Systematic Analysis of Residues in Conserved Region 3 of the Human Papillomavirus 16 E7 Oncoprotein

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Abstract

Although remarkable biological diversity is exhibited by viruses, as obligate intracellular parasites, they rely on host cell functions. As such, viruses typically must overcome a set of host barriers that prevent infection. For human papillomaviruses (HPV) one of these barriers is the state of terminal differentiation of the host cell. For that purpose HPVs encode two major oncoproteins, E6 and E7, which combine their efforts to effectively uncouple cellular differentiation from the cell cycle arrest. The E7 proteins have no intrinsic enzymatic activity or DNA binding ability, but they bind and manipulate numerous host proteins. E7 is a modular oncoprotein and contains three conserved regions (CR), of which CR3 is a highly structured zinc-binding domain, and is the primary focus of this thesis. In order to study this highly structured portion of E7, we created a panel of surface-exposed mutants with the primary aim of preserving the structure of CR3, but disrupting potential cellular interactions. Although it was known that E7 CR3 exists as a dimer, the functional significance of dimerization had not been established. Using our panel of novel E7 mutants, we established that E7 does not need to dimerize in order to transform primary baby rat kidney cells. Furthermore, we utilized the same panel of surface-exposed mutants to characterize the contribution of CR3 in deregulation of one of the most vital interacting partners of E7, the retinoblastoma tumor suppressor (pRb). We establish that CR3 binds pRb independently of the well characterized high-affinity binding site in vivo, and show that this interaction is functionally important in overcoming the cell cycle arrest. We also find that E7 has additional mechanisms for targeted pRb degradation, aside from the known pathway. Additionally, we identify a novel binding partner of E7, the p190RhoGAP, and characterize this interaction using the surface-exposed mutants. We find that the interaction of E7 with p190RhoGAP contributes to deregulation of the RhoA GTPase and the actin cytoskeleton, and therefore likely represents an important aspect of HPV induced tumorigenesis. Considered together, these studies have expanded our knowledge of known processes and illuminated novel pathways utilized by HPV E7 in carcinogenesis.
Keywords

Human papillomavirus (HPV), early protein 7 (E7), conserved region 3 (CR3), structure, dimerization, transformation, retinoblastoma tumor suppressor (pRb), actin cytoskeleton, RhoA GTPase, p190RhoGAP

Co-Authorship Statement

Chapter 2 of this thesis was published in Journal of Virology 85(19): 10048-57, October 2011. Experiments presented in FIG. 2-2A, B, C were performed by K. Hung, and the experiments for the transformation data presented in Tables 2-3 and 2-4 were carried out in collaboration with P. Massimi. HPV16 E7 CR3 modeling was carried out in collaboration with Dr. Gary Shaw.

Chapter 3 of this thesis was published in Journal of Virology 86(24) 13313-23, December 2012. I was involved in performing all of the experiments.
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<th>Meaning</th>
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<tbody>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>BPV</td>
<td>Bovine papillomavirus</td>
</tr>
<tr>
<td>BRK</td>
<td>Baby rat kidney</td>
</tr>
<tr>
<td>bs</td>
<td>Base pair</td>
</tr>
<tr>
<td>cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical intraepithelial neoplasia</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>CR</td>
<td>Conserved region</td>
</tr>
<tr>
<td>CRPV</td>
<td>Cottontail rabbit papillomavirus</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbacco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>E1</td>
<td>Early protein 1</td>
</tr>
<tr>
<td>E1A</td>
<td>Early protein 1A</td>
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<td>E2</td>
<td>Early protein 2</td>
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<tr>
<td>E4</td>
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<td>Early protein 5</td>
</tr>
<tr>
<td>E6</td>
<td>Early protein 6</td>
</tr>
<tr>
<td>E6AP</td>
<td>E6 associated protein</td>
</tr>
<tr>
<td>E7</td>
<td>Early protein 7</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>Food and drug administration</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GBD</td>
<td>GTP-binding domain</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>KD</td>
<td>Knockdown</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>L1</td>
<td>Late protein 1</td>
</tr>
<tr>
<td>L2</td>
<td>Late protein 2</td>
</tr>
<tr>
<td>LCR</td>
<td>Long control region</td>
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<tr>
<td>LDS</td>
<td>Lithium dodecyl sulfate</td>
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<tr>
<td>MD</td>
<td>Middle domain</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>ONPG</td>
<td>Ortho-nitrophenyl-β-galactoside</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>p190</td>
<td>p190RhoGAP</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>Pap</td>
<td>Papanicolaou</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDB</td>
<td>Protein data bank</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>pRb</td>
<td>Retinoblastoma tumor suppressor</td>
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<tr>
<td>PV</td>
<td>Papillomavirus</td>
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<tr>
<td>RBD</td>
<td>Rhotekin binding domain</td>
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<tr>
<td>RMS</td>
<td>Root mean square</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SV40</td>
<td>Simian vacuolating virus 40</td>
</tr>
<tr>
<td>TAg</td>
<td>Large tumor antigen</td>
</tr>
<tr>
<td>URR</td>
<td>Upstream regulatory region</td>
</tr>
<tr>
<td>VLP</td>
<td>Viral-like particle</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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Chapter 1 INTRODUCTION

1.1 Preface

The origin of viruses is unclear. However, viruses which are obligate intracellular parasites have shaped the history and evolution of their hosts. Nearly all living organisms are known to have viral parasites. The medical significance of human viral infections has altered our history and has resulted in remarkable efforts on the part of virologists to study, understand and eventually eradicate these agents when associated with disease. However, the field of virology has not only contributed to understanding and eradication of viruses and life-threatening diseases such as smallpox, but has been instrumental in revealing new fundamental principles of life processes in general. Many of the concepts and tools of modern day molecular biology were derived from the study of viruses. As well, essential cellular processes such as splicing were first described either when viruses were used as tools to dissect the affected host pathways or were first described in viruses themselves.

Nowhere is the concept of using viruses as tools better illustrated than with tumor viruses and studies of cancer development and progression. Tumor viruses attack common cellular pathways, which makes them powerful tools for understanding how multiple complex pathways are integrated. The unique mechanisms employed by each viral system to alter cellular functions serve as effective molecular probes for the identification of new targets and pathways that drive tumorigenesis.

Herein, the focus will be directed to advancing our understanding of human papillomaviruses (HPV) and their contribution to human cancers. Primarily, the author focuses on one of the main papilloma oncogenes, early protein 7 (E7), and investigates: 1) the importance of E7 structure and dimerization as it relates to function, 2) the interaction between the E7 oncogene and the retinoblastoma tumor suppressor, and 3) the identification and characterization of a novel interaction between E7 and p190RhoGAP. It is the sincere hope of the author that during the course of these studies using E7, we have learned more about important cellular regulatory pathways and their role in carcinogenesis.
1.2 General Introduction

Viruses are diverse. This diversity makes each virus fascinating in its own way. Many of these viruses, however, present a significant health and economic burden; for instance, World Health Organization (WHO) International Agency for Research on Cancer estimated that in the year 2002, 17.8% of the global cancer burden was due to infections, with 12% being caused by viruses. The principal agents are the bacterium *Helicobacter pylori* (5.5% of all cancer), HPVs (5.2%), the hepatitis B and C viruses (4.9%), Epstein-Barr virus (1%), and human immunodeficiency virus (HIV) together with the human herpes virus 8 (0.9%) (177). Due in part to their association with cancer, viruses such as HPVs have become major drivers of research. Studies of these viruses allow us to better understand the mechanisms of viral infection and production with the primary goal of preventing infection and developing antiviral therapies. Additionally, viruses redirect host cellular processes for viral production and antagonize host antiviral defense systems. Therefore, viruses can serve as powerful model systems and tools to discover and elucidate regulatory pathways and cellular processes.

Specifically, when studying tumor initiation and development, molecular studies using DNA tumor viruses have proven exceptionally valuable. DNA tumor viruses are part of the extended family of DNA viruses; although all DNA viruses share a common form of genetic material, specific characteristics, such as size, host species and tissue tropism, vary widely. As their name implies, DNA tumor viruses are capable of inducing malignant growth. Here we focus on papillomaviruses (PVs), small DNA tumor viruses which have a marked tropism for epithelial cells.

1.2.1 Introduction to papillomaviruses

Warts and their infectious nature were recognized by ancient Greeks and Romans (112). Once the viral nature of human warts was demonstrated in the early 1900s, PVs were consequently identified in a variety of vertebrate species in addition to humans (31). They are highly species specific and infection typically induces the formation of benign lesions
of the skin (warts or papillomas) or mucous membranes (condylomas). The first papillomavirus was identified in 1933 by Richard Shope, who recognized the virus as the etiologic agent responsible for cutaneous papillomas in wild cottontail rabbits (201) – this was the first DNA tumor virus identified. Today, 118 PV types have been completely described, and a yet higher number of presumed new types have been detected by sequencing of subgenomic amplicons (46).

The identification of the first PV by Richard Shope, and the realization that in addition to causing benign papillomas, some warts induced by the cottontail rabbit papillomavirus (CRPV) would undergo malignant transformation, set CRPV as an important model of viral tumorigenesis (194, 220). However, efforts to study PVs in conventional cell culture were, and are to this day, hampered by the fact that their replication is associated with the differentiation process of the infected epithelium. Discovery of polyomaviruses in the 1950s, viruses which can replicate in cultured cells and induce transformation in vitro, led to replacement of CRPV by polyomaviruses as model tumor viruses (112). However, the advent of molecular cloning in the 1970s initiated a resurgence of research on PVs. Application of molecular techniques allowed cloning and sequencing of PV genomes and subsequent identification of putative open reading frames (ORFs). Improved tools to study PVs renewed the interest in the field; the attention grew even more heightened when research focused on genital-mucosal HPVs identified the medical importance of this virus. This began with the observation that the histological appearance of cervical dysplasia in Papanicolaou (Pap) smears resembled that of condylomata acuminata of the vulva (162), and represented signs of HPV infection of the cervix. Combined with several lines of evidence primarily from research conducted by Dr. Harald zur Hausen and colleagues, led to the proposal in the early 1980s, that HPV infection might be involved in cervical carcinoma (163, 259). In 1985, Dr. zur Hausen identified and sequenced two new HPVs present in cervical cancer cell lines, providing critical new tools to study HPV induced human cancers (198). By the early 1990s, it was widely accepted that HPV was the etiologic agent for cervical carcinoma. zur Hausen’s work in this field and his initial hypothesis that HPV plays an important role in cervical cancer earned him the Nobel Prize in Physiology and Medicine in 2008. Identification of
HPV as a major risk factor for anogenital cancers has undoubtedly been pivotal in combating cervical cancer.

1.2.2 HPV infection and cancer

Cervical cancer is the second most frequent cancer in women and the leading cause of cancer-related deaths, especially in women in developing countries (55, 207). It is estimated that each year there are close to 500,000 new cervical cancer cases attributed to HPV infection, and approximately 270,000 deaths (178, 238). In addition, it is estimated that 20% of head and neck cancers are due to HPV infection (38, 51, 102, 258). In the United States, the incidence of HPV positive head and neck cancer cases has increased by approximately 2% per year since the early 1970s (81), in contrast to the decreasing incidences of other head and neck cancers due to decreased smoking and alcohol consumption. Furthermore, HPV infection is associated with cancers of other genital areas, such as vulvar, vaginal, penile, and anal cancer, as well as with nonmelanoma skin cancer (88, 96, 101, 124, 155, 175, 182).

Although the incidence of cervical cancer in the developed world has decreased steadily due to successful implementation of the Pap smear screening program, cervical cancer remains a devastating and costly disease. It is especially devastating in developing countries, where 84% of all new cervical cancers are being diagnosed (178). In developed countries, despite successful screening strategies and a decrease in deaths associated with cervical cancer, direct medical costs associated with the prevention and treatment of HPV-related anogenital warts and cervical disease are staggering. For instance, in United States approximately 11,000 women are diagnosed with cervical cancer and 4,000 die from it each year (111). However, it is estimated that costs associated with prevention and treatment are approximately $4.0 billion per year (122).

Genital HPV infection is usually acquired through sexual contact and is extremely common. In one study conducted in the United States it was demonstrated that 25% of women between the ages of 14 and 19 years and 45% of women between the ages of 20
and 24 years were HPV-positive (65). It is further estimated that at least 80% of both men and women in the United States will be infected with HPV at some point in their lives (136). Fortunately, most HPV infections are temporary and have little long-term significance. Ninety percent of infections are cleared within 2 years (14, 108, 240, 242). However, the risk of developing precancerous lesions of the cervix and, eventually invasive cervical cancer, occurs when the infection persists. The progression of HPV infection to cervical cancer is accompanied by a sequence of histological changes. Cervical intraepithelial neoplasia (CIN) is a histologic abnormality of the cervical epithelium and is associated with HPV infection, with potential to progress to cancer (214, 236). CIN is classified into three grades. Mild dysplasia is defined as CIN1, and generally represents the morphologic abnormality associated with transient HPV infections. In CIN1, only a few cells are considered abnormal. On the other hand, malignant precursors of squamous cervical carcinoma include moderate and severe dysplasia, defined as CIN2 and CIN3, respectively. In CIN2 approximately 50% of cells are abnormal, whereas in CIN3 virtually all of the cells are considered abnormal (36, 138). Data indicates that 70-90% of CIN1 lesions undergo spontaneous regression; on the other hand, rates of progression to invasive cancer among CIN2 and CIN3 have been estimated at 57% and 70%, respectively (236).

1.2.3 HPV vaccines

Two prophylactic vaccines that prevent primary infection with HPV are commercially available. Gardasil, developed and marketed by Merck & Co was approved by the Food and Drug Administration (FDA) in the United States as well as by Health Canada in 2006; Cervarix was developed by GlaxoSmithKline and approved for use by the FDA and Health Canada in 2009 and 2010, respectively. Both vaccines protect against initial infection with HPV16 and HPV18, which are associated with 70% of cervical cancers. Gardasil, on the other hand, also protects against HPV6 and HPV11, which cause 90% of genital warts.
Since the vaccine is prophylactic and provides little benefit to individuals already infected with HPV, the vaccine is recommended primarily for those women who have not yet been exposed to HPV (238). In most countries, the vaccines are primarily approved for use in females, but are approved for males ages 9-26 in the United States, United Kingdom, South Korea and Australia. In Canada, Gardasil has been approved for use in males 9-26, as well as women up to the age of 45.

Although it is clear that HPV vaccines are effective in preventing HPV infection and cervical dysplasia in women, and most likely cervical and other HPV associated cancers, implementation of vaccination programs and uptake of the vaccine has been an ongoing issue. In the United States in 2010, only 32% of girls aged 13 to 17 years had received the three doses of the HPV vaccine (125). The same study also reported that overall trends in declining cancer death rates continue, in both men and women, but that an increase in incidence rates for some HPV-associated cancers (oropharynx and anus) are on the rise. Taken together with the fact that both Gardasil and Cervarix protect only against two HPV types associated with cancer, it is clear that additional prevention efforts for HPV-associated cancers are needed. Understanding the exact mechanisms by which HPV oncoproteins promote tumorigenesis will provide novel insight into basic biological processes, but will at the same time provide knowledge that may contribute to the development of better therapeutic and preventative options.

1.3 Biology of HPVs

1.3.1 PV classification

PVs belong to Papillomaviridae family, which is further classified into genus, species, types, subtypes and variants. Historically, PVs have often been classified according to the host species and the site or disease with which they are associated. Currently, sequencing of viral genomes allows for phylogenetic classification based on homology of the L1 gene, which is the most highly conserved PV gene. The broadest category is the genus; there are 12 genera, each of which is designated by a letter of the Greek alphabet. Within
a given genus, the L1 sequences of all members share more than 60% nucleotide identity. Each genus is comprised of several species that further group together separate types. A species is designated for those PVs in a given genus that share 60% to 70% identity. Furthermore, a type within a species has 71 to 89% identity with other types within the given species. Subtypes and variants exist within a type. Subtypes are defined as PVs which share 90% to 98% identity, whereas variants have greater than 98% identity. To date, more than 100 different types of HPVs have been genetically characterized (46).

Despite a similar genomic organization, different HPVs display tropism for epithelia at distinct anatomical locations. Approximately 40 different HPVs preferentially infect the anogenital and oral mucosa. These belong to the Alpha PVs and are termed “low-risk” or “high-risk” according to the propensity of the lesions that they cause to progress to malignancy. Conversely, the viral oncoproteins E6 and E7 encoded by the high- or low-risk viruses are referred to as high- or low-risk E6 and E7, respectively. Low-risk viruses, such as types 1, 6 and 11, cause benign mucosal warts that usually regress with time. In contrast, infection with high-risk HPV types such as 16, 18, 31, and 45 (which combined account for approximately 80% of HPV-positive cancers) can cause epithelial lesions that often progress to invasive cell carcinomas. Amongst the high-risk genotypes, HPV16 is most often associated with cancer, and is responsible for approximately half of all cases of cervical cancer (170). The causal role that HPV16 plays in a variety of human cancers has made this high-risk genotype the most studied and well documented amongst all HPVs and is the main focus of this thesis.

1.3.2 Viral structure

PVs are small, non-enveloped, icosahedral DNA viruses. The viral particles are 52 to 55 nm in diameter, consisting of a single molecule of double-stranded circular DNA of approximately 8,000 base pairs (bs). Within the capsid, viral DNA is found associated with cellular histones to form a chromatin-like complex (75, 183). Cryo-electron microscopy has revealed that the virus consists of 72 pentameric capsomers. The capsid consists of two structural proteins, the major capsid protein L1, and the minor protein L2
which makes up 20% of the icosahedral shell. Virus-like particles (VLPs) can be produced by expressing recombinant L1 alone or expressing the combination of L1 and L2 using yeast or mammalian systems (98, 193, 256). The ability of the capsid proteins to assemble into VLPs has been the basis of the prophylactic HPV vaccines.

The genomes of many of the animal and human PVs have been sequenced. The genomic organization of all types of PVs is remarkably similar, as is the function of many of the encoded proteins. All open reading frames (ORF) are located on the same strand of viral DNA. Transcriptional studies confirm that only one strand serves as a template for transcription and that the ORFs are transcribed into polycistronic mRNA (198, 206).

The coding strand contains eight designated translational ORFs that can be classified either as early (E) or late (L), based on the location in the genome and expression pattern (FIG.1-1). Of the early proteins, the two best studied are E6 and E7 due to their oncogenic potential. The other oncoprotein is encoded by ORF E5. Although E5 is the main transforming protein in bovine papillomavirus (BPV), its importance in human cancers it not well understood. The other major PV proteins include E1, E2, E4, and two late, structurally important capsid proteins, L1 and L2. The early region is expressed in nonproductively infected cells; this region encodes non-structural, viral regulatory proteins as well as proteins important for DNA replication. The L1 and L2 proteins are expressed as part of the productive viral infection and belong to the late region. Additionally, a region of approximately 1,000 bp in size, which contains no ORFs, is termed the upstream regulatory region (URR) or alternatively the long control region (LCR). This portion of the PV genome contains the origin of replication, a variety of cis elements which regulate DNA replication, and elements important for transcriptional control.

Transcription of papillomavirus DNA is complex because it is tightly linked to the differentiation state of the infected cell and because of the presence of multiple promoters and alternate splice patterns. The LCR of HPV16 genome contains the major promoter active in non-terminally differentiated cells (P97). This promoter directs the expression of
FIG. 1-1. Schematic representation of the genomic organization of HPV16. The 7.9kb genome of HPV16 can be divided into three functional regions: the early region (E), which encodes six early proteins E1 to E7; the late region (L), encoding the viral capsid proteins (L1 and L2); and a long control region (LCR). Adapted from Crosbie and Kitchener, Clinical Science (2006), 110:543–552.
several early gene products, including the E6 and E7 oncogenes. Alternatively, upon the activation of the differentiation dependent promoter P$_{670}$ (HPV16), increased expression of late gene products L1 and L2, as well as E4 and E1 mRNA, follows (93, 135, 195). The mechanisms by which PV mRNAs are ultimately translated from polycistronic messages are still poorly understood. Termination-reinitiation, leaky scanning, and shunting, have all been previously suggested as potential mechanisms (189, 204, 212, 223).

1.3.3 Overview of the viral life cycle

As mentioned previously, PVs are unique in that their life cycle is tightly linked to the differentiation program of the epithelium. The expression of viral gene products is closely regulated as the infected basal cell differentiates and migrates toward the surface of the epithelium. It is believed that initial infection requires access to the cells in the basal layer, which may occur through a break in the stratified epithelium or via microscopic abrasions in the skin (FIG.1-2). Cell binding and entry is an area of active research, but controversy exists as to the nature of the cell surface receptor that is required for viral attachment and entry. The $\alpha$6 integrin, heparin and cell-surface glycosaminoglycans have all been suggested to play an important role (22, 74, 129, 200). However, most PVs seem to enter the cell via clathrin-dependent receptor-mediated endocytosis (42). Following infection and uncoating within the endosomes, the L2 protein and the genome escape into the cytoplasm, and enter the nucleus where the genome is maintained as a low copy episome. It has been suggested that the viral genome is maintained in the basal layer at approximately 10-200 copies per cell, and that the viral
**FIG. 1-2. The HPV life cycle.** Following virus entry through a cut or micro-abrasion in the epithelium, HPV infection is established at the basal layer of the epithelium. The viral genome is maintained and early proteins are expressed at low levels for viral genome maintenance. In normal cells, as the basal cells divide and migrate away from the basement membrane, they differentiate and withdraw from the cell cycle. However, differentiation and cell cycle arrest are effectively uncoupled in HPV-infected cells. This allows the viral life cycle to go through successive stages of genome amplification, virus assembly, and virus release, with a concomitant shift in expression patterns from early to late genes. As infected cells move toward the apical surface, dead epithelial cells are shed, releasing infectious virions into the extracellular environment. Adapted from Hebner and Laimins, Reviews in Medical Virology (2006), 16:83-97.
proteins are expressed at low levels (44, 213). The viral E1 and E2 proteins function to maintain the viral DNA as an episome and to enable segregation of the genomes during cell division (239, 247). The basal layer of the epithelium is comprised of undifferentiated progenitor cells that are used to replenish the outer layers of the epithelium as cells are shed from the topmost layer. Upon replication, one of the daughter cells migrates away from the basal layer. Under normal circumstances in the uninfected epithelium, basal cells exit the cell cycle soon after migrating into the suprabasal cell layers and undergo a process of terminal differentiation (FIG.1-2). With the exception of the viral E1 and E2 proteins, which play roles in origin recognition and unwinding (52, 147), PVs lack all the remaining enzymes necessary for viral DNA replication. Hence, PVs rely extensively on the host replication machinery for a productive life cycle. Importantly, this means that the virus must maintain a cellular environment that is conducive for viral DNA replication. The E6 and E7 oncoproteins are thought to work together to drive the infected cell into the S-phase of the cell cycle, ultimately uncoupling terminal differentiation from cell cycle arrest. Both E6 and E7 have functions that stimulate cell cycle progression and both can associate with important regulators of the cell cycle; some of the key functions for E6 and E7 will be discussed in later sections.

PV late gene expression, synthesis of capsid proteins, vegetative viral DNA synthesis and assembly of viral particles occurs only in terminally differentiated layers of the mid and upper epithelium (FIG.1-2). The late promoter is located within the E7 open reading frame and it is thought that its upregulation leads to increased expression of proteins involved in viral DNA replication, as well as L1 and L2 capsid proteins, but without direct effect on the expression of E6 and E7 proteins, which are necessary for S-phase entry (164). PVs are non-lytic, and the virus progeny is not released until the infected cells reach the epithelial surface and are shed into the environment. It is still not completely understood how the differentiation program of the host cell triggers or activates the productive life cycle of PVs. Activation of transcription from the late promoter, and increased expression of viral replication proteins, E1 and E2, along with the late, structural genes are important. It is likely that critical cellular factors necessary for efficient late gene expression are upregulated upon differentiation. In this way, the differentiation state of the host cell is thought to drive progression of the virus infection.
The unique differentiation-dependent PV life cycle and species specificity has made propagation of this virus in the laboratory difficult, significantly hampering PV research efforts. However, many of the technical difficulties have been overcome. Initial approaches utilized nude mice with xenografts from HPV infected epithelial tissue (137). Although this method allowed for successful propagation of several HPVs, it remains technically challenging and is not suited for routine virus isolation. Much more success has been achieved through the use of organotypic “raft” cultures. Initially developed by Asselineau et al., this system is designed to mimic epithelial differentiation \textit{in vitro} (8), such that the complete virus life cycle is recapitulated in cultured cells. Most recently, transient-transfection-based system was developed which is able to encapsidate the PV genome, independently of viral DNA replication and epithelial differentiation (186). Although this system is able to produce higher yields of infectious virus than traditional methods, this technique is relatively new, and organotypic raft cultures remain the method of choice for most laboratories actually growing virus.

1.4 Functions of the Viral Proteins

1.4.1 E1 and E2 replication proteins

Upon infection, among the first viral proteins to be expressed are the replication factors, E1 and E2. E1 proteins are approximately 68 kDa in size; they function in origin recognition and exhibit both ATPase and 3’-5’ helicase activities (24, 199, 246). E1 is required for both initiation and elongation of viral DNA synthesis (147). On its own, E1 binds the origin with weak affinity, but this binding is facilitated by complex formation with E2 (52, 80, 151, 218). The E2 binding sites are located adjacent to E1 recognition sequences, such that E2 acts to load E1 onto the origin. Once bound, E1 proteins assemble into hexamers that have a much higher affinity for the DNA. E1 has been shown to interact with a number of cellular factors, including the DNA polymerase \(\alpha\), thus recruiting the cellular DNA replication machinery to the viral origin of replication (20, 176). Several other host proteins have been found to associate with E1, including
histone H1, chromatin remodeling complex component SWI/SNF5, and cyclins A and E (37, 142, 154, 219).

The E2 protein is important for DNA replication, transcriptional regulation and long-term plasmid maintenance. The E2 proteins are approximately 50 kDa in size and function as dimers. The N-terminus contains a transactivation domain, while the C-terminus encodes a DNA binding domain and interacts with E1 (89, 158). Although E2 is not essential for viral DNA replication, it does greatly enhance the ability of E1 to bind to the origin of replication, and thus serves as an auxiliary factor that promotes assembly of the initiation complex at the origin.

E2 was first described as a transcriptional activator, capable of activating viral transcription via E2 response elements found in the viral DNA (209, 210). The full-length E2 protein can function as a transactivator or a repressor, depending on the location of E2-binding sites and expression levels. For example, at low concentrations, E2 activates early-genes, at high concentrations it represses activation by interfering with the binding of transcription factors such as TFIID and Sp1 to their recognition sequences which overlap the E2 binding site (222). The two shorter forms of E2, called E2TR and E8/E2, have been described as repressors. They competitively inhibit the transactivation function of the full-length E2TA isoform (139, 140), as they contain the DNA binding domain but not the transactivating domain. In high-risk HPVs, such as HPV16, upstream of the early promoter (P97) lie four E2-binding sites. The basal activity of the P97 promoter is repressed by full-length E2, although the mechanism by which this is achieved is not yet clear. The importance of E2 in HPV induced cancers is illustrated by the finding that in HeLa cervical carcinoma cells (HPV18 positive), introduction of heterologous expression vectors for E2 results in suppression of transcription of the endogenous E6 and E7 genes, leading to senescence (54, 225).

In addition, E2 is also required for long-term episomal maintenance of viral genomes (15, 202). In the presence of E2, plasmids encoding viral E2 binding sites associate with mitotic chromosomes. This chromatin attachment function facilitates stable episomal maintenance of the viral plasmids (121). The cellular protein Brd4 (bromodomain
containing protein 4) mediates the association of bovine papillomavirus 1 (BPV1) E2 with the mitotic chromosomes, and the interaction has been shown to be conserved (247). In addition, some HPV E2 proteins have also been shown to associate with mitotic spindles (230).

1.4.2 E4 and E5 late proteins

Although the mRNA for HPV E4 and E5 proteins is expressed during the early phases of the viral life cycle, these proteins are only the third and fourth ORFs on the polycistronic transcript. Since HPVs are thought to use a leaky ribosome-scanning mechanism to translate proteins from this polycistronic message, efficient synthesis of E4 and E5 is unlikely to occur (189). In contrast, upon differentiation and activation of the late promoter, E4 and E5 are expressed as the first and second ORF, respectively. It is therefore, most likely that these two proteins are primarily synthesized in the late phases of the viral life cycle. The E4 proteins from the high-risk HPV types are associated with keratin networks in cells, and were shown to induce keratin network collapse upon overexpression (53). Thus, it has been suggested that E4 proteins may play a role in viral egress. In addition, E4 proteins have also been implicated in regulating gene expression, initiation of translation, and cell cycle regulation (40), although these roles are not well understood.

The E5 proteins are small hydrophobic proteins, with largely unknown function. They localize to endosomal membranes, the Golgi and cellular membranes (33). E5 has been best characterized in BPVs; it acts as the primary transformation protein, and associates with the platelet-derived growth factor (PDGF) receptor (197). However, HPV E5 proteins share little homology with BPV E5, and therefore may function by acting through different cellular targets. In spite of lack of homology, a number of studies have examined transforming activities of the HPV E5 proteins. It has been demonstrated that HPV E5 affects the epidermal growth factor (EGF) receptor, primarily by increasing the phosphorylation and by inhibiting degradation of the EGF receptor (33, 192, 216, 217). In the context of tissue culture and animal studies, it has been shown that HPV16 E5 can
alter the growth and differentiation of epithelial cells and can induce tumors in transgenic mice (84). Other viral-host interactions described for E5 include its association with the 16 kDa subunit of the vacuolar ATPase, which contributes to the inhibition of the acidification of endosomes (33, 192, 216); there is also evidence that E5 may be involved in downregulation of major histocompatibility complex (MHC) class II antigen expression (252). Interestingly, E5 was also recently described as an oligomeric channel-forming protein, placing it within the virus-encoded “viroporin” family (235). However, the role and contribution of E5 to cancer is still not clear. Although E5 may participate in deregulating cell growth, it presumably functions in this same manner in benign papillomas and is not essential to maintain the cancerous phenotype.

1.4.3 L1 and L2 structural proteins

As mentioned previously, L1 and L2 are the two structural proteins of PVs. The L1 protein is the major capsid protein and has a molecular weight of 55 kDa. The L2 protein is not as abundant as L1 and migrates between 72 and 78 kDa (34). 80% of the capsid consists of L1 pentamers. L1 alone or with L2 can assemble into virus like particles (VLP) (77, 193, 256). Aside from forming the viral capsid, these proteins also have other essential roles. For instance, upon entry into the cell, L2 escapes together with the viral genome into the cytoplasm, and then enters the nucleus (41). L2 was shown to interact with endoplasmic reticulum proteins, an interaction which suggests that L2 may also play a role in transport of the viral genomes into the nucleus (21). In addition, it also mediates endosomal escape after viral uncoating, such that cleavage of L2 at a furin consensus site is necessary (190). Furthermore, L2 may play an important role in localizing capsid components to the site of virion assembly. It was proposed that L2 enters the nucleus via nuclear localization signal sequences and that this occurs independently from L1. It then interacts with E2 bound to viral genomes and L1 pentamers, bringing together these components for virion assembly (43). L2 also has a DNA binding domain at its amino terminus. This region is necessary for L2 to facilitate pseudovirion genome encapsidation (191, 255).
1.4.4 The E6 transforming protein

Only two viral proteins, E6 and E7, are consistently expressed in HPV-positive cervical cancers (231). The E6 and E7 proteins contribute to tumor initiation, but also play a role in malignant progression via multiple mechanisms, either independently or in cooperation with each other. The HPV E6 proteins are approximately 150 amino acids in length and contain four zinc-binding motifs (Cys-X-X-Cys) (12, 94, 95). E6 proteins have transcriptional activation properties; they also interact with a variety of important cellular targets, modulating apoptosis, G-protein signaling, immune recognition, telomerase activity, chromosome stability, cell adhesion, polarity and epithelial differentiation, among others (reviewed in (110)). The first transforming ability identified for the high-risk HPV E6 proteins was its ability to complex with p53 (234). The p53 protein plays many roles in the cell, including cell-cycle regulation and activation of repair pathways in response to DNA damage and improper stimulation of DNA synthesis (171). Normally present at low levels and transcriptionally inactive, cellular damage triggers an increase in p53 protein levels and activation via post-translational modifications. Once activated, p53 can induce growth arrest in the G1 phase of the cell cycle, or lead to apoptosis. This allows for either DNA damage repair before initiation of a new round of DNA replication, or the removal of the damaged cell (131, 134). The importance of p53 in guarding the integrity of the cell is exemplified by the observation that approximately one-half of all human cancers harbor mutations in the p53 gene. Importantly, unlike most other human cancers, cervical cancers generally harbor wild-type p53, illustrating the fact that the virally encoded oncogenes effectively overcome the functions of wild-type p53 (35, 196).

Through the interaction with p53, E6 blocks the transcriptional functions of this tumor suppressor, leading to deregulation of p53-dependent gene expression (165). In addition, high-risk E6 proteins promote ubiquitin-dependent degradation of p53 by forming a complex with the cellular ubiquitin-protein ligase E6AP (116, 117). Therefore, the levels of p53 in E6 expressing cells, or in HPV-positive cervical cancer cell lines are typically two- to threefold lower in comparison with primary cells (196). E6AP cannot on its own associate with p53 and induce its degradation; instead, E6 must first bind to the N-
terminal substrate recognition domain of E6AP before p53 can be bound and ubiquitinated (117, 118). In addition, E6 interaction with p53 can inhibit the binding of p53 to its site-specific DNA sequences (141), and it can also induce conformational changes in the p53 protein, which in turn leads to an inhibition of p53 DNA binding ability (226). The ultimate consequence of E6-induced degradation and blocked function of p53 is the inhibition of apoptotic signaling that would otherwise eliminate the HPV infected cell.

Although many of the cellular effects of E6 occur through the interaction with p53, E6 performs a number of p53-independent functions which are relevant to transformation. For instance, transgenic mice expressing E6 from the K14 epithelial-specific promoter develop epidermal hyperplasia in a p53-null background (208). Another interesting p53-independent function of E6, which is high-risk HPV E6 specific, involves the X-(S/T)-X-(V/I/L)-COOH motif at the extreme C-terminus. This motif mediates binding with cellular PDZ domain containing proteins. E6 is thought to act as a bridge between the PDZ domain proteins and E6AP, facilitating ubiquitination of these targets and mediating their degradation. Some of the PDZ domain proteins implicated as E6 targets are the human homologue of the Drosophila melanogaster discs large tumor suppressor (hDlg), and the human homologue of the Drosophila melanogaster scribble tumor suppressor (hScrib) (182, 369). Both hDlg and hScrib are tumor suppressors targeted by E6 for degradation. hScrib is involved in epithelial tight junctions, and mediates adhesion of basal cells to the extra-cellular matrix (ECM). Similarly, hDlg is involved in epithelial tight junctions, cell-to-cell junctions and epithelial polarity. It has been suggested that binding of E6 to PDZ proteins modulates the early viral functions such as proliferation and maintenance of the viral copy number.

Overcoming senescence signals in somatic cells is critical to cellular immortalization and carcinogenesis. Another important function of HPV E6 that contributes to cellular immortalization is the ability of this oncoprotein to modulate telomerase activity (86, 149). E6 specifically activates the catalytic component of telomerase, hTERT (86, 123). To modulate telomerase activity, E6 proteins depend on E6AP (17, 85); the E6/E6AP
heterodimers increase transcription of hTERT at least in part by targeting NFX1-91, a transcriptional repressor of hTERT, for polyubiquitination and degradation (86).

1.5 Characteristics of the Papillomavirus E7 Oncoproteins

The E7 gene is expressed early after infection, and its transcription is upregulated as the host epithelial cell undergoes differentiation. In these cells, E7 and E6 are tasked with reprogramming the cellular environment to make it permissive for viral replication. E7 proteins are small, acidic polypeptides; the HPV16 E7 protein is 98 amino acids in length. The amino terminus of HPV16 E7 has both sequence and functional homology to a small portion of conserved region (CR) 1 and to the entire CR2 of adenovirus (Ad) E1A and related sequences in the simian vacuolating virus 40 large tumor antigen (SV40 TAg) (FIG.1-3). Based on amino acid sequence homology within E7 proteins, E7 can be separated into three conserved regions in an analogous fashion to Ad E1A (FIG.1-4). CR1 spans residues 2-15, CR2 spans residues 16-37 and CR3 spans residues 38-98. The CR2 homology domain includes a Leu-X-Cys-X-Glu (LXCXE, where X is any amino acid) motif, which is the canonical binding site for the retinoblastoma susceptibility locus product (pRb) and the related pocket proteins, p107 and p130 (68, 169). Immediately adjacent to this motif is a consensus phosphorylation site for casein kinase II (CKII) (11, 78). The carboxyl terminus of HPV16 E7 contains a zinc-binding domain that is composed of two Cys-X-X-Cys motifs separated by 29 amino acids (C$_{58}$-X-X-C$_{61}$-X$_{29}$-C$_{91}$-X-X-C$_{94}$) (180). Interestingly, the E6 proteins contain four of these Cys-X-X-Cys motifs and it has been hypothesized that the two oncoproteins may be evolutionarily related. The zinc-binding domain of E7 is a highly structured dimer, with topology unique to E7. Binding of zinc is vital for maintaining the structural integrity of the protein and providing a dimerization interface (32, 148, 159, 174).

E7 is found predominantly in the nucleus, although cytoplasmic pools of E7 can also been detected in vivo (205). It lacks a prototypical nuclear localization sequence and enters the nucleus through a novel Ran-dependent pathway (6). There are low steady-
FIG. 1-3. Sequence alignment between conserved regions (CR) 1 and 2 of HPV16 E7, adenovirus type 5 E1A (Ad5 E1A) and simian vacuolating virus 40 large tumor antigen (SV40 TAg). Conserved amino acids are shaded and the boxed region indicates the consensus LXCXE motif that targets pRb and pocket proteins p107 and p130.
FIG. 1-4. Schematic depiction of HPV16 E7. The positions of conserved region (CR) 1-3 in HPV16 E7 is depicted. Locations of the Cys-X-X-Cys (CXXC) motifs which participate in coordinating zinc ion are also indicated.
state levels of E7 in the cell, which can be attributed to the relatively short half-life of this protein (less than 2 hours) (205). E7 is also targeted for degradation by the ubiquitin-dependent proteasomal pathway that involves conjugation of ubiquitin to the amino terminus of E7 (188). Interestingly, HPV16 E7 is known to aberrantly migrate on SDS polyacrylamide gels at an apparent molecular weight of 18-20 kDa, as opposed to its predicted size of approximately 11 kDa. This has been found to be caused primarily by the acidic nature of the amino terminal CR1 domain of HPV16 E7 and the aberrant migration is not shared by HPV6 E7 proteins (103, 168).

1.5.1 Biological activities and molecular targets of E7

Studies in the 1980s revealed that the E7 protein of high-risk HPVs is the major transforming protein (18, 130, 185, 224, 232). The E7 protein of HPV16 has been studied the most extensively as it was the first high-risk HPV oncogene to be discovered (130, 185, 232, 248), but also because of the medical relevance of HPV16. E7 of high-risk HPVs possesses several properties characteristic of an oncogene. Specifically, E7 alone can transform cultured rodent fibroblasts (18, 185) and bypass serum starvation-induced growth arrest (181). In co-operation with an activated ras oncogene, E7 can transform primary rodent fibroblasts (185), and in the presence of E6, it can efficiently immortalize primary human keratinocytes (100). Low-risk E7 proteins have markedly decreased transforming and immortalizing activities (13, 99).

In low-grade HPV-induced lesions, the HPV genome is maintained as an episome. However, during the malignant progression of the high-risk HPV-associated lesions, the viral genome is often found integrated into the host genome. This is a key event in HPV-induced carcinogenesis, and is accompanied by termination of the viral life cycle and loss of portions of the HPV genome. Viral tumorigenesis can therefore be regarded as an accidental by-product of virus propagation. Integration often disrupts the region encoding the viral E2 transcriptional regulator, leading to loss of the repressive actions of E2 and resulting in deregulated overexpression of the E6 and E7 oncoproteins (30). In fact, both E6 and E7 are consistently expressed in malignant tissue and are required for the
induction and maintenance of the transformed phenotype, as it has been shown that re-expression of E2 represses E6 and E7 transcription in addition to causing rapid growth arrest and senescence in cervical cancer cell lines (91, 92).

1.5.1.2 Association of E7 with pRb and activation of E2F-dependent transcription

In normal epithelial cells, the differentiation program is tightly linked to withdrawal from the cell cycle. The HPV E7 protein is tasked with the critical role of altering the cellular environment into a DNA replication competent state that supports viral replication. The E7 proteins lack any intrinsic enzymatic or DNA binding activity and it is widely accepted that its biological activities are primarily exerted through interactions and subversions of cellular regulatory proteins. The binding of E7 to pRb is the most studied interaction between this viral protein and a cellular target, although the precise mechanism of how E7 deregulates pRb is still not fully established. This topic will be discussed further in Chapter 3; here the author provides a summary of our state of knowledge concerning E7 and pRb interaction as it appeared at the onset of the studies described in Chapter 3.

The best studied function of pRb is the ability of this tumor suppressor to regulate G1/S entry and progression whereby it modulates the activities of E2F transcription factors (reviewed in (82)). The E2F transcription factors exist as heterodimers that contain an E2F (E2F1-6) and a DP (DP1-2) subunit; they act as critical regulators of genes necessary to transition from the G1 to S phase of the cell cycle. Different E2F transcription factors have similar DNA sequence specificity, but show differences in their ability to force quiescent cells into the cell cycle and in their relative occupancy of various cellular promoters in G1 and S cells (227). Typically, the association of pRb with E2F blocks the transition through the G1/S checkpoint of the cell cycle until the cell receives a signal to divide (FIG.1-5). In normal cells, disruption of pRb/E2F repressor complex is triggered by cdk4/6 and cdk2 mediated pRb phosphorylation in late G1. Once dissociated from pRb, E2F acts as a transcriptional activator, leading to entry and progression into S phase. In addition to blocking E2F-dependent transcription prior to S phase entry, pRb can also
remodel chromatin in neighboring regions to silence transcription. For instance, pRb interacts with chromatin modifiers, such as the histone deacetylases (HDAC1 and HDAC2), histone demethylases (RBP2), DNA methyl transferase (DNMT1), a helicase (Brg1, Brm), and histone methyl transferases (Suv39h1, RIZ and Suv4-20h1/h2), to create a repressive chromatin environment at target genes (reviewed in (221)). In HPV infected cells, E7 binds to the hypophosphorylated form of pRb through the LXCXE motif, the primary high-affinity binding site found in CR2 (67, 169). This binding causes disruption of the pRb/E2F repressor complex, resulting in uncontrolled G1 exit and S-phase entry (FIG.1-5). Although the LXCXE motif is the primary binding site for pRb, sequences in both CR1 and CR3 are also important for deregulating the pRb pathway (90, 115). Structural analysis of the binding sites of the LXCXE containing peptide from the CR2 region of HPV E7 (143) and a high affinity transactivation peptide from E2F (245), shows that the viral peptide binds on the B domain of pRb almost 30Å away from the binding site of the transactivation (TA) domain of the E2F peptide. The latter is formed by a groove between the A and B domains of pRb; hence the LXCXE motif cannot be solely responsible for disrupting the pRb/E2F complex. Indeed, other experiments have shown that additional sequences are necessary to disrupt pRb/E2F complex, although the mechanism still remained unknown. CR1 and CR3 residues facilitate the displacement of E2F (104, 113, 244), while several residues within CR3 also provide a second low-affinity pRb binding site (148, 179). However, the LXCXE independent interaction between pRb and CR3 of E7 has only been demonstrated in vitro, and the biological significance of this interaction was unknown prior to the work undertaken in this thesis.

While E7 acts similarly to SV40 TAg and Ad E1A in terms of binding of pRb and displacement of E2F, unlike other viral oncoproteins, E7 does not depend solely on stoichiometric interaction with pRb for inactivation of its function. Instead, the HPV E7 proteins also destabilize pRb through proteosomal degradation (23, 127). E7 associates with cullin 2 E3 ubiquitin ligase complex. This interaction presumably leads to pRb ubiquitination and subsequent proteosomal degradation (115). Sequences in both CR1 and CR3 regions of E7 have been shown as important for cullin 2 binding, and therefore may play an important role in pRb degradation (115). The ability of E7 to degrade pRb
FIG. 1-5. Relaxation of the G1/S checkpoint by HPV proteins. High-risk E7 proteins interfere with pRb function, thus abrogating the pRb/E2F transcriptional repressor complex. This causes an increased expression of E2F responsive cell-cycle regulators, including Cdc25, cyclin E and cyclin A, and replication enzymes. HPV16 E7 proteins can also associate with and abrogate the inhibition of cdk2 activity by interacting with the cyclin-dependent kinase inhibitors, p21^{Cip1} and p27^{Kip1}. These activities contribute to the ability of HPV to create and/or maintain an environment in the differentiating host cell that is conducive for viral replication. In uninfected cells, proliferation signal leads to a cascade of events, ultimately activating cyclin-dependent kinases which phosphorylate pRb, releasing the E2F/DP1 transcription factors. Under certain circumstances in which p53 is activated, p53 can induce transcription of p21^{Cip1} and p27^{Kip1} which in turn inhibit cyclin-dependent kinases and therefore prevent phosphorylation of pRb.
has important consequences for both the host cell and the viral life cycle. E7 proteins are expressed at much lower levels than their cellular target proteins, suggesting that stoichiometric inhibition of pRb would not be favourable for the virus. By eliminating pRb in an enzymatic fashion, low steady-state levels of E7 protein are sufficient to impair the entire spectrum of biological activities of the pocket proteins, while activating E2F transcription. Furthermore, low-risk HPV E7 proteins can also efficiently bind to pRb, but are unable to target pRb for degradation (87, 90). Due to these observations, E7’s oncogenic potential may correlate more closely with the ability to induce pRb proteolysis than merely with its pRb-binding potential (87, 90). However, this assumption has been weakened by the observation that destabilization of the pocket proteins is not sufficient to overcome cell cycle arrest (104) or efficiently transform some cell lines (10). These observations highlight the importance of additional cellular targets of E7 involved in stimulating cell cycle progression, and also emphasize that further studies are necessary to precisely understand how E7 deregulates the pRb pathway.

1.5.1.3 Effects of E7 on other components of the cell cycle machinery

Although replication of the HPV genome depends on the ability of the infected differentiating keratinocytes to support DNA synthesis, certain aspects of the viral life cycle, such as the synthesis of capsid proteins, require the milieu of a differentiated host cell. For this reason, it is important that E7 does not reverse or alter the differentiated phenotype, but rather, uncouples differentiation from proliferation. There are a number of mechanisms by which E7 achieves this goal and contributes to cell cycle dysregulation.

E7 associates with and regulates the expression of several cell cycle regulators besides pRb. Importantly, E7 associates with both positive regulatory subunits, cyclins, and negative regulators, cyclin-dependent kinase inhibitors (CKIs), to regulate the activity of cyclin dependent kinases (cdks), which are the motors behind cell cycle division (FIG. 1-5). The expression of cyclins E and A, the regulatory subunits of cdk2, is under the control of E2F; as a result of inactivation of pocket proteins by E7 and increased E2F dependent transcription, E7 expressing cells maintain higher levels of cyclin E and A
(250). HPV16 E7 also causes an increase in the transcription of the Cdc25A phosphatase, which leads to further activation of Cdk activity (132). In addition, E7 can directly associate with cyclinA/Cdk2 and cyclinE/Cdk2 complexes, resulting in increased cdk2 activity (172). HPV16 E7 also abrogates the inhibition of cdk2 activity by interacting with CKIs p21$^{\text{Cip1}}$ and p27$^{\text{Kip1}}$ (83, 126, 249). Levels of p21$^{\text{Cip1}}$ are increased due to protein stabilization in E7 expressing cells (126), however cdk2 remains active despite the increase in p21$^{\text{Cip1}}$. Interestingly, p21$^{\text{Cip1}}$ has been implicated in coupling cell cycle arrest and differentiation in keratinocytes (2, 49, 167). Thus, the ability of E7 to interfere with the capacity of CKIs to block cell cycle progression is critical to the ability of HPV to uncouple differentiation from DNA synthesis. The culmination of these activities resulting from HPV16 E7 expression is the inappropriate phosphorylation of substrates that allows cells to transition the G1/S boundary. As a consequence, differentiating cells are induced to enter and sustain the replication-competent cellular milieu necessary for virus propagation.

1.5.1.4 Transcriptional regulation by E7

The organization of eukaryotic chromatin has a major impact on gene expression (243). The E7 protein can associate with chromatin-modifying enzymes, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs) to regulate gene expression. Specific modifications of the exposed amino terminal tails of histones, which include acetylation, phosphorylation, methylation and ubiquitination, alter their biochemical properties, ultimately affecting their ability to interact with DNA and cellular proteins that read the "histone code" (145). While acetylation of specific lysine residues neutralizes the positively charged histones, deacetylases favor the formation of compact, repressive chromatin (145). The ability of E7 to interact with histone-modifying enzymes has the potential to affect the accessibility and expression of specific chromosomal loci. HPV16 E7 is able to indirectly associate with HDAC-1 via the interaction with Mi2$^\beta$ of the NuRD (nucleosome remodeling and histone deacetylation) chromatin remodeling complex (26). pRb and the related pocket proteins, p107 and p130
are also capable of associating with HDAC-1 through sequences that are distinct from the E7 binding domain, and it is therefore presumed that E7 associates with HDAC-1 independently from pRb (25, 50, 152). As mentioned in previous sections, the formation of pRb/E2F/HDAC complexes represses E2F responsive promoters and transcription of S-phase specific genes. In HPV expressing cells, it is not known whether E7 competes with pRb for HDAC-1 or if E7/pRb/E2F/HDAC inhibitory complexes are formed. In either event, the consequence of the interaction between E7 and HDACs increases the levels of E2F-mediated transcription in differentiating keratinocytes and is important for sustained viral replication (150).

The idea that E7 has the ability to epigenetically modify the genomes of infected cells on a global scale has not been explored in much detail, but likely represents an important and interesting possibility if other viral oncoproteins serve as an example. Individual genes or entire sets of genes may be epigenetically deregulated to promote the “hallmarks of cancer” such as self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, increased proliferation potential, sustained angiogenesis, and capability of metastasis and invasion (73, 97). Other DNA tumor viruses have the potential to alter histone modifications on a global level, eventually resulting in a cellular environment that is conducive for viral replication but which may also display many of the “hallmarks of cancer”. Specifically, Ad5 E1A targets p300/CBP (69), and this interaction is important for cellular transformation (3, 187). Binding of Ad5 E1A to p300 and CBP results in global hypoacetylation of histone H3 lysine 18 (109) and concomitant relocalization of these HATs to the promoter regions of a limited number of genes that promote cell growth and division, hyperacetylation of H3 lysine 18, and gene-specific transcriptional activation (76). These studies indicate that cellular transformation by E1A is mediated through concerted epigenetic reprogramming following interaction with HATs such as p300 and CBP. Considering these findings and the observation that HPV16 E7 is also able to interact with various HATs including p300, CBP, and p300/CBP-associated factor (pCAF) (9, 19, 114), and that the acetylation of histone H3 is increased at E2F-responsive promoters in HPV16 expressing human foreskin keratinocytes (251), it may be hypothesized that E7 also has the potential to globally and epigenetically modify cellular gene expression. Epigenetic reprogramming of host cells by E7 has recently been
demonstrated at the level of histone methylation (160). E7 causes a dramatic decrease in repressive trimethyl marks on lysine 27 of histone 3. This is caused by transcriptional induction of the KDM6A and KDM6B histone 3 lysine 27-specific demethylases, and specifically leads to increased expression of the cervical carcinoma biomarker p16$^{INK4A}$.

Additionally, E7 interacts with many other transcriptional regulators; E7 interacts with several members of the AP-1 family of transcription factors, including c-Jun, c-Fos, JunB and JunD, and activates transcription of their responsive promoters (7). E7 also binds the TATA-binding protein (TBP) (156) and several TBP associated factors (TAFs), including TBP-associated factor-110 (157). Furthermore, HPV16 E7 targets multiple members of the E2F transcription factor family, including the transcriptional activator, E2F1 and the repressor, E2F6 (119, 161); interaction with E2F1 can enhance E2F1-mediated transcription (119). E2F1 plays a role in mediating the transcriptional control of the E2F6 gene, which is upregulated at the G1/S phase transition in order to repress E2F-responsive promoters, thereby directing appropriate cell cycle exit and differentiation (153). E2F6 is therefore an attractive target for E7 to deregulate; HPV16 E7 associates with E2F6 and abrogates its ability to function as a transcriptional repressor (161). The interaction and deregulation of E2F6 is presumably necessary to ensure that the cells remain in an S-phase-competent state for the viral life cycle to occur. These specific interactions with E7 can have profound implications on the induction of malignancy, as several AP-1 proteins and E2F1 have been implicated as potent transforming agents (1).

The activities of E7 described in previous sections complement one another and primarily allow enhanced expression of cellular proteins which are rate-limiting for cellular DNA synthesis. Together, the co-ordinated deregulation of the host cell cycle creates and maintains an environment that is conducive for viral replication and the full viral life cycle in differentiated keratinocytes where E7 expression is indispensable (79).
1.5.1.5 Deregulation of the p53 function by E7

The levels and half-life of p53 are increased in E7 expressing cells, suggesting that E7 may perturb p53 degradation (48, 127). However, the mechanism of p53 stabilization in E7 expressing cells is still unclear; it is established that it is independent of p14ARF, an inhibitor of mdm2-mediated p53 degradation (16, 253). Mdm2 is the ubiquitin ligase that mediates p53 degradation in normal cells, however it has been established that mdm2-mediated p53 degradation is defective in high-risk HPV expressing cervical cancer cells (105). On the other hand, despite the high levels of p53 in E7 expressing cells, the transcriptional activity of p53 is not increased (128). On the contrary, E7 expression can inhibit p53 transcriptional activity in reporter assays (72). The increased levels of p53 are transcriptionally “inert” (71), and the augmented steady-state levels of p53 targets, such as p21Cip1, appear to be the consequence of protein stabilization (128, 173). Additionally, HPV16 E7 interferes with p53-mediated G1 growth arrest signaling in response to DNA damage (47, 107), although the mechanistic details are unknown. One major consequence of increased levels of p53 is impaired growth and increased susceptibility of E7-expressing cells to apoptosis (71, 127). To counteract this, HPV infected cell express the E6 proteins. As discussed previously, E6 proteins use several mechanisms to interfere with p53 functions, including recruiting the cellular E3 ubiquitin ligase E6AP, which leads to efficient proteasomal degradation of p53.

1.5.1.6 Genomic instability

HPV E6 and E7 proteins are necessary for maintenance of the transformed phenotype, but they are not sufficient to fully transform cells. Expression of E6 and E7 in human keratinocytes causes cellular immortalization, but these cells do not form tumors when injected into nude mice. Additional oncogenic events are necessary for malignant progression to occur. Genomic instability is a defining hallmark of many human tumors and has been observed in premalignant high-risk HPV-associated lesions (62, 106, 237). In addition, most HPV positive malignancies have numerous chromosomal imbalances,
including gains or losses of whole chromosomes (aneuploidy) and chromosomal rearrangements (257).

High-risk, but not low-risk E6 and E7, can independently induce genomic instability in normal cells (237). Induction of genomic instability is thought to be an early event in HPV-induced cancers, occurring before the viral genome is integrated into the host chromosomes (59). The expression of high-risk E6 and E7 induces numerous mitotic defects, including multipolar mitoses, anaphase bridges and aneuploidy (58). Multipolar mitoses are characteristic of most high-risk HPV lesions and are associated with abnormal centrosome numbers (61, 241). It has been demonstrated that expression of E7 rapidly induced centrosome amplification, which correlates with cell division errors and occurs before the detection of genomic instability (59, 60). E7 induces multiple rounds of centrosome synthesis in a single S phase from a single maternal centriole; this function of E7 appears to be dependent on high levels of cdk2 activity, and therefore is at least in part dependent on the ability of E7 to deregulate pRb (56, 57). However, E7 can also induce centrosome abnormalities in mouse embryonic fibroblasts deficient in pRb family members, albeit at much lower levels (64).

In addition, E6 and E7 can also induce genomic instability through the induction of DNA damage and the activation of the ATM-ATR pathway (ataxia telangiectasia-mutated-ATM and RAD3-related DNA damage repair pathway) (63, 133). Activation of the ATM DNA damage response was shown to be important for differentiation-dependent viral genome amplification. However, the activation of DNA damage pathways also contributes to malignant progression. An important aspect of the ATM-ATR DNA damage response is the induction of cell cycle checkpoints at S or G2-M. E7 can abrogate these checkpoints to promote mitotic entry by inducing degradation of claspin (211), a key regulator of the ATR-CHK1 DNA damage-signaling pathway that is activated in response to replication stress. It is thought that the accelerated degradation of claspin by E7 in G2-M may cause cells to initiate checkpoint recovery even in presence of DNA damage, potentially leading to genomic instability. Taken together, E7 has the potential to induce genomic instability via multiple mechanisms, ultimately contributing to tumor progression by generating cancerous cells at a greater frequency.
1.5.2 Structural organization of E7 proteins

Although some of the major biological activities of E7 were described in previous sections, E7 has the potential to affect many other cellular processes. The effects exerted by E7 on the host cell are primarily carried out by binding numerous cellular factors. Understanding the structure of the HPV E7 proteins can provide great insight into the mechanisms behind E7’s ability to target a broad spectrum of cellular targets. In addition, the structure could also significantly aid in planning mutational studies that mechanistically address E7 functions and interactions. Potentially, structural studies could also help explain the functional differences between the high-risk and low-risk E7 proteins, and the malignant progression of lesions induced by high-risk HPVs. Ultimately, this information may suggest new antiviral strategies to interfere with the action of E7.

With those aims in mind, a solution structure of the high-risk HPV45 E7 protein was solved using Nuclear Magnetic Resonance (NMR) spectroscopy (FIG.1-6) (174). Experiments using full-length HPV45 E7 and a construct representing its CR3 region revealed that the N-terminus (amino acids, 1-54) of this oncoprotein is unstructured and flexible in solution, whereas the C-terminus (residues 55-106) folds autonomously into a well-structured zinc-binding domain. Results obtained from the NMR analysis are consistent with the intrinsic disorder of high-risk E7 proteins predicted by bioinformatic studies (229) and with a previously reported secondary structure of E7 proteins (228). The N-terminal region of E7 represents an example of an intrinsically disordered region that may undergo a localized conformational change upon interaction with its biological target (66). This is not surprising given the fact that intrinsic protein disorder is very common in cancer associated proteins, with approximately 79% of cancer-associated and 66% of cell-signaling proteins containing predicted regions of disorder of 30 residues or longer (120). The dynamic nature of the N-terminus may aid HPV E7 proteins in fulfilling some of their biological functions. In disordered domains, protein function is traced to short sequences called “linear motifs”. Indeed, the N-terminus of E7 contains multiple functional linear motifs that contribute to binding of cellular targets, with the LXCXE motif being a prime example (29, 78, 146, 188, 203). Linear motifs within the
FIG. 1-6. Representative 3-dimensional structure of the HPV45 CR3 dimer. A structural schematic of the HPV45 E7 (residues 55-106) CR3 dimer. CR1 and CR2 are not shown as they are intrinsically disordered. Structures of protomers are coloured in green and blue, and the alpha helices and beta sheets are labeled in the top left structure. Different views were generated by rotating the top left structure down 90° for TOP view, up 90° for BOTTOM view, and left or counter clockwise 90° for side view. The structures were generated from the PDB file #2F8B using PyMOL software. (Structure was originally published in Oncogene (2006), 25:5953-5959.)
intrinsically disordered N-terminus may be beneficial by providing multiple binding sites, with high specificity but low affinity, extending the capacity of the interaction surface to bind diverse proteins and enhancing protein association rates (254). Indeed, the localization of the LXCXE motif within an intrinsically disordered domain is thought to provide the fast, diffusion-controlled interaction that allows viral proteins to outcompete physiological targets of pRb (27).

The solution structure of the C-terminal domain of HPV45 E7 is virtually identical to the X-ray crystallography structure of a CR3 construct derived from the low-risk HPV1 E7 protein (148). Both of these E7-CR3 domains form a well-structured zinc-binding domain with a unique \( \beta_1\beta_2\alpha_1\beta_3\alpha_2 \) topology containing a zinc binding fold that is not found in any other solved structures (FIG.1-6); however, it has been proposed that the fold arose from a host PHD domain (45), which is also involved in protein-protein interactions. This region contains a C4-type zinc finger which co-ordinates one molecule of zinc ion. The distance between the two Cys-X-X-Cys motifs is too large to form a classical zinc finger structure that was first proposed for the \textit{Xenopus} transcription factor IIIA (TFIIIA) (144, 166).

Both structural studies demonstrate that E7 assembles as a roughly globular, obligate zinc-dependent dimer. Conversely, other studies have suggested that E7 CR3 may adapt a range of conformations that contribute to the protein interaction repertoire of E7. It was shown that E7 CR3 protomers modulate binding to the AB domain of the retinoblastoma protein (28, 29) and are able to bind zinc, and yet other studies have shown that CR3 can also form large structural oligomers (4, 5, 39). It is therefore unclear whether E7 dimerization is of functional importance.

Although the co-ordinated zinc ions are not directly involved in E7 dimer formation, they are important for maintaining the folded state of the protomeric CR3. Indeed, the removal of zinc ions causes unfolding of CR3 and formation of large oligomeric structures. E7 forms a stable dimer through interactions of \( \alpha_1 \) helices of each protomer and \( \beta \)-sheet interactions between the \( \beta_2 \) and \( \beta_3 \) strands of opposing protomers. Dimerization leads to the formation of a contiguous hydrophobic core that is further stabilized by a subset of
residues that form intersubunit contacts. A sequence alignment of the CR3 domains from E7 proteins previously predicted that the E7 dimer is maintained in large part by a hydrophobic core, based on the observations that 7 hydrophobic residues in CR3 exhibit more than 90% conservation, and 5 more exhibit 70% conservation (228). Based on sequence analysis of more than 200 E7 sequences, the highest degree of sequence conservation within the CR3 region, aside from the invariant zinc-coordinating cysteins, belongs to the residues that form the hydrophobic dimerization interface (27). These findings suggest that the structural features of CR3 are likely to be very highly conserved among E7 proteins.

A large number of cellular targets have been reported to interact with E7 via the C-terminus; however, most studies have not attempted to map the residues on the surface which are necessary for the interaction with the particular target. On the contrary, studies which have endeavored to carry out mutational and mapping studies have frequently utilized mutants within CR3 which target residues critical for the formation of the hydrophobic core and/or dimerization. These studies include mapping of p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1}, TBP, TAF110, Mi\textalpha 2 and pCAF (9, 26, 83, 126, 156, 157, 249). The loss of interaction or function observed in these studies could likely be attributed to gross structural effects upon E7 or to the loss of dimerization rather than to impairment of specific functions. This issue has consistently been raised with mutations in the coordinating cysteines that destroy protein stability (32, 184, 233) and impair transformation and transactivation functions (70, 159, 184, 215). To explore the function of CR3, a thorough structure-function analysis will need to be performed using a set of reagents which take into consideration that the CR3 domain of E7 is highly structured, and which aim to preserve the folded state.

1.6 Thesis Overview

Here, we created a panel of mutations in the CR3 region of HPV16 E7, targeting putative surface-exposed residues, and I utilize this set of novel E7 mutants to further characterize the role of CR3. By targeting surface-exposed residues of CR3 instead of the buried,
hydrophobic and structurally important residues, this set of reagents aims to preserve the structure, but disrupt interactions with cellular targets. In Chapter 2, I describe these reagents. We also analyzed our novel collection of mutants, as well as mutants targeting predicted hydrophobic core residues of the HPV16 E7 dimer, in order to answer the question: is dimerization of E7 functionally important? We assessed the ability of E7 mutants to dimerize using a yeast-two hybrid approach, and also assessed this same set of mutants for their transformation capability in baby rat kidney cell in cooperation with activated ras. Our data shows that some mutants of HPV16 E7 CR3 fail to dimerize, yet are still able to transform baby rat kidney cells. Our results also identify several novel E7 mutants that abrogate transformation. Overall, this data indicates that E7 does not need to exist as a stable dimer in order to cause cellular transformation.

I further carry on with these studies using the panel of surface-exposed mutants in order to carefully assess the role of CR3 in deregulating the activity of the retinoblastoma tumor suppressor. Prior to the study described in Chapter 3, evidence was presented that CR3 contributes to pRb binding \textit{in vitro} and may be involved in pRb degradation. I utilized our panel of mutants to systematically analyze the molecular mechanisms by which CR3 contributes to deregulation of the pRb pathway. The data indicates that despite differences in the ability to interact with cullin 2, all CR3 mutants degrade pRb comparably to wild-type E7, suggesting that cullin 2 may not be the only mechanism utilized by E7 to target pRb for degradation. Furthermore, I identified two specific patches of residues on the surface of CR3 that contribute to pRb binding, independently of the high-affinity CR2 binding site, and show for the first time that CR3 can bind pRb \textit{in vivo}. Mutants within CR3 that affect pRb binding are less effective than the wild-type E7 in overcoming pRb induced cell cycle arrest, illustrating for the first time that this secondary LXCXE independent binding site may be functionally important.

In Chapter 4 we identify a novel interacting partner of E7, p190RhoGAP, using a mass spectrometry approach. p190RhoGAP is a direct negative regulator of RhoA; it stimulates the GTPase activity of RhoA to convert the bound GTP to GDP, therefore keeping RhoA in its inactive form. I show that CR3 of E7 is necessary and sufficient to associate with p190RhoGAP, and hence utilize the panel of surface-exposed mutants in
CR3 to study this interaction. I identified two mutants within CR3 of E7 that completely abolish binding to p190RhoGAP and showed that the E7 proteins of multiple HPV types also bind p190RhoGAP. Functional data indicates that expression of E7 leads to decreased levels of active RhoA. As RhoA regulates the cytoskeleton, contributing to cellular migration, I also determined if E7 influences the integrity of the cytoskeleton. Using fluorescence microscopy, my data indicates that E7 expressing cells have reduced levels of F-actin, whereas the two mutants unable to associate with p190RhoGAP do not exhibit this reduced phenotype. These results suggest that E7 may be utilizing p190RhoGAP to regulate RhoA levels, altering the cytoskeleton and ultimately controlling cell attachment and migration.

Taken together, these studies highlight the importance of CR3 of HPV E7, and illustrate that this panel of novel surface-exposed mutants can be effectively utilized to study this region of E7. It is the hope of the author that we have gained valuable insight into the structure-function relationship of E7 and have advanced our knowledge on how E7 deregulates the function of at least some of its interacting partners. Importantly, our work also emphasizes that further studies are needed to fully elucidate certain pRb independent mechanisms of E7 function.

1.7 References


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2.1 Introduction

More than 100 human papillomavirus (HPV) types have been described, and more are presumed to exist (16). HPVs induce papillomas in the cutaneous and mucosal epithelia, where specific HPV types often preferentially infect distinct anatomical sites. HPVs associated with lesions that can progress to carcinogenesis are classified as “high-risk” types, the most common of which is HPV16. In contrast, HPVs associated with benign warts that regress with time are termed “low-risk” viruses (50). Persistent infection by high-risk HPVs is associated with 99.7% of all human cervical cancer cases (15), other genitourinary cancers, and an increasingly growing number of oral cancers (21).

The viral E6 and E7 oncoproteins are consistently expressed in HPV-induced cancers and are necessary to maintain malignant cell growth (3, 6, 29, 42, 43). Repression of their transcription by re-expression of the viral E2 protein induces rapid growth arrest and senescence of cervical cancer cells (22, 23). The HPV E7 oncoprotein binds the product of the retinoblastoma susceptibility locus (pRb) and the related family members p107 and p130 (17). These proteins function as tumor suppressors, maintaining control of the G1/S checkpoint of the cell cycle by binding to the E2F family of transcription factors and by recruiting transcriptional repressor complexes to the E2F-responsive genes (10, 36). Besides pRb, the E7 oncoproteins from high-risk HPV types abrogate the inhibitory activities of the cyclin-dependent kinase inhibitors p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} by directly binding these factors (19, 48). E7 interacts with the pCAF acetyltransferase, the Mi2\beta subunit of the NuRD histone deacetylase complex, the S4 component of the 26S proteasome, the HIF-1\alpha and E2F6 transcription factors, and many other important cellular proteins (4, 7, 8, 11, 35).

The N-terminal region of E7 displays sequence similarity to other viral oncoproteins, namely, to a portion of conserved region 1 (CR1) and all of CR2 of adenovirus (Ad) E1A
Based on the amino acid sequence similarity between different HPV types, the HPV16 E7 protein can also be separated into three conserved regions, namely, CR1 (amino acids 2 to 15), CR2 (amino acids 16 to 37), and CR3 (amino acids 38 to 98). Binding of many cellular targets of E7 has been mapped to CR3, including a secondary low-affinity pRb binding site (32, 38). This C-terminal zinc binding region of E7 is highly structured and composed of two Cys-X-X-Cys motifs separated by 29 or 30 amino acids, whereas the amino-terminal region (CR1/CR2) is unstructured (34). High-resolution three-dimensional structures of the zinc binding region of E7 have been reported for HPV1 and HPV45 (32, 37). E7 CR3 exhibits a unique $\beta_1\beta_2\alpha_1\beta_3\alpha_2$ topology that is not present in any other known zinc binding proteins. The co-ordinating zinc ions are important for maintaining a folded state of CR3, as removal of zinc causes unfolding of the tertiary structure and formation of large oligomeric complexes (2). E7 CR3 also forms a stable dimer involving the $\alpha_1$ helices of the protomers and the $\beta_2$ sheet of one protomer and $\beta_3$ sheet of the other, with dimerization leading to the formation of a hydrophobic core. However, whether E7 exists as a functional dimer in vivo or can function as a protomer has not been established conclusively. Furthermore, it is now known, based on existing structures of E7, that the most highly conserved residues of CR3 are in fact structural components of the protomer core and dimer interface.

The information from structural studies has necessitated a reexamination of much of the existing literature regarding the highly structured CR3 portion of E7. Most of our understanding of this region is based on the analysis of mutants that target the most highly conserved amino acids, which are not surface-exposed and are not available for interaction with cellular targets but are likely important structural components. It is thus conceivable that the loss of protein interactions and/or functions observed in studies utilizing these mutants may be attributed to structural effects on E7 rather than to the impairment of specific functions. For instance, this issue has been raised consistently because of mutations in the invariant zinc co-ordinating cysteines that decrease protein stability (39, 44, 46) and impair transformation and transactivation functions (18, 34, 39). To explore in detail the functions of CR3, a structure-function analysis requires mutations that preserve the overall structure of this region. In addition, many studies have used short deletion mutants within this region and have reported apparent multiple overlapping
interactions with diverse cellular targets, which might simply reflect global disruption of this particular region (25, 27, 28).

Our aim, therefore, was 2-fold: (i) to create a panel of mutations targeting surface-exposed residues within CR3 of E7 and (ii) to establish whether E7 must exist as a dimer for its function. Utilizing the existing structures of HPV1 and HPV45 E7 CR3, we modeled the structure of CR3 of the HPV16 E7 protein. Based on the modeled structure, 24 side chains within CR3 were identified as at least 25% solvent exposed and were chosen as targets for systematic mutagenesis. These residues are likely to be accessible for interaction with cellular proteins and hence may contribute to E7 function. We utilized a substitution strategy in which amino acid residues were replaced with residues of opposite charge, preserving solvent interaction, and thus protein structure, but potentially disrupting specific intermolecular protein interactions. We also targeted the highly conserved hydrophobic core residues for mutagenesis. With this panel of mutants and an analysis of transformation potential, we determined that E7 does not need to exist as a dimer to transform primary baby rat kidney cells (BRK cells), and we identified several new mutants of E7 that abrogate transformation.

2.2 Materials and Methods

2.2.1 Plasmid construction

Two methods of site-directed mutagenesis were utilized to construct all mutations targeting the surface-exposed residues, namely, megaprimer (31) and overlap extension (26) methods. A list of primer sequences used is provided in Tables 2-1 and 2-2. The panel of CR3 mutants was generated with flanking EcoRI and SalI restriction sites and then inserted into the multiple cloning site of the pBAIT (49) yeast expression vector, generating constructs to be used in the yeast two-hybrid assay (LexA DNA-binding-domain fusions). The coding sequence for each mutation in E7 was subsequently subcloned, using EcoRI and SalI, into the same site of a modified pJG4-5+ vector (Clontech) for expression as a fusion with the synthetic B42 transcriptional activation
Table 2-1. List of surface-exposed HPV16 E7 mutants and oligonucleotides utilized in PCR

<table>
<thead>
<tr>
<th>E7 construct</th>
<th>Oligos (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>GTCGAATTCTAGCATGGAGATACACCTACATTGC (F, JMO 680)</td>
</tr>
<tr>
<td></td>
<td>CGTGCACCTTTTTTGAAGAAGAGATGGGG (R, JMO 713)</td>
</tr>
<tr>
<td>Y52A</td>
<td>GAACCGGACAGAGCCATGCAATATTTGTAACCTTTGTTG (F, JMO 722)</td>
</tr>
<tr>
<td></td>
<td>GCAAAAGTGTTAATTTGCAATGGAAGCCTGTCGTCGTGTTG (R, JMO 702)</td>
</tr>
<tr>
<td>N53D</td>
<td>CCGGACAGAGCCATGCAATATTACCTTTTGTGAATGTGG (F, JMO 719)</td>
</tr>
<tr>
<td></td>
<td>CTTGCAACACACAGTTACAATATCTGTAATGGGCTCTGCTCGG (R, JMO 701)</td>
</tr>
<tr>
<td>V55T</td>
<td>GGACAGAGCCCATCATAACTTACCTTTTGTGAATGTGGAAC (F, JMO 720)</td>
</tr>
<tr>
<td></td>
<td>GTCAACACTTGCAACAAAAGGTGTAATATTGTGAATGGGCTCGTCC (R, JMO 700)</td>
</tr>
<tr>
<td>F57A</td>
<td>CCAATTACAAATTTGTAACCTTTTGTGAACGTGATCTACCTAAGG (F, JMO 717)</td>
</tr>
<tr>
<td></td>
<td>GAACGCTAGAGCTCAACACTTGCAACAAAAGGTGTAATATTGGAAC (R, JMO 698)</td>
</tr>
<tr>
<td>C59S</td>
<td>CCCATTTAAATTTTGAACCTTTTGTGAACGTGATCTACCTAAGG (F, JMO 717)</td>
</tr>
<tr>
<td></td>
<td>GAACGCTAGAGCTCAACACTTGCAACAAAAGGTGTAATATTGGAAC (R, JMO 698)</td>
</tr>
<tr>
<td>K60E</td>
<td>CCGAAGCGTACGATCATGCAACACTTGCAACAAAAGGTGTAATATTGGAAC (R, JMO 698)</td>
</tr>
<tr>
<td>D62K</td>
<td>GTAACACTTTTGTGAACCTTTTGTGAACGTGATCTACCTAAGG (F, JMO 717)</td>
</tr>
<tr>
<td></td>
<td>GAACGCTAGAGCTCAACACTTGCAACAAAAGGTGTAATATTGGAAC (R, JMO 698)</td>
</tr>
<tr>
<td>S63D</td>
<td>GCACACACCGTATCCCACAGCTCAGGAACAGGTGTAATATTGGAAC (R, JMO 687)</td>
</tr>
<tr>
<td>T64D</td>
<td>GCTTTGTCAGCAACAGCAAGACAGATGCAACACTTGCAACAAAAGGTGTAATATTGGAAC (R, JMO 695)</td>
</tr>
<tr>
<td>R66E</td>
<td>GCCTTTGACACACACAATCTCAAGGGTAAATCTGTAAGCTCACTTG (F, JMO 723)</td>
</tr>
<tr>
<td></td>
<td>GCCTTTGACACACACAATCTCAAGGGTAAATCTGTAAGCTCACTTG (R, JMO 686)</td>
</tr>
<tr>
<td>T72D</td>
<td>CCAAAAGCTAGATGTCTAGGTGCTTTTGTACGCACACAC (R, JMO 694)</td>
</tr>
<tr>
<td>H73E</td>
<td>CCAAAAGCTAGATGTCTAGGTGCTTTTGTACGCACACAC (R, JMO 685)</td>
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<td>V74T</td>
<td>GGTCTTTGCAAGTCACTTACGTTGACGTTTGAACGTGATCTACCTAAGG (R, JMO 693)</td>
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</tr>
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<td>E80K/D81K</td>
<td>CGTACTTTGAAAAAGCTGTTAATGGG (F, JMO 724)</td>
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<td></td>
<td>GCCATATTAAGCTTATAAAATGGG (R, JMO 714)</td>
</tr>
<tr>
<td>M84S</td>
<td>CGTGCTGACTTATGTTTTTGGAGAACAGATGGACACACACATGCGTAGCTAGGTACCTAACAGCT (R, JMO 690)</td>
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<tr>
<td>G85A</td>
<td>GCTTGACCTTTAGTTTTTGGAGAACAGATGGACACACACATGCGTAGCTAGGTACCTAACAGCT (R, JMO 689)</td>
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<tr>
<td>T86D</td>
<td>GCTTGACCTTTAGTTTTTGGAGAACAGATGGACACACACATGCGTAGCTAGGTACCTAACAGCT (R, JMO 688)</td>
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<tr>
<td>P92A</td>
<td>CGTGCTGACTTATGTTTTTGGAGAACAGATGGACACACACATGCGTAGCTAGGTACCTAACAGCT (R, JMO 684)</td>
</tr>
<tr>
<td>I93T</td>
<td>CGTGCTGACTTATGTTTTTGGAGAACAGATGGACACACACATGCGTAGCTAGGTACCTAACAGCT (R, JMO 683)</td>
</tr>
<tr>
<td>QKP96-98EEA</td>
<td>CGTGCTGACTTATGCTTTTGGAGAACAGATGGACACACACATGCGTAGCTAGGTACCTAACAGCT (R, JMO 682)</td>
</tr>
</tbody>
</table>

* JMO = Joe Mymryk Oligonucleotide

Mutants in bold were created by the megaprimer method. Otherwise, mutants were generated by overlap extension.
Table 2-2. List of hydrophobic core HPV16 E7 mutants and oligonucleotides utilized in PCR

<table>
<thead>
<tr>
<th>E7 construct</th>
<th>Oligos (5’ – 3’)</th>
<th>Forward (F, JMO #)</th>
<th>Reverse (R, JMO #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L65A</td>
<td>GTACGCACAAACCGAGCTGTAGAGTCACAC</td>
<td>(R, JMO 204)</td>
<td></td>
</tr>
<tr>
<td>V69A</td>
<td>GTCTACGTGTGTACTTTGTGCGCACAACCGAAGC</td>
<td>(R, JMO 205)</td>
<td></td>
</tr>
<tr>
<td>I76A</td>
<td>GGTCTTCCAAAGTACGAGCGTCTACGTGTGTCG</td>
<td>(R, JMO 206)</td>
<td></td>
</tr>
<tr>
<td>L79A</td>
<td>CCCATTAACAGGTCTTCCGCAGTACGAAATGTCTAC</td>
<td>(R, JMO 207)</td>
<td></td>
</tr>
<tr>
<td>LLMG82-85AAAD</td>
<td>CCTAGTGATCCGCTGCGCATCTTCCAAAGTAC</td>
<td>(R, JMO 208)*</td>
<td></td>
</tr>
<tr>
<td>L87A</td>
<td>GGGCACACAATTCCCGGCGGTTCATATAACAGGCTC</td>
<td>(R, JMO 209)</td>
<td></td>
</tr>
<tr>
<td>I89A/V90A</td>
<td>GAGAACAGATGGACATGCGCTCTAGTGTCGC</td>
<td>(R, JMO 210)</td>
<td></td>
</tr>
</tbody>
</table>

*Primer designed to produce LLM82-84AAA mutation

Mutants were created by the megaprimer method.
domain. For recombinant protein production and purification, the CR3 region of each E7 construct was cloned into the pGEX4T-1 (Invitrogen) vector, modified to contain the tobacco etch virus (TEV) protease recognition sequence between a glutathione S-transferase (GST) tag and the protein of interest. For transformation assays, E7 mutations were subcloned into the BamHI and XhoI restriction sites of a modified pCMV-Neo-Bam mammalian expression vector. Constructs for the del21-24, L67R, C58G/C91G, and C91G mutations were obtained from K. Munger (Harvard Medical School, Boston, MA), K. H. Vousden (National Cancer Institute at Frederick, Frederick, MD), and D. Galloway (Fred Hutchinson Cancer Research Center, University of Washington) and subcloned as necessary. The pCMV-pRb construct was obtained from F. Dick (University of Western Ontario, London, Ontario, Canada) and subcloned into the pBAIT vector for yeast two-hybrid experiments.

2.2.2 Cell culture and transfection
Saos2, HEK293, and U2OS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/ml). For pRb degradation assays, Saos2 cells were seeded into 6-well plates at 3 × 10^5 cells per well and transfected 24 h later with 2 μg of pRb, with or without 0.2 μg of E7 expression plasmid and with 0.1 μg of green fluorescent protein (GFP) expression plasmid as a control, using FugeneHD (Roche) according to the instructions provided by the manufacturer. The amount of plasmid DNA was balanced with empty pCMV vector where necessary. The amounts of pRb, GFP, and actin were assessed by western blotting at 48 h post-transfection. For cycloheximide treatment experiments, HEK293 cells were seeded into 6-well plates at a density of 3.5 × 10^5 cells per well 24 h before transfection. For each mutant, the cells were transfected with 2 μg of E7 pCMV-Neo-Bam expression plasmid along with 0.1 μg of GFP expression plasmid, using the calcium phosphate method (33). At 24 h post-transfection, the cells were treated with 0.5 μg/μl cycloheximide for 0, 15, 30, 60, and 120 min. For immunofluorescence experiments, U2OS cells were seeded on coverslips in 6-well dishes at 1.2 × 10^5 cells per
well. The cells were transfected with 2 µg of E7 expression plasmid using calcium phosphate method (33).

2.2.3 Yeast two-hybrid assay

Yeast two-hybrid analysis was performed in strain L-40 [MATa his3A200 trip1-90 leu2-3,112 ade2 lys2-801am LYS2:(lexAop) 4-HIS3 URA3:(lexAop)8-lacZ Gal4] (provided by D. Mangroo, University of Guelph). Standard yeast culture medium was prepared using previously described methods (1). Yeast transformation was carried out using a modified lithium acetate procedure (20). Transformed yeast cells were plated on selective medium plates and grown at 30°C for 2 to 4 days.

The method for assaying β-galactosidase activity in yeast has been described previously (1). Briefly, transformed yeast colonies were grown in selection medium overnight at 30°C, and a 1.5-ml aliquot of culture was used for the assay. The cells were pelleted and resuspended in 1 ml of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol, pH 7.0), and the optical density at 600 nm (OD600) was determined. To 300 µl of resuspended culture, the following was added: 700 µl Z buffer, 40 µl of 0.1% SDS, and 20 µl of chloroform. The samples were mixed and incubated at 30°C for 15 min. Two hundred microliters of the substrate ortho-nitrophenyl-β-d-galactopyranoside (ONPG; Bioshop) (4 mg/ml) was added to each sample and mixed. The reaction mixtures were incubated at 30°C and, when necessary, terminated by addition of 400 µl of 1 M Na2CO3. The samples were centrifuged for 10 min to remove cellular debris before measuring the absorbance at 420 nm. The β-galactosidase activity was calculated in Miller units by utilizing the following formula: \( \frac{\text{OD}_{420}}{\text{OD}_{600} \times 0.3 \text{ ml [culture volume] \times reaction time [min]}} \). For the reported dimerization values, the numbers were corrected for any observed autoactivation for each individual mutant. For assessing binding between pRb and E7, pRb was fused to the LexA DNA binding domain (bait), and the described E7 constructs were used as prey.
2.2.4 Dimerization *in vitro* and GST pull-down assays

Recombinant GST-tagged wild-type E7 CR3 and mutants were expressed in and purified from the BL21 strain of *Escherichia coli* per protocols provided by the affinity resin manufacturer (Amersham). To obtain untagged E7 CR3, purified recombinant protein was incubated with \( \sim 0.2 \mu g/ml \) TEV protease overnight at 4°C. The purified protein concentration was determined using the Bio-Rad assay and was verified by running 5 \( \mu g \) of purified protein in a gel and staining it with Coomassie blue (see FIG. 2-3B) prior to using the samples in the assay. The ability of E7 CR3 to dimerize was determined by analyzing the capacity of E7 CR3 to interact with GST-E7 CR3. GST pull-down assays were set up at protein concentrations well below the reported dimerization dissociation constant \( (K_d) \) of 1.1 \( \mu M \) (14). Briefly, 600 nM E7 CR3 was incubated at room temperature for 1 h in a total volume of 500 \( \mu l \) of buffer (20 mM phosphate, pH 7.0, 200 mM NaCl, 1% Tween 20, 3 \( \mu M \) \( N \)-ethylmaleimide); 300 nM GST-E7 CR3 was then added and incubated for an additional 10 min, following by the addition of 20 \( \mu l \) of 50% glutathione Sepharose. GST pull-down assays were carried out for 1 h at 4°C, and the samples were then washed three times with wash buffer (20 mM phosphate, pH 7.0, 200 mM NaCl, 1% Tween 20) and examined by western blotting for untagged E7 CR3.

2.2.5 Antibodies

The following antibodies were used: rabbit anti-Lex A (Upstate) at a 1:500 dilution, rat anti-hemagglutinin (anti-HA) (Roche) at 1:2,000, and rabbit anti-glucose-6-phosphate dehydrogenase (anti-G6PD), used as a loading control for yeast samples, at 1:80,000 (Sigma). For detection of E7 CR3 in *in vitro* dimerization assays, the membranes were blotted with CR3-specific donkey antibody (C20; Santa Cruz) at 1:200. For detection of pRb in the degradation assay, mouse anti-pRb (G3-245; BD Pharmingen) was used at 1:500. GFP was detected using mouse anti-GFP (Living Colors; Clontech) at 1:2,000, and E7 was detected with mouse anti-E7 (8C9; Invitrogen) at 1:200. Horseradish peroxidase (HRP)-conjugated donkey anti-goat secondary antibody was used at 1:10,000 (Santa Cruz). HRP-conjugated goat anti-rabbit (Jackson Laboratories), goat anti-rat
(Pierce), and rabbit anti-mouse (Jackson Laboratories) secondary antibodies were used at 1:100,000, 1:20,000, and 1:100,000, respectively.

2.2.6 Western blotting

To determine expression levels of each E7 mutant in yeast cells, yeast colonies were isolated from selection plates and inoculated into 5 ml of selective liquid medium. Cultures were grown at 30°C overnight; the cell pellet was resuspended in 250 μl of lysis buffer (25 mM Tris, pH 7.5, 125 mM NaCl, 2.5 mM EDTA, 1% Triton X-100) and an equal volume of acid-washed glass beads (Sigma). Samples were mixed vigorously for five cycles of 3 min of vortexing and 3 min on ice. Cellular debris was removed by centrifugation, and 15 μg of protein extract was resolved in NuPAGE Novex Bis-Tris 4 to 12% polyacrylamide gels (Invitrogen). The resolved protein samples were transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham); the samples containing LexA DNA fusions were blotted with rabbit anti-LexA, and the B42 fusions were blotted with rat anti-HA. Mammalian cells were lysed in either NP-40 lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 0.1% NP-40) or directly in 2× SDS sample buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol [DTT], 4% SDS, 20% glycerol, 0.2% bromophenol blue), and the cell extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) or in NuPAGE Novex Bis-Tris 4 to 12% polyacrylamide gels and blotted onto 0.45-μm-pore-size nitrocellulose membranes (Schleicher and Schuell) or PVDF membranes. Typically, the membranes were blocked at room temperature for 1 h in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T). The blots were incubated with the appropriate primary antibodies diluted in TBS-T. The incubation time was 1 h at room temperature or overnight at 4°C. After extensive washing, the blots were developed with enhanced chemiluminescence (ECL) or ECL Plus reagent (GE Healthcare) according to the manufacturer's instructions. Protein band intensities were quantitated where possible, using the ImageJ quantification program.
2.2.7 E7 transformation assay

The ability of wild-type and mutated E7 constructs to transform baby rat kidney (BRK) cells in co-operation with activated EJ-ras was assessed in a colony-forming assay, as described previously (33). Briefly, 9-day old BRK cells were transfected with EJ-ras alone or together with wild-type or indicated E7 mutant. The cells were placed under selection for 2-3 weeks, and the formation of colonies was observed. Due to the large number of mutants created in this study, transformation experiments were carried out in small groups, together with respective wild-type controls. Each mutant was tested independently at least three times and sometimes up to five times.

2.2.8 Immunofluorescence and microscopy

Cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) and were permeabilized with 0.1% Triton X-100 in PBS. Primary antibody (anti-E7; 8C9) was incubated for 2 to 3 h at 37°C, washed extensively in PBS, and incubated for 1 h at 37°C with a secondary anti-mouse antibody conjugated to rhodamine (Molecular Probes). Samples were washed several times with PBS and water and were mounted with Vectashield mounting medium (Vector Laboratories) on glass slides. Slides were analyzed with a Leica DMLB fluorescence microscope with a Leica photo camera. The data were collected with a ×40 objective lens.

2.3 Results

2.3.1 HPV16 E7 CR3 model and identification of surface-exposed residues

The dimeric CR3 structures for HPV45 (Protein Data Bank [PDB] accession no. 2F8B) and HPV1A (PDB accession no. 2B9D) (32, 37) have been determined, and they are very similar despite representing a high-risk and a low-risk HPV type, respectively. Indeed, these structures are superimposable to a backbone root mean square (RMS) deviation of
FIG. 2-1. HPV16 E7 CR3 surface-exposed mutants. (A) Multiple sequence alignment of CR3 regions from representative supergroup A HPVs. Perfectly conserved residues are highlighted in black, with less conserved residues shaded in progressively lighter shades of gray, corresponding to the degree of conservation. The numbering scheme corresponds to HPV16 E7 CR3, starting with residue 49. Residues that are involved in zinc coordination are indicated with asterisks at the bottom of the alignment. Highly conserved hydrophobic core residues which are predicted to stabilize the dimer are labeled with ovals. The panel of mutations created for the purposes of the described studies is labeled at the top and bottom of the alignment. (B and C) Ribbon diagrams of the HPV16 model showing the positions of substitutions introduced into the protein. The model was produced using the PDB co-ordinates of HPV45 (2F8B) and a sequence alignment between the two proteins. The figures show the dimeric HPV16 model (A) and a view across a portion of the dimerization interface (B). The surface-exposed side chains where substitutions were made are shown as sticks (pink), and the bound zinc atoms are indicated as spheres (cyan). The two protomers are shaded differently, and substitutions are shown on only one protomer for clarity.
1.61 Å. The CR3 structure for HPV16 E7 has not been determined. Utilizing the existing structures of HPV45 and HPV1A E7 CR3 (32, 37), we generated a structural model of CR3 for the HPV16 E7 protein (FIG. 2-1B and C). The HPV45 structure served as a template for construction of our HPV16 model, based on the observation that both HPV16 and HPV45 belong to the supergroup A of HPVs and are commonly associated with human cancers. Additionally, the sequences of HPV16 and HPV45 share $\sim 43\%$ identity and $73\%$ similarity, whereas HPV16 and HPV1A share $\sim 37\%$ identity and $\sim 67\%$ similarity. A homology method was chosen to generate the model, as it can produce high-quality structural predictions when the target (HPV16) and the template (HPV45) are closely related (5).

The CR3 dimer of HPV45 was analyzed by the web server program VADAR (http://redpoll.pharmacy.ualberta.ca/vadar/), which compiles more than 15 different algorithms for analyzing and assessing peptide and protein structures from atomic coordinate data (47). The HPV45 E7 dimer was analyzed to determine the accessible surface area of the protein and the chi 1 angles. Appropriate residues in HPV45 were then swapped to match those present in HPV16. Often, the quality of a homology model is complicated by the presence of alignment gaps, indicating that a structural region may be present in the target but not in the template, or vice versa. This was not an issue in this case, as the lack of gaps in the alignment of CR3s from the HPV45 and HPV16 E7 proteins made direct substitution of residues possible. The structure was refined by manually adjusting the chi 1 angles in the HPV16 model to superimpose with those in HPV45, followed by 500 steps of energy minimization to relieve poor van der Waals interactions. Atomic overlaps and unnatural strains in the structure were removed, and strong hydrogen bonds were reinforced, while weak ones were broken. As expected from the high level of sequence similarity, the predicted HPV16 E7 CR3 structure superimposes with that of HPV45 E7, with an RMS deviation of 0.34 Å.

The CR3 region of E7 has a specific folded tertiary structure, and therefore various surfaces of the folded conformation of this protein region are available to mediate specific interactions with target cellular proteins. To identify likely residues that may participate in binding of cellular targets, we first identified all amino acid residues whose
side chains were at least 25% surface-exposed. This analysis resulted in identification of 24 residues within CR3 of HPV16 E7, and from these, we chose to construct 19 point mutations, one double mutation (E80K/D81K), and one mutation targeting the last three amino acids (QKP96-98EEA) of the protein (FIG. 2-1). The overall aim of the substitution scheme was to target residues in a manner that would help retain the folded state of the CR3 protomer and dimer and preserve solvent interactions. Given the structure of CR3, mutating polar or charged amino acids by the conventional strategy of alanine-scanning mutagenesis would likely disrupt interaction of these residues with solvent and could lead to precipitation or aggregation. Consequently, we chose to devise an alternative mutational strategy; in general, basic or acidic amino acids were replaced with residues of opposite charge, with the polarity and size of the side chain group being maintained. In the case of proline, glycine, phenylalanine, and tyrosine, there was no clear substitution choice, and these amino acids were replaced with alanine.

In addition to targeting surface-exposed residues, our analysis also included substitutions in the most highly conserved residues within CR3, some of which have been characterized extensively in the literature (11, 12, 30). Based on HPV45 E7 structural studies, the side chains of I73, L75, V77, L84, L87, L90, F91, L95, F97, and V98 contribute to formation of a small hydrophobic core in each protomer (37). The dimer interface is stabilized by contacts of residues V77, E78, T86, L87, L90, L95, and F97; hence, the dimer interface is stabilized by some of the hydrophobic core residues, including V77, L87, L90, L95, and F97. The corresponding hydrophobic core residues in HPV16 CR3 include L65, L67, V69, I76, L79, L82, L83, L87, I89, and V90. As in HPV45, these were also predicted from our model to form the hydrophobic core, and some may contribute to stabilization of the dimer. Alanine mutations targeting these highly conserved hydrophobic residues were therefore perfect candidates to establish whether dimerization of E7 is necessary for function.
2.3.2 Dimerization properties of E7 CR3 mutants in yeast two-hybrid analyses

It is possible that despite our mutational strategy, some of the mutations in E7 CR3 may affect the ability of E7 to properly fold and/or dimerize. Therefore, the entire panel of surface-exposed mutants as well as the E7 mutants that targeted the hydrophobic core residues were tested for the ability to dimerize. The yeast two-hybrid test has been utilized previously to demonstrate that E7 can form dimers \textit{in vivo} (13, 51). HPV16 E7 has been reported to possess an intrinsic ability to activate transcription (51) and interacts with transcriptional regulators such as TATA-binding protein (TBP) (41), which could confound this analysis. However, fusion of wild-type E7 with the LexA DNA binding domain activated transcription of a LexA-responsive reporter gene very poorly in the absence of an interacting prey (FIG. 2-2A). Similarly, expression of wild-type E7 fused to the B42 activation domain as prey without an interacting bait had no effect on reporter gene activity. This was also confirmed for each of the mutants in CR3 of E7 (data not shown). Coexpression of wild-type E7 as both bait and prey resulted in a dramatically higher reporter gene activity, indicating dimerization (FIG. 2-2A). Based on these results, intrinsic transcriptional activation activity by E7 does not contribute significantly to the ability of the E7 homodimer to initiate transcription in the yeast two-hybrid assay. Each mutant was tested for dimerization when coexpressed as a LexA or B42 fusion (FIG. 2-2B and C). Dimerization of each mutant was calculated relative to wild-type E7 dimerization, with values corrected for the intrinsic transactivation activity of each mutant E7 protein. Mutants with transcriptional activity of <45% of wild-type E7 activity were considered impaired for dimerization. From these data, it is apparent that the majority of mutants with mutations predicted by our model as likely to influence dimerization were unable to dimerize (FIG. 2-2C). In particular, all but one E7 mutation targeting the hydrophobic core residues and zinc co-ordinating cysteines resulted in <45% activity in the yeast two-hybrid assay relative to the wild-type protein (FIG. 2-2C). This analysis also identified mutations in surface-exposed residues that appeared to have decreased dimerization potential; these included the V55T, F57A, R66E, R77E, M84S, T86D, and possibly G85A mutations (FIG. 2-2B).
**FIG 2-2. E7 dimerization in vivo.** (A) Controls for the yeast two-hybrid assay. L-40 yeast cells were cotransformed with a LexA DNA binding domain (bait) and a B42 activation domain (prey). The combination of vectors (bait/prey) used is indicated below each data bar, with “vector” representing an empty vector and “WT” indicating a vector expressing a wild-type HPV16 E7 fusion. Indicated constructs were expressed in L-40 yeast cells containing an integrated LexA-responsive β-galactosidase reporter. Cell extracts were prepared and assayed for β-galactosidase activity. (B) Dimerization of mutants with mutations targeting predicted surface-exposed residues as determined by the yeast two-hybrid assay. HPV16 E7 mutants were expressed as fusions to the LexA DNA binding domain and the B42 activation domain in L-40 yeast cells, and β-galactosidase activity was assessed. The upper horizontal line is set at 45% of wild-type activity, corresponding to a predicted 5-fold increase in $K_d$; the lower horizontal line corresponds to a predicted 10-fold increase in $K_d$ and is set at 28% of wild-type activity. We considered any mutation with <45% of wild-type activity to be impaired for dimerization. The same criterion also applies to panel c. (C) Dimerization of mutants with mutations targeting predicted hydrophobic core residues and zinc co-ordinating cysteines as assessed by the yeast two-hybrid assay. (D) Expression levels of LexA and B42 fusion proteins of dimerization-impaired E7 mutants. Yeast extracts cotransformed with either wild-type E7 or the E7 mutant as both LexA (bait) and B42 (prey) fusions were tested for protein expression by western blotting using anti-LexA and anti-HA antibodies. An anti-G6PD blot served as a loading control.
Alterations in the metal-binding cysteines have repeatedly been reported to interfere with E7 protein stability, and misfolded proteins are generally degraded quickly in the cell (13, 39, 46). To determine whether the loss of transcriptional activity in the yeast two-hybrid assay was truly due to a defect in the ability to dimerize or simply caused by a lack of sufficient protein expression, western blots were conducted on yeast extracts for mutants putatively impaired in dimerization (FIG. 2-2D). Most of these mutants were expressed at levels comparable to the wild-type level, although slightly decreased expression was observed for some of the bait or prey fusions (e.g., for the V55T, I76A, G85A, R77E, I89A/V90A, and C91G mutants). Decreased expression of bait fusions with the C58G/C91G and L67R mutants was observed consistently, whereas the prey counterparts were expressed as well as the wild-type protein. The underlying reasons for this are unclear, although significant differences in the sizes of the bait and prey fusions were most likely a contributing factor, in conjunction with the structural differences between the DNA binding or activation domain and the particular mutation.

2.3.3 Dimerization of E7 mutants in vitro

As an alternative method to assess dimerization, we tested the ability of E7 CR3 to dimerize in vitro by using the purified recombinant protein. We focused on the surface-exposed E7 mutants that appeared to be impaired for dimerization by the yeast two-hybrid assays. We also included the G85A mutant in this analysis, as this mutant was shown to have 54% ± 13% of wild-type activity in the yeast two-hybrid assay and could fall into the category of proteins impaired for dimerization. We utilized a simple GST pull-down assay where one E7 protomer was expressed and purified as a GST-tagged fusion and the other was protease cleaved to release CR3. The ability of the two proteins to interact with each other was monitored by examining the ability of resin-immobilized GST-CR3 to pull-down soluble recombinant CR3 as determined via western blotting (FIG. 2-3A). As expected, a mutant in which the zinc co-ordinating cysteine residues (C58G/C91G) were targeted failed to dimerize in vitro. Of the seven surface-exposed E7 mutants with decreased dimerization based on the yeast two-hybrid results, six also
FIG. 2-3. Dimerization of E7 CR3 in vitro. (A) Predicted surface-exposed mutants identified in the yeast two-hybrid assay as impaired for dimerization (>55% decrease in activity) were tested for the ability to form dimers in vitro. Both E7 CR3 fused to GST and that cleaved with TEV protease to obtain E7 CR3 alone were purified. A 300 nM concentration of GST-tagged wild-type or mutant E7 CR3 was mixed with 600 nM E7 CR3. The ability of the wild-type or mutant to dimerize was assessed by determining the amount of recovered E7 CR3 in a GST pull-down assay. The proteins were resolved by SDS-PAGE, and the amount of E7 CR3 was determined by western blotting. The bar graph shows band intensities for the representative western blot, as determined by densitometry. (B) Coomassie blue-stained gels of purified GST-E7 CR3 (top) and TEV-cleaved E7 CR3 (bottom), used as a loading control for the in vitro dimerization assay.
showed decreased binding in vitro (V55T, F57A, R77E, M84S, G85A, and T86D mutants). One mutant, the R66E mutant, seemed to be slightly better at binding than the wild-type protein in this system.

2.3.4 Transformation potential of E7 mutants

HPV16 E7 can transform rodent cells in co-operation with activated ras or extend the life span of primary human cells in co-operation with the viral E6 oncogene (24, 40). These activities require CR1 and CR2. Although not as well characterized, CR3 also plays a role, as certain mutations in CR3 can lead to defective immortalization/transformation activities (25, 30, 34, 39). We tested this panel of E7 CR3 mutants for the ability to transform primary BRK cells in co-operation with activated ras. Most mutations targeting surface-exposed residues had no effect on transformation. However, the E7 M84S and QKP96-98EEA mutants had consistently decreased transformation potentials (Table 2-3). Interestingly, a number of mutants of E7 demonstrated increased transformation potential, including the V55T, T64D, R66E, and P92A mutants. For the mutants we originally predicted to be part of the hydrophobic core, we identified two different phenotypes. The L67R, V69A, and L79A mutants showed decreased transformation, whereas the L65A, I76A, LLMG82-85AAAD, L87A, and I89A/V90A mutants all behaved like the wild-type (Table 2-4). Both mutations in E7 targeting cysteines (C58G/C91G and C91G) that co-ordinate zinc gave a decreased transformation potential. This is in agreement with previous studies (30, 34) and can be expected based on the important structural roles of these residues.

To determine if the two novel mutants identified in the transformation assay as having a decreased ability to transform cells (M84S and QKP96-98EEA mutants) were still able to target pRb, we tested their potential to degrade and bind the retinoblastoma protein (FIG. 2-4A and B). Utilizing the pRb null cell line Saos2, we demonstrated that both M84S and QKP96-98EEA mutants were able to efficiently target pRb for degradation (FIG. 2-4A). Additionally, employing a yeast two-hybrid approach where pRb was expressed as bait and the E7 wild-type or mutant was expressed as prey, we showed that both the M84S
Table 2-3. Ability of E7 mutants with mutations in predicted surface-exposed residues to transform primary BRK cells in co-operation with activated ras, with a summary of their dimerization potential

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mean (±SD) no. of colonies</th>
<th>Transformation phenotype</th>
<th>Mean dimerization level (±SD) in vivo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dimerization level in vitro&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutant</td>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y52A</td>
<td>9.33 ± 3.85</td>
<td>12.00 ± 3.00</td>
<td>Transforms</td>
<td>2.05 ± 0.06</td>
</tr>
<tr>
<td>N53D</td>
<td>22.60 ± 17.20</td>
<td>26.33 ± 10.33</td>
<td>Transforms</td>
<td>1.11 ± 0.21</td>
</tr>
<tr>
<td>V55T</td>
<td>20.00 ± 9.16</td>
<td>13.00 ± 3.46</td>
<td>Increased ability</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>F57A</td>
<td>13.33 ± 8.08</td>
<td>14.00 ± 1.73</td>
<td>Transforms</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>C59S</td>
<td>14.20 ± 6.68</td>
<td>12.00 ± 2.50</td>
<td>Transforms</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>K60E</td>
<td>19.75 ± 9.32</td>
<td>16.25 ± 3.40</td>
<td>Transforms</td>
<td>0.62 ± 0.01</td>
</tr>
<tr>
<td>D62K</td>
<td>19.00 ± 9.84</td>
<td>13.20 ± 3.83</td>
<td>Transforms</td>
<td>2.70 ± 0.03</td>
</tr>
<tr>
<td>S63D</td>
<td>15.25 ± 10.50</td>
<td>15.00 ± 4.69</td>
<td>Transforms</td>
<td>1.88 ± 0.74</td>
</tr>
<tr>
<td>T64D</td>
<td>20.60 ± 8.32</td>
<td>12.00 ± 3.00</td>
<td>Increased ability</td>
<td>1.28 ± 0.24</td>
</tr>
<tr>
<td>R66E</td>
<td>22.33 ± 6.80</td>
<td>12.00 ± 3.00</td>
<td>Increased ability</td>
<td>0.43 ± 0.05</td>
</tr>
<tr>
<td>T72D</td>
<td>9.66 ± 3.51</td>
<td>11.66 ± 3.51</td>
<td>Transforms</td>
<td>1.05 ± 0.07</td>
</tr>
<tr>
<td>H73E</td>
<td>14.50 ± 7.77</td>
<td>15.00 ± 0.00</td>
<td>Transforms</td>
<td>1.07 ± 0.02</td>
</tr>
<tr>
<td>V74T</td>
<td>11.00 ± 1.00</td>
<td>12.00 ± 3.00</td>
<td>Transforms</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td>R77E</td>
<td>15.00 ± 4.24</td>
<td>13.50 ± 2.12</td>
<td>Transforms</td>
<td>0.08 ± 0.00</td>
</tr>
<tr>
<td>E80K/D81K</td>
<td>11.33 ± 4.93</td>
<td>11.00 ± 3.46</td>
<td>Transforms</td>
<td>0.68 ± 0.15</td>
</tr>
<tr>
<td>M84S</td>
<td>7.66 ± 5.50</td>
<td>12.00 ± 3.00</td>
<td>Decreased ability</td>
<td>0.42 ± 0.08</td>
</tr>
<tr>
<td>G85A</td>
<td>6.00 ± 4.08</td>
<td>10.25 ± 4.27</td>
<td>Transforms</td>
<td>0.54 ± 0.13</td>
</tr>
<tr>
<td>T86D</td>
<td>10.00 ± 4.24</td>
<td>13.62 ± 4.46</td>
<td>Transforms</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>P92A</td>
<td>17.00 ± 6.08</td>
<td>11.00 ± 3.46</td>
<td>Increased ability</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td>I93T</td>
<td>11.50 ± 5.00</td>
<td>12.75 ± 2.87</td>
<td>Transforms</td>
<td>1.37 ± 0.10</td>
</tr>
<tr>
<td>QKP96-98EEA</td>
<td>5.25 ± 2.98</td>
<td>14.50 ± 4.12</td>
<td>Decreased ability</td>
<td>1.70 ± 0.05</td>
</tr>
<tr>
<td>del21-24</td>
<td>2.66 ± 2.31</td>
<td>13.00 ± 3.46</td>
<td>Does not transform</td>
<td>ND</td>
</tr>
<tr>
<td>C58G/C91G</td>
<td>4.46 ± 1.52</td>
<td>12.00 ± 3.00</td>
<td>Decreased ability</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>ras only</td>
<td>1.50 ± 1.73</td>
<td>12.00 ± 3.00</td>
<td>Does not transform</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, not determined;  
<sup>b</sup> −, little or no dimerization; +, intermediate dimerization; +++, similar to wild-type.
Table 2-4. Transformation properties of E7 mutants with mutations in predicted hydrophobic core residues and in the zinc co-ordinating cysteines, with a summary of dimerization potential *in vivo*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mean (±SD) no. of colonies</th>
<th>Transformation phenotype</th>
<th>Mean dimerization level (±SD) <em>in vivo</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutant</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>C58G/C91G</td>
<td>4.46 ± 1.52</td>
<td>12.00 ± 3.00</td>
<td>Decreased ability</td>
</tr>
<tr>
<td>L65A</td>
<td>12.00 ± 9.93</td>
<td>12.75 ± 2.87</td>
<td>Transforms</td>
</tr>
<tr>
<td>L67R</td>
<td>8.00 ± 3.36</td>
<td>16.25 ± 3.40</td>
<td>Decreased ability</td>
</tr>
<tr>
<td>V69A</td>
<td>7.33 ± 3.51</td>
<td>14.16 ± 5.29</td>
<td>Decreased ability</td>
</tr>
<tr>
<td>I76A</td>
<td>13.00 ± 6.97</td>
<td>12.00 ± 3.46</td>
<td>Transforms</td>
</tr>
<tr>
<td>L79A</td>
<td>4.00 ± 3.60</td>
<td>12.00 ± 3.00</td>
<td>Decreased ability</td>
</tr>
<tr>
<td>LLMG82-85AAAD</td>
<td>7.50 ± 7.45</td>
<td>11.25 ± 2.87</td>
<td>Transforms</td>
</tr>
<tr>
<td>L87A</td>
<td>11.00 ± 7.87</td>
<td>10.25 ± 4.27</td>
<td>Transforms</td>
</tr>
<tr>
<td>I89A/V90A</td>
<td>10.50 ± 6.19</td>
<td>10.25 ± 4.27</td>
<td>Transforms</td>
</tr>
<tr>
<td>C91G</td>
<td>5.25 ± 4.27</td>
<td>13.75 ± 4.27</td>
<td>Decreased ability</td>
</tr>
</tbody>
</table>
and QKP96-98EEA mutants were able to bind pRb comparably to the wild-type. Furthermore, these two mutants did not show any changes in subcellular localization (FIG. 2-4D) as determined by immunofluorescence. To further investigate whether the introduced mutations could cause instability in the protein, HEK293 cells were transfected with wild-type E7, L67R or QKP96-98EEA mutant expression plasmids. The cells were treated with cycloheximide for 0 to 120 min at 24 h post-transfection, and the amount of E7 was assessed by western blotting. The L67R mutant, which targets the hydrophobic core and fails to dimerize, and perhaps causes structural defects in the protein, showed a decreased half-life compared to the wild-type (FIG. 2-4C). In contrast, the QKP96-98EEA mutant appeared to be more stable than wild-type E7. Hence, instability of the QKP96-98EEA mutant was unlikely to contribute to the decreased transformation potential that we observed.

2.4 Discussion

Previous studies on the E7 proteins of HPV16 and HPV18 have indicated that the C-terminus is required to maintain a stable and functional structure of the protein (9, 13, 34, 51). CR3 contains two Cys-X-X-Cys motifs necessary for zinc binding and subsequent folding of the protomer into a unique viral $\beta_1\beta_2\alpha_1\beta_3\alpha_2$ topology. Although the Zn-binding Cys-X-X-Cys motifs do not directly mediate the interprotomer contacts, folding of the individual protomers is necessary to create the dimer interface surface. Specifically, dimerization is mediated by interactions between hydrophobic residues of the $\alpha_1$ helices of the protomers and the formation of an intermolecular two-stranded antiparallel $\beta$-sheet between the $\beta_2$ strand of one protomer and the $\beta_3$ strand of the other. This forms a continuous hydrophobic core stabilizing the dimeric form of the E7 protein. Point mutations in either of the metal-binding motifs have consistently been shown to significantly reduce the ability of E7 to transform a variety of rodent cells (34, 46) and to immortalize primary human keratinocytes (30). It has not been established definitively if these defects are caused by perturbations in protomer structure that affect binding to
FIG. 2-4. Further characterization of a select panel of mutants. (A) E7 mutants with decreased transformation potential. The M84S and QKP96-98EEA mutants, as well as the CR2 del21-24 deletion mutant, which targets the LXCXE motif, were assessed for the ability to target pRb for degradation. Saos2 cells were transfected with a pRb expression plasmid and vectors expressing the indicated E7 mutant; pRb levels were determined by western blotting at 48 h post-transfection. (B) The ability of E7 mutants to bind pRb was also assessed in a yeast two-hybrid assay. pRb was expressed as bait, with the indicated E7 mutant as prey. β-Galactosidase activity was measured and reported relative to that of wild-type E7. (C) The L67R mutant, with a mutation in the hydrophobic core of E7 which leads to decreased dimerization, and the QKP96-98EEA mutant, which dimerizes but fails to transform cells, were transfected into HEK293 cells together with a GFP expression plasmid. At 24 h post-transfection, cells were treated with cycloheximide for the indicated times and the levels of E7 determined by western blotting. (D) Cellular localization of M84S and QKP96-98EEA mutants was assessed by immunofluorescence in U2OS cells.
specific factors or arise through a loss of dimerization. Indeed, it was not yet known if E7 must exist as a dimer for its transforming activity. We therefore sought to resolve this issue. Our approach was to utilize the available structural information on E7 proteins (32, 37) and to model the structure of HPV16 E7 CR3. This would allow the identification of residues contributing to the formation of the hydrophobic core, and therefore to dimer stabilization, and also those surface residues which are solvent exposed and available for interaction with E7 target proteins (FIG. 2-1). This analysis resulted in construction of 21 novel mutations in the putative surface-exposed residues and 8 mutations targeting the highly conserved residues that likely form the hydrophobic core. Some of the latter residues have previously been targeted for mutagenesis (12, 30). As negative controls, we also mutated the zinc co-ordinating Cys-X-X-Cys motifs. In total, we created 28 novel mutants and studied their properties in conjunction with several other previously described mutants, such as the L67R mutant. This new panel of E7 CR3 mutants has the potential for many other future applications in this field of study beyond the dimerization properties we assessed.

Early work that addressed the potential of HPV16 E7 to dimerize in vivo utilized the yeast two-hybrid system (13, 51). Employing our extensive panel of mutants and the yeast two-hybrid assay, we tested our bait and prey samples in five combinations: empty vector-mutant E7, mutant E7-empty vector (to determine any possible autoactivation by E7), wild-type E7–mutant E7, mutant E7–wild-type E7, and mutant E7–mutant E7. Although relatively insignificant, any observed autoactivation of the reporter by E7 was subtracted from the reported values (FIG. 2-2). Yeast two-hybrid data obtained when the mutant E7–mutant E7 combination was tested (FIG. 2-2) were considered the most biologically relevant.

E7 dimerizes with a reported $K_d$ of 1.1 $\mu$M (14). Based on an expression level equivalent to the $K_d$, a mutation leading to a 5-fold increase in $K_d$ would result in an $\sim$45% change in activity in our system with respect to wild-type E7; an increase in $K_d$ of 10-fold would result in $\sim$28% of wild-type activity. Using a 5-fold increase in $K_d$ as a guide, we considered any E7 CR3 mutant with $<45\%$ of the wild-type E7 activity to be impaired for dimerization. Using this criterion, all but one of the mutants with mutations predicted to
be in the hydrophobic core of the protomer were impaired in the ability to dimerize (FIG. 2-2C).

Based on the criterion of <45% of wild-type E7 activity, we identified six mutants with mutations in putatively surface-exposed residues with a reduced ability to dimerize, with the possibility that the G85A mutant also falls into this category (FIG. 2-2B). The apparent inability of these mutant E7 proteins to dimerize could also result from decreased expression, and this could have been the case for several of these proteins (e.g., the V55T, G85A, and R77E mutants). Interestingly, two of the mutants with reduced dimerization had mutations affecting residues G85 and T86, which map close to the junction between the two protomers. These residues may be involved in mediating intersubunit contacts, suggesting that despite their being solvent exposed, a mutation at any of these residues may interfere with stable dimer formation. Additionally, the relatively large positively charged side chain of arginine at position 77 was targeted for mutagenesis in a previous dimerization study and was replaced with a single hydrogen in glycine (13). In that report, the R77G mutant was able to interact strongly with wild-type E7, and it also retained transforming and transcriptional activities in a previous mutagenic analysis conducted in mammalian cells (39). In this study, R77 was replaced with the oppositely charged amino acid glutamate, which should retain solvent interaction. This more dramatic alteration greatly reduced dimerization in vivo. However, this may have been related to decreased expression, as it exhibited only a slight reduction in vitro (FIG. 2-3). However, this residue is highly conserved across all HPV E7 proteins examined, suggesting that it could play an important nonstructural role (FIG. 2-1).

As for the remaining surface-exposed mutations in mutants impaired for dimerization (V55T, F57A, R66E, and M84S mutants) (FIG. 2-2D), these residues are not predicted to lie close to the dimer interface, and a role in stabilizing the dimer is not a likely reason for the lack of interaction between the two protomers. These mutations in E7 may therefore cause other structural defects in the protein. When tested for dimerization in vitro using recombinant protein, all mutants except the R66E mutant had reduced binding (FIG. 2-3). This provides additional evidence that these mutations in E7 perturb folding of the protomer, which leads to a reduction in the ability to dimerize. It is not known why
the *in vitro* and *in vivo* assays differed with respect to the R66E mutant. Further biochemical analysis of this mutant is necessary to establish if the introduced change in the protein leads to structural alterations and perhaps aggregation or higher-order oligomerization as opposed to dimerization.

We assessed the ability of wild-type E7 or our novel collection of mutants to transform primary BRK cells in co-operation with *ras*. Mutations targeting Cys-X-X-Cys motifs have been shown in similar assays to cause impairment in transformation (30, 34), and these mutations served as controls for the assay. Additionally, we also included the del21-24 mutant as another control for these experiments. The del21-24 mutation disrupts the primary pRb binding site in CR2 of E7. It has been studied extensively in the literature and used previously in similar assays (30, 34, 39). Table 2-3 summarizes the transformation results for the entire panel of surface-exposed mutants, many of which behave like the wild-type in this assay. Some have increased transformation potential (V55T mutant, T64D mutant, etc.), indicating that these mutants may perhaps be better at targeting certain cellular proteins/pathways necessary for transformation. Additionally, two mutations in E7, M84S and QKP96-98EEA, were identified as causing decreased transformation ability. These two mutants retained the capacity to both bind and degrade pRb (FIG. 2- 4A and B), and subcellular localization also remained comparable to that of wild-type E7 ( FIG. 2-4D). Additionally, as illustrated by cycloheximide treatment experiments, the QKP96-98EEA mutation did not contribute to any destabilizing effects on the protein, as mutations that target the hydrophobic core might (FIG. 2-4C). Together, this evidence suggests that the M84S and QKP96-98EEA mutations disrupt interaction with other important cellular targets necessary for transformation, whose identities remain to be elucidated.

Furthermore, Table 2-4 summarizes transformation results for the hydrophobic core mutants. As expected, the C58G/C91G and C91G mutants had a decreased ability to transform cells. The L67R, V69A, and L79A mutants also had decreased transforming potential. In contrast, a number of mutants with mutations in this region still transformed cells as effectively as wild-type E7, including the L65A, I76A, LLMG82-85AAAD, L87A, and I89A/V90A mutants. Importantly, our aim was to establish whether E7 must
exist as a dimer to function in oncogenic transformation. Correlating transformation ability with dimerization (Table 2-3 and 2-2) established two important phenotypes: those mutants that fail to dimerize and have decreased transformation potential (M84S, C58G/C91G, C91G, and L79A mutants) and, notably, numerous mutants that fail to dimerize yet still transform cells as well as wild-type E7 (F57A, R77E, G85A, T86D, I76A, L87A, and I89A/V90A mutants). The second class of mutants clearly illustrates that E7 does not need to exist as a dimer in order to transform BRK cells. This conclusion is also reinforced by the V55T mutation, which results in a protein that is impaired for dimerization yet exhibits an increased potential to transform. Our results also raise the intriguing possibility that additional, as yet unidentified cellular targets of E7 remain to be identified that contribute to E7's transforming activity through interaction with the CR3 region.

In summary, our data strongly suggest that dimerization of E7 is not required for oncogenic transformation of primary rodent cells. In addition, our systematic and rational construction of a new collection of CR3 mutants by use of structural information should provide the field with useful reagents for tackling future questions regarding protein interaction surfaces in CR3 and the functional consequences of abrogating these interactions.

2.5 References


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Chapter 3  CONSERVED REGION 3 OF HUMAN PAPILLOMAVIRUS 16 E7 CONTRIBUTES TO DEREGULATION OF THE RETINOBLASTOMA TUMOR SUPPRESSOR

3.1 Introduction

Papillomaviruses are a group of DNA viruses that infect the skin and mucosal tissues of most vertebrates. More than 100 human papillomavirus (HPV) types have been identified but far more are presumed to exist (21). A subset of HPVs is associated with lesions that frequently progress to cancer and these HPVs are classified as “high-risk” types. Persistent infection by high-risk HPVs is related to 99.7% of all human cervical cancer cases (20), other genitourinary cancers, and an increasing number of oral cancers (31). Although recently developed vaccines protect individuals from the two most frequently carcinogenic HPV types, HPV16 and HPV18, they offer limited protection against other cancer-causing HPVs and provide no benefit to individuals with a pre-existing history of infection (13, 28, 48). The development of other therapies to treat HPV induced malignancy may be facilitated by a greater understanding of the mechanisms of virally-mediated cellular transformation.

The viral E6 and E7 oncoproteins are consistently expressed in HPV-induced cancers and are necessary to maintain malignant cell growth (2, 4, 37, 49, 51). Repression of their transcription by re-expression of the viral E2 regulatory protein induces rapid growth arrest and senescence of cervical cancer cells, suggesting that these cancer cells are "addicted" to E6 and E7 (33, 34). The HPV E7 protein is a multifunctional oncoprotein that interacts with a multitude of cellular factors (3, 6, 8, 11, 53). One of the main activities of E7 is to induce terminally differentiated cells to enter the cell cycle. This is accomplished in part by E7’s association with the product of retinoblastoma susceptibility locus (pRb) and the related pocket protein family members p107 and p130 (25). The interaction of E7 with pRb is essential for transformation and abrogation of the anti-proliferative signals in cervical cancer (26, 47). One of the ways in which pRb has an essential role in proliferation control is through binding to and regulating its key effectors, the E2F family of transcription factors. E2F coordinates the transcription of
genes that are necessary for cell cycle progression (10, 24, 43). During the cell cycle, as cells progress from the $G_1$ to $S$ phase, the sequential phosphorylation of pRb by cyclin/cyclin-dependent kinase complexes causes the release of E2F from pRb and activation of genes required for the entry into $S$ phase (19, 41, 50).

The E7 protein contains three conserved regions (CR), termed CR 1, 2 and 3. CR1 and CR2 are thought to be intrinsically disordered, while CR3 has a defined three dimensional structure comprised of a unique $\beta 1\beta 2\alpha 1\beta 3\alpha 2$ fold (40, 45). All three regions are required for abrogation of epithelial cell quiescence and contribute to cellular transformation (5, 26, 47). The role of the LXCXE motif within CR2 is well established and is necessary for the abrogation of anti-proliferative signals and oncogenic transformation (26, 47). The LXCXE motif functions as a high-affinity binding site for pRb and related family members. However, other regions of E7 are also important for deregulating the pRb pathway and E7 is known to exert its activity on pRb through multiple mechanisms. Firstly, E7 binds the hypo-phosphorylated form of pRb in complex with E2F, and blocks the association of E2F and pRb. Although the LXCXE motif in CR2 is necessary for interference with pRb-E2F binding (14, 44), a requirement for both the CR1 and CR3 regions has also been established (35). Precisely how these two regions contribute is not known. Secondly, E7 inactivates a fraction of the cellular pool of pRb by targeting it for proteasome-mediated degradation (9, 32, 36). Both the LXCXE motif and CR1 are necessary for efficient reduction of pRb steady-state levels (29, 38), while the role of CR3 has remained largely elusive. One study has shown that small changes within CR3 do not disrupt the pRb degradation capacity of E7 (35), but it has also been hypothesized that CR3 may contribute to pRb degradation due to the observation that mutations in this region lead to reduced association with the cullin 2 E3 ubiquitin ligase complex (36, 38). Binding of the cullin 2 complex to E7 is mediated via its Zer1 subunit and is currently the only known mechanisms by which HPV16 E7 targets pRb for destruction by the proteasome (36, 53). As the interaction of cullin 2 is exclusive to HPV16 E7, the mechanism by which other HPV E7 types degrade pRb remains unclear (36, 53). Lastly, strong evidence has been presented that E7 CR3 contributes to pRb binding independently of the LXCXE motif, but the functional significance of this interaction is unknown (15, 40, 46).
Despite the large number of biological activities that are attributed to CR3, the role of this domain of E7 in deregulating the pRb pathway is still unclear. The study presented here was aimed at determining the role of E7 CR3 sequences in deregulating the pRb pathway. We examined the involvement of surface-exposed residues of HPV16 E7 CR3 in binding pRb, targeting it for degradation and influencing pRb's ability to induce cell cycle arrest. Although CR3 is necessary for association with the cullin 2 complex and specific CR3 mutants affect cullin 2 interaction, none of these mutants were impaired for pRb degradation. However, we identified two specific patches on the surface of CR3 which contribute to pRb binding independently of the LXCXE motif in CR2. Additionally, we show for the first time that the CR3-pRb interaction is functionally important in overcoming pRb induced cell cycle arrest.

3.2 Materials and Methods

3.2.1 Plasmids

The surface-exposed mutants of E7 were previously generated by site-directed mutagenesis and their construction has been described in Chapter 2. For pRb degradation assays, E7 mutations were subcloned into the BamHI and XhoI restriction sites of a modified pCMV-Neo-Bam mammalian expression vector. Constructs for the del21-24 and C58G/C91G, and C91G mutations were obtained from D. Galloway (Fred Hutchinson Cancer Research Center, University of Washington) and K. H. Vousden (National Cancer Institute at Frederick, Frederick, MD), respectively, and subcloned as necessary. For degradation assays carried out in H1299 cells, full-length E7 mutants were sub-cloned into the pCMV-Neo-Bam expression vector with a tandem N-terminal flag-hemagglutinin tag, using BamHI and XhoI restriction sites. For expression in yeast, full-length E7 was cloned into a modified pJG4-5+ vector (Clontech) using EcoRI and SalI or XhoI sites (52), or in the case of HPV16 E7 CR3 region, using PvuII and XhoI. HPV6, 11 and 18 CR3 regions were PCR amplified using the following primers:

HPV6-F-CGAATTCGGAAGTGGACGGACGACAAGATTCA
HPV6-R-GATCCTCGAGTTAGGTCTTCCGCGCGACGAG
HPV11-F-CGAATTCAAGGTGAACAAACAGACGCA
HPV11-R-TATGTCGACTTTAGGTTTGTGTCGCAGATGGG
HPV18-F-CGAATTGATGAGTTAATCATCAACAT
HPV18-R-CCGCTCGAGTTACTGCTGGGATGC

PCR products were cloned into EcoRI and XhoI or SalI sites of the modified pJG4-5+. For expression of GFP-fused constructs, either full-length or PCR amplified fragments of E7 were cloned into EcoRI and XbaI sites of pCAN-myc-EGFP. Either wild-type or mutant CR3 region of HPV16 E7 (residues 39-98) was subcloned from their respective pJG4-5+ plasmids into pCAN-myc-EGFP. Alternatively, fragments 1-39, 1-57, and 39-58 were first PCR amplified using the following primers:

1-39 F-GTCGAATTCATGCATGGAGATACACCTACATTGC
1-39 R-ATTATCTAGAATCTCGAGTTAATCTATTTCATCCTCCTC
1-57 F-CGAATTCGTAATCATGCATGGAGATAC
1-57 R-CCTCTCGAGCTAAAAGGTTACAATATTGTAATG
39-58 F-ATTAGAATTCGATGTCgccGATGGGACAA
39-58 R-ATTATCTACAATCTCGAGTTAACAAAGGTTACAATATT

and subsequently cloned into the EcoRI and XbaI sites of pCAN-myc-EGFP. For recombinant protein production and purification, the CR3 region of each E7 construct was cloned into the pGEX4T1 (Invitrogen) vector, modified to contain the tobacco etch virus (TEV) protease recognition sequence between the glutathione S-transferase (GST) tag and the protein of interest. The pCMV-pRb, pGEX4T1-pRbC and pScodon-pRbABC constructs were previously described (12, 22, 23). Residues 379-792 of pRb were PCR amplified and cloned into the BamHI site of pGEX4T1. pBB14-Ub9-EGFP has been described previously (39). pCMV-HA-cullin 2 was a gift from P. Branton (McGill University, Montreal, Canada); pcDNA-HA-pRb was a gift from J. DeCaprio (Dana Farber Cancer Institute, Harvard Medical School, Boston, USA) and pcDNA-His-βgal has been described previously (42). The PSH1834 reporter plasmid for yeast two-hybrid assays was recovered from the EGY48 yeast strain (Clontech) and contains eight operator sequences that respond to LexA. The pCMV-HA-p21Cip1 plasmid has been described previously (16).
3.2.2 Cell culture, transfection and pRb degradation assay

Human Saos2, HT1080 and H1299 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/ml). H1299 control and cullin 2 knockdown (KD) cells have been previously described (17, 18), and were maintained in media supplemented with 1 µg/ml of puromycin. For pRb degradation assays, Saos2 cells were seeded into 6-well plates at 3 x 10^5 cells per well and transfected 24 h later with 2 µg of pRb, with or without 0.2 µg of E7 expression plasmid and with 0.1 µg of green fluorescent protein (GFP) or β-galactosidase (βgal) expression plasmid as a control, using FugeneHD (Roche) according to the instructions provided by the manufacturer, unless otherwise noted. The amount of plasmid DNA was balanced with empty pCMV vector where necessary. The amounts of pRb, GFP, and actin were assessed by western blotting 48 h post-transfection. Assessment of pRb degradation in H1299 control or cullin 2 KD cells was carried out similarly, where 0.2 µg of HA-pRb expression plasmid was co-transfected with an increasing amount of wild-type or mutant Flag-HA-tagged E7 expressing plasmid (20, 200, 2000 ng) using X-tremeGENE HP (Roche). The amount of pRb, E7 and actin was determined 24 h post-transfection by western blot. For co-immunoprecipitation experiments, HT1080 cells were seeded into 10-cm plates at 2 x 10^6 cells per plate and transfected 24 h later with 8 µg of total DNA with X-tremeGENE HP (Roche), according to the manufacturer’s instructions. 24 h post-transfection cells were collected for co-immunoprecipitation experiments. For cycloheximide treatment experiments, HT1080 cells were seeded into 6-well plates 24 h before transfection. For each mutant, the cells were transfected with 2 µg of E7 pCMV-Neo-Bam expression plasmid along with 0.1 µg of GFP expression plasmid, using X-tremeGENE HP. At 24 h post-transfection, the cells were treated with 0.5 µg/µl cycloheximide for 0, 15, 30 and 60 min.

3.2.3 Antibodies

The following antibodies were used: rat anti-hemagglutinin (anti-HA) (Roche) at 1:2,000, and rabbit anti-glucose-6-phosphate dehydrogenase (anti-G6PD), used as a loading
control for yeast samples, at 1:80,000 (Sigma). For detection of pRb in the degradation assay, mouse anti-pRb (G3-245; BD Pharmingen) was used at 1:500 or mouse anti-pRb hybridoma lysate (clone C36) at 1:4. GFP was detected using rabbit anti-GFP (Living Colors; Clontech) at 1:2,000. Cullin 2 was detected using rabbit polyclonal anti-cul2 antibody at 1:10,000 (Bethyl Laboratories). Myc tagged constructs were detected with mouse anti-myc hybridoma lysate (clone 9E10), used at 1:200. Mouse anti-β-galactosidase (anti-β-gal) (Promega) and rabbit anti-actin (Sigma) were used at 1:2,000. HRP-conjugated goat anti-rabbit (Jackson Laboratories), goat anti-rat (Pierce), and rabbit anti-mouse (Jackson Laboratories) secondary antibodies were used.

3.2.4 Co-immunoprecipitation and western blot analysis

Cells were transfected in a 1:1 ratio with the myc-GFP fusion and hemagglutinin (HA)-tagged binding partner (cullin 2, pRb or p21Cip1). Cells were harvested at 24 h post-transfection by scraping and washed once with 1× phosphate-buffered saline (PBS). Cells were lysed in NP-40 (50 mM Tris, pH 7.8, 150 mM NaCl, 0.1% NP-40) lysis buffer supplemented with 1× mammalian protease inhibitor cocktail (Sigma). Typically, 1 mg of cell lysate was mixed with 100 μl of anti-myc hybridoma (clone 9E10) or 1 μl of anti-GFP antibody and 100 μl of 10% slurry of protein A-Sepharose resin (Sigma) and incubated at 4°C for 1-2 h with nutating. Immunoprecipitates were washed three times with lysis buffer and re-suspended in 2× lithium dodecyl sulfate (LDS) sample buffer, and boiled for 5 min. Samples were then separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (GE), and blocked in 5% nonfat milk in Tris-buffered saline-Tween 20. Western blot analyses were carried out with mouse anti-myc hybridoma clone (9E10) or rat monoclonal anti-HA (clone 3F10 Roche).

3.2.5 Yeast two-hybrid assay

Yeast two-hybrid analysis was performed in strain W303-1a MAT1a [leu2-3,112; his3-11,15; trp1-1; ura3-1; ade2-1, can1-100]. Standard yeast culture medium was prepared
using previously described methods (1). Yeast transformation was carried out using a modified lithium acetate procedure (30). Yeast cells were transformed with the reporter plasmid, and pRb and E7 expression plasmids, then plated on selective medium plates and grown at 30°C for 2 to 4 days. The method for assaying β-galactosidase activity in yeast has been described previously (1).

3.2.6 GST pull-down assays

Recombinant GST-tagged E7 CR3 (residues 39-98) and GST-tagged fragments of pRb (pRbAB and pRbC) were expressed in and purified from the BL21-RIL strain of *Escherichia coli* and pRbABC from BL21-Gold strain bacteria, per protocols provided by the affinity resin manufacturer (Amersham). For GST pull-down experiments with GST-CR3, Saos2 cells were transfected with pRb expression plasmid, and lysates prepared 48 h post-transfection in NP-40 lysis buffer. Approximately 400 µg of Saos2 extract was used per reaction, and mixed with an increasing amount of GST-CR3, 600 µl of NP-40 lysis buffer and 20 µl of 50% glutathione sepharose. Reactions were incubated at 4°C for 1 h, and then washed two times with the lysis buffer. Samples were re-suspended in 2x LDS sample buffer, boiled for 5 min, then separated by SDS-PAGE and examined by western blotting for the amount of associated pRb. For GST pull-down assay with purified recombinant GST-pRbABC, -pRbAB or -pRbC, HT1080 cells were transfected with myc-GFP-E7 39-98 expression plasmid. Lysates were prepared 24 h post-transfection in NP-40 lysis buffer. Approximately 1 mg of lysate was mixed with an increasing amount of GST-pRb fragment and 20 µl of 50% glutathione sepharose. Reactions were incubated and samples process as indicated above. Western blot analysis was used to assess the amount of associated myc-GFP-E7 39-98 by blotting with anti-myc antibody.
3.2.7 *In vitro* pRb binding assay

To obtain untagged E7 CR3 (residues 39-98), purified recombinant GST-tagged wild-type E7 CR3 and mutants were incubated with ~0.2 µg/ml TEV protease overnight at 4°C. The purified protein concentration was determined using the Bio-Rad assay and was verified by running 0.5 µg of purified protein on a gel and silver staining (SilverQuest Staining Kit, Invitrogen) prior to using the samples in the assay. The ability of E7 CR3 to associate with GST-pRbABC was assessed at protein concentrations well below saturation levels (FIG. 3-7A). Briefly, 0.6 µM E7 CR3 (residues 39-98) was incubated with 0.3 µM GST-pRbABC at room temperature for 1 h in a total volume of 300 µl of phosphate buffer (20 mM phosphate, pH 7.0, 200 mM NaCl, 1% Tween 20, 2 mM DTT) and 20 µl of 50% glutathione sepharose. Samples were washed three times with phosphate buffer, boiled in 2x LDS sample buffer for 5 min and separated by SDS-PAGE. Gels were silver stained to determine the amount of associated E7 CR3.

3.2.8 Cell cycle analysis

6 x 10⁵ or 1 x 10⁶ Saos2 cells were seeded into 6- or 10-cm dishes, respectively. Cells were transfected with 1.5 µg of each Us9-EGFP expression plasmid (membrane bound GFP) and pRb, and 3 µg of untagged, full-length E7 (pCMV-Neo-Bam plasmids) with FugeneHD (Roche). 24 h post-transfection, cells were transferred to either 10- or 15-cm dishes (depending on the size of the original dish), using phosphate buffered saline supplemented with 10 mM EDTA (PBS-EDTA). 48 h after the transfer to a new dish, cells were collected with PBS-EDTA solution and washed once with PBS. Cell pellets were re-suspended in 300 µl of 50% FBS solution (in PBS), and fixed by drop-wise addition of 900 µl of ice-cold 70% ethanol. Samples were incubated on ice for at least 2 h, and then washed two times with PBS. Cells were re-suspended in and stained with 300 µl of propidium iodide staining solution (1% FBS, 10 µg/ml propidium iodide, 0.25 mg/ml RNase A in PBS). Cell cycle data was collected by flow-cytometry on FACSCalibur (BD Biosciences). DNA content was determined based on propidium
iodide staining. Data was analyzed using FlowJo software and percent of cells in $G_1$ phase was determined using the Watson Model.

3.3 Results

3.3.1 CR3 mutations

To examine the role that HPV16 E7 CR3 plays in pRb deregulation, we utilized the panel of putative surface-exposed mutants within CR3, from here on referred to as surface-exposed mutants. These have been previously described by our group, and also in Chapter 2 of this thesis (52). Briefly, these mutations target residues within CR3 whose side chains are predicted to be at least 25% surface-exposed, but not those that would participate in stabilizing the structural features of E7 (40, 45, 52). Hence, these mutations were constructed with the dual aims of preserving the structure of the CR3 region, while disrupting the interaction surfaces available for cellular targets. In total we included in these studies 19 point mutants, one double mutant, and one mutant targeting the last three amino acids of CR3 (FIG. 3-1). As controls, we also utilized the deletion mutant, del21-24, which removes the LXCXE motif in CR2, and the C58G/C91G mutant which targets two critical zinc coordinating cysteines in CR3, and is thought to grossly perturb the folded structure of this region of E7.

3.3.2 Contribution of HPV16 E7 CR3 to pRb degradation

The LXCXE motif and certain residues within CR1 of E7 were previously shown to be important for pRb degradation (32, 35). Other studies have also examined the role of CR3 and have found no contribution of any single residue to pRb degradation (7, 35). However, another study has shown that specific residues within CR3 contribute to cullin 2 complex binding, currently the exclusive mechanism known to be used by HPV16 E7 to degrade pRb (36). These findings led us to re-examine the role of CR3 in pRb degradation. Our first aim was to map the surface of CR3 which is required for pRb
degradation utilizing the panel of surface-exposed mutants. Our approach was to test full-length wild-type E7 or E7 mutants for their ability to degrade pRb exogenously expressed in pRb-null Saos2 osteosarcoma cells. Co-transfection of pRb with increasing amount of wild-type E7 led to a dose-dependent decrease in pRb levels (FIG. 3-2A). As expected, co-transfection of the del21-24 E7 mutant, which removes the high affinity LXCXE site, did not reduce pRb levels. However, all 21 of the CR3 mutants that we tested retained the ability to degrade pRb comparably to wild-type E7 under non-saturating conditions (FIG. 3-2B).

3.3.3 Contribution of HPV16 E7 CR3 to cullin 2 binding and the role of cullin 2 in E7 mediated pRb degradation

The above findings led us to question whether the pRb degradation phenotypes observed could be due to the fact that each of these surface-exposed mutants maintains the ability to associate with the cullin 2 complex. This seemed unlikely, as others previously showed that mutations CVQ68-70AAA, del79-83 and C91S lose the ability to associate with the cullin 2 complex (36). Before testing the individual mutants for interaction with cullin 2, we first determined which portions of E7 mediated binding using GFP fused to fragments of E7 corresponding to residues 1-39 (CR1 and CR2), 39-58 (N-terminus of CR3) and 39-98 (CR3). In co-immunoprecipitation experiments, neither residues 1-39 nor 39-58 of E7 could on their own interact with the cullin 2 complex. In contrast, residues 39-98 (CR3) of E7 were necessary and sufficient for this interaction (FIG. 3-3A). To test the properties of the surface-exposed mutants for cullin 2 binding, we expressed the entire panel in the context of the 39-98 fragment fused to GFP. In co-immunoprecipitation experiments with co-expressed HA-cullin 2, all mutants retained at least some ability to associate with this complex (FIG. 3-3B). However, mutants such as N53D, V55T and S63D had a substantially reduced capacity to associate with the cullin 2 complex. Nonetheless, as described above, all of these mutations retained the ability to target pRb for degradation. These results suggest that either the relatively weak interaction with cullin 2 is sufficient for pRb degradation, or that other, as yet unidentified mechanisms are utilized for the degradation process. In addition to assessing the ability of our panel of
FIG. 3-1. Schematic representation of HPV16 E7 and the position of mutations targeting residues within CR3. The conserved regions (CR1, 2 and 3) are depicted as boxes. The amino acid sequence of CR3 and the mutations used in this study are indicated. Also indicated are del21-24, which removes the LXCXE motif in CR2 of E7, and the C58G/C91G mutant, which targets zinc coordinating cysteine residues within CR3.
FIG. 3-2. All E7 CR3 mutants retain the ability to degrade pRb. (A) Dose dependent degradation of pRb by wild-type E7 in Saos2 osteosarcoma cells. Saos2 cells were transfected with 1 µg of pRb expression plasmid, 0.1 µg GFP expression plasmid, and the indicated amount of wild-type E7 expression plasmid; 48 h post-transfection cells were collected and samples analyzed by western blotting for pRb, GFP and actin levels. (B) pRb degradation by E7 mutants. Full-length wild-type E7 or the indicated mutants were transfected with pRb and GFP expression plasmids at 1:10 ratio of E7 to pRb, and 48 h post-transfection levels of pRb were analyzed. The blot is a representative image; the bar graphs represent quantified pRb levels normalized to GFP and actin levels. The relative pRb levels are representative of five independent experiments.
FIG. 3-3. Contribution of the cullin 2 complex to E7 induced pRb degradation. (A) CR3 of E7 is necessary and sufficient to bind cullin 2. HT1080 cells were transfected with equal ratio of HA-tagged cullin 2 expression plasmid and either myc-GFP or myc-GFP-fused E7 fragment (as indicated). 24 h post-transfection, cell lysates were subjected to immunoprecipitation with anti-myc antibody, followed by western blotting for cullin 2 with anti-HA antibody. (B) Cullin 2 binding capacity of CR3 mutants. HT1080 cells were co-transfected with HA-tagged cullin 2 and either myc-GFP or myc-GFP-fused E7 39-98 wild-type or indicated mutant. Co-immunoprecipitation was carried out as described above. (C) The C58G/C91G mutant retains the ability to degrade pRb. Saos2 cells were transfected with 1 µg pRb, 0.1 µg β-galactosidase and the indicated amounts of full-length wild-type or C58G/C91G mutant E7. Samples were collected 48 h post-transfection and the steady state levels of pRb were analyzed by western blotting. The %pRb indicates the relative amount of remaining pRb in each lane. (D) Cullin 2 levels in H1299 control and H1299 cullin 2 knockdown (KD) cells. The levels of endogenous cullin 2 present in H1299 control and H1299 cullin 2 KD cells were analyzed using anti-cullin 2 antibody. (B) Dependence of E7 on cullin 2 for pRb degradation. pRb degradation assays were conducted in cullin 2 KD and control cells for indicated E7 mutants, as described in the Materials and Methods.
surface-exposed mutants to bind the cullin 2 complex, we also included in these experiments the C58G/C91G mutant, which abrogates zinc co-ordination within CR3 and presumably disrupts proper folding of this domain (FIG. 3-3B). A similar mutation, C91S, in the context of full-length E7 was previously shown to disrupt cullin 2 binding (36). Unexpectedly, we found that C58G/C91G associated with cullin 2 at least as well as wild-type E7 residues 39-98 (FIG. 3-3B). When tested for the ability to target pRb for degradation in the Saos2 cell assay, the C58G/C91G mutant in the context of full-length E7 degraded pRb in a dose-dependent manner that was indistinguishable from wild-type E7 (FIG. 3-3C). The C91G mutation was similarly tested and behaved identically to C58G/C91G (data not shown). These findings suggest that both cullin 2 binding and pRb degradation function independently of correct folding of CR3. To further study the role of cullin 2 in the pRb degradation process, we compared the ability of the four E7 CR3 mutants identified to have reduced association with cullin 2 (N53D, V55T, R66E and T72D) to degrade pRb in H1299 cells in which cullin 2 was stably knocked down (FIG. 3-3E). Each mutant degraded pRb in cullin 2 knockdown cells similarly to control cells, further suggesting that E7 is utilizing a cullin 2 independent mechanism to degrade pRb.

### 3.3.4 CR3 binds pRb independently of the LXCXE motif in vitro and in vivo

Several reports have suggested that CR3 may contribute to deregulation of pRb function by providing a secondary lower-affinity binding site. For example, full-length E7 binds pRb more strongly than mutants retaining the LXCXE motif, but lacking CR3 (46). More recently, it was demonstrated in two independent studies that CR3 binds pRb in vitro (15, 45). We first confirmed that purified GST-CR3 of E7 was sufficient to independently pull-down pRb from cell lysates in a dose dependent manner (FIG. 3-4A). Utilizing the reciprocal approach, we also demonstrated that recombinant large pocket of pRb (GST-pRbABC) could associate with GFP-CR3 from cell lysates obtained by overexpressing GFP-CR3 in HT1080 cells (FIG. 3-4B). Additionally, the small pocket of pRb (pRbAB) was sufficient for this interaction, consistent with a previous report (15). In contrast, the C-terminus of pRb (pRbC) was not sufficient to interact with CR3 of E7. As CR3 has
FIG. 3-4. E7 CR3 interacts with pRb independently of the LXCXE motif in vitro. (A) GST-CR3 associates with pRb from cell lysates. Increasing amounts of GST-CR3 (residues 39-98) were incubated with ~400 µg of Saos2 cell lysate (previously transfected with pRb expression plasmid), and analyzed for the amount of associated pRb via western blotting. (B) GFP-CR3 (residues 39-98) associates with the large and small pocket of pRb. HT1080 cells were transfected with myc-GFP or myc-GFP-fused E7 39-98. 24 h post-transfection cell lysates were prepared and ~1 mg was used in each GST pull-down reaction. 5 µg of GST was incubated with 1 mg of myc-GFP-E7 39-98 lysate as a control. 5 µg of GST-pRbABC (large pocket), GST-pRbAB (small pocket) and GST-pRbC (C-terminus of pRb) was incubated with 1 mg of myc-GFP lysate as a control. Increasing amount of GST-pRb fragments (0.5, 1 or 5 µg) was incubated with 1 mg of myc-GFP-E7 39-98 lysate. Samples were washed and then analyzed by western blotting for the amount of associated myc-GFP-E7 39-98. Bottom panel is the Ponceau stain of the membrane, illustrating the input levels of GST-pRb fragments. The left side of the Ponceau stain indicates the position and size of the ladder in kDa.
never been reported to interact with pRb \textit{in vivo}, we examined the ability of GFP-CR3 to co-immunoprecipitate HA-pRb. As expected, an N-terminal fragment of E7 (residues 1-57; which contains the high-affinity LXCXE binding site for pRb) was found to associate strongly with pRb by co-immunoprecipitation (FIG. 3-5A). Importantly, the CR3 region of E7 (residues 39-98) was also sufficient to co-immunoprecipitate pRb, albeit at a substantially lower level than the full-length E7 protein or the LXCXE containing N-terminal fragment. Yeast two-hybrid analysis utilizing E7 fragments spanning residues 1-57 or 39-98 as prey and full-length pRb as bait showed that CR3 also interacted with pRb independently of the N-terminus. In these assays, residues 39-98 bound pRb with approximately 30% of the activity observed with full-length E7 (FIG. 3-5B).

3.3.5 CR3s from other HPV types also interact with pRb

Utilizing co-immunoprecipitation experiments we determined that full-length E7 from the high-risk HPV18 and low-risk HPV6 and HPV11 also associated with pRb in mammalian cells co-transfected with E7 and pRb (FIG. 3-5C), or utilizing endogenous pRb (data not shown). This was expected as each of the E7 proteins contains a high-affinity LXCXE motif. We next tested whether the E7 CR3 regions from other HPV types could interact \textit{in vivo} with pRb independently of the LXCXE binding site in CR2. In a yeast two-hybrid analysis, low-risk HPV6 and HPV11 E7 CR3 regions also interacted with pRb, whereas little or no activity was detected for the high-risk HPV18 CR3 (FIG. 3-5D). Similarly to the co-immunoprecipitation experiments from mammalian cells, each of the full length E7 proteins was also able to bind pRb in the yeast two-hybrid assay. These results indicate that while all the E7 proteins tested could bind pRb, the ability to interact with pRb via CR3 may not be a universal property of all E7 proteins.
FIG. 3-5. E7 CR3 interacts with pRb independently of the LXCXE motif in vivo. (A) E7 CR3 interacts with pRb in vivo. HT1080 cells were co-transfected with equal amount of HA-tagged pRb and myc-GFP or myc-GFP-E7 fragments. 24 h post-transfection cell lysates were prepared and subjected to immunoprecipitation with anti-myc antibody, followed by western blotting for HA-pRb. (B) HPV16 E7 CR3 interacts with pRb in a yeast two-hybrid assay. Yeast cells were transformed with either empty vector, full-length E7 or the two indicated E7 fragments as prey proteins, and along with pRb as bait and a LexA responsive β-galactosidase reporter plasmid. Following transformation, yeast cell were analyzed for β-galactosidase activity. Data is presented as percent binding relative to full-length E7. (C) E7 proteins from other HPV types also associate with pRb. HT1080 cells were co-transfected with equal amount of HA-tagged pRb and myc-GFP or myc-GFP-E7 from HPV16, 18, 6 or 11. 24 h post-transfection, immunoprecipitation and western blotting was carried out as described above. (D) CR3 regions from other HPV types also associate with pRb independently from the LXCXE motif. The C-terminal portions of HPV16, HPV18, HPV11 and HPV6 were tested for the ability to associate with pRb similarly to what was described in (B). Data is represented as experimentally determined β-galactosidase activity.
3.3.6 Mapping the binding surface for pRb in CR3

We initially used the yeast two-hybrid system as a rapid method to identify candidate residues from HPV16 E7 that are involved in binding pRb. We cloned our panel of mutants into a yeast two-hybrid prey vector as fragments expressing only residues 39-98, and assessed their capacity to bind pRb. With this approach, we identified a number of mutants with statistically significant difference in pRb binding. Specifically, V55T, F57A, R66E, M84S, and I93T were increased and Y52A, N53D, C59S, S63D, T64D, T72D, R77E and G85A were decreased. The most significant increase in binding was observed for R66E, which displayed 10 times higher β-galactosidase activity than the wild-type CR3. In contrast, Y52A, N53D, C59S and G85A, all showed more than 50% reduction in activity (FIG. 3-6B). It should be noted that for the yeast two-hybrid analysis, CR3 mutants were previously assessed for any auto-activation potential (52). Although R66E had greatly increased pRb binding potential in the yeast two-hybrid, this was not due to enhanced auto-activation. To rule out the possibility that differential expression played a role in the observed changes in binding capacities, we tested the majority of CR3 mutants which displayed increased or decreased binding (mutations with $p \leq 0.05$), for expression in yeast cells. All mutants with decreased binding capacity were expressed comparably to wild-type, except for S63D which was expressed at a slightly lower level (FIG. 3-6C). Similarly, mutants with increased binding for pRb were expressed comparably to wild-type, with the possibility that the slight increase in binding seen with the D62K and I93T mutants could be the result of increased expression. It should be noted that full-length E7 wild-type or mutants were also assessed for the capacity to associate with pRb in a yeast two-hybrid (FIG. 3-6A). With this approach, we found that none of the mutants exhibited a substantially reduced ability to associate with pRb, suggesting that defects in binding via CR3 are masked by the high affinity pRb binding site within CR2.
FIG. 3-6. Identification of CR3 residues involved in binding pRb using the yeast two-hybrid system. (A) Yeast two-hybrid analysis of the binding capabilities of full-length E7 mutants and pRb. Yeast cells were transformed with full-length pRb expression plasmid as bait and full-length wild-type or mutant E7 as prey, in addition to the LexA responsive β-galactosidase reporter plasmid. Data is shown relative to wild-type E7 and is representative of four independent experiments. (B) Yeast two-hybrid analysis of the binding capabilities of E7 CR3 mutants. Yeast cells were transformed with full-length pRb expression plasmid as bait and wild-type or mutant fragment of E7 spanning residues 39-98, in addition to the LexA responsive β-galactosidase reporter plasmid. Data is represented relative to wild-type E7 39-98. **p≤0.01, *p≤0.05. (C) Expression level of E7 CR3 mutants in yeast cells. The same yeast cultures used for the analysis of the pRb-CR3 interaction were also analyzed by western blotting for E7 CR3 protein expression levels.
3.3.7 Confirmation of the pRb binding properties of E7 CR3 mutants *in vitro*

Our initial analysis using the yeast two-hybrid system identified a number of residues that appeared to contribute to the interaction of HPV16 E7 CR3 with pRb. Interestingly, most of these residues map to two independent patches on the surface of CR3. Specifically, residues Y52, N53, V55, F57, C59, S63, T64, R66, and T72 map to one region, referred to as patch 1; residues R77, M84 and G85 map to a second region, referred to as patch 2. However, results from yeast two-hybrid assays may be influenced by the presence of additional factors in yeast. In order to conclusively determine if the identified residues contribute to pRb binding, we set up an *in vitro* GST pull-down assay utilizing affinity purified recombinant GST-fused large pocket of pRb and increasing molar amounts of purified wild-type CR3 (E7 39-98). In this assay, CR3 associated with pRb in a dose dependent manner (FIG. 3-7A). This analysis established appropriate protein concentrations for the assay, ensuring that the reactions were carried out at levels of CR3 below saturation. For all subsequent experiments, GST pull-down assays were set up with 0.3 µM pRbABC, and a two-fold molar excess of wild-type or mutant CR3 (0.6 µM).

To confirm the contribution of patch 1 to pRb binding, we focused on mutants of E7 with the most significantly altered binding (p≤0.01) as determined by the yeast two-hybrid system (FIG. 3-6B). These included Y52A, N53D, V55T, F57A, C59S, S63D, T64D, R66E and T72D. Of these mutants, all but R66E bound pRb at reduced levels *in vitro* (FIG. 3-7C and 7D). Taken together, the data from these two independent types of analyses clearly indicate that patch 1 contributes to pRb binding by CR3.

Patch 2 overlaps residues previously reported to contribute to pRb interaction, including R77, E80 and D81 (45). In agreement, E80K/D81K retained only ~25% of the binding capacity for the large pocket of pRb in our *in vitro* tests. In contrast, R77E showed a substantial increase in pRb binding and G85A bound comparably to wild-type (FIG. 3-7C and 7D). These data suggest that negative charges present within patch 2 contribute to pRb interaction, as previously proposed (45).
FIG. 3-7. Mapping the pRb binding surface on CR3 in vitro. (A) CR3 associates with the large pocket of pRb (pRbABC) in a dose dependent manner. 0.3 µM GST-pRbABC was incubated with increasing amount of purified CR3 (residues 39-98), and the amount of associated CR3 was determined by silver staining. (B) Representative gel image of CR3-pRb binding as quantified in (C). (C) Patch 1 residues most significantly contribute to pRb binding. Purified CR3 wild-type or indicated mutant was assessed for the ability to bind pRb in a GST pull-down assay using 0.3 µM GST-pRbABC and 0.6 µM CR3. Data is presented relative to wild-type protein and is representative of a minimum of three independent assays. **p≤0.001, *p=0.032. (D) Based on the in vitro analysis patch 1 and patch 2 residues significantly contributed to pRb binding. Colored in blue are patch 1 residues Y52, N53, V55, F57, C59, S63, T64 and T72 and R77, E80 and D81 within patch 2.
3.3.8 The CR3-pRb interaction is important for E7 to overcome pRb induced cell cycle arrest

Although all surface-exposed mutants of E7 CR3 can target pRb for degradation, our data demonstrate that specific residues contribute to pRb binding. To determine the functional significance of this interaction, we assessed patch 1 and patch 2 mutants for their ability to overcome cell cycle arrest induced by re-introduction of pRb into pRb-null Saos2 cells. Re-introduction of pRb into Saos2 cells results in ~80% of cells arresting in G1 phase of the cell cycle (FIG. 3-8A). Co-expression of full-length wild-type E7 reverses this arrest with ~40% of the cells remaining in G1. The change in percentage of cells in G1 in the presence or absence of wild-type E7 was normalized to 100% (FIG. 3-8A). Although the LXCXE deletion mutant del21-24 was impaired for inducing G1 exit, it still retained 27% of wild-type activity. We tested all CR3 mutants that were assessed for pRb binding \textit{in vitro} for their ability to overcome this pRb induced cell cycle arrest. Of those mutants located within patch 1, we found that all except C59S, R66E and T72D had a significantly reduced ability to overcome cell cycle arrest. Their activity ranged from 60-77% of wild-type E7. C59S and T72D showed a modest decrease in the ability to bind pRb \textit{in vitro} but behaved similarly to wild-type E7 in their ability to overcome cell cycle arrest. R66E retained pRb binding in both the yeast two-hybrid and the \textit{in vitro} studies, and overcame cell cycle arrest similarly to wild-type E7. This finding is consistent with previous analysis of R66A in similar functional assays (35). Of the patch 2 mutants, R77E behaved like wild-type E7, but G85A and E80K/D81K retained only about 60% of wild-type capacity to overcome the G1 arrest. The reduced ability of the mutants to overcome the pRb induced cell cycle arrest was not related to a reduction in their stability (FIG. 3-8B). We also tested selected mutants for their ability to interact with p21\textsuperscript{Cip1} by co-immunoprecipitation, as this regulator of G1 exit has previously been reported to interact with HPV16 E7 CR3 (27). However, no correlation was apparent between binding p21\textsuperscript{Cip1} and ability to overcome a pRb induced cell cycle arrest (FIG. 3-9). Taken together, CR3 mutants with reduced ability to bind pRb were typically less able to overcome a pRb induced cell cycle arrest.
FIG. 3-8. CR3-pRb interaction is functionally important for overcoming cell cycle arrest. (A) Mutants with reduced capacity to associate with pRb via CR3 have defects in overcoming the cell cycle arrest. Mutants analyzed for pRb binding in vitro were also assessed for their ability to overcome cell cycle arrest induced by re-expression of wild-type pRb in Saos2 cells. The percentage of cells in G1 phase of the cell cycle was determined by flow cytometry. Control cells represent the normally cycling Saos2 population. Saos2 cells expressing pRb only, but not E7 are labeled as “pRb only”. For all other samples, cells express pRb and either the full-length wild-type or indicated mutant E7 protein. Data is representative of a minimum of three independent experiments. ***p ≤ 0.001, **p ≤ 0.01 and *p ≤ 0.05 relative to wild-type E7 sample. Relative activity of E7 mutants in overcoming the cell cycle arrest was calculated and is indicated below the bar graph. (B) Analysis of E7 mutant stability. HT1080 cells were transfected with the expression plasmid for the indicated E7 mutants together with GFP and 24 h post-transfection were treated with cycloheximide for 15, 30 or 60 min. The levels of E7 were analyzed by western blotting.
**FIG. 3-9. Association of E7 mutants with p21^{cip1}.** HT1080 cells were co-transfected with expression plasmids for HA epitope tagged p21^{cip1} and wild-type full-length E7 or indicated mutant fused to a myc-GFP tag. 24 h post-transfection, the cell were lysed, and immunoprecipitation was performed with anti-GFP antibody, followed by western blotting.
3.4 Discussion

Although the role of HPV E7 in cellular transformation and cancer progression has been extensively studied, we still do not fully understand how E7 deregulates one of its most vital interacting partners, the retinoblastoma tumor suppressor (pRb). Precise assessment of E7-pRb protein interactions appears fundamental to understanding virally mediated subversion of cell cycle control and may allow novel shared features of viral and cellular pRb protein interaction partners to be uncovered. The objective of this study was to more precisely determine the role of E7 CR3 sequences in deregulating the pRb pathway.

For these studies, we utilized an extensive panel of surface-exposed mutants of HPV16 E7 CR3, which target residues that are accessible for interaction with cellular partners (FIG. 3-1). Unlike previous work, we avoided using deletion mutants or mutants which target highly conserved, structurally important hydrophobic residues of E7 CR3 (52). This approach allowed us to examine the role of each individual residue on the surface of CR3 in deregulating pRb function. Utilizing this panel of mutants, we first tested whether any of the introduced changes on the surface of E7 CR3 reduced its capacity to target pRb for degradation. Unexpectedly, the entire panel of 21 surface-exposed mutants was as effective as the wild-type HPV16 E7 protein in targeting pRb for degradation (FIG. 3-2B). The only mutant that was unable to induce pRb degradation was the del21-24, which cannot associate with pRb via the LXCXE motif located in CR2. This is most consistent with a previous report that even mutants with substantial deletions in CR3 were still able to destabilize pRb (35).

The ability of all the CR3 mutants to degrade pRb was unexpected, as this region is necessary for interaction with the cullin 2 E3 ubiquitin ligase, which facilitates ubiquitination of pRb and subsequent proteasome-mediated degradation (36, 53). In agreement with other studies, we show that CR3 confers association with cullin 2 (FIG. 3-3A). Our finding that mutations in the zinc coordinating cysteine residues of CR3 still bind cullin 2 suggests that this interaction occurs independently of the correct folding of CR3, perhaps via a linear interaction motif. Although some CR3 mutations result in reduced binding capacity for cullin 2, these mutants still degrade pRb as efficiently as
wild-type E7 under non-saturating conditions. We found this to be the case in the Saos2 degradation assay, as well as in cells in which cullin 2 levels were diminished by stable expression of a shRNA targeting cullin 2. This suggests that a cullin 2 independent mechanism may be utilized for pRb degradation. This is certainly possible, as the ability of HPV16 E7 to bind cullin 2 is not shared by the E7 proteins of any other HPV type so far tested (53).

Nearly 20 years ago, it was suggested that CR3 may also function as a secondary lower affinity binding site for pRb (46). However, this interaction was only recently confirmed by two independent reports (15, 45). Importantly, we have extended those in vitro studies to demonstrate that the interaction of CR3 with pRb occurs in mammalian cells in vivo by utilizing co-immunoprecipitation experiments (FIG. 3-5A). We also found that CR3 from other HPV types, specifically the low-risk HPV11 and HPV6, associate with pRb (FIG. 3-5D). Interestingly, CR3 from HPV18 E7 did not bind pRb, suggesting that this interaction may be present in some, but not all E7 proteins. To establish the functional consequences of the pRb-CR3 interaction, we first sought to identify the surface of CR3 that binds pRb. We assessed our panel of surface-exposed CR3 mutants for their pRb binding properties utilizing the yeast two-hybrid assay. This identified two specific patches on the surface of CR3 that contribute to pRb binding. We confirmed these data in vitro using purified recombinant proteins and GST pull-down experiments with the large pocket of pRb (pRbABC) fused to GST and purified wild-type or mutant CR3. Although both yeast two-hybrid and in vitro interaction assays yield similar results for the majority of mutants, the two assays responded differently for a subset of the mutants. It is possible that additional factors present in yeast, but not in the fully recombinant system influence the results. Nevertheless, these studies identified residues that comprise patch 1, which forms one continuous surface on CR3 and significantly contributes to pRb binding (FIG. 3-7D). These include residues Y52, N53, V55, F57, C59, S63, T64 and T72. Consistent with a previous report, we also identified residues within patch 2 that also contribute to pRb binding (40). Although we tested other mutants of E7 that are found within patch 2 region, we found that only R77E and E80K/D81K had an impact on binding. This suggests that negative charges in this region contribute to pRb binding via electrostatic interactions, as previously proposed (45).
We have established that all CR3 mutants maintain the ability to degrade pRb, but that specific residues in this region of E7 have reduced binding to pRb. We therefore wanted to determine whether CR3 contributes functionally to deregulating the pRb pathway by targeting the remaining pools of undegraded pRb. Previous work has shown that CR3 of E7 is important for pRb-E2F complex interference, but that small alterations in CR3 do not lead to significant changes in the ability of E7 to interfere with pRb-E2F complex formation (35). Therefore, our approach was to assess the role of the CR3-pRb interaction more globally, by determining the ability of CR3 mutants with decreased pRb binding to overcome cell cycle arrest induced by re-introduction of pRb into pRb-null Saos2 cells. We found that there is a high degree of correlation between the ability of E7 to bind pRb via the CR3 region and the ability of the corresponding full-length mutants to overcome cell cycle arrest. Most notably, mutations in residues within patch 1 that exhibited the most significant decrease in pRb binding also displayed a significant decrease in the ability to overcome pRb induced cell cycle arrest. Additionally, mutants in patch 2 were also examined. E80K/D81K had a reduced ability to overcome cell cycle arrest, which correlated with its reduced ability to bind pRb in vitro. Importantly, this data establishes that the ability of HPV16 E7 to induce pRb degradation is not sufficient to fully overcome a pRb induced cell cycle arrest. The data suggest that the remaining pool of undegraded pRb is still partially able to block cell cycle entry, and that this is overcome by the CR3-pRb interaction.

The cell cycle analysis also identified the G85A mutant as potentially interesting. Although, this mutant binds pRb similarly to wild-type E7 in vitro, it has a reduced ability to overcome cell cycle arrest. This mutant maintains the ability to interact with p21Cip1 (FIG. 3-9), but it is possible that it may have lost the capacity to interact with another important cellular target which is necessary for E7 to drive the cells into the S phase of the cell cycle.

Taken together, we have identified a new surface of HPV16 E7 that contributes to binding to pRb, which we have called patch 1. We also observed that negative charges located in patch 2 likely contribute to electrostatic interactions with pRb. Our data illustrate for the first time that the ability of E7 to bind pRb via CR3 is important in
overcoming the cell cycle arrest functions of pRb. In light of these findings, we suggest that the CR3 domain of E7 may be a candidate for targeted inactivation by small molecule compounds. Specifically, development of small molecules that bind patch 1 of CR3 may disrupt the ability of E7 to perturb pRb function and have utility in the treatment of papillomas or HPV induced cancers.

3.5 References


Chapter 4  ASSOCIATION OF THE HUMAN PAPILLOMAVIRUS E7 ONCOPROTEINS WITH THE P190RHOGAP

4.1 Introduction

Human papillomaviruses (HPVs) are small, double-stranded DNA viruses, which induce papillomas in cutaneous and mucosal epithelia, and are also the etiological agents of many cervical and other anogenital cancers (20, 23, 34, 49, 66). More than 100 HPV types have been described, and many more are presumed to exist (12). Specific HPV types often preferentially infect distinct anatomical sites. HPVs associated with lesions that can progress to carcinogenesis are classified as “high-risk” types, the most common of which is HPV16. In contrast, HPV types associated with benign warts that regress with time are termed “low-risk” viruses (65).

The ability of HPVs to induce cellular immortalization and transformation is attributed primarily to the viral oncoproteins E6 and E7, which are consistently expressed in HPV-induced cancers (3, 24, 36, 64). While E6 prevents apoptosis by inducing the degradation of the tumor suppressor p53 through the proteasome system, E7 disrupts cell cycle regulation by binding and inactivating the retinoblastoma tumor suppressor (pRb) (37, 59). In addition, both E6 and E7 alter other cellular signaling pathways by interacting with a number of cellular proteins and deregulating their function, thereby enhancing the carcinogenic potential of the cell (6, 18, 19, 29, 39, 63). To date, E7 has been reported to interact with well over fifty cellular factors, although the biological significance of many of these interactions is unknown (10).

Here, we demonstrate a novel interaction of HPV E7 with p190RhoGAP (p190), a Rho family GTPase activating protein (GAP). p190 is a multi-domain protein (47, 48) that includes an N-terminal GTP-binding domain (GBD), a middle domain (MD) containing critical phosphorylation sites and multiple protein–protein interaction motifs, and a C-terminal region that includes the GAP domain. p190 belongs to a large family of proteins that stimulate the intrinsic GTPase activity of the Rho proteins, leading to Rho inactivation. Rho family GTPases serve as molecular switches, cycling between active GTP-bound and inactive GDP-bound states, and transducing signals from the
extracellular environment to elicit cellular responses such as changes in gene expression, morphology, and migration (25, 46). Of the known Rho proteins, Cdc42, Rac1, and RhoA are the most thoroughly characterized (51). Active RhoA stimulates the formation of focal adhesions and stress fibers (11). Induction of these structures is thought to occur via the concerted activities of the RhoA effectors mDia and Rho kinase/ROCK/ROK, but may involve other targets (32, 57). Rho kinase stimulates myosin-based contractility by directly and indirectly elevating phosphorylation of the regulatory myosin light chain (31). The resulting activation of myosin triggers myosin filament formation and bundling of filamentous actin into stress fibers. The tension exerted by stress fibers is responsible for the maturation of focal complexes into focal adhesions by aggregating integrins and their associated proteins (11). Precise regulation of RhoA is crucial for efficient cell migration, and although some RhoA activity is required for migration, possibly to maintain sufficient adhesion to the substrate, high RhoA activity inhibits movement (40).

Through inactivation of Rho, p190 plays a critical role in regulating actin cytoskeleton dynamics, and also negatively controls tumor growth, transformation, metastasis, invasion and angiogenesis, strongly suggesting that p190 may function as a tumor suppressor (2, 7, 16, 43, 56, 60). Therefore, p190 appears as an attractive target for a viral oncoprotein such as HPV E7. In this study, I characterize the interaction of E7 with p190. I show that the C-terminal region of E7 is necessary and sufficient to associate with p190, but that expression of E7 does not alter the total steady state level of p190 or the level of active p190. Conversely, expression of E7 leads to decreased levels of active RhoA, as well as actin stress fibers. I map the interaction of p190 to the C-terminal portion of E7 and utilize a panel of surface-exposed mutants to identify two mutants unable to bind p190. Employing these two mutants, I demonstrate that the loss of the ability of E7 to bind p190 results in inability of the viral oncoprotein to deregulate RhoA levels and actin stress fibers. In addition, I show that p190 interacts with E7 proteins from many other HPV types, including high- and low-risk E7 proteins, suggesting that this interaction is evolutionarily important. Collectively, my results indicate that E7 may be utilizing p190 to deregulate the RhoA pathway, potentially contributing to the transformation, migration and invasion of HPV positive cancers.
4.2 Materials and Methods

4.2.1 Plasmids

The surface-exposed mutants within CR3 of HPV16 E7 were previously generated by site-directed mutagenesis and described elsewhere (53). GFP-fused E7 constructs for HPV16, HPV6, HPV11 and HPV18, either full-length or PCR amplified fragments, were previously described (52). E7 genes from HPV31, HPV33, HPV39, HPV45, HPV52, HPV55, HPV58, HPV59, HPV67 and HPV74 were PCR amplified from the viral genomes and cloned into the pCANmycEGFP vector using EcoRI and XbaI restriction sites. HPV genomes were obtained as plasmids from the sources indicated in Table 4-1. All primer sequences used for HPV E7 cloning are listed in the Table 4-1. HA-tagged p190RhoGAP (p190) full-length and GBD (amino acids 1-266) were kind gifts from S. Parsons (University of Virginia, Charlottesville, USA) and were previously described (50). p190 MD (amino acids 379-1183) and GAP (aa 1180-1513) were PCR amplified using the following primers:

MD-F-ACTGTGGATCCTGGTTTGTTGTACTTT
MD-R-ACTGTGAATTCTCACAGCTCATCATCACT
GAP-F-ACTGTGGATCCGATGATGAGCTGGGA
GAP-R-ACTGTGAATTCTCAAGAAGACAACTGATT

then cloned into the backbone of the pKH3 vector using BamHI and EcoRI restriction sites. pGEX4T3-RhoA-Q63L was purchased from Addgene.

4.2.2 Cell culture and transfection

Human CaSki, C33A and HT1080 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/ml). U2OS-neo and U2OS-E7 cell were previously described (14) and were maintained as indicated above with the addition of 500 µg/ml of G418 to cell culture media. For co-immunoprecipitation experiments, HT1080 cells were seeded into 10-cm plates at 2 x 10^6 cells per plate and transfected 24 h later with 8 µg of total DNA
### Table 4-1. HPV genome source and primers for cloning of HPV E7 genes

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with X-tremeGENE HP (Roche), according to the manufacturer’s instructions. Twenty four hours post-transfection cells were collected for co-immunoprecipitation experiments. For RhoGTP assays, U2OS cells were transfected at ~40% confluence with a total of 8 µg of DNA with X-tremeGENE HP, according to the manufacturer’s instructions. Cells were collected and lysed 48 h post-transfection and process as indicated below.

4.2.3 Antibodies

For western blots, rat anti-hemagglutinin (anti-HA) (Roche) was used at 1:2,000, and for co-immunoprecipitation experiments 100 µl of mouse anti-HA hybridoma lysate (12CA5 clone) was utilized per sample. GFP was detected using rabbit anti-GFP (Living Colors; Clontech) at 1:2,000 and E7 using the mouse anti-E7 (8C9, Invitrogen) at 1:200. Myc tagged constructs were detected with mouse anti-myc hybridoma lysate (clone 9E10), used at 1:200. Rabbit anti-actin (Sigma) was used at 1:2,000. Rabbit anti-p190 antibody (Bethyl Laboratories) was used at 1:3,000 and mouse monoclonal anti-RhoA at 1:500 (Cytoskeleton). Monoclonal phospho-tyrosine was used at 1:500 (Upstate). HRP-conjugated goat anti-rabbit (Jackson Laboratories), goat anti-rat (Pierce), and rabbit anti-mouse (Jackson Laboratories) were used as secondary antibodies.

4.2.4 Co-immunoprecipitation and western blot analysis

Cells were transfected in a 1:1 ratio of the myc-GFP fusion (full-length E7 or indicated fragment) and hemagglutinin (HA)-tagged binding partner (full-length p190 or indicated fragments). Cells were harvested at 24 h post-transfection by scraping and washed once with 1× phosphate-buffered saline (PBS). Cells were lysed in NP-40 (50 mM Tris, pH 7.8, 150 mM NaCl, 0.5% NP-40) lysis buffer supplemented with 1× mammalian protease inhibitor cocktail (Sigma). Typically, 1 mg of cell lysate was mixed with 100 µl of anti-myc hybridoma or 25 µl of anti-HA hybridoma and 100 µl of a 10% slurry of protein A-Sepharose resin (Sigma) and incubated at 4°C for 1-2 h on a nutator. Immunoprecipitates
were washed three times with lysis buffer and re-suspended in 2× lithium dodecyl sulfate (LDS) sample buffer, then boiled for 5 min. Samples were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (GE), and blocked in 5% nonfat milk in Tris-buffered saline-Tween 20. Western blot analyses were carried out with mouse anti-myc or rat monoclonal anti-HA (clone 3F10 Roche) antibodies. For detection of p190 interaction with E7, CaSki cell lysates were subjected to immunoprecipitation using 1 µg of anti-E7 antibody (8C9).

4.2.5 RhoGTP assay

Cellular RhoGTP was measured by pull-down with the Rho binding domain (RBD) of Rhotekin fused to GST (GST-RBD), as suggested by the manufacturer (Cytoskeleton). Briefly, clarified cell lysates were prepared from two 10-cm dishes for each sample by lysing in 800 µl of cell lysis buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 0.5 M NaCl, 2% Igepal, supplemented with protease inhibitors) and were then incubated with 30 to 50 µg of GST-RBD bound to glutathione-Sepharose beads for 1 h at 4ºC. Beads were washed once with wash buffer (25 mM Tris pH 7.5, 30 mM MgCl₂, 40 mM NaCl), and analyzed by immunoblotting for RhoA. Densitometry of digital images was carried out using Image J, and the experiments were performed three independent times.

4.2.6 p190 RhoGAP assays

Recombinant GST-RhoA-Q63L was produced in Escherichia coli BL21 (DE3). Briefly, transformed E. coli cells were grown to an OD₆₀₀ of 0.6-0.7, and then induced overnight at 16ºC with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). GST-RhoA-Q63L was purified as per protocols provided by the affinity resin manufacturer (BioBasic). To determine levels of active p190, clarified lysates prepared in lysis buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 0.5 M NaCl, 2% Igepal, supplemented with protease inhibitors) were incubated with 15 or 30 µg of GST-RhoA-Q63L for 1 h at 4ºC. The beads were washed two times with wash buffer (25 mM Tris pH 7.5, 30 mM MgCl₂, 40 mM NaCl) and
analyzed by immunoblotting with anti-p190 antibody. To assess the phosphorylation status of p190 in E7 expressing cells, U2OS cells were transfected with 2 μg of HA-p190 and 6 μg of GFP or GFP-E7. 48 h post-transfection, the lysates were prepared in NP-40 lysis buffer, and subjected to immunoprecipitation with anti-HA antibody, followed by immunoblotting as indicated.

4.2.7 Immunofluorescence and image analysis

Cells were fixed with 3.7% formaldehyde for 20 min at room temperature, then permeabilized with 0.2% Triton X-100 for 20 min at room temperature. Samples were extensively washed with 1x phosphate-buffered saline (PBS), pH 7.4, and once with 0.1 M glycine (in PBS). The slides were then incubated with Alexa-568 phalloidin (Invitrogen) at 1:100 (in PBS) for 1 h at 37°C in a humidity chamber. The slides were washed extensively with PBS, and mounted on glass slides with ProLong Antifade with DAPI (4’,6-diamidino-2-phenylindole) mounting medium (Invitrogen). Imaging was performed using a Nikon Eclipse Ti-S fluorescence microscope equipped with a QImaging Retiga 1300-coded monochrome 12-bit camera. Images were captured using Velocity software, version 7.0. Confocal images were acquired with a Fluoview 1000 laser scanning confocal microscope (Olympus Corp), using the x60 Plan Apochromat 1.42 oil objective. Multicolor images were acquired in the sequential acquisition mode to avoid cross-excitation. Quantification of phalloidin levels was carried out using Image J, as previously described (54). Briefly, the cell perimeter was marked and integrated densities or integrated densities per area were calculated. Data is representative of a minimum of 75 cells for each sample. Experiments were performed three independent times.
4.3 Results

4.3.1 HPV16 E7 associates with p190RhoGAP

We initially observed that p190 associates with HPV16 E7 in mass spectrometry experiments performed on cell lysates prepared from HEK293 cells transfected with epitope tagged E7 that were immunoprecipitated for the epitope tag (data not shown). p190 was an intriguing interacting partner for E7 due to previous observations that p190 functions as a tumor suppressor, leading us to hypothesize that deregulation of p190 contributes to the oncogenic properties of E7. As an initial step in confirming this novel binding partner of E7, we utilized two human cervical cancer cell lines; CaSki cells are HPV16 positive and express E6 and E7 oncoproteins, whereas C33A cells are HPV negative, and served as a negative control. We carried out co-immunoprecipitation experiments using extracts from both CaSki and C33A cells, and anti-E7 antibody, and then performed a western blot with anti-p190 antibody. This experiment confirmed that p190 associates with E7 under endogenous condition (FIG. 4-1). Interestingly, a recent study using mass spectrometry identified 1079 cellular proteins targeted by various tumor virus oncoproteins; in that study, p190 was also identified specifically as a target of HPV16 E7 (45).

4.3.2 C-terminal sequences of HPV16 E7 are necessary and sufficient to bind p190RhoGAP

We next identified which residues within E7 are important for interaction with p190. Firstly, we utilized co-immunoprecipitation experiments and two fragments of HPV16 E7 fused to myc-GFP. Residues 1-39 span the intrinsically disordered CR1 and CR2 of E7, whereas residues 39-98 contain CR3 of E7, the highly structured zinc-binding domain. We co-transfected human fibrosarcoma HT1080 cells with HA-tagged p190 and the individual myc-GFP-E7 constructs, and carried out co-immunoprecipitation experiments using anti-HA antibody. These experiments revealed that CR3 of E7 is both necessary and sufficient for interaction with p190 (FIG. 4-2A). In addition, we carried out these
FIG. 4-1. HPV16 E7 associates with p190RhoGAP (p190). Lysates from HPV16 positive (CaSki) and HPV negative (C33A) cells were prepared and immunoprecipitated for E7 as described in materials and methods. Samples were immunoblotted as indicated.
FIG. 4-2. Conserved region 3 (CR3) of HPV16 E7 and middle domain (MD) of p190 are sufficient for interaction. (A) CR3 of HPV16 E7 (residues 39-98) is necessary and sufficient to associate with p190. HT1080 cells were transfected with full-length HA-tagged p190 and indicated GFP or GFP-E7 fusion construct (E7, E7 1-39, E7 39-98 are all GFP fusions). 24 h post-transfection, cell lysates were prepared and subjected to co-immunoprecipitation as indicated. (B) The middle domain of p190 is sufficient to associate with E7 (residues 39-98). HT1080 cells were transfected with indicated HA-tagged p190 constructs and GFP or GFP-E7 39-98. Samples were subjected to co-immunoprecipitation as indicated.
experiments in a reciprocal manner, immunoprecipitating for E7 and blotting for p190, with a similar result (data not shown). Furthermore, we also mapped the region of p190 which is sufficient to associate with HPV16 E7. We utilized three fragments of p190; the GTP-binding domain (GBD), residues 1-266, the middle domain (MD), residues 397-1183 and GTPase activating protein domain (GAP), residues 1180-1513. Co-immunoprecipitation experiments with E7 residues 39-98 revealed that the middle domain, a region of p190 which is known to contain many protein-protein interaction motifs, is sufficient to associate with E7 (FIG. 4-2B) (28, 30, 58).

4.3.3 Expression of HPV16 E7 decreases RhoA-GTP levels and leads to reduced stress fiber formation

In order to begin to understand the biological significance of the E7-p190 interaction, we first wondered whether expression of E7 leads to changes in steady-state levels of p190 or whether it could influence activation of this GAP. We compared the steady-state levels of p190 in human osteosarcoma U2OS cells transfected with E7 or GFP and found that there was no difference between the two samples (FIG. 4-3A). Thus, the interaction of E7 with p190 likely does not stimulate p190 degradation as observed for pRb. To assess the status of p190 activation in E7 expressing cells, we carried out two types of experiments; since p190 is a direct negative regulator of RhoA, we assessed the ability of this GAP to associate with RhoA in presence of E7. For that purpose, we utilized a constitutively active mutant of RhoA, Q63L, fused to GST, and carried out GST-pull-down experiments. We found that there was no significant difference in the ability of p190 to associate with active RhoA in the presence of E7 when compared to the control (FIG. 4-3B), suggesting that E7 may not impact the ability of p190 to associate with active RhoA. In addition, we assessed the amount of phosphorylated p190 in E7 expressing cells by immunoprecipitating p190 from control and E7 expressing cells, followed by western blotting with a phospho-tyrosine antibody (FIG. 4-3C). We found no evidence that there were any changes in phosphorylation status of p190 upon expression of E7.
FIG. 4-3. HPV16 E7 deregulates RhoA GTPase activity. (A) Expression of E7 does not affect steady-state levels of p190. U2OS cells were transfected with GFP or GFP-E7 expression vectors. 48 h post-transfection, equal amount of cell lysates was subjected to immunoblotting as indicated. (B) E7 does not affect association of p190 with RhoA. U2OS cells were transfected and processed as indicated in (A), and lysates were subjected to GST-pull-down using purified GST or GST-RhoA-Q63L (constitutively active RhoA), following by immunoblotting as indicated. (C) E7 does not affect p190 phosphorylation levels. U2OS cells were transfected with 2 μg of HA-p190 and 6 μg of GFP or GFP-E7 expression plasmids. 48 h post-transfection, lysates were subjected to immunoprecipitation using anti-HA antibody, then immunoblotted as indicated. (D) Expression of E7 in U2OS-neo and U2OS-E7 cells. Equal amount of cell extract from U2OS-neo and U2OS-E7 cells was analyzed by immunoblotting as indicated. (E) Expression of E7 affects RhoA-GTP levels. Equal amounts of cell extract from U2OS-neo and U2OS-E7 cells were subjected to GST-pull-down with GST-RBD in order to assess the levels of RhoA-GTP, as described in materials and methods. (F) Exogenously expressed E7 affects RhoA-GTP levels. U2OS cells were transfected with GFP or GFP-E7 expression vectors. 48 h post-transfection, cell lysates were prepared and analyzed for RhoA-GTP levels as described in materials and methods section.
FIG. 4-4. HPV16 E7 disrupts actin stress fibers. (A) U2OS-neo and U2OS-E7 cell were stained with phalloidin-Alexa 568 to visualize F-actin. The nuclei were stained with DAPI; images were acquired on the confocal microscope. (B) Quantification of phalloidin intensity in U2OS-neo and U2OS-E7 cells; ***p<0.001. Data are representative results from three independent experiments. (C and D) U2OS cells were transfected with GFP or GFP-E7, and 24 h later cells were processed as described above (A and B, respectively); ***p<0.001
In contrast, when we assessed the levels of active RhoA (RhoA-GTP), the direct target of p190, in U2OS cells stably expressing E7, we found a significant decrease in RhoA-GTP levels using a GST-RBD pull-down assay (FIG. 4-3D and E). Similarly, we found that expression of exogenous E7 in U2OS cells can also lead to decreased RhoA-GTP levels (FIG. 4-3F).

It has been well established that organization of actin filaments is controlled by the Rho family members (Rho, Rac, and Cdc42) (5, 26). RhoA regulates the assembly of actin stress fibers and focal contacts through the activation of the downstream effectors mDia and ROCK kinase (1, 57). Due in part to the observation that E7 expression leads to decreased RhoA-GTP levels and due to a recent report that the low-risk HPV38 E7 disrupts actin stress fibers by lowering the level of F-actin (62), we hypothesized that HPV16 E7 may also disrupt actin stress fibers. We therefore assessed the matched U2OS-neo and U2OS-E7 cell lines for F-actin by staining with Alexa-568 conjugated phalloidin. This staining revealed that U2OS-neo cells contained much more actin stress fibers than did U2OS-E7 cells. The actin stress fibers were more frequently present throughout the cytoplasm of U2OS-neo cells, while such an organization was more frequently missing or altered in U2OS-E7. In those cells, F-actin was localized mainly at the periphery of the cells in a cortical pattern (FIG. 4-4A). Quantification of the actin stress fiber loss is presented in FIG. 4-4B, and a statistically significant reduction was observed in E7 expressing cells. Similarly, expression of exogenous HPV16 E7 fused to GFP in U2OS cells leads to statistically significant disruption of actin stress fibers compared to cells expressing GFP only (FIG. 4-4B and D).

4.3.4 HPV16 E7 mutants V55T and R66E do not associate with p190RhoGAP

The reduction of actin stress fiber formation by HPV16 E7 could occur independently of its interaction with p190. We questioned whether the interaction of E7 with p190 could be contributing to RhoA deregulation and actin stress fiber formation. We utilized a panel of E7 mutants targeting CR3 residues which are surface-exposed (53), to map the surface of E7 required for p190 interaction and determine if these residues correlated with the
FIG. 4-5. Residues V55 and R66 in HPV16 E7 are required for association with p190. (A) HT1080 cells were transfected with full-length HA-tagged p190 and GFP or GFP fused to residues 39-98 of HPV16 E7 containing the indicated mutations. Co-immunoprecipitation experiments were carried out 24 h post-transfection, as indicated. (B) HT1080 cells were transfected with full-length HA-tagged p190 and full-length GFP-E7 wild-type, V55T or R66E mutants, and samples were processed for co-immunoprecipitation as indicated in (A). (C) V55 and R66 residues form a patch on the surface of HPV16 E7 CR3.
FIG. 4-6. Interaction with p190 is required for HPV16 E7 to deregulated RhoA-GTP levels and actin stress fibers. (A) HPV16 E7 mutants unable to bind p190 do not effectively reduce RhoA-GTP levels. U2OS cells were transfected with expression plasmids for GFP or the indicated GFP-E7 fusions. Forty eight hours post-transfection, RhoA-GTP levels were assessed as indicated in the materials and methods section. The bar graph represents normalized (to total RhoA) RhoA-GTP levels. (B) HPV16 E7 mutants unable to bind p190 do not efficiently disrupt actin stress fibers. U2OS cells were transfected with the indicated plasmids, and visualized for F-actin with phalloidin-Alexa 568. Nuclei were stained with DAPI. (C) Quantification of phalloidin staining in transfected U2OS cells. Phalloidin intensity was quantified as described in the materials and methods; data are representative of three independent experiments; **p=0.002, ***p<0.001
ability to alter F-actin. Initially, we co-transfected full-length p190 and twenty one point mutants of E7 in the context of residues 39-98, and carried out co-immunoprecipitation experiments. This approach revealed that two mutants, V55T and R66E completely lost binding to p190 (FIG. 4-5A). Additionally, we confirmed these findings using the V55T and R66E in the context of full-length E7 (FIG. 4-5B). Interestingly, V55T and R66E form a small patch on the surface of E7 CR3 (FIG. 4-5C). The identification of mutants unable to bind p190 provided us with the necessary tools to study the significance of E7-p190 interaction.

4.3.5 Mutants of E7 unable to associate with p190RhoGAP do not effectively deregulate RhoA and stress fiber formation

In order to establish whether the interaction of E7 with p190 could be contributing to deregulation of the RhoA pathway, we utilized the two mutants, V55T and R66E, which failed to associate with p190. We tested them for their effects on RhoA-GTP levels in transfection-based assays similarly to what was described for wild-type E7 (section 4.3.3). We found that both V55T and R66E had decreased ability to deregulate RhoA-GTP levels as compared to wild-type E7 (FIG. 4-6A). Like wild-type E7, neither mutant affected the steady state levels of p190. In addition, U2OS cells transfected with GFP-E7 V55T or R66E, were also assessed for levels of F-actin by staining the cells with phalloidin. We found that F-actin levels in these cells correlated with RhoA-GTP levels; both V55T and R66E had more stress fibers, with quantified phalloidin levels resembling the cells expressing GFP only (FIG. 4-6B and C). These data strongly suggest that the association of E7 with p190 is important for deregulating the RhoA pathway.

4.3.6 The interaction of E7 with p190 is evolutionarily conserved

Since this is the first report illustrating that HPV16 E7 associates with p190, we next aimed to establish whether the E7 proteins of other HPVs similarly bind p190. For that purpose, we cloned thirteen other E7 oncoproteins from their respective genomes. These
FIG. 4-7. The E7 proteins of multiple HPV species interact with p190. (A) E7 proteins from other HPV species and types associate with p190. HT1080 cells were transfected with HA-tagged p190 and GFP or GFP-E7 fusions, as indicated. 24 h post-transfection, cell lysates were prepared and subjected to co-immunoprecipitation using anti-HA antibody. These samples were immunoblotted as indicated. (B) Multiple sequence alignment of HPV E7 CR3. The residue numbering indicated on the top line corresponds to HPV16 E7. Darker shading corresponds to more highly conserved residues. The position of residues V55 and R66 is indicated at the bottom of the alignment (*).
included low-risk species 10 types HPV6, HPV11, HPV55 and HPV74, high-risk species 7 types HPV18, 39, 45 and HPV59, and high-risk species 9 types HPV31, HPV33, HPV52, HPV58 and HPV67. We co-transfected these E7 oncoproteins together with p190 into HT1080 cells and carried out co-immunoprecipitation experiments. We found that all tested E7 proteins, except for HPV59, associate with p190, illustrating that this interaction is highly evolutionarily conserved (FIG. 4-7A). Low-risk species 10 E7 oncoproteins seem to bind p190 with the highest affinity. On the other hand, high-risk species 7 E7 proteins, which also include HPV59, appear to bind p190 with the lowest affinity. Interestingly, this low affinity binding is illustrated further by the observation that in reverse immunoprecipitation experiments, we could not detect the interaction between species 7 E7 proteins and p190 (data not shown). What is more intriguing is the observation that species 7 E7 oncoproteins contain Q or E at the position corresponding to residue R66 in HPV16. An arginine at this position is perfectly conserved across all the other tested E7 proteins, and was also one of the two residues identified as important for p190 binding in HPV16 (FIG. 4-7B and 5A). This confirms the importance of R66 in this interaction but residual binding in species 7 E7 proteins suggests that they may bind p190 in a slightly different manner. Overall these experiments illustrate that association with p190 is an evolutionarily conserved target of the E7 proteins of multiple HPV species and may likely represent an important target for HPV infection.

4.4 Discussion

The viral life cycle, and ultimately cell transformation by these pathogens, depends on the ability of the virus to alter a number of normal cellular functions, including cell cycle regulation, leading to uncontrolled cell proliferation. Remodeling of the actin cytoskeleton is another key event accompanying viral replication and the cell transformation process (41, 42, 55). Many pathogens, including viruses, have evolved gene products to engage and subvert the actin cytoskeleton, and in particular, the Rho-family GTPase signaling system (15, 22, 38). The actin cytoskeleton is highly dynamic and is primarily manipulated by members of the Rho-family GTPases that control signal
transduction pathways linking membrane receptors to the cytoskeleton. Rho-family GTPases regulate several cellular processes, including F-actin polymerization, cell polarity, and cell migration. Among the known Rho-family GTPases, RhoA is responsible for the formation of stress fibers. Rous sarcoma virus, simian virus 40, polyomaviruses, adenoviruses as well as human papillomavirus have all been shown to encode viral gene products which manipulate the Rho-family GTPase signaling pathway (9, 17, 21, 35, 61). Of particular relevance for this report is the observation that the cutaneous beta HPV38 E7 protein affects actin stress fiber formation. This event is associated with enhanced cell proliferation, suggesting a key role of actin filament remodeling during cell transformation induced by HPV38 (62). Disruption of actin stress fibers was shown to be dependent on inhibition of RhoA activity (62). In addition, the mucosal high-risk HPV16 E7 has been implicated in deregulating both RhoA and Rac1 GTPases (9, 61). Specifically it was shown that RhoA activity is inhibited by E6/E7 expression and that this induces cell migration by enhancing the cytoplasmic accumulation of p27Kip1 (9). Rac1 is also overexpressed in recurrent respiratory papilloma tissue, leading to deregulation of the Rho-family GTPase signaling pathway and resulting in elevated cyclooxygenase 2 expression (61). Moreover, possible contribution and importance of the RhoA pathway in HPV induced transformation is further illustrated by recent findings that primary human keratinocytes can be effectively immortalized by treatment with the Rho kinase inhibitor (Y27632) (8).

In this report, we show that HPV16 E7 interacts with a potent inhibitor of RhoA, the p190RhoGAP protein (4). Interestingly, this interaction was independently identified in a recent study as one of several GAPs targeted by viral proteins (45). Aside from the association of p190 with HPV16 E7, Epstein-Barr virus proteins and SV40 small t-antigen were shown to associate with Cdc42GAP, whereas HPV11, 6 and 8 E7 proteins also bind MgcRacGAP (45), although the functional consequence of these interactions is not known. We show that E7 binds p190 under endogenous conditions in HPV16 positive cervical cancer cells (FIG. 4-1). We also show that the E7 proteins of diverse species and types, including high- and low-risk, all bind p190, suggesting that it is an important target of E7 (FIG. 4-7A). Co-immunoprecipitation experiments revealed that E7 CR3 was necessary and sufficient to interact with p190 (FIG. 4-2A). Additionally, we showed that
the middle domain of p190 was sufficient to associate with E7 residues 39-98 (FIG. 4-2B). Interestingly, this region of p190 contains many protein-protein interaction motifs and contains the major site of tyrosine phosphorylation (Tyr 1105) (27, 28, 33, 44). Among the interactions made by this portion of p190, phospho-tyrosine 1105 serves as the major binding site for p120RasGAP (44). Phosphorylation of tyrosine 1105 in p190 by Src family kinases and other tyrosine kinases results in activation of p190’s RhoGAP activity, and concomitant inhibition of p120RasGAP activity of bound p120. This phosphorylation event therefore co-regulates signaling through the Ras (upregulated) and Rho (downregulated) pathways. Targeting of the middle domain by E7 could potentially have an impact on the ability of p190 to associate with other binding partners including tyrosine kinases and p120RasGAP.

In order to begin to decipher the functional consequences of the E7-p190 interaction, we first assessed whether there are any effects on steady state level of p190 by exogenous introduction of E7. We did not find any significant changes to the steady state levels of p190 (FIG. 4-3A), suggesting that E7 is not targeting p190 for degradation, nor impacting the expression levels of this GAP. However, treatment of E7-expressing cells with cycloheximide and assessment of p190 half-life in comparison to control cells would illustrate whether E7 has an impact on p190 turn-over. Although, the steady state levels of p190 do not change in cells expressing E7, we wondered if E7 was affecting the levels of active p190. To assess this, we first utilized a constitutively active mutant of RhoA (Q63L) which specifically associates with active p190, and carried out GST-pull-down experiments. This approach quantitatively recovers total active p190 in the cell lysate. In these experiments, we did not find any significant changes in active p190 levels when compared to the control, suggesting that E7 may not be affecting p190 activation and/or the ability of this GAP to bind RhoA (FIG. 4-3B). Despite similar overall levels of active p190, it remains possible that E7 is affecting p190 activation spatially and/or temporally. For instance, kinase mediated activation of p190 upon phosphorylation of tyrosine 1105 does not necessarily lead to increased catalytic activity of p190, but rather leads to increased association with p120RasGAP and increased localization of p190 to the cell periphery where it can associate with RhoA. For this reason, we also determined the level of p190 phosphorylation in E7 expressing cells, but found no significant change with
respect to control cells (FIG. 4-3C). Taken together, these finding suggest that E7 does not impact p190 activation. Perhaps E7 is impacting the association of p190 with its binding partners, such as p120RasGAP, and hence localization to the membrane. Therefore, it would be interesting to pursue future studies to assess the capacity of p190 to associate with partners such as p120RasGAP. Furthermore, determining the levels of Ras-GTP and Rac1-GTP would allow us to assess whether the interaction of E7 with p190 is manipulating multiple small GTPase pathways.

It has been well established in the literature that E7 transformation requires activation of the Ras pathway (13). However, the impact of E7 on the RhoA GTPase has not been investigated. A recent report has shown that human keratinocytes expressing HPV16 E7 have reduced RhoA-GTP levels, and that this is due to E7-induced localization of p27\(^{kip1}\) to the cytoplasm where it can bind and dysregulate RhoA (9). Additionally, a report examining cutaneous HPV38 E7 has clearly established that E7 alone is sufficient to reduce RhoA-GTP levels. We therefore investigated whether HPV16 E7 is also sufficient to induce a reduction in RhoA-GTP. For this purpose, we utilized U2OS cells stably expressing HPV16 E7 and we performed pull-down experiments using GST-RBD. Like the previous two reports, we found that cells expressing HPV16 E7 have a significant reduction in RhoA-GTP (FIG. 4-3E). In order to rule out any cell line specific effects on these observations, we also expressed E7 exogenously, and carried out the same experiments with similar findings (FIG. 4-3F). Because RhoA is the main GTPase regulating the actin cytoskeleton, we also assessed the status of stress fibers in E7 expressing cells. We stained for F-actin and examined U2OS-neo and U2OS-E7 cells for the appearance and abundance of actin stress fibers (FIG. 4-4A). E7 expressing cells showed fewer and more disorganized actin stress fibers, and also more frequently exhibited strong staining around the cell periphery. Quantification of phalloidin staining (FIG. 4-4B) revealed that E7 expressing cells have reduced F-actin levels, and this correlated with the observations presented in FIG. 4-4A. It would have been useful to include an inhibitor of RhoA in these experiments, in order to illustrate that the observed changes in F-actin levels occurred as a result of RhoA. However, these findings are in agreement with the observation that E7 expressing cells have reduced RhoA-GTP levels (FIG. 4-3E). Furthermore, analysis of actin stress fibers and phalloidin intensity in cells
transfected with HPV16 E7 revealed similar findings to those observed for stable cell lines, and once again correlated with reduced RhoA-GTP levels (FIG. 4-4D and FIG. 4-3F).

We questioned whether the interaction of E7 with p190 could be contributing to reduced RhoA-GTP levels and stress fibers. For that purpose, we utilized a panel of E7 mutants targeting surface-exposed residues in CR3 of E7 (53). Since we had established that CR3 of E7 is necessary and sufficient to associate with p190, our aim was to identify mutants of E7 which are unable to bind p190. Using co-immunoprecipitation experiments, we identified two mutants, V55T and R66E that were unable to bind p190 (FIG. 4-5A and B). When transfected into U2OS cells, these mutants had a significant reduction in the ability to downregulate RhoA-GTP (FIG. 4-6A), as well as actin stress fibers (FIG. 4-6B and C). V55T and R66E RhoA-GTP levels and quantified phalloidin levels more closely resembled those found in cells expression GFP rather than cells expressing E7. These data strongly suggest that the interaction of E7 with p190 contributes to deregulation of the RhoA pathway. However, the mechanism by which E7 utilizes p190 for this function remains unknown.

Since E7 deregulation of the RhoA pathway and the actin cytoskeleton could contribute to carcinogenesis, we also wondered if the interaction with p190 was conserved across multiple HPV species and types. We tested the ability of 13 different E7 proteins to associate with p190. These included four low-risk, species 10 E7s, four high-risk, species 7 E7s, including HPV18, and five other high-risk species 9 E7s which are most highly related to HPV16. Using co-immunoprecipitation assays, we found that the interaction with p190 is highly conserved, with all HPV E7 proteins tested interacting with p190, except for species 7 HPV59 (FIG. 4-7A). In general, the E7 proteins from species 7 HPVs interacted with p190 with the lowest affinity of all the E7 proteins tested. Interestingly, residues V55 and R66 (HPV16 E7) which we identified to be necessary for interaction with p190 in HPV16 E7 are highly conserved (FIG. 4-7B), with the exception of the weakly interacting species 7 members. Members of species 7 contain an acidic residue (E or Q) at the position corresponding to R66, which may account for their reduced interaction.
In conclusion, we have identified that p190, an upstream negative regulator of RhoA, is a novel interacting partner of the E7 proteins of multiple HPV types. Our data strongly suggests that p190 is utilized by E7 to regulate the RhoA GTPase and alter the actin cytoskeleton. Our findings suggest that E7’s deregulation of RhoA influences the growth and migration properties of HPV infected and transformed cells, which may be exploited for therapy.

4.5 References


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Chapter 5    GENERAL DISCUSSION

5.1 Thesis Summary

Much of the existing literature examining the role of the E7 CR3 domain has utilized mutations that target the most highly conserved amino acid residues in this domain. The logical reasoning behind the construction and use of such mutants at the time rested on the assumption that the most highly evolutionarily conserved residues must be functionally important. However, this assumption does not take into consideration that the evolutionary conservation of amino acid residues may also be the consequence of structural integrity. Indeed, X-ray crystallography and NMR studies of CR3 region from two different E7 proteins have revealed that the most highly conserved residues participate in the formation and stabilization of E7 dimer (21, 27). This therefore raises the question of whether the observed defects for many of these mutants were truly functional or whether they could be due to structural impairments.

In the studies described in previous chapters, our primary aim was to utilize mutants which are less likely to impact the structure of CR3. In Chapter 2, we describe the construction of 21 novel HPV16 E7 CR3 mutants. We used the existing structures for HPV45 and HPV1 to model the structure of HPV16 E7 CR3, and identify putative surface-exposed residues which were subsequently targeted for mutagenesis. We therefore, targeted residues which are likely available to interact with host proteins and are less likely to be structurally important. In Chapter 2, in addition to the panel of novel surface-exposed residues, we also utilized mutants which target the highly conserved hydrophobic residues that are not exposed to solvent. We first assessed the dimerization potential of each solvent exposed and hydrophobic core mutant; we then correlated dimerization data with transformation capacity of each construct in order to establish whether dimerization was functionally important.

In Chapter 3, we utilized the same panel of putative surface-exposed mutants to assess the role that CR3 plays in deregulating the retinoblastoma tumor suppressor (pRb). Although evidence has suggested that CR3 is necessary to deregulate pRb, precisely how
it contributes to this process has not been established. In order to better understand the contribution of CR3 to pRb deregulation, we assessed the role of surface-exposed CR3 mutants in binding to pRb, overcoming its function and inducing its degradation.

In Chapter 4, we identified a novel interacting partner of E7, p190RhoGAP (p190). In this chapter, we illustrate how the panel of surface-exposed mutants can be effectively used as tools to study not only existing interactions, but to dissect and decipher the importance of novel interacting partners, such as p190. We showed that CR3 of E7 is necessary and sufficient for this interaction. We then went on to show that two residues in CR3 are important for p190 binding, and we utilized these mutants to assess the role of E7-p190 interaction on RhoA related functions.

Overall, the work completed here expands our knowledge of E7 function and illustrates that the panel of surface-exposed residues we generated are valuable tools. Our analysis of the structure-function of E7 illustrates for the first time that dimerization is not necessary for the transformation process. Our analysis of the role of CR3 in deregulating pRb function adds another layer to this process which was previously unknown, illustrating that the interaction of CR3 with pRb is functionally important. Lastly, identification of p190 as a novel interacting partner of E7 highlights that E7 deregulates the RhoA pathway and the cytoskeleton, a largely unexplored niche in the HPV E7 literature.

5.2 Structure-Function Analysis of Amino Acid Residues of Human Papillomavirus 16 E7 Conserved Region 3

The HPV16 E7 C-terminus contains two Cys-X-X-Cys motifs which are involved in binding to a zinc ion and mediating proper folding and dimerization of this viral protein. Point mutations in either one or both of the metal-binding motifs significantly reduce or abolish the ability of E7 to transform a variety of rodent cells (23, 33) and to immortalize primary human keratinocytes (19). Based on these observations, it was suggested that dimerization of E7 may be important for at least some of its biological activities.
Subsequently, studies with chimeric proteins, composed of CR1 and CR2 of E7 and of the C-terminal region of Ad E1A, which also contains two Cys-X-X-Cys motifs, showed that this chimeric protein retains transforming activity, but no longer dimerizes (5). These findings indicated that the zinc-binding motifs are more likely required for E7 to maintain a proper folded state, rather than play a direct role via dimerization, for its transforming activity. The debate over whether or not native E7 must exist as a dimer to function had remained.

Early work that addressed the potential of HPV16 E7 to dimerize in vivo used the yeast two-hybrid system (7, 36). Unfortunately, the extent of the studies was limited to only four mutations in CR3: L67R, R77G, C91G and C58S/C91S. Each of the four mutants was co-expressed in *Saccharomyces cerevisiae* with the full-length wild-type E7 protein, and protein-protein interactions were analyzed by assessing the activity of a reporter gene. Three of the four tested mutations (L67R, C91G and C58S/C91S) in the carboxyl terminus had lost their ability to bind to the wild-type E7 protein. In contrast, the R77G mutant interacted strongly with wild-type E7. Based on the structure of CR3, it is now understandable why the mutation at R77, which targets a solvent exposed residue, would not affect dimerization of E7. The other mutations are buried (L67) or are necessary to coordinate the zinc ion (C58, and C91) in the protomer. Nevertheless, observations from these studies led the authors to propose that the CR3 region of E7 is a major determinant for dimerization of this protein.

The studies discussed above primarily utilized mutations in highly conserved amino acid residues of CR3. These residues were thought to have a critical role in E7 function, because of their strong conservation among E7 proteins from different HPV types. We now know that most of these mutations disrupted the structural integrity of E7. At the time, it was difficult to overcome this obstacle, since no structure of E7 had been solved. However, with the availability of structures for E7 from HPV1 and HPV45 E7, it was possible for us to accurately model the CR3 structure for the highly related HPV16 E7 protein. With the model for HPV16 E7 CR3 at hand, the experiments conducted in Chapter 2 represent the first structure-based mutational analysis of the dimerization properties of this viral protein.
Using our proposed structure, substitution mutations could be introduced in all solvent-exposed residues in CR3. Our rational mutagenesis strategy aimed to maintain solvent interaction of each mutant, hopefully preserving the structure of CR3. Although this was a sensible approach, our strategy could potentially lead to abrogation of existing or introduction of new post-translational modification sites. For instance, mutagenesis of residue V55, C59, V74 or M84 to a threonine or serine could result in a protein that can now be targeted for phosphorylation. Alternatively, a substitution in residues such as T64, S63, T72, or T86 to an aspartic acid has the potential of creating a “phospho-mimic,” resulting in an E7 mutant protein that constitutively acts like its phosphorylated wild-type counterpart. Additionally, introductions of new lysine residues such as in the case of E80K/D81K could result in a new ubiquitination site. The above mentioned impact on post-translational modifications should be kept in mind when examining the results obtained using the panel of surface-exposed mutants.

In Chapter 2 we tested the new panel of surface-exposed mutants, alongside mutations targeting highly conserved CR3 residues for dimerization. Overall, this analysis resulted in evaluation of an extensive number of residues for their involvement in maintaining E7 as a properly folded protomer and/or their participation in stabilizing the dimer. The yeast-two hybrid system is a relatively simple and quick assay, ideally suited to screen a large number of mutants for their ability to dimerize. In addition, it has been used by two other groups for this purpose (7, 23). Our yeast two-hybrid data for the hydrophobic, structurally important residues was in perfect agreement with our hypothesis. As expected, all but one of the ten tested mutants targeting zinc-coordinating cysteines or the highly conserved hydrophobic residues failed to dimerize. The yeast two-hybrid data for the novel panel of surface-exposed mutants also identified some mutations which failed to dimerize, but also some which dimerized better than the wild-type (FIG. 2-2). This unexpected finding prompted us to verify if the surface-exposed mutants truly fail to dimerize using an in vitro approach. In this system we found that all mutants tested, except for one (R66E) had reduced dimerization (FIG. 2-3). This data provides evidence that some residues on the surface of E7 can perturb folding of the protomer, and hence lead to reduction in the ability to dimerize. It is also important to note that some of these
residues are located close to the interface of the two protomers, and could therefore participate in stabilizing the dimer.

Having extensively assessed dimerization potential of each mutant, we also assessed the transformation potential in cooperation with activated \textit{ras}. We assessed the ability of wild-type or mutant E7 in cooperation with \textit{ras} to form colonies. We found that some mutants targeting surface-exposed residues were better transformers than the wild-type E7 in this assay, and we identified two mutants with reduced capacity to transform (M84S and QKP96-98AAA). Throughout this work, we did not identify any clear mechanisms as to why these two mutants have reduced transformation potential. It is likely that these two mutants have lost the capacity to associate with other targets of E7 which are important for transformation and remain to be identified.

Importantly, targeting of highly conserved hydrophobic residues resulted in greater frequency of decreased transformation capacity, with five out of ten mutants tested having decreased transformation (Table 2-2). However, when we compared the dimerization and transformation data, we were able to conclude that E7 does not require dimerization in order to transform. Correlating these two sets of data established two important phenotypes: those mutants that fail to dimerize and have decreased transformation potential (M84S, C58G/C91G, C91G, and L79A mutants) and, notably, numerous mutants that fail to dimerize yet still transform cells as well as wild-type E7 (F57A, R77E, G85A, T86D, I76A, L87A, and I89A/V90A mutants). The second class of mutants clearly illustrates that E7 does not need to exist as a dimer in order to transform BRK cells. However, assessing our panel of mutants in other functional assays such as anchorage independent growth in soft-agar or the capacity of these to cooperate with E6 to induce immortalization of primary human keratinocytes may reveal other phenotypic differences not observed in BRK transformation assays.

Since the data in Chapter 2 strongly supports that dimerization of E7 is not required for oncogenic transformation of primary rodent cells, this begs the question of whether E7 exits a monomer \textit{in vivo} and whether the monomer maintains the same fold as that illustrated in structural studies for HPV1 and HPV45 CR3 dimers. If E7 was to form a
monomer, would the hydrophobic core which stabilizes the dimer and in the case where it is exposed to solvent result in unfavourable interactions, forcing E7 to assume new structure? Although we carry out thorough structure-function analysis in Chapter 2, further analysis would be useful. For instance, utilizing structural prediction software to determine the putative structures of each E7 mutant could be usefully in assessing whether any of the mutations can lead to structural changes. Furthermore, the transformation studies, although usefully for understanding the role of E7 as an oncoprotein, do not provide much information about the role of these mutants in viral life-cycle. Examining the role of E7 mutants in key events of the viral life-cycle, including immortalization, episomal maintenance, late promoter activation and infectious virion synthesis, as previously described, may reveal and identify those with defects that warrant further study (4).

In conclusion, Chapter 2 describes a panel of novel mutants of E7 which will, without a doubt, serve the HPV field in many future studies. Our systematic and rational construction of a new collection of CR3 mutants using structural information provided us with useful reagents for tackling future questions regarding protein interaction surfaces in CR3 and the functional consequences of abrogating these interactions. We further illustrate the usefulness of this panel of mutants in Chapters 3 and 4.

5.3 The Role of Conserved Region 3 in Deregulating the Function of the Retinoblastoma Tumor Suppressor

In Chapter 3, we illustrate how the panel of surface-exposed mutants we previously created can be used to address some of the ongoing questions in the field. The role of HPV E7 as an important contributing factor to cellular transformation and cancer progression has been clearly illustrated in the literature. E7’s role in binding and deregulating the retinoblastoma tumor suppressor (pRb) in the transformation process is also clearly illustrated; however, we still do not fully understand how E7 deregulates this crucial protein. E7 has an impact on pRb in multiple ways; one of these is by binding pRb and dissociating the pRb-E2F complexes. E7 also targets pRb for proteasome
degradation. However, the role of each E7 conserved region in each pRb deregulation mechanism was not well established. For instance, it is known that the E7 C-terminus is involved in E2F competition and that it binds pRb independently from the LXCXE motif. Prior to the study described in Chapter 3, CR3 had also been implicated in pRb degradation (16-18, 22, 35). CR3 was therefore suggested as an important component of multiple mechanisms by which E7 deregulates pRb. Our overall aim with the study presented in Chapter 3 was to precisely evaluate the role of CR3 in deregulating pRb function.

For the purposes of the studies described in Chapter 3, we utilized our panel of surface-exposed mutants which target the residues that are accessible for interaction with cellular partners. Contrary to most, if not all, previous studies where the role of CR3 in deregulating pRb was examined, we avoided using deletion mutants or mutants which target the highly conserved and structurally important residues of E7. Our panel of mutants allowed us to examine the role of each individual residue on the surface of CR3 in deregulating pRb. We first asked whether any of the introduced changes on the surface of E7 can lead to a reduced capacity to target pRb for degradation. We hypothesized that CR3 is important for pRb degradation based on the finding that certain mutations in CR3 lead to the loss of cullin 2 binding (18). At the time, the interaction of E7 with the cullin 2 E3 ubiquitin ligase was proposed as the primary mechanism by which E7 targets pRb for ubiquitination and proteasome dependent degradation. With our studies, we have found that the entire panel of surface-exposed mutants was as effective as the wild-type E7 in inducing pRb degradation. This data was most consistent with the previous report where even large deletion mutants in CR3 were found to effectively destabilize pRb (16). Our findings prompted us to wonder whether degradation was due to the fact that the entire panel of surface-exposed mutants maintains the interaction with cullin 2. For that purpose, we tested our set of mutants for cullin 2 binding, and found that a number of mutants had reduced association with cullin 2. These findings suggested that either low levels of cullin 2 binding were sufficient for degradation, or that E7 uses more than one mechanism to degrade pRb. With these questions in mind, we utilized a cell line in which cullin 2 was stably knocked down, and tested some of the mutants with reduced cullin 2 binding for their capacity to degrade pRb. In these experiments, we found that the tested
mutants behaved identically in knocked down and control cells. These observations suggest that HPV16 E7 is using other mechanisms to degrade pRb. This is also supported by the observation that only HPV16 E7 interacts with cullin 2 (34), yet other HPV E7 proteins also degrade pRb and illustrates the necessity to further investigate this activity of E7. Perhaps, experiments utilizing co-immunoprecipitation of E7 and associated binding partners followed by mass spectrometry analysis could identify additional targets with which E7 is associating to target pRb for degradation.

E7 CR3 was also suggested to binding to pRb independently from the high-affinity LXCXE motif, albeit only in vitro prior to the study described in Chapter 3. We aimed to better understand how CR3 contributes to pRb binding and whether there were any functional consequences from this interaction. For that purpose, the availability of the panel of CR3 surface-exposed mutants proved essential. We first confirmed that CR3 of E7 can independently associate with pRb utilizing in vitro GST pull-down assays, and mapped this interaction to the small pocket of pRb (pRbAB). More importantly, we also established that this interaction occurs in mammalian cells in vivo by utilizing co-immunoprecipitation experiments. We found that CR3 from other HPV types, specifically the low-risk HPV11 and HPV6 also associate with pRb independently from the LXCXE motif in CR2 in the yeast two-hybrid, illustrating that this interaction may be a conserved feature of the E7 proteins from multiple HPV types.

To understand what, if any, functional consequences may occur as a result of the pRb-CR3 interaction, we first sought to identify the surface of CR3 that binds pRb. We assessed our panel of surface-exposed mutants for their pRb binding properties utilizing the yeast two-hybrid assay. The yeast two-hybrid analysis identified two specific patches on the surface of CR3 that appeared to contribute to pRb binding. Similarly to the dimerization studies described in Chapter 2, the yeast two-hybrid method was a useful tool in narrowing down the potential surfaces; however, because the interaction in yeast cells may be influenced by a variety of factors, we set to confirm the identified surfaces in vitro using recombinant purified protein. We set up GST pull-down experiments at protein concentrations well below binding saturation. With this system, we found that the majority of patch 1 mutants tested behaved similarly to what was observed in the yeast
two-hybrid, although several mutants were found to have different pRb binding properties in the two systems. For instance, although R66E was shown to have greatly increased binding potential in the yeast two-hybrid, *in vitro* it was found to behave similarly to wild-type CR3. It is unclear why this discrepancy was observed, but it is most likely the result of differences between these two vastly different systems, where the yeast cell environment and hence the measured β-galactosidase activity may be influenced by the presence of yeast cellular factors.

In addition, the *in vitro* experiments were also carried out for patch 2 mutants. We focused on the three mutants that were shown to have reduced binding in the yeast two-hybrid: T72D, R77E, and G85A. T72D was confirmed to have slightly reduced binding ability, but mutations R77E and G85A were found to behave differently to what was determined in the yeast two-hybrid. G85A was found to act like the wild-type protein; R77E had increased binding capacity, and as such was found to act in perfect agreement with previous reports for this mutant and pRb binding (21). The mutant E80K/D81K maps in close proximity to patch 2 and was included as a control for these experiments. A similar mutation was found to lead to greatly decreased pRb binding (21); indeed, we have confirmed that the double mutant behaves similarly to previous report, illustrating that our *in vitro* system functions similarly to other published experiments. With the above data, we have concluded that patch 2 mutants do not form a specific surface on CR3 that contributes to pRb binding, but rather that individual residues in this area on the surface of CR3 could contribute to binding. More specifically, we hypothesize that electrostatic potential in this region contributes to pRb binding based on the observation that charge reversal in this area impacts binding.

Having established the surfaces of CR3 which contributes to pRb binding, and having determined that all CR3 mutants can target pRb for degradation, we wondered whether the CR3-pRb interaction could contribute to deregulating pRb function. Previous work has shown that CR3 of E7 is important for pRb-E2F complex interference, but that alterations in CR3 do not lead to significant changes in the ability of E7 to interfere with pRb-E2F complex formation (17, 28). Specifically, although peptides containing only the LXCXE binding motif can associate with pRb, CR3 sequences are necessary to disrupt
pRb-E2F complex. However, mutations E46A, H51A, DST62-64AAA, R66A, LRL65-67AAA, CVQ68-70AAA, THV72-74AAA, R77A, Δ75–77, and Δ79–83 all have wild-type activity in blocking pRb-E2F binding (16). Therefore, we did not expect to find any significant impact of our panel of mutants on pRb-E2F complex disruption. Our approach to assessing the contribution of CR3 to pRb deregulation was to examine the capacity of all patch 1 and patch 2 mutants to overcome cell cycle arrest that is initiated by re-introduction of pRb into pRb-null Saos2 cells. We included mutants with reduced pRb binding capacity and those that behave like wild-type, since they could serve as appropriate controls. With this analysis, we have shown for the first time that there is a high degree of correlation between the ability of E7 to bind pRb via the CR3 region and the ability of the corresponding full-length mutants to overcome cell cycle arrest. Most notably, mutations in residues within patch 1 with the most significant decrease in pRb binding also show a significant decrease in the ability to overcome a pRb induced cell cycle arrest. We also identified two potentially interesting candidate residues from the cell cycle analysis: S63D and G85A. Although, both of these had reduced pRb binding capacity in the yeast two-hybrid, in vitro both bound similarly to wild-type E7. The reduced ability of these two mutants to overcome cell cycle arrest may suggest that they have lost the capacity to interact with other important cellular targets, which contribute to E7 mediated induction of S phase of the cell cycle.

Taken together, our data illustrates for the first time that the ability of E7 to bind pRb via CR3 is important in overcoming the functions of pRb, although the mechanism remains unknown. In addition, our studies on the contribution of CR3 to pRb degradation process suggest that E7 has evolved additional mechanisms for this process, contrary to previous literature. Overall, the studies described in Chapter 3 highlight the need for further analysis and elucidation of mechanisms by which E7 can deregulate a critical tumor suppressor and ultimately contribute to carcinogenesis.
5.4 E7 Associates with a Novel Binding Partner, p190RhoGAP and Deregulates the RhoA Pathway

In Chapter 4, we illustrate how the panel of surface-exposed CR3 mutants can be used to study novel interacting partners of E7. These studies also highlight the potential importance of previously unexplored pathways in HPV life-cycle and HPV induced carcinogenesis, specifically the RhoA pathway and deregulation of this GTPase. The remodelling of the actin cytoskeleton is a key event accompanying the cell transformation process (29-31). Many pathogens, including viruses, encode gene products to engage and subvert the actin cytoskeleton (8, 14, 25), in particular by targeting Rho GTPases.

Indeed, Rous sarcoma virus (RSV), simian vacuolating virus 40 (SV40), adenovirus (Ad), Epstein-Barr virus (EBV), Hepatitis B virus (HBV) and human papillomavirus (HPV) all encode proteins which impact the cytoskeleton and contribute to transformation process. The first prominent changes in transformed cells are the rounded cell shape, with motile blebs, bulging pseudopodia, loss of contact inhibition and lack of stress fibers (13, 24, 32). The first link between viral infection, cell morphology and changes in actin cytoskeleton was observed in early 1970s with the description of transformation (12). Since then, the research has focused on how viral infection induces dramatic cytoskeletal reorganization and how these changes are related to viral life-cycle and neoplastic properties of viral infections. Work with RSV, the causative agent of chicken tumors, revealed that the viral src (v-src) gene product induces actin filament reorganization (20). v-SRC induces the loss of stress fibers, not by depolymerizing F-actin but rather by reorganizing actin polymers so that canonical stress fibers are not formed (9). In addition, the small t-antigen of the SV40 also engages the actin cytoskeleton during cell transformation. This viral oncoprotein alone is responsible for the loss of actin filaments in rat cells (15). It was demonstrated that SV40 small t-antigen affects protein phosphatase 2A (PP2A), resulting in the loss of tight junctions as well as rearrangements of F-actin networks in epithelial cells (26). These include Rac-induced membrane ruffling and formation of lamellipodia, Cdc42-initiated formation of filopodia, and loss of Rho-dependent stress fibers. In addition, Ad E1A proteins also disrupt actin stress fibers and interact with Rac-Cdc42 pathway in transformed rodent cells (3). These
interactions lead to a disorganization of the actin cytoskeleton with increased filopodia and lamellipodia, and therefore enhanced cell motility and loss of contact inhibition (11).

Although there is evidence that E6/E7 expressing cells induce cytoskeletal changes, the mechanistic details have largely remained unknown. In Chapter 4, we identified p190RhoGAP (p190) as a novel interacting partner of E7 by co-immunoprecipitation followed by mass spectrometry experiments. Based on previous observations in the literature, we found this RhoGAP to be a potentially interesting target. It has been well established that p190 functions as a major negative regulator of RhoA and therefore actin cytoskeleton remodelling and cell migration. In addition, a recent report finds that HPV38 E7 affects actin stress fiber formation, and that this event is dependent on inhibition of RhoA activity. In Chapter 4, our main objective was to characterize this novel interaction and determine its functional significance. The main points we addressed included mapping the interaction surfaces on E7 and p190, followed by assessing the functional implications of this interaction. We hypothesized that HPV16 E7 could be using the interaction with p190 to deregulate RhoA and the actin cytoskeleton, potentially enhancing the migration of E7 expressing cells.

We first established that the E7-p190 interaction occurs under endogenous conditions. In addition, we found that E7 CR3 was necessary and sufficient for this interaction, and that the middle domain of p190 bound to CR3. To begin to understand what impact E7 may have on p190, we first assessed if expression of E7 leads to changes in steady state levels of p190; we found no changes. We also found no change in the amount of active p190 by two different approaches. We did not find any change in phosphorylation of p190, or the overall level of active p190 based on the ability of this GAP to associate with constitutively active RhoA. These results suggested that E7 is not affecting activation of p190. Based on the recent report which shows that human keratinocytes expressing HPV16 E6 and E7 have reduced RhoA-GTP levels (6), the direct downstream target of p190, we next wondered if expression of E7 is sufficient to induce this RhoA phenotype. We found in two different systems that E7 expression is indeed sufficient to reduce RhoA-GTP levels. Furthermore, because RhoA is the main GTPase regulating the actin cytoskeleton, we assessed the status of stress fibers in E7 expressing cells. In agreement
with reduced RhoA-GTP levels, E7 expressing cells showed fewer and more disorganized actin stress fibers, with strongest staining around the periphery of the cell. Quantification of phallloidin staining revealed that E7 expressing cells have reduced F-actin levels. These findings correlate with reduced RhoA-GTP levels.

In order to assess the contribution of the p190 interaction on the observed cytoskeleton and RhoA phenotype induced by E7, we utilized the panel of E7 mutants targeting surface-exposed residues in CR3 of E7. Our aim was to map the surface of E7 required to associate with p190, ideally identifying mutants of E7 which are unable to bind p190. Indeed, we found that V55T and R66E are unable to bind p190. In cells expressing these mutants, R66E, and to a lesser extent V55T, had a significant reduction in the ability to downregulate RhoA-GTP, as well as actin stress fibers. These results suggest that E7 is utilizing p190 to deregulate RhoA, however, the mechanism by which this occurs remains unknown.

The relevance of Rho pathway in HPV induced cancers is highlighted by recent findings that treatment of primary human keratinocytes with Y27632, a Rho kinase inhibitor, greatly increases long term proliferation and enables them to efficiently bypass senescence and become immortalized. These findings suggest that deregulation of the Rho pathway by the viral oncoproteins may have a similar impact on primary human keratinocytes and may be an important early event in HPV induced cancers.

Although much effort has been extended on understanding the role of viral oncoproteins in tumor development, it is important to remember that the main function of HPV oncoproteins E6 and E7 is to facilitate completion of the viral life-cycle. Keeping that in mind, an interesting question to address would be the role of p190 in viral life-cycle. For instance, it has been established that RhoA inactivation by p190RhoGAP regulates cell spreading and migration by promoting membrane protrusion and polarity (1). Conversely, the importance of E6 and cell polarity in HPV life-cycle has been illustrated in organotypic raft culture studies showing that viruses defective in E6-cell polarity interactions lead to fewer cells that are capable of supporting viral DNA replication and produce less progeny viral DNA (reviewed in (2)). Hence, whether p190RhoGAP
dependent inactivation of RhoA in E7 expressing cells is important in viral life-cycle remains an intriguing question.

Overall, data presented in Chapter 4, not only illustrates the usefulness of the surface-exposed mutants of E7 in studying novel E7 targets, but also uncovers an as yet unexplored interaction of HPV E7 with a host pathway that is highly relevant to human cancer and potentially the viral life-cycle and opens the door for further studies.

5.5 Concluding Remarks

The study of viruses including small DNA tumor viruses such as HPVs, serves two main purposes; firstly, since many viruses cause disease, it is useful to understand the molecular mechanisms of viral pathogenesis in order to effectively combat these pathogens by developing anti-viral therapies. Since tumor suppressors such as pRb and p53 are most often maintained in their wild-type form in HPV positive cancers, targeting of viral oncoproteins to inhibit their functions holds great therapeutic potential. The work in this thesis identifies a novel pRb targeting mechanism; understanding the means of pRb dysregulation and the binding surfaces may help in identification of small molecule antagonists of pRb-E7 interactions as recently attempted (10). Secondly, the tremendous selection pressure exerted on viruses by the host leads to fascinating innovation at the molecular level by these pathogens. Therefore, viruses can be viewed as important biochemical tools to identify and probe essential host pathways. It is in this way that viral oncoproteins such as E7 have truly been of value, and studies of this viral protein have propelled our understanding of many cellular processes, including those which are essential to tumor development, and will likely continue to do so.
5.6 References


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Publications


**Selected Conference Presentations**

2012 – *DNA Tumor Virus Meeting, Montreal, Canada*
Oral presentation - Conserved region 3 of human papillomavirus 16 E7 contributes to deregulation of the retinoblastoma tumor suppressor

Poster presentation - The Role of the C-terminal Region of HPV 16 E7 Oncoprotein in Targeted Deregulation of pRb Tumor Suppressor

2011 – *DNA Tumor Virus Meeting, Trieste, Italy*
Oral presentation - Systematic analysis of the amino acid residues of human papillomavirus 16 E7 conserved region 3 involved in dimerization and transformation

2010 – *Infection and Immunity Research Forum, London, Canada*
Poster presentation - Systematic analysis of the roles of solvent-exposed residues in the CR3 region of the HPV 16 E7 protein

2010 – *DNA Tumor Virus Meeting, Madison, U.S.A.*
Poster presentation - Systematic analysis of the roles of solvent-exposed residues in the CR3 region of the HPV 16 E7 Protein

2010 – *Oncology Research and Education Day, London, Canada*
Poster presentation - Systematic analysis of the roles of solvent-exposed residues in the CR3 region of the HPV 16 E7 Protein

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Poster presentation - Systematic analysis of the roles of solvent-exposed residues in the CR3 region of the HPV 16 E7 Protein

2009 – DNA Tumor Virus Meeting, Oxford, England
Poster presentation - Systematic analysis of the roles of solvent-exposed residues in the CR3 region of the HPV 16 E7 Protein

2009 – Oncology Research and Education Day, London, Canada
Poster presentation - Systematic analysis of the roles of solvent-exposed residues in the CR3 region of the HPV 16 E7 Protein

2008 – Canadian Society of Plant Physiologists, 50th meeting, Ottawa, Canada
Poster presentation - The interconversion of ACC Deaminase and D-cysteine desulphhydrase by directed mutagenesis

2007 – Graduate Student Research Conference, Waterloo, Canada
Oral presentation - Identification and Characterization of ACC Deaminase in Tomato Plants

Invited Lectures
2012 - HPV Infection & Cancer – Deregulation of the Retinoblastoma Tumor Suppressor by E7. Seminar in acceptance of the John A. Thomas Award
Location: The University of Western Ontario, London, Ontario.

Recent Awards and Accomplishments

2012, Ontario Graduate Scholarship. Value = $15,000
• Awarded to the top graduate students across all disciplines in the Province of Ontario

2012, Queen Elizabeth II Graduate Scholarship in Science and Technology – Declined. Value = $15 000
• Awarded to the top graduate students across all disciplines in the Province of Ontario

2012, John A. Thomas Award. Value = $1 000 CAD
• Awarded to the top Ph.D. Candidate in the Schulich School of Medicine and Dentistry during their last year in their program from the areas including Bacteriology, Virology, Immunology and Genetics

2011, Canada Graduate Scholarships – Michael Smith Foreign Study Supplement. Value = $6 000
• Awarded to CIHR award holders to conduct research abroad

2010 Infection and Immunity Research Forum. Value = $100 CAD
• Awarded to the graduate poster presentation judged to be the strongest within its category

2009-2012, Canadian Institutes of Health Research Frederick Banting and Charles Best Canada Graduate Scholarship Doctoral Research Award. Value = $105 000 over 3 years ($35 000/yr)
• Awarded to the top national Ph.D. candidates participating in health research

2009, Ontario Graduate Scholarship – Declined. Value = $15 000 CAD
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2008-2012, Schulich/Western Graduate Scholarship. Value = ~$7 500/year
• For maintaining the academic average above 80%

2008, Microbiology and Immunology Entrance Award. Value = $5 000
• Awarded to top entering students in the program

2008, E.B. Dumbroff Award. Value = $1 000
• Awarded for outstanding work in Master of Science program and thesis in plant sciences

2006-2008, University of Waterloo Graduate Studies Scholarship. Value = $6 000
• For maintaining the academic average above 80%

2006, University of Waterloo Graduate Entrance Award. Value = $1 000
• Awarded to top entering students in the program

2003-2006, Dean’s Honor List.
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2011, Biology of Prokaryotes (M&I 2100a, lab), University of Western Ontario
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