5R79, E5A54, E5V36 and E5R30 have the same N-terminus and lack increasing portions of the C-terminus; E5Del-1 has a complete C-terminus but lacks the first hydrophobic domain. To investigate if any such interaction exists, we performed Co-IP experiments. HPV-16 E5 mutants and HLA-A2 were separately transcribed/translated, *in vitro,* in the presence of ^{35}S-labelled methionine and were then kept separately or mixed together as described before. The protein samples were precipitated with three different antibodies: mAb HC10, mAb HA and E5 N-terminus antiserum. The samples were then loaded into consecutive wells in the SDS-PAGE gels. The results show that there was no significant precipitation in the absence of antibody (Figure 28, lanes 3, 5, 7; Figure 29, lanes 2, 4; Figure 30A, lanes 3, 5, 7; Figure 30B, lanes 3, 5; Figure 31A, lanes 2, 4; Figure 31B, lanes 3, 5, Figure 31C, lanes 3, 5 and Figure 32, lanes 3, 5).

The HC10 antibody precipitated HLA heavy chain but not E5 mutants (Figure 28, lane 6, Figure 31A, lane 5 and Figure 32, lane 4), and the HA antibody and E5 N-terminus antiserum precipitated E5 mutants (Figure 30A, lane 4; Figure 30B, lane 4 and Figures 31B and 31C, lanes 4). We found that E5R79, E5A54 and E5R30 were all co-precipitated with HLA-A2 by HC10 antibody against HLA heavy chain (Figure 28, lane 2; Figure 29, lane 3 and Figure 31A, lane 3). Moreover, HLA-A2 co-precipitated with E5R30 using either HA antibody or

E5N-terminus antiserum against E5 (Figures 31B and 29C, lanes 2), and E5V36 co-precipitated with either HA antibody or E5N-terminus antiserum against E5 (Figures 30A, and 30B, lanes 2). When the E5Del-1 and HLA-A2 proteins were mixed together, the E5Del-1 protein did not precipitate using HC10 antibody (Figure 32, lane 2).

There were additional bands, particularly in lane 2 of Figure 28 and lane 3 of Figure 29. These bands appeared to derive from the transcription reactions (see corresponding "input" lanes) and were considered background.

As E5Del-1 mutant does not interact with HLA-A2 heavy chain and E5V36 and E5R30 mutants interact, we conclude that the physical interaction between E5 and HLA-A2 heavy chain takes place between the first hydrophobic domain of HPV-16 E5 and HLA heavy chain.

28. IP and Co-IP of HPV-16 E5 R79 and HLA heavy chain

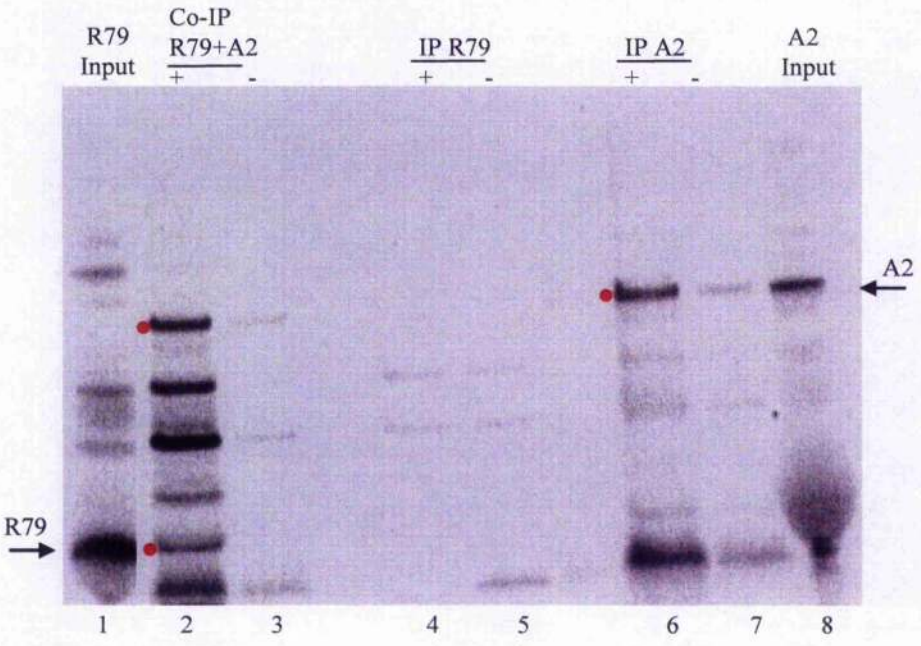


Figure 28: For Co-IP, HPV-16 E5 R79 mutant and HLA-A2 were translated and labelled with [^{35}S]methionine and precipitated with HC10 antibody against HLA heavy chain. Lane 1 is E5R79 input (5µl). Lanes 2 and 3 are Co-IP with HLA-A2+E5R79 with or without HC10 antibody. Lanes 4 and 5 are IP-E5R79 with or without HC10 antibody. Lanes 6 and 7 are IP-A2 with or without HC10 antibody and lane 8 is HLA-A2 input (5µl).

The E5R79 and MHC class I heavy chain bands are indicated by red dots and the other bands originate from the translated E5R79 protein (see input lanes) and may represent multimers of E5R79.

29. IP and Co-IP of HPV-16 E5 A54 and HLA heavy chain

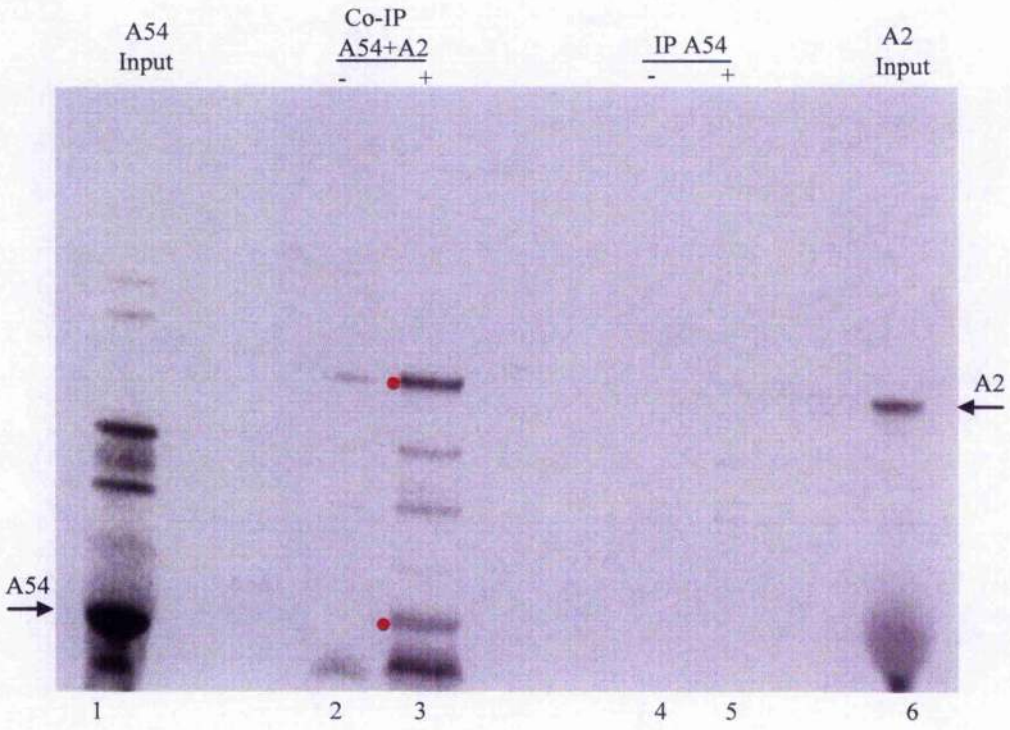


Figure 29: For Co-IP, HPV-16 E5 A54 mutant and HLA-A2 were translated and labelled with [^{35}S]methionine and precipitated with HC10 antibody against HLA heavy chain. Lane 1 is E5A54 input (5µl). Lanes 2 and 3 are Co-IP with HLA-A2+E5A54 with or without HC10 antibody. Lanes 4 and 5 are IP-E5A54 with or without HC10 antibody and lane 6 is HLA-A2 input (5µl).

The E5A54 and MHC class I heavy chain bands are indicated by red dots and the other bands originate from the translated E5A54 protein (see input lanes) and may represent multimers of E5A54.

30. IP and Co-IP of HPV-16 E5 V36 and HLA heavy chain

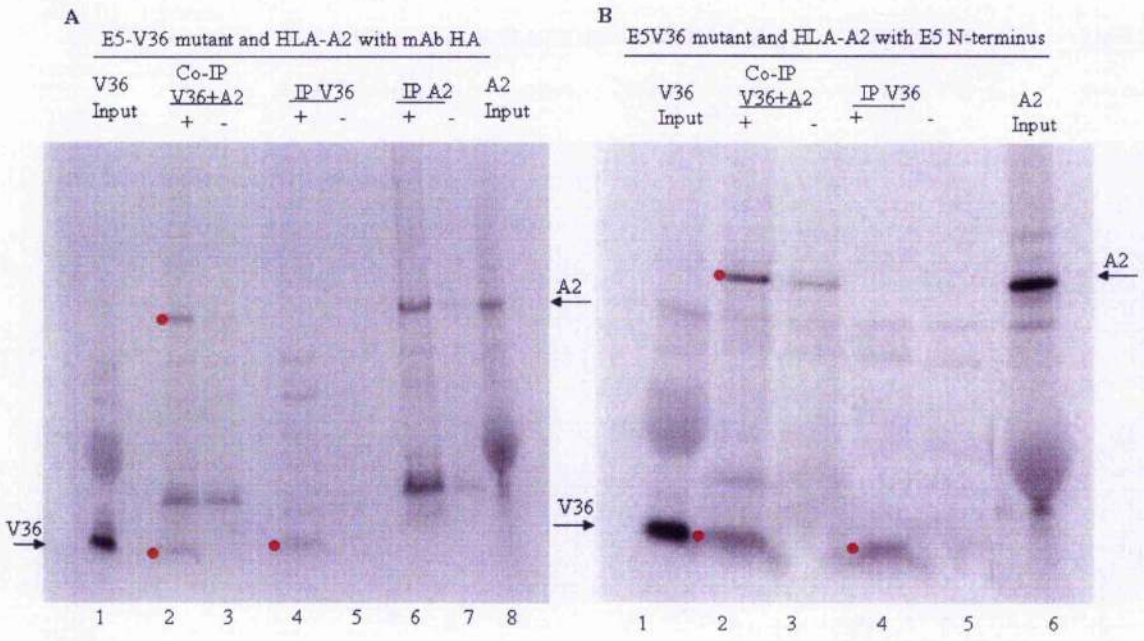


Figure 30: For Co-IP, HPV-16 E5 V36 mutant and HLA-A2 were translated and labelled with [^{35}S]methionine and precipitated with two different antibodies: HC10 antibody against HLA heavy chain and E5N-terminus antiserum against E5 protein.

A panel: immunoprecipitated with HA antibody against E5HA-tag. Lane 1 is E5V36 input (5µl). Lanes 2 and 3 are Co-IP with HLA-A2+E5V36 with or without HA antibody. Lanes 4 and 5 are IP-E5V36 with or without HA antibody and lane 6 is HLA-A2 input (5µl).

B panel: immunoprecipitated with E5 N-terminus antiserum against E5. Lane 1 E5V36 is input (5µl). Lanes 2 and 3 are Co-IP with HLA-A2+E5V36 with or without E5 N-terminus antiserum. Lanes 4 and 5 are IP-E5V36 with or without E5 N-terminus antiserum and lane 6 is HLA-A2 input (5µl).

The E5V36 and MHC class I heavy chain bands are indicated by red dots and the other bands originate from the translated E5V36 protein (see input lanes) and may represent multimers of E5V36.

Figure 31: For Co-IP, HPV-16 E5 R30 mutant and HLA-A2 were translated and labelled with [^{35}S] methionine and precipitated with three different antibodies: HC10 antibody against HLA heavy chain, and HA antibody and E5N-terminus antiserum against E5.

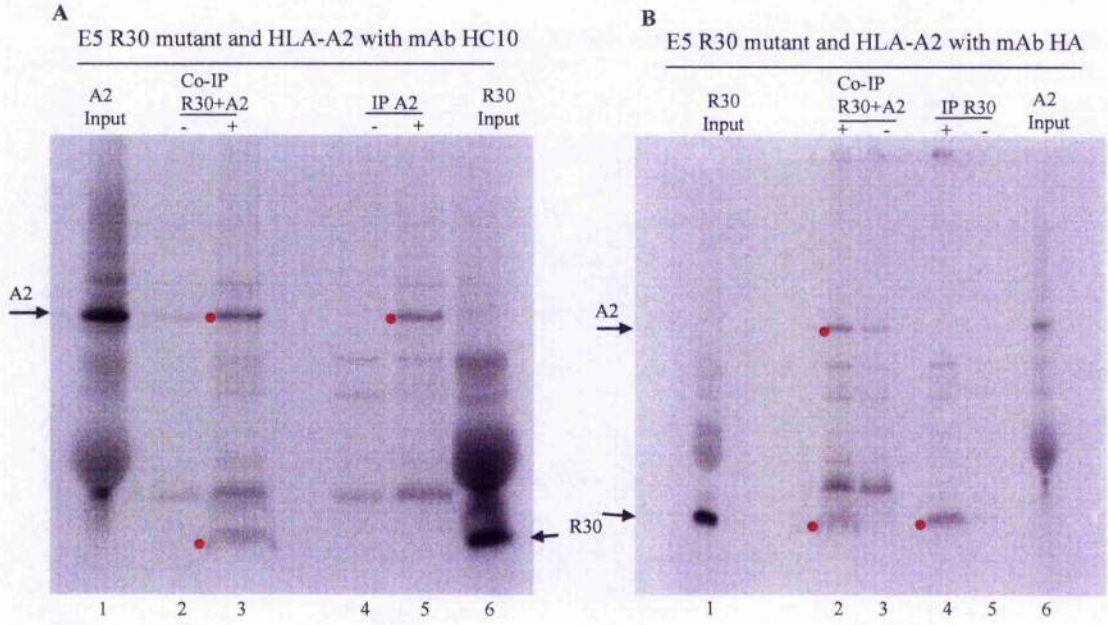
A panel: immunoprecipitated with HC10 antibody against HLA heavy chain. Lane 1 is HLA-A2 input (5μl). Lanes 2 and 3 are Co-IP with HLA-A2+E5R30 with or without HC10 antibody. Lanes 4 and 5 are IP-E5R30 with or without HC10 antibody and lane 6 is E5R30 input (5μl).

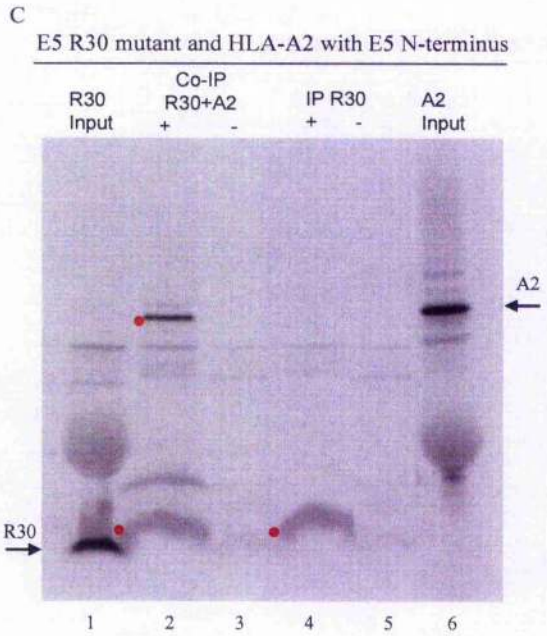
B panel: immunoprecipitated with HA antibody against E5HA-tag. Lane 1 is E5R30 input (5μl). Lanes 2 and 3 are Co-IP with HLA-A2+E5R30 with or without HA antibody. Lanes 4 and 5 are IP-E5R30 with or without HA antibody and lane 6 is HLA-A2 input (5μl).

C panel: immunoprecipitated with E5 N-terminus antiserum against E5. Lane 1 is E5V36 input (5μl). Lanes 2 and 3 are Co-IP with HLA-A2+E5R30 with or without E5 N-terminus antiserum. Lanes 4 and 5 are IP-E5R30 with or without E5 N-terminus antiserum and lane 6 is HLA-A2 input (5μl).

The E5R30 and MHC class I heavy chain bands are indicated by red dots and the other bands originate from the translated E5R30 protein (see input lanes) and may represent multimers of E5R30.

31. IP and Co-IP of HPV-16 E5 R30 and HLA heavy chain





32. IP and Co-IP of HPV-16 E5 Del-1 and HLA heavy chain

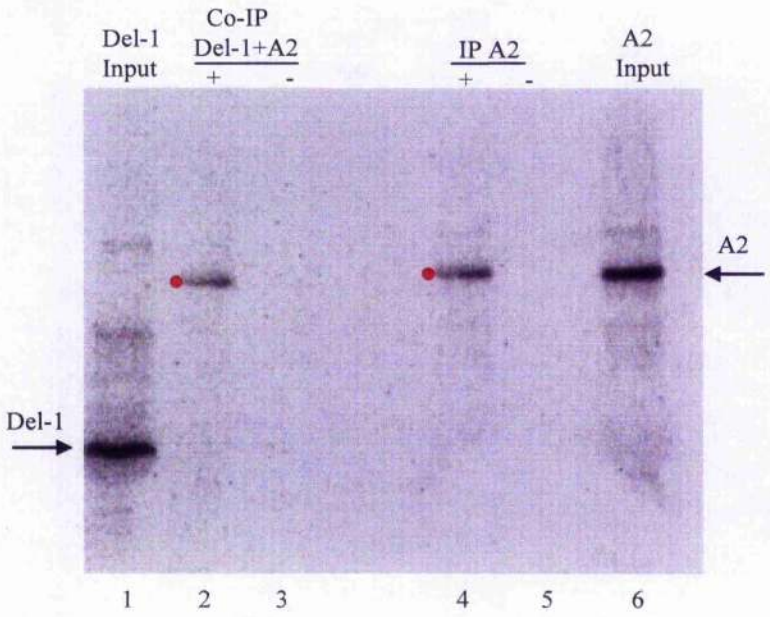


Figure 32: For Co-IP, HPV-16 E5 Del-1 mutant and HLA-A2 were translated and labelled with [^{35}S]methionine and precipitated with HC10 antibody against HLA heavy chain. Lane 1 is E5Del-1 input (5µl). Lanes 2 and 3 are Co-IP with HLA-A2+E5Del-1 with or without HC10 antibody. Lanes 4 and 5 are IP-A2 with or without HC10 antibody and lane 6 is HLA-A2 input (5µl).

The MHC class I heavy chain bands are indicated by red dots.

3.3.5 IP and Co-IP of HPV-16 E5 wild type, HPV-16 E5 Del-1 mutant and HLA-A2

We found that HPV-16 E5 wild type and HPV-16 E5 mutants (E5R79, E5A54, E5V36 and E5R30), which have the same N-terminus, physically interact with HLA heavy chain, but the mutant HPV-16 E5 Del-1 does not. There were non-specific bands in addition to HLA-A2 and HPV-16 E5. To decrease the number of non-specific bands, we performed Co-IP experiment using unlabelled HLA-A2. HPV-16 E5 wild type and HPV-16 E5 Del-1 were separately transcribed/translated, *in vitro,* in the presence of ^{35}S-labelled methionine, but HLA-A2 was transcribed/translated without ^{35}S-labelled methionine and was then kept separately or mixed together as described above. The protein samples were precipitated with mAb HC10 and loaded into consecutive wells in the SDS-PAGE gels.

Figure 33A, lane 4 shows that there is one band corresponding to HPV-16 E5 wild type but no bands corresponding to HLA-A2 because this is not labelled. Figure 33B, lane 4 shows that there is no band corresponding to E5Del-1. These results confirm that HPV-16 E5 wild type physically interacts with HLA heavy chain but HPV-16 E5 Del-1 mutant does not.

33. IP and Co-IP of HPV-16 E5/ HPV-16 E5 Del-1 and HLA- A2

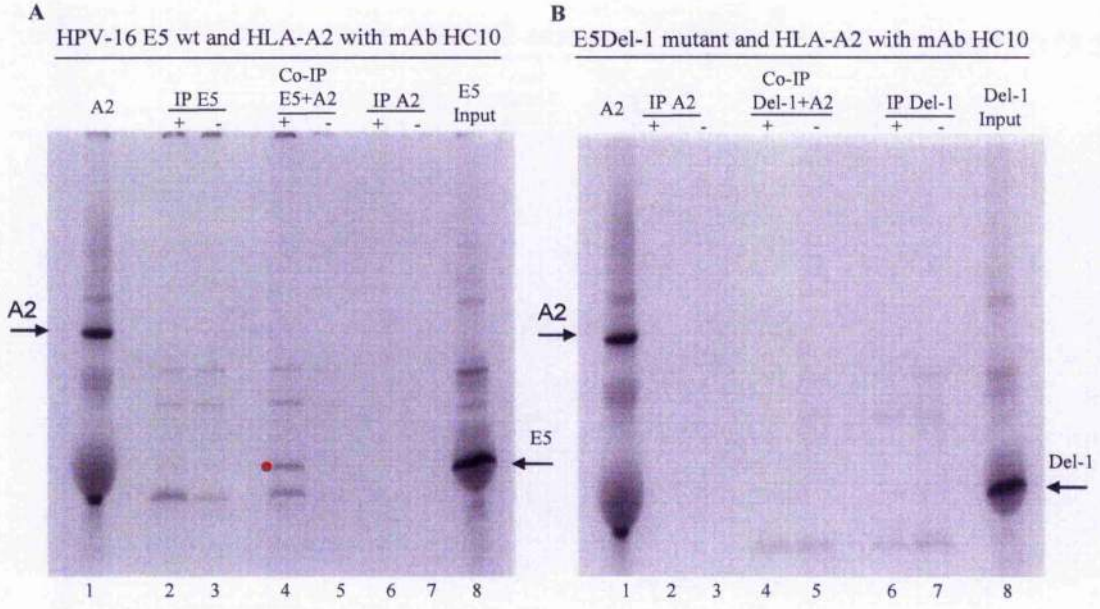


Figure 33: For Co-IP, HPV-16 E5 wild type and HPV-16 E5 Del-1 mutant were translated and labelled with [^{35}S]methionine but HLA-A2 was not labelled and precipitated with HC10 antibody against HLA heavy chain.

A panel: Co-IP, HPV-16 E5 wild type and HLA-A2 precipitated with HC10 antibody. Lane 1 is HLA-A2 input (5µl). Lanes 2 and 3 are IP-E5 with or without HC10 antibody. Lanes 4 and 5 are Co-IP with HLA-A2+E5 with or without HC10 antibody. Lanes 6 and 7 are IP-A2 with or without HC10 antibody and lane 8 is E5 input (5µl).

B panel: Co-IP, E5Del-1 mutant and HLA-A2 precipitated with HC10 antibody. Lane 1 is HLA-A2 input (5µl). Lanes 2 and 3 are IP-A2 with or without HC10 antibody. Lanes 4 and 5 are Co-IP with HLA-A2+E5Del-1 with or without HC10 antibody. Lanes 6 and 7 are IP-E5Del-1 with or without HC10 antibody and lane 8 is E5Del-1 input (5µl).

The E5 band is indicated by red dot.

3.3.6 Competition between HPV-16 E5 wild type and HPV-16 E5 Del-1 mutant for HLA-A2 binding

To confirm the above results, a competition experiment was designed between E5 wild type and E5Del-1. If E5Del-1 does not bind to MHC heavy chain, E5Del-1 will not inhibit the binding of E5 wild type to MHC heavy chain. If E5Del-1 binds to MHC heavy chain, it will prevent the E5 binding. To this end, Co-IP of HPV-16 E5 wild type, HPV-16 E5 Del-1 and HLA-A2 were performed as described in section 2.2.32.2. HPV-16 E5 Del-1 was mixed with HLA-A2 and then HPV-16 E5 wild type and HC10 antibody were added as previously described. As expected, E5Del-1 did not co-precipitate with HLA-A2 (Figure 34, lane 2), whereas E5 wild type did (Figure 34, lane 4). E5 wild type co-precipitated with HLA-A2 even in the presence of E5Del-1 (Figure 34, lane 6), showing that E5Del-1 did not compete with E5 wild type for binding to HLA-A2. These results also confirm that HPV-16 E5 wild type physically interacts with HLA heavy chain but HPV-16 E5 Del-1 mutant does not.

34. IP and Co-IP of HPV-16 E5 Del-1 and HLA-A2/HPV-16 E5 wild type

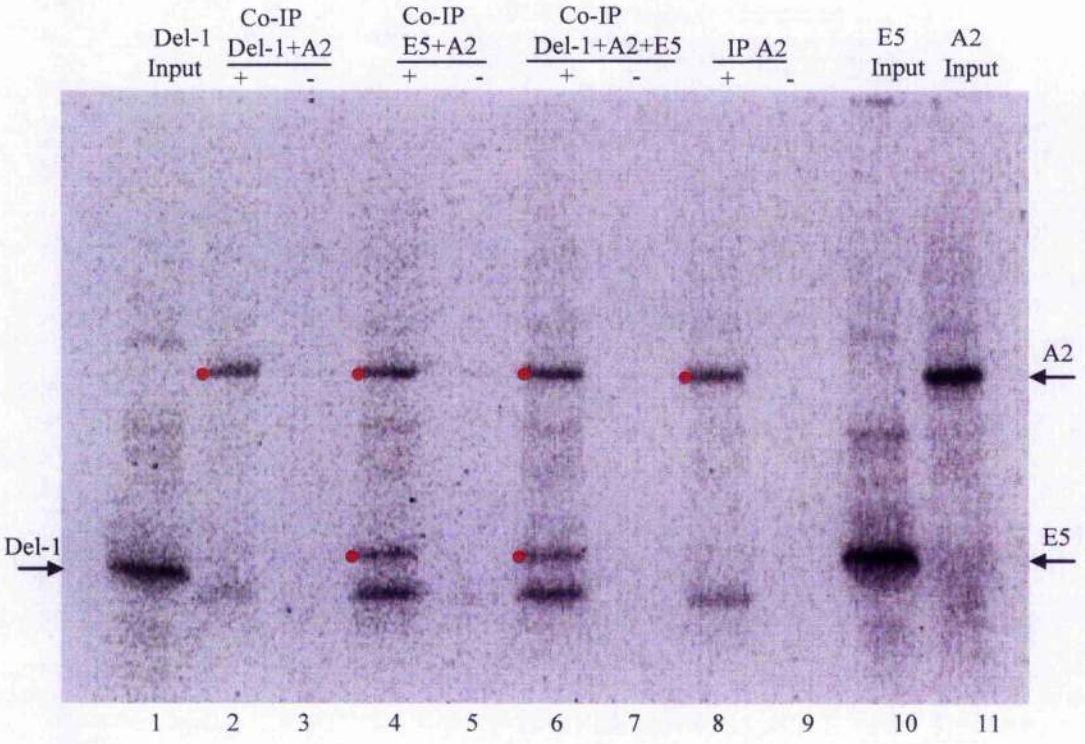


Figure 34: For Co-IP, HPV-16 E5 wild type, HPV-16 E5 Del-1 mutant and HLA-A2 were translated and labelled with [^{35}S]methionine, competed as described above and precipitated with HC10 antibody against HLA heavy chain.

Lane 1 is E5Del-1 input (5µl). Lanes 2 and 3 are Co-IP with A2+E5Del-1 with or without HC10 antibody. Lanes 4 and 5 are Co-IP with A2+E5 wild type with or without HC10 antibody. Lanes 6 and 7 are Co-IP with A2+E5Del-1+E5 wild type with or without HC10 antibody. Lanes 8 and 9 are IP-A2 with or without HC10 antibody. Lane 10 is E5 input (5µl) and lane 11 is HLA-A2 input (5µl).

The E5 and MHC class I heavy chain bands are indicated by red dots.

3.3.7 Competition between HPV-16 E5 wild type and HPV-16 E5 mutants (R30 and V36) for binding to HLA-A2

To confirm the above results, a similar competition experiment was performed with E5V36 and E5R30 mutants (also E5 wild type; Dr.Ashrafi, personal communication). As expected, E5V36 and E5R30 co-precipitated with HLA-A2 (Figures 35 and 36, lanes 5). Furthermore, the presence of E5V36 and E5R30 mutants, either labelled or not labelled, prevented the co-precipitation of E5 wild type with HLA-A2 (Figures 35 and 36, lanes 5 and 7). These results show that both E5V36 and E5R30 mutants compete with E5 wild type and that therefore the binding to HLA-A2 takes place in the first hydrophobic domain of E5.

35. IP and Co-IP of HPV-16 E5 V36 and HLA-A2/HPV-16 E5 wild type

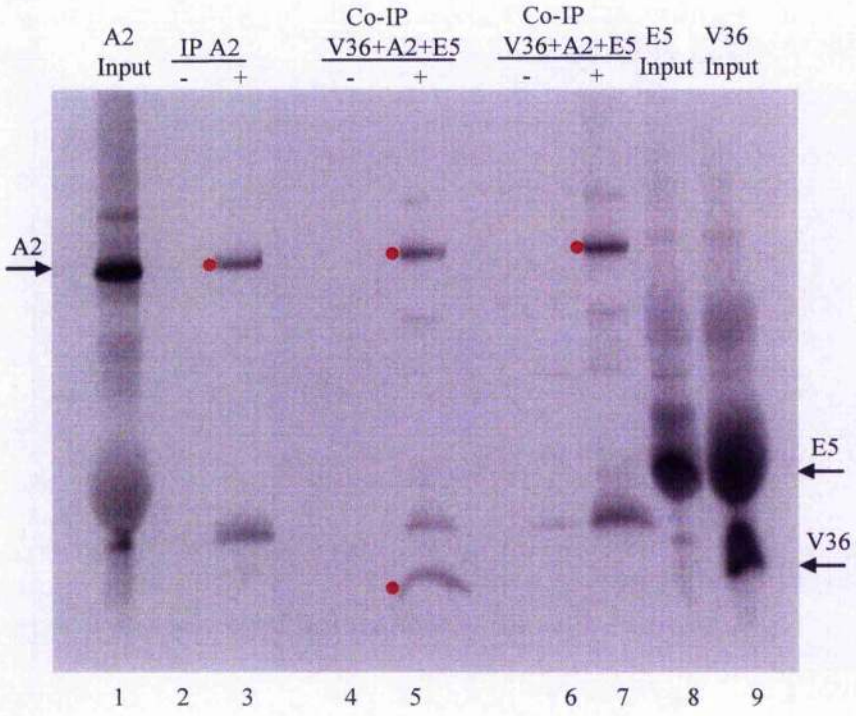


Figure 35: For Co-IP HPV-16 E5 wild type, HPV-16 E5 V36 mutant and HLA-A2 were translated and labelled or not labelled with [^{35}S]methionine, competed as described above and precipitated with HC10 antibody against HLA heavy chain.

Lane 1 is HLA-A2 input (5µl). Lanes 2 and 3 are IP-A2 with or without HC10 antibody. Lanes 4 and 5 are Co-IP with HLA-A2+E5V36+E5 wild type with or without HC10 antibody. Lanes 6 and 7 are Co-IP with A2+E5V36+ E5 wild type with or without HC10 antibody. E5V36 is labelled in lane 5 and not labelled in lane 7. Lane 8 is E5 wild type input (5µl) and lane 9 is E5V36 input (5µl).

The E5V36 and MHC class I heavy chain bands are indicated by red dots.

36. IP and Co-IP of HPV-16 E5 R30 and HLA-A2/HPV-16 E5 wild type

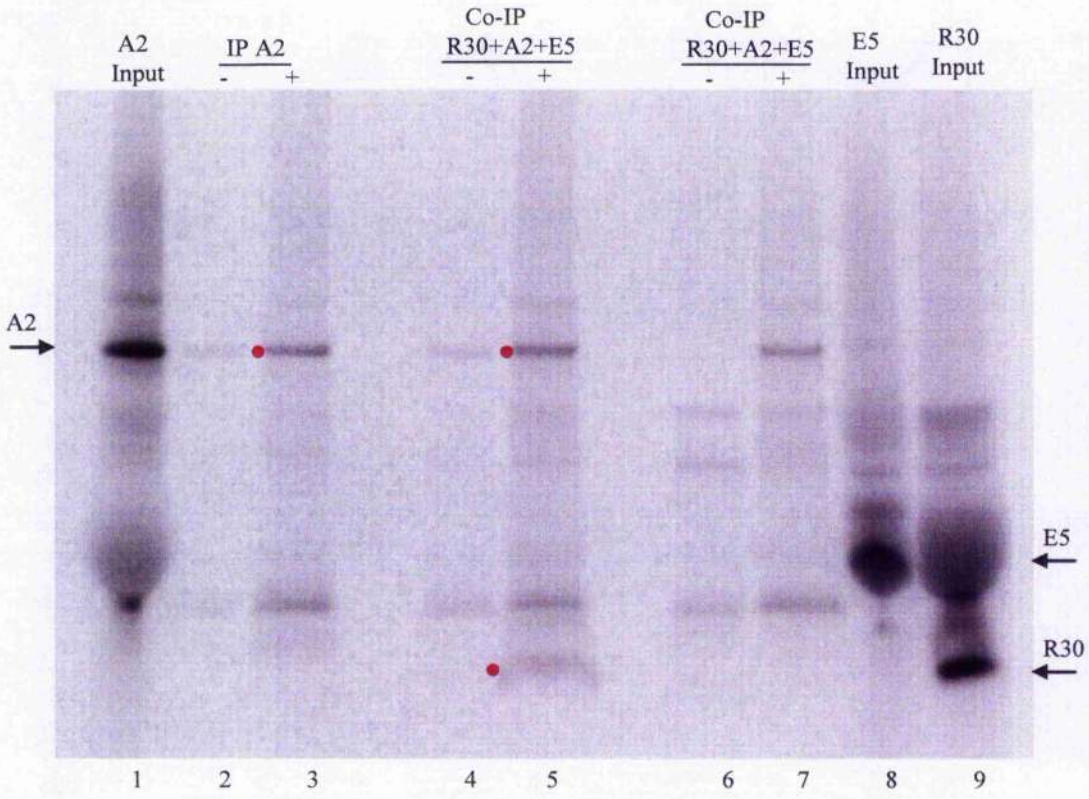


Figure 36: For Co-IP HPV-16 E5 wild type, HPV-16 E5 R30 mutant and HLA-A2 were translated and labelled or not labelled with [^{35}S]methionine, competed as described above and precipitated with HC10 antibody against HLA heavy chain.

Lane 1 is HLA-A2 input (5µl). Lanes 2 and 3 are IP-A2 with or without HC10 antibody. Lanes 4 and 5 are Co-IP with HLA-A2+E5R30+E5 wild type with or without HC10 antibody. Lanes 6 and 7 are Co-IP with HLA-A2+E5R30+E5 wild type with or without HC10 antibody. E5R30 is labelled in lane 5 and not labelled in lane 7. Lane 8 is E5 wild type input (5µl) and lane 9 is E5R30 input (5µl).

The E5R30 and MHC class I heavy chain bands are indicated by red dots.

3.3.8 Conclusion

We found that HPV-16 E5 wild type physically interacts with HLA heavy chain using four different antibodies: HA antibody, HC10 antibody, E5N-terminus antiserum and E5C-terminus antiserum (Figure 27). We determined that HPV-16 E5 mutants (R79, A54, V36 and R30) which have the same N-terminus, physically interact with HLA heavy chain, similar to HPV-16 E5 wild type (Figures 28, 29, 30 and 31), but the HPV-16 E5 Del-1 mutant, which lacks the first hydrophobic domain, does not (Figure 30). Also the results of competition experiments between E5 wild type and three mutants (E5Del-1, E5V36 and E5R30) confirm that the E5V36 and E5R30 physically interact with HLA heavy chain (Figures 35 and 36) but E5Del-1 does not (Figure 34). We conclude that the first hydrophobic domain of the HPV-16 E5 protein is critical for binding to HLA heavy chain. As determined before, HPV-16 E5 down-regulates MHC class I on the cell surface and the first hydrophobic domain is important to this down-regulation. It appears that the interaction of E5 with HLA heavy chain is necessary for down-regulation of MHC class I.

3.4 HPV-16 E5 does not down-regulate non-classical MHC I

3.4.1 Introduction

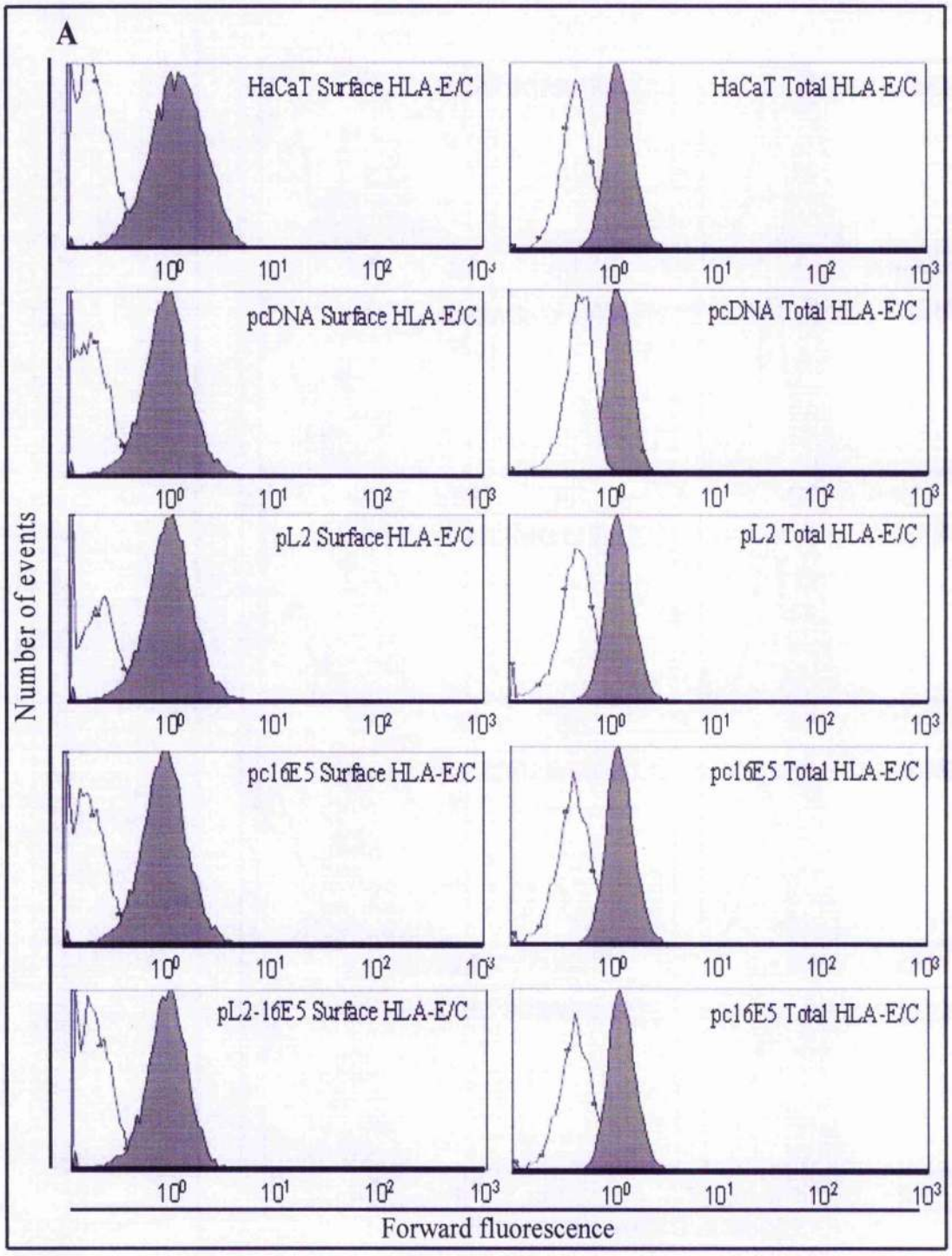
Like the classical MHC class I loci, the non-classical MHC class I genes that include HLA-E, HLA-F, and HLA-G molecules are highly transcribed in many tissues, but they display only limited genetic variation. These molecules show homology to the classical MHC I molecules but generally have limited polymorphism, low cell surface expression, and more restricted tissue distribution. While classical MHC I molecules (HLA-A and B) are the main presenters of antigenic peptides to CTL, HLA-C and non classical HLA-E inhibit NK cell-mediated lysis by interacting with inhibitory NK receptors; NK killing is part of the innate immune response. Inhibitory receptors prevent NK killing if MHC class I molecules are present but lysis of the target cell occurs if MHC class I is absent. Thus, classical MHC I negative virus-infected cells would be killed by NK (Yokoyama *et al.,* 1998; Lee *et al.,* 1998; Braud *et al.,* 1999), unless non-classical MHC I molecules are present. It has been shown that several viruses are capable of inhibiting classical MHC I but do not inhibit non-classical MHC I thus escaping both CTLs and NK cells. It has been demonstrated that the HIV Nef and HCMV US10/UL40 proteins down-regulate HLA-A/B but do not down-regulate HLA-C/E, which are mostly involved in positive selection of inhibitory receptors on NK cells (Cohen *et al.,* 1999; Tomasec *et al.,* 2000; Furman *et al.,* 2002). As a part of understanding how HPV-16 E5 could selectively down-regulate MHC class I molecules, we determined the levels of HLA-C and E in the HPV-16 E5 expressing cells. The effect of E5 on HLA-C/E was investigated by FACS, by immunofluorescence microscopy and by western blots.

3.4.2 Detection of HLA-C and non-classical HLA-E by FACS

We investigated whether HPV-16 E5 selectively down-regulates surface MHC class I. Using

FAC analysis, we determine the levels of HLA-C/E in HaCaT parental cell, cells carring empty vector (pcDNA and pL2) or cell expressing HPV-16 E5 (pc16E5 and pL2-16E5). Cells were stained with mAb DT9 that recognizes both HLA-C and HLA-E, and were analyzed by FACS for surface and total HLA-C/E following the previously described protocol (see section 2.2.25). This results show that the shift in forward fluorescence was small (Figures 37A and 37B), in agreement with the observation that human fibroblasts have little HLA-E (Tomasec *et al.*, 2000), but it was consistently higher than background (secondary antibody only; Figure 37A). Importantly, there were no significant differences between the control and E5-expressing cells (Figure 37B).

37. Detection of HLA-C/E by FACS



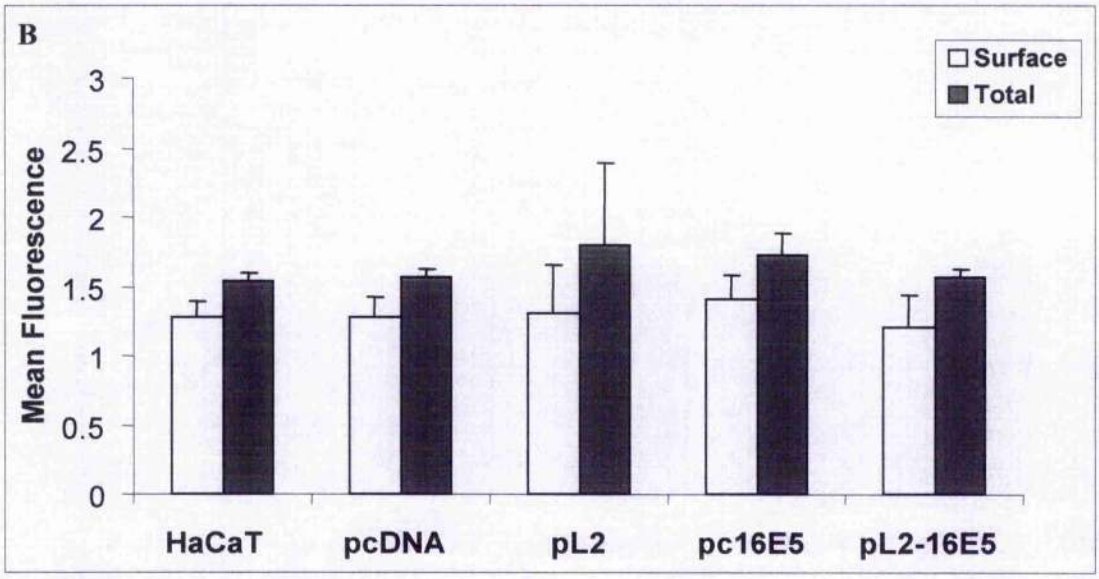


Figure 37: HPV-16 E5 does not down-regulate non-classical MHC I on the cell surface. HaCaT parental cells, pcDNA and pL2 cells or pc16E5 and pL2-16E5 cells (three cell lines of each) were stained with mAb DT9, specific for HLA-C/E and analysed for expression of surface and total HLA-C/E by FACS. **A)** Representative FACS profiles of single cell lines. Dotted line and open histogram, forward fluorescence with secondary antibody only; solid histogram, forward fluorescence with primary and secondary antibody. **B)** The mean fluorescence from at least three experiments performed on three cell lines was calculated from FACS analysis. A background of 0.4 (the reading of cells stained with only secondary antibody) was subtracted in all cell lines.

3.4.3 Localisation of HLA-C/E by immunofluorescence microscopy

The cellular localisation of HLA-C/E was investigated by immunofluorescence. The cells were aliquoted into 24-well plates containing coverslips at $1x10^4$ cells per well, grown and stained with mAb DT9 to show the localization of non-classical MHC I as previously described (see section 2.2.26). For the detection of HLA-C/E, the control HaCaT cells carrying empty vectors and cells expressing HPV-16 E5 were incubated with mAb DT9 and analysed under the confocal microscope. We determined that there were no differences between the staining patterns of HLA-C/E in control cells and in E5 cells (Figure 38), and therefore we conclude that expression of E5 does not lead to any appreciable decrease in surface HLA-C/E, in agreement with the FACS data.

38. Detection of HLA-C/E by immunofluorescence microscopy

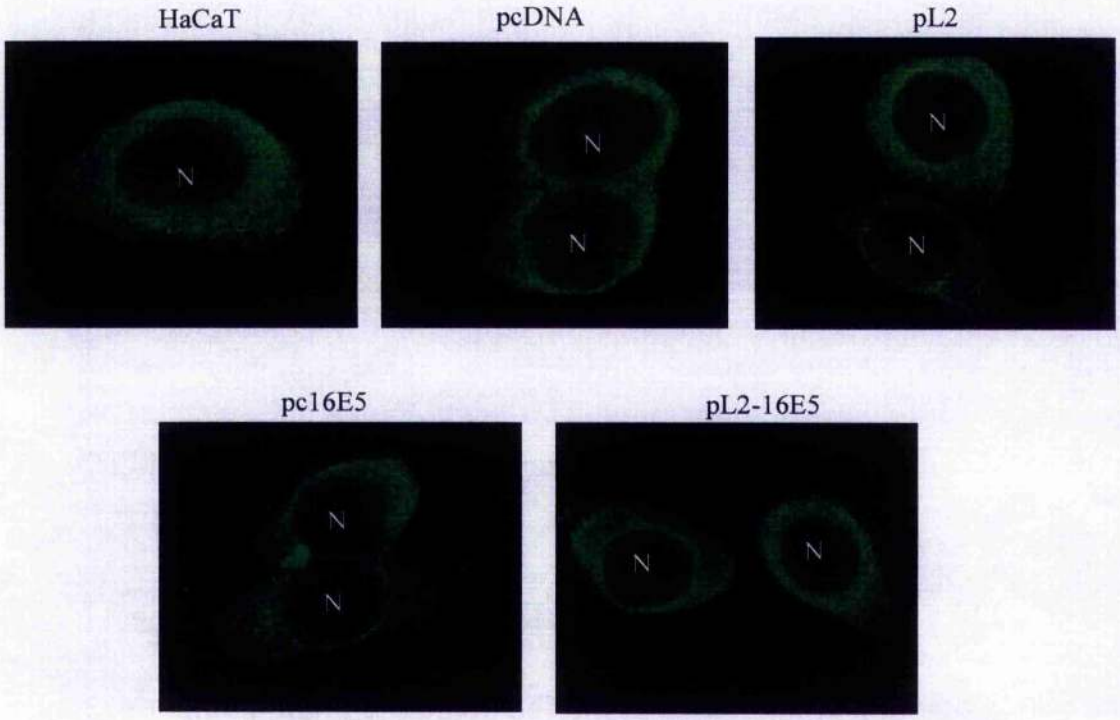


Figure 38: HaCaT parental cells, cells carrying empty vector (pcDNA and pL2) or cells expressing HPV-16 E5 (pc16E5 and pL2-16E5) were stained with mAb DT9, specific for HLA-E/C. The slides were mounted in Citifluor^{TM} and analysed with a Leica TCS SP2 fluorescence confocal microscope at 488nm wavelength. Images were acquired using Leica confocal software. N=nucleus

3.4.4 Detection of non-classical MHC I by Western Blots

The results above showed that, similarly to HIV Nef and HCMV US3/UL40, HPV-16 E5 did not have any effect on the levels of the HLA-C and non-classical HLA-E. Here we investigated the level of HLA-E heavy chain using mAb MEM-E/02, specific for HLA-E. We determined that there were no significant differences between control cells and E5 cells (Figure 39). These results confirm that HPV-16 E5 does not affect the expression of HLA-E/C. We cannot however discriminate between HLA-C and HLA-E on the cell surface because mAb DT9 recognises both molecules, and mAb MEM-E/02, specific for HLA-E, does not function in FACS or immunofluorescence.

39. Detection of non classical MHC class I by Western Blots

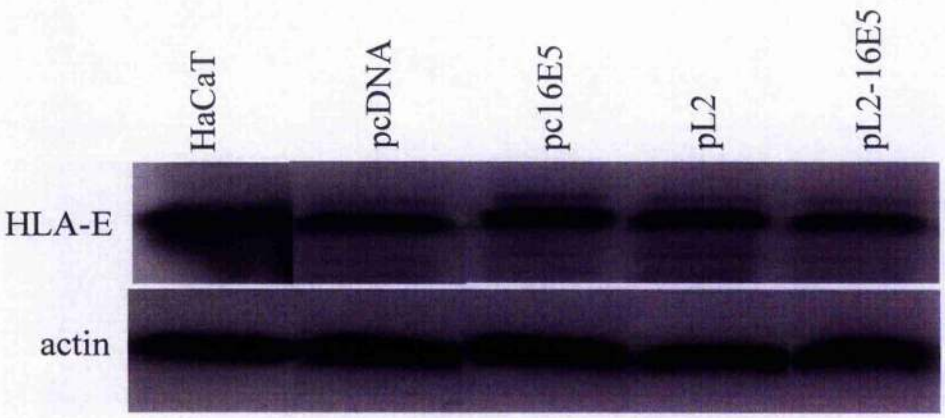


Figure 39: For western blots detection of non classical HLA-E heavy chain, protein lysates from one line each of HaCaT parental cells, cells carrying empty vector (pcDNA and pL2) or cells expressing E5 (pc16E5, pL2-16E5) was probed with mAb MEM-E/02, specific for HLA-E, or mAb AB-1 (anti-actin) to determine the levels of the HLA-E.

3.4.5 Conclusion

To determine whether HPV-16 E5 could selectively downregulate MHC class I molecules, we determined the levels of HLA-C/E in parental, control and E5-expressing HaCaT cell lines. The results show that there were no differences between the staining patterns of HLA-C/E in the control cells and in the E5-expressing cells; therefore, we conclude that expression of E5 does not lead to any appreciable decrease in surface HLA-C/E using FACS and immunofluorescence microscopy (Figures 37 and 38). Similarly, we did not detect any significant differences between HLA-E levels in the control cells and the E5-expressing cells by immunoblotting with mAb MEM-E/02, specific for HLA-E (Figure 39). The ease of HLA-C/E detection by immunofluorescence and immunoblotting compared with FACS is likely attributable to the different affinities of the two antibodies for HLA (mAb DT9 and mAb MEM-E/02) and to the greater sensitivity of mAb DT9 in immunofluorescence. These results confirm that HPV-16 E5 does not down-regulate the surface expression of the HLA-C or non-classical HLA-E.

Chapter Four

Discussion

4 Discussion

4.1 Introduction

Viruses that infect humans have evolved mechanisms of avoiding the immune response. A component of cellular immunity, CTL control viral infection by recognizing virus-encoded peptides presented on the surface of the infected cells by MHC class I molecules. MHC class I plays an important role in alerting the immune system to virally infected cells and presents peptide fragments derived from intracellular proteins. To avoid detection by CTL, viruses encode protein that inhibits the expression of the MHC complex on the surface of infected cells (Hewitt, 2003). Down-regulation of surface MHC class I potentially exposes the virus-infected cell to attack by NK cells, which possess the ability to recognise and lyse virally infected cells, and the expression of "self" MHC molecules inhibits NK lysis of target cells (Ploegh, 1998).

In human, many types of papillomaviruses cause benign infection but specific types of HPV, particularly types 16 and 18, are associated to malignancy, which in turn appears to depend on several factors, including the genetic background of the host (Breitburd *et al.*, 1996; Krul *et al.*, 1999), environmental cofactors (Castellsague *et al.*, 2002), and the ability of the virus to avoid immune clearance (Frazer *et al.*, 1999). The immune system plays a crucial role in determining the clinical outcome of HPV disease, as demonstrated by the increased persistence and enhanced neoplastic progression of HPV infections in hosts with cell-mediated immune deficiencies (Heard *et al.*, 2000; Harwood *et al.*, 2000). However, even in immunocompetent individuals, HPVs persist for a significant period of time before activation of the host immune system. This lack of recognition suggests that the host immune system escapes, or disables to recognize HPV infection. HPVs can impair the immune response indirectly via the nature of the virus life cycle (Frazer, 1996), and by direct interference with

the host antiviral immune mechanisms, including the IFN response and MHC class I antigen presentation to CTLs (O'Brien and Campo, 2003).

It has been shown (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002) that down-regulation of MHC class I by BPV E5 takes place at several steps of the MHC pathway, including transcription of the MHC class I heavy chain mRNA, expression of MHC class I heavy chain, and transport of the MHC class I complex to the cell surface. However, the amino acid analysis of the HPV E5 proteins and BPV-1 E5 has revealed that they are very different. But some limited sequence similarities have been suggested between the HPV and BPV-1 E5 proteins such as a few hydrophobic residues (Bubb *et al.*, 1988). The C-terminal portion of BPV-1 E5, which appears to be important for biological function, does not have an identifiable similar region in the HPV E5s. In contrast to BPV-1 E5, where E5 is the main oncoprotein, little is known about the biological activity of HPV-16 E5.

4.2 Down-regulation of MHC class I by HPV-16 E5

The MHC class I molecules are essential for presentation of foreign peptides to the host CTL and play a critical role in immune surveillance of many pathogens (Tortorella *et al.*, 2000; Hewitt, 2003). The MHC class I complex consists of a heavy chain containing the peptide binding site and β_2M, which assemble in the lumen of the ER, where peptides are loaded onto the MHC class I heavy chain in a pH-dependent process. The complex proteins are transported to the GA, where dissociation of MHC class I from TAP takes place, and then transported to the cell surface for presentation to CTL (York and Rock, 1996). As MHC class I travel through the GA, and the E5 protein of papillomavirus interferes with the functions of the GA and endosomes (Schapiro *et al.*, 2000; Bravo *et al.*, 2004), it was expected that the transport of the MHC class I complex would be disrupted in E5 expressing cells. We have

shown that HPV-16 E5 wild type down-regulates MHC class I on the cell surface, reducing the level of surface MHC class I to approximately half of that in the control cells (Figure 8). Additionally, we found that MHC class I was retained in the GA (Figure 9). In this respect, HPV-16 E5 is similar to BPV E5. However, HPV-16 E5 does not affect the morphology of the GA (Figure 10, central panels) while BPV-4 E5 affects the structure of the GA, leading to Golgi swelling and fragmentation (Marchetti *et al.*, 2002). Despite the similarities, there are also differences between the extent to which BPV-4 E5 and HPV-16 E5 interfere with the MHC class I pathway. BPV-4 E5 down-regulates transcription of the MHC class I heavy chain gene, promotes degradation of the translated polypeptide and blocks the transport of the MHC class I complex to the cell surface (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002 and 2006). In contrast, HPV-16 E5 does not inhibit expression of the heavy chain (Figure 11), but retains the complex in the GA and reduces its transport to the cell surface without completely abolishing it. Prevention of transport of MHC class I to the cell surface is a property shared by papillomaviruses E5. It has been reported that also HPV2a E5 can inhibit the transport of MHC class I to the cell surface (Cartin and Alonso, 2003). Therefore, down-regulation of surface MHC class I appears to be a property of many, if not all, papillomavirus E5 proteins. Moreover, whereas the transport of MHC class I to the cell surface was irreversibly inhibited in cell expressing BPV-4 E5, treatment with β-IFN increased the transport of MHC class I to the cell surface in cell expressing HPV-16 E5. The reason for this difference is not known but it can be speculated that the increased production of MHC class I heavy chain by IFN is sufficient to overcome the inhibitory effect mediated by the low levels of HPV-16 E5. In contrast, as BPV E5 also inhibits transcription and promotes degradation of the MHC class I heavy chain (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2006), IFN treatment is insufficient to restore MHC class I expression to normal levels. These observations are consistent with the

hypothesis that there is a correlation between oncogenicity and immune evasion (O'Brien and Campo 2003). As discussed earlier, BPV E5 is a more effective transforming protein than HPV-16 E5 (Venuti and Campo, 2002) and therefore would be predicted to have a greater effect on MHC class I down-regulation (and other immune evasion mechanisms) than HPV-16 E5. In HPV-16, E6 and E7, which are two major transforming proteins would complement the inhibitory effect of the lesser transforming protein E5 on the MHC class I pathway. HPV-16 E7 can repress the MHC class I heavy chain gene promoter (Georgopoulos *et al,.* 2000) thus likely replacing the inhibitory action of BPV on the same promoter, and HPV-11 E7 can bind directly to TAP, thus further contributing to the down-regulation of MHC class I (Vambutas *et al.,* 2001). Furthermore, both HPV E6 and E7 can inhibit the type I IFN pathway, thus preventing the IFN-mediated release of E5-induced blockage in MHC class I trafficking (Ronco *et al.,* 1998; Barnard *et al.,* 2000).

4.3 Down-regulation of MHC class I by the first hydrophobic domain of HPV-16 E5

We found that HPV-16 E5, which has three hydrophobic domains, down-regulates MHC class I on the cell surface. It was important to determine the domain of the HPV-16 E5 protein responsible for down-regulation of surface MHC class I. We used deletion mutants of HPV-16 E5 lacking each of the hydrophobic domains (Figure 16). We found that the level of surface MHC class I was not different between the cells expressing HPV-16 E5 Del-1 and HaCaT parental or no E5 cells. In contrast, the level of surface MHC class I was reduced in the cells expressing HPV-16 E5 mutants (R79, A54, V36 and R30), similar to HPV-16 E5 wild type (Figures 23A, 23B and 24). So, these results confirmed that the first hydrophobic domain of HPV-16 E5 is important for down-regulation of surface MHC class I. We could not detect HPV-16 E5 protein in HaCaT cells, because E5 is hydrophobic and small and difficult

to detect in cells, and therefore we used indirect methods of detection such as RNA expression. We have done Taq-Man RT-PCR to show the level of E5 RNA expression in the cell transfected with HPV-16 E5 wild type and all mutants. Whereas, we could detect E5 RNA in the cell transfected with HPV-16 E5 wild type and mutants (E5R79, E5A54, E5V35, E5R30), but we could not detect E5Del-1 RNA, because the E5Del-1 mutant lacks the first hydrophobic domain, which is the best target for the probe (Figure 18). Therefore, we have done semi-quantitive RT-PCR to show the expression of E5 RNA in the cell transfected HPV-16 E5 wild type and mutants including E5Del-1. The results showed that the expressions of E5 RNA were approximately the same level in the cells transfected with HPV-16 E5 wild type and mutants including E5Del-1 (Figures 20, 21 and 22). Also, HPV-16 E5 wild type and all mutants were expressed as GFP-fusion proteins visualise their cellular localization. We found that GFP-HPV-16 E5 wild type and GFP-HPV-16 E5 mutants including GFP-E5Del-1 localized in the GA, as does BPV-4 E5 (Marchetti *et al.*, 2002), whereas GFP alone is expressed throughout the cell (Figures 25 and 26). These results suggest that the localization of the HPV-16 E5 protein in the GA is important but not sufficient for down-regulation of surface MHC class I, because the only mutant that failed to down-regulate surface MHC class I was still localized in the GA. Another difference between HPV-16 E5 and BPV-4 E5 is that the C-terminus of BPV-4 E5 is important for the down-regulation of MHC class I (Marchetti *at al.*, 2006), whereas, it is the first hydrophobic domain of HPV-16 E5 which is important to reduce surface MHC class I as shown by the failure of E5Del-1 mutant to retain MHC class I in the membrane compartment.

4.4 How does HPV-16 E5 down-regulate MHC class I?

We found that HPV-16 E5 down-regulates MHC class I on the cell surface and the first hydrophobic domain of HPV-16 E5 is important for this down-regulation. We also found that HPV-16 E5 and the residual MHC class I co-localized in the GA, which is similarly to BPV-4 E5 (Marchetti *et al.,* 2002). This complete co-localization would suggest an interaction between the HPV-16 E5 protein and MHC heavy chain. Indeed, this is the case and the HPV-16 E5 protein and HLA-A2 protein co-precipitate, *in vitro,* using four difference antibodies (Figures 27 A-D). In addition, the HPV-16 E5 protein also interacts "*in vitro*" with HLA-A1 and HLA-B8 (Dr.Ashrafi and Dr.Marchetti, personal communications). These results reveal that HPV-16 E5 wild type physically interacts with MHC class I heavy chain in non-dependently of HLA heavy chain. Also, we determined that HPV-16 E5 mutants (R79, A54, V36 and R30), which have the same first hydrophobic domain, physically interact with MHC heavy chain, similarly to HPV-16 E5 wild type (Figures 28, 29, 30 and 31) but the HPV-16 E5 Del-1 mutant, which truncates the first hydrophobic domain of HPV-16 E5 wild type, does not interact with MHC heavy chain (Figure 32). This interaction is not an artifact, as E5 is precipitated by HC10 antibody against MHC heavy chain even when HLA-A2 is not labelled (Figure 33A), and HPV-16 E5 Del-1 mutant does not interact with MHC heavy chain (Figure 33B). We confirmed by competition experiments that the first hydrophobic domain of the HPV-16 E5 protein is necessary for binding to MHC heavy chain (Figures 34, 35 and 36). Also, we determined that this interaction is not an artifact, as E5V36 and E5R30 mutants can compete with E5 wild type for binding to MHC class I heavy chain (Figure 35 and 36, lane 7). It has been demonstrated that HPV-16 E5 wild type and MHC heavy chain interact also in HaCaT-16 E5 cells but HPV-16 E5 Del-1 mutant does not interact in the HaCaT-E5Del-1 cells (Ashrafi *et al.,* Submitted). Therefore, these results suggest that down-regulation of

MHC class I in the cell expressing HPV-16 E5 takes place by physically interaction between MHC heavy chain and the first hydrophobic domain of the HPV-16 E5 protein, and this interaction may impair the function of the MHC class I. In contrast, down-regulation of MHC class I in the cell expressing BPV-4 E5 is caused by physically interaction between MHC class I protein and the C-terminus of the BPV-4 E5 protein (Marchetti *et al.*, 2006).

4.5 HPV-16 E5 does not down-regulate non-classical MHC I

While classical MHC I molecules (HLA-A and B) are the main presenters of antigenic peptides to CTL, HLA-C and non-classical HLA-E inhibit NK cell-mediated lysis by interacting with inhibitory NK receptors. Inhibitory receptors prevent NK killing if MHC class I molecules are present but lysis of the target cell occurs if MHC class I is absent. Thus, classical MHC I negative virus-infected cells would be killed by NK cells (Ploegh, 1998; Natarajan *et al.*, 2002), unless non-classical MHC I molecules are present. Human NK cells express multiple receptors that interact with MHC class I molecules to inhibit or activate their effector function. Two types of receptors are specific for self MHC class I molecules to inhibit NK cells, including KIRs that predominantly recognize classical MHC I, and the lectin-like heterodimer CD94/NKG2 receptors that recognize HLA-E which is non-classical MHC I molecule (Yokoyama *et al.*, 1998; Lee *et al.*, 1998; Braud *et al.*, 1999). Recognition of the class I molecules by their inhibitory receptors inhibits NK-mediated cell lysis, which would occur in the absence of classical HLA-C and non-classical HLA-E. To investigate whether HPV-16 E5 could down-regulate non-classical MHC I molecules, we determined the effect of HPV-16 E5 on HLA-C and non-classical HLA-E. Our experiments establish that the expression of HLA-C and non-classical HLA-E in HaCaT control cells or cells expressing HPV-16 E5 are at the same level in the cell surface and total (Figures 37 and 38). These

results confirm that HPV-16 E5 does not down-regulate the expression of non-classical MHC I. Likewise, it has been determined that BPV E5 protein down-regulates bovine classical MHC I but does not affect non-classical MHC I (Marchetti and Araibi, personal communication). We conclude that HPV-16 E5 selectively inhibits surface expression of HLA-A and B but does affect neither the synthesis of HLA-C and non-classical HLA-E nor the transport of them to the cell surface. NK cells of patients with HPV-induced anogenital lesions are incapable of specific killing HPV-16-infected cells (Malejczyk *et al.,* 1989), although the mechanism by which this occurs is as yet unknown. Moreover, concordant with loss of MHC class I molecules that present viral peptides to the CTL, there is a very low frequency of HPV-specific HLA-A-restricted CTLs in patients infected with HPV-16, an order of magnitude lower than those found in other viral infections, including influenza A and EBV (Youde *et al.,* 2000). It is not yet known whether these observations are the consequence of E5 expression; however, experiments to establish the functional outcome of E5 expression on CTL and NK cell recognition of HPV-transfected cells are in progress. Regardless, our results support the hypothesis that E5 plays a major role in immune evasion by HPV. It is interesting to note that HPV-16 E5 has also been reported to protect HaCaT cells from tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand- mediated apoptosis and this effect correlates with the level of E5 expression (Kabsch and Alonso, 2002). FasL binds to the Fas receptor (CD96) leading to activation and clustering of the death receptors (Walczak *et al.,* 2000). TRAIL interacts with several cellular soluble receptors, the TRAIL receptors 1 and 2, (Emery *et al.,* 1998) that contain intracellular death domains (DDs) to transmit the apoptotic signal (Kischkel *et al.,* 2000; Sprick *et al.,* 2000). Fas and TRAIL receptors are expressed on a broad panel of normal epithelial cells (Wiley *et al.,* 1995). Down-regulation of Fas expression is a common abnormality in gynecological cancers (Das *et*

al., 2000), whereas the expression of TRAIL receptors is not reduced in cervical cancer (Ryu *et al.*, 2000) compared to normal tissue. Therefore, E5 can disrupt several critical components of the cell-mediated immune response to viruses, which may contribute to the establishment and persistence of HPV infection. The down-regulation of MHC class I has been observed in CIN (Bontkes *et al.*, 1998) and in cervical carcinomas (Keating *et al.*, 1995). However, the down-regulation of MHC class I in cervical carcinomas, which often do not express E5, is common to other cancer types and therefore unlikely to be due to E5. No correlation was made between MHC class I down-regulation and E5 expression in CIN, and this point warrants further investigation.

4.6 HPV-16 E5 binds to 16K subunit c and HLA-A2 heavy chain

The E5 protein is able to interact with several different cellular proteins including epithelial growth factor and 16K subunit c/ductin. It has been reported that BPV E5 (Goldstein *et al.*, 1990) and HPV E5 (Conrad *et al.*, 1993) bind to 16K subunit c, which is a component of the V0 sector of the H^+ V-ATPase. The binding of the E5 polypeptide to 16K subunit c has led to the suggestion that the function of the V-ATPase might be impaired. The V-ATPase is a universal transmembrane proton pump of eukaryocytes and is responsible for acidification of endomembrane components (e.g. Golgi, endosomes, lysosomes, and certain secretory vesicles) (Finbow *et al.*, 1997). As shown previously (Rodriguez *et al.*, 2000) the second hydrophobic domain of the HPV-16 E5 protein is critical for interaction with the 16K subunit c of the V-ATPase. The interaction of E5 and 16K subunit c takes place to increase the pH of the GA (Schapiro *et al.*, 2000), which was attributed to a malfunction of the vacuolar H+ ATPase (Briggs *et al.*, 2001). The acidification of the Golgi may impair the MHC complex, which presents viral peptides to the host CTL through ER and the GA. The retention of MHC

class I in the GA by BPV-4 E5 is due at least in part to the impeded acidification of the endomembrane components, as retention in the GA and down-regulation of surface MHC class I are also caused by monensin, an inhibitor of V-ATPase and the acidification of the GA (Marchetti *et al.*, 2002). The same mechanism may support the retention of MHC class I in the GA by HPV-16 E5. However, given the selective down-regulation of HLA-A and B by HPV-16 E5, the lack of the GA acidification cannot be the whole explanation and other mechanisms must come into play. These results lead us to conclude that HPV-16 E5 down-regulates surface MHC class I by interaction with 16K subunit c that inhibits acidification of the GA, and by binding to MHC class I heavy chain.

4.7 Similarities between HPV-16 E5 and other viral protein

CTL recognize virus-infected cells through the specific interaction of their T-cell receptor with an MHC class I molecule presenting a viral peptide. Peptides, generated by the proteasome in the cytoplasm, are translocated by TAP into the ER where they assemble in ternary complexes and are transported to the cell surface for presentation to CTL (York and Rock, 1996). Interference with the assembly and/or trafficking of the MHC class I complex can contribute to the persistence of a virus. It has been reported that several viral proteins induce chronic infections by targeting or modulating the host's immune system. E3/19K adenovirus protein binds to MHC class I in the ER and prevents its transport to the cell surface. Murine CMV glycoprotein, gp34, also interacts with the heavy chain β_2M complex in the ER and has been recently shown to target MHC class I for degradation in the lysosomes. Infected cell protein (ICP) 47, a protein encoded by herpes simplex virus, inhibits the TAP transporter (Tortorella *et al.*, 2000). More recently, it was demonstrated that the K3 and K5 proteins encoded by Kaposi's sarcoma-associated herpesvirus down-regulate MHC class I

from the cell surface (Ishido *et al.,* 2000). It has been demonstrated that the HIV Nef and HCMV US10/UL40 proteins down-regulate HLA-A and B by interaction but do not down-regulate HLA-C and non-classical HLA-E, which are mostly involved in positive selection of inhibitory receptors on NK cells (Cohen *et al.,* 1999; Tomasec *et al.,* 2000; Furman *et al.,* 2002). We found that the HPV-16 E5 protein binds to MHC heavy chain in the GA and prevents the transport of MHC class I to the cell surface but does not inhibit HLA-C and non-classical HLA-E thus potentially escaping both CTLs and NK cells. Thus HPV-16 E5 joins numerous other viral proteins in the avoidance of the host immune response.

Future works

We found that HPV-16 E5 inhibits the transport of MHC class I to the cell surface, reducing the level of surface MHC class I to approximately half of that in the control cells. MHC class I is essential for presentation of foreign peptides to the host CTL and play a critical role in immune surveillance of many pathogens (Tortorella *et al.*, 2000). We also found that down-regulation of MHC class I is due to the first hydrophobic domain of HPV-16 E5 by physical interaction with HLA heavy chain. We determined that HPV-16 E5 down-regulates selectively HLA-A and B but does not inhibit HLA-C and non-classical HLA-E thus escaping both CTLs and NK cells. Future work should concentrate on the effects of HPV-16 E5 on the MHC class I.

Firstly, it should be investigated which amino acid(s) within the first hydrophobic domain of the HPV-16 E5 protein is/are essential for the down-regulation of surface MHC class I. It has been shown that the HIV Nef and HCMV US10/UL40 proteins down-regulate selectively HLA-A/B by physical interaction but do not down-regulate HLA-C and non-classical HLA-E (Cohen *et al.*, 1999; Tomasec *et al.*, 2000; Furman *et al.*, 2002). It has been also shown that the di-leucine amino acid motif of the HIV Nef protein is important in the Nef-function and in the interaction of Nef with adaptor protein (AP) complexes (Coleman *et al.*, 2006). The first hydrophobic domain of HPV-16 E5 has four di-leucine motifs (Table 6). A deletion or point mutation analysis will identify which amino acid(s) is/are responsible.

Table 6. Amino acid sequences of BPV-1 E5, BPV-4 E5, HPV-16 E5 and HPV-18 E5 proteins

BPV-1 E5	1 MPNLWFLLFL GLVAAMQLLL LLFLLLFFLV YWDHFECSCT GLPF 44
BPV-4 E5	1 MSLWLIYVLL LFWCAFNFLA LLFAIIVYLL LI**STITRLDG WD** 42
HPV-16 E5	1 **MTNRDTASTT LLACFLLCFC VLLCVCLLIR** PLLLSVSTYT SLIILVLLLW ITAASAFRCF IVYIIFVYIP LFLIHTHARF LIT 83
HPV-18 E5	1 MLSLIFLFCF CVCMYVCCHV PLLPSVCMCA YAWVLVFVYI VVITSPATAF TVYVFCFLLP MLLLHIHIL SLQ 83

Table 6: Amino acid sequences of BPV-1 E5, BPV-4 E5, HPV-16 E5 and HPV-18 E5 proteins Bold: domains know to be critical for interaction with MHC class I heavy chain and underline sites are potential leucine motifs.

Secondly, as HPV-16 E5 physically interacts with HLA heavy chain, it is important to investigate which site of HLA heavy chain is involved in this interaction. A deletion or point mutation analysis will identify which amino acid(s) is/are responsible.

Thirdly, it has been shown that expression of HPV-16 E5 increased the pH of the Golgi, which is thought to be a result of its binding to 16K subunit c (Schapiro *et al.*, 2000; Bravo *et al.*, 2004). The acidification of the Golgi impairs the transport of the MHC complex. Our results showed that HPV-16 E5 physically interacts with HLA heavy chain and this interaction is associated with the down-regulation of the MHC complex. This suggests that HPV-16 E5 down-regulates surface MHC class I both by interaction with 16K subunit c of the V-ATPase and by binding to HLA heavy chain. It is important to investigate that HPV-16 E5 physically interacts with both 16K subunit c and HLA heavy chain at the same time.

Fourthly, lack of surface MHC I in infected epithelial cells expressing E5 would allow evasion of cytotoxic T lymphocyte killing and thus establishment of viral infection, and the presence of non-classical MHC I would allow escape from NK killing. CTL and NK killing assays of E5-expressing cells would clarify this point.

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