The Pennsylvania State University
The Graduate School
Department of Biochemistry and Molecular Biology

JC VIRUS LARGE T ANTIGEN INTERACTS WITH THE F-BOX PROTEIN β-TRANSDUCIN REPEAT CONTAINING PROTEIN (βTrCP), THEREBY INFLUENCING VIRAL DNA REPLICATION

A Dissertation in
Biochemistry, Microbiology and Molecular Biology

by
Marta M. Reviriego-Mendoza

© 2011 Marta M. Reviriego-Mendoza
Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

May 2011
The dissertation of Marta M. Reviriego-Mendoza was reviewed and approved* by the following:

Richard J. Frisque  
Professor of Molecular Virology  
Dissertation Advisor  
Chair of Committee

Ross Hardison  
Professor of Biochemistry and Molecular Biology

Jean Brenchley  
Professor of Microbiology and Biotechnology

Gary Perdew  
Professor in Agricultural Sciences

Robert Paulson  
Associate Professor of Veterinary and Biomedical Sciences

Anthony Schmitt  
Assistant Professor of Molecular Immunology and Infectious Diseases

Scott Selleck  
Professor of Biochemistry and Molecular Biology  
Head of the Biochemistry and Molecular Biology Department

*Signatures are on file in the Graduate School
ABSTRACT

JC virus (JCV) is a small DNA tumor virus that causes the fatal demyelinating disease progressive multifocal leucoencephalopathy (PML) in immunocompromised patients and has been detected in certain human cancers. Lytic infection and transformation of cultured cells by JCV require five tumor proteins, which interact with factors regulating critical cellular processes. In this study, I have identified an interaction between large T Antigen (TAg) and beta-transducin repeat containing protein 1 and 2 (βTrCP1/2), the substrate recognition factors of the Skp1-Cul1-F box (SCFβTrCP) ubiquitination complex. Immunofluorescence analyses revealed that TAg and βTrCP1, as well as βTrCP2, co-localize in the cytoplasm, and a functional SCF complex is essential for the cytoplasmic translocation of TAg and βTrCP1. These interactions involve a phosphodegron (DpSGX\textsubscript{2-4}pS) found in βTrCP substrates. TAg stability is unaltered, suggesting that TAg is a pseudo-substrate. Site-directed mutagenesis revealed that serine 640 within the destruction motif is essential for binding. βTrCP targets several proteins involved in distinct cellular pathways including Wee1, p53, PDCD4, β-catenin and IκB. I analyzed the effect of the TAg-βTrCP interaction upon β-catenin and p53. I was unable to demonstrate that TAg elevates β-catenin levels as previously reported, and a mutant TAg unable to bind βTrCP, also had no detectable effect on β-catenin stability. I did, however, observe that wild type and mutant TAgS differentially influence p53 levels. Cells expressing mutant TAg yielded higher levels of p53 than wild type TAg expressing cells. However, control experiments did not support my prediction that βTrCP is responsible for the observed differences in p53 levels. DNA replication assays revealed that
mutations within TAg’s destruction motif exhibit defects at late times of the viral replication cycle, highlighting possible roles for βTrCP in JCV DNA replication and infectious virion production. For the first time, the JCV TAg has been linked to the cellular destruction machinery. Proteasomal degradation pathways are essential for the proper spatial and temporal regulation of cellular functions, including cell cycle regulation, differentiation and proliferation. Further analyses are necessary to identify βTrCP substrates that may be affected by the TAg-βTrCP complex essential to viral function.
# TABLE OF CONTENTS

**LIST OF FIGURES** ........................................................................................................... viii

**LIST OF TABLES** ............................................................................................................... x

**ABBREVIATIONS** ............................................................................................................... xi

**ACKNOWLEDGEMENTS** ................................................................................................... xiii

**CHAPTER 1** Introduction ................................................................................................... 1

- JCV Genome ................................................................................................................. 3
- JCV early tumor proteins ............................................................................................. 5
  - Large T antigen .................................................................................................... 7
  - Small t antigen (tAg) .......................................................................................... 11
  - T'\textsubscript{165}, T'\textsubscript{136} and T'\textsubscript{135} ............................................. 13
- The ubiquitin proteasome system ............................................................................... 15
- Viruses interfere with the destruction machinery of the cell .................................. 17
- The functions of the F-box protein beta-transducin repeat containing proteins
  (βTrCP) and their role in mammalian cells ............................................................ 19
- Achievements ............................................................................................................. 24

**CHAPTER 2** JC virus large T antigen interacts with the F-box protein beta-transducin
repeat containing protein 1 and 2 (βTrCP1 and 2) and localizes in the cytoplasm ......... 27

**ABSTRACT** ...................................................................................................................... 28

**INTRODUCTION** ......................................................................................................... 29

**MATERIALS AND METHODS** ....................................................................................... 32

- DNA constructs ........................................................................................................ 32
- Cell culture ............................................................................................................... 32
- Transfections .......................................................................................................... 34
- Antibodies ............................................................................................................ 34
- Cell extracts .......................................................................................................... 35
- Immunoprecipitation, GST pull down experiments and Western blots analysis .... 35
- Phosphatase treatment ......................................................................................... 36
- Immunofluorescence staining .......................................................................... 36
- RT-PCR .................................................................................................................. 37

**RESULTS** ....................................................................................................................... 38

- JCV TAg interacts with βTrCP1 ............................................................................. 38
- SV40 TAg does not interact with βTrCP1 ............................................................. 39
- Phosphorylation is required for the TAg-βTrCP1 interaction ............................ 44
Serine 640 in the JCV TAg is essential for binding βTrCP1 .........................45
TAg interacts with βTrCP2 ........................................................................49
TAg co-localizes with βTrCP in the cytoplasm and depends on the
ability of βTrCP to interact with the rest of the degradation complex...51
JCV early tumor proteins elevate the levels of the exogenously-
expressed GST-tagged proteins..............................................................55

CHAPTER 3 Mutation of the TAg destruction motif affects viral DNA
replication and p53 levels but not β-catenin levels .................................58

ABSTRACT ..........................................................................................59
INTRODUCTION ..................................................................................60
MATERIALS AND METHODS ...............................................................63
DNA constructs .................................................................................63
Cell culture ..........................................................................................64
Transfections and cell extracts .........................................................64
Antibodies............................................................................................65
Immunoprecipitation and Western blot experiments .......................65
MG132 treatment..............................................................................67
DpnI Replication Assay.......................................................................67

RESULTS ............................................................................................69
Wild type and mutant TAgS do not alter the levels of the βTrCP substrate
β-catenin .............................................................................................69
Mutations of serine 640 and 644 increase the levels of endogenous p53 .....72
Mutation of serine 640 within the TAg destruction motif inhibits JCV
DNA replication ..................................................................................80

CHAPTER 4 Discussion .......................................................................84

CHAPTER 5 Future Experiments ..........................................................96

Analysis of the interaction of SV40 TAg with βTrCP1 and βTrCP2 ..........97
Experimental approach .......................................................................98
Analysis of the phosphorylation status of wild type and mutant TAgS by mass
spectrometry (MS) .............................................................................98
Experimental approach .....................................................................99
Analysis of the effects of the TAg destruction motif mutant on p53 levels ......100
Experimental approach .....................................................................100
Investigation of the effect of the TAg-βTrCP interaction on specific βTrCP
natural substrates ............................................................................101
Experimental approach .....................................................................101
Identification of new substrates affected by the TAg-βTrCP interaction ....106
Experimental approach .....................................................................105
Examination of the effects of the TAg-βTrCP interaction on JCV DNA
replication ..........................................................................................108
Experimental approach: .............................................................................................................. 109

REFERENCES ................................................................................................................................. 111

APPENDIX A: Small t antigen of JC virus localizes in the nucleus and cytoplasm and interacts with the Rb family proteins p130 and p107 .................. 132

APPENDIX B: Identification and characterization of mefloquine efficacy against JC virus in vitro ................................................................................................................................. 143

APPENDIX C: Sources of Content .................................................................................................. 184
LIST OF FIGURES

Figure 1-1. JCV Genome. .................................................................................................................5

Figure 1-2. Model describing the mechanism by which Polyomaviruses drive cells into S phase. ..........................................................................................................................8

Figure 1-3. Structure and Functional Domains of JCV Tumor Proteins. ..................15

Figure 1-4. Mechanism of βTrCP-degradation of targeted substrates. ....................22

Figure 2-1. JCV TAg interacts with βTrCP1. .................................................................40

Figure 2-2. SV40 and JCV TAg sequence comparison of the destruction motif........41

Figure 2-3. SV40 TAg does not interact with βTrCP1. ..............................................43

Figure 2-4. Phosphatase treatment abolishes the TAg-βTrCP1 interaction.........45

Figure 2-5. Amino acids serine 640 and 644 influence TAg binding to βTrCP1......47

Figure 2-6. TAg double mutants that include the S640A mutation are impaired in βTrCP1 binding. ............................................................................................................................48

Figure 2-7 JCV TAg interacts with βTrCP2 .................................................................50

Figure 2-8. TAg and βTrCP1 co-localize mainly in the cytoplasm........................52

Figure 2-9. Co-localization of TAg and βTrCP. .........................................................54

Figure 2-10. Expression levels of GST-βTrCP1 and GST-βTrCPΔF in the presence of the T proteins..............................................56

Figure 2-11. Analysis of the mRNA levels of GST and GST-βTrCP in the presence or absence of the T proteins.................................................................57

Figure 3-1. TAg does not alter β-catenin levels......................................................70

Figure 3-2. Levels of endogenous β-catenin in the presence of WT or mutant TAg in RKO and PHFG cells. .................................................................71

Figure 3-3. TAg does not affect the exogenous myc-β-catenin levels.............74
Figure 3-4. Mutations of serines 640 and 644 of TAg alters p53 levels. .......................... 76

Figure 3-5. Expression of βTrCPΔF in the presence of TAg does not affect p53 levels. .......................................................................................................................... 78

Figure 3-6. Mutations at serines 640 and 645 of TAg negatively affects JCV DNA replication ........................................................................................................... 82

Figure 4-1. Sequence alignment of JCV, BKV and MCV large T antigen.................. 85

Figure 4-2. Diagram depicting the hypothesized nucleo-cytoplasmic shuttling of βTrCP1 and TAg........................................................................................................... 90

Figure A-1 Protein sequence alignment of JCV, BKV and SV40 tAg sequences harboring LXCXE domains....................................................................................................... 134

Figure A-2: JCV tAg interacts with the Rb proteins p130 and p107....................... 136

Figure A-3. tAg localizes in the cytoplasm and in the nucleus............................. 139
LIST OF TABLES

Table 1-1. Association of DNA tumor viruses with cellular degradation machineries. .............................................................. 17

Table 1-2. βTrCP reported substrates................................................................. 23

Table 1-3. Cancers associated with βTrCP deregulation. ................................. 24

Table 2-1. Site directed mutagenesis and RT-PCR primers............................... 33

Figure 2-11. Analysis of the mRNA levels of GST and GST-βTrCP in the presence or absence of the T proteins........................................ 57

Table 3-1. JCV relative DNA replication activity in PHFG cells.......................... 83
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Adenomatosis polyposis coli</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein kinase 1</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase 3</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>Hsc70</td>
<td>Heat-shock cognate protein 70</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
</tr>
<tr>
<td>JCV</td>
<td>John Cunningham Virus</td>
</tr>
<tr>
<td>MCV</td>
<td>Merkel Cell Virus</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute</td>
</tr>
<tr>
<td>Pex5</td>
<td>Peroxin 5</td>
</tr>
<tr>
<td>PHFG</td>
<td>Primary human fetal glial</td>
</tr>
<tr>
<td>p. i.</td>
<td>post-infection</td>
</tr>
<tr>
<td>PML</td>
<td>Progressive Multifocal Leucoencephalopathy</td>
</tr>
<tr>
<td>p. t.</td>
<td>post-transfection</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma susceptibility gene product</td>
</tr>
<tr>
<td>SCF</td>
<td>SKP-Cullin F-box</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>βTrCP</td>
<td>beta-Transducin repeat containing protein</td>
</tr>
<tr>
<td>βTrCPΔF</td>
<td>beta-Transducin repeat containing protein-delta-F dominant negative</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>VLCFA</td>
<td>Very long chain fatty acid</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-int</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Richard (Dick) Frisque, for the opportunity he has given to me to study my Ph. D. in his lab and for his guidance and mentorship throughout these 5 years. I especially thank him for the freedom he has given me in working on my projects and allowing me to pursue my ideas. I strongly believe that such independence has made my passion for sciences to grow stronger, and has helped me become a better scientist.

I thank the Frisque lab members, Ramya and Brigitte. Ramya and I went through our Ph. D journey together at Dick’s lab, where we have shared many painful, funny, and happy moments. I have really enjoyed her companionship through this trip with her, and thank her. I also like to thank Brigitte. She has helped me with my experiments throughout my years in the lab, and has taught me secret ways to make the results look beautiful. She has also listened to me and my stories, and has always given me great advice in both the professional and personal level. We have shared great moments together, and she has become a very important person and friend in my life. I will greatly miss her, thus I will have to come visit her periodically!

I would like to thank my committee members for their guidance and help throughout my studies. I also thank Dr. Reese for giving me the opportunity in his lab (my first lab experience) as an exchange student in 2003. He taught me to give the first steps and to think as a scientist. I thank him and his wife Debbie for all the good moments I have
spent with them and for their help. I will miss them. I thank Myriam Attar, my friend, for her companionship and help, and for being there for me. She is a very special friend for me and I know our friendship will last. I am very grateful to the Biochemistry and Molecular Biology Department at Penn State for believing in me, and giving me the opportunity to study my Ph. D. with them.

Finally, I want to give a special thank you to my family. Mike, my husband and best friend, I thank you for your patience and support throughout all these years, and for always being alongside me, no matter what. I am extremely lucky to have you. I love you. Thank you mother (Marisol), father (Paco), brother (Paquito) and Avita (aka grandma) for helping me to become who I am, both professionally and personally. I know I wouldn’t have been able to make it without you! You all made me strong. Thank you for your help, for all your guidance and, especially for all your love.
CHAPTER 1

INTRODUCTION
Polyomaviruses are a family of small, non-enveloped icosahedral DNA viruses capable of inducing tumors in vivo. Members of this family include five human viruses, JCV, BKV, WUV, KIV and Merkel cell virus (MCV), the monkey virus, simian virus 40 (SV40) and the mouse polyomavirus (mPyV) (1, 2). SV40, the most intensively studied virus in this group, was isolated in 1960 from rhesus monkey kidney cells, used to produce polio vaccine. Less than two years after its discovery, SV40 was found to cause tumors in hamsters, a frightening observation since millions of people had likely been infected with the virus after receiving the poliovirus vaccine. Over the next several decades hundreds of laboratories examined the oncogenic potential of this virus, but it wasn’t until the development of sensitive PCR techniques that SV40 was linked to certain human tumors including medulloblastomas, glioblastomas, mesotheliomas, osteosarcomas and non-Hodgkins lymphomas (3).

JCV was first isolated from humans in 1971, and was named after a patient, John Cunningham, who succumbed to the fatal demyelinating brain disease, progressive multifocal leucoencephalopathy (PML). JCV causes persistent infections in over 50% of the human population, and it is believed that the virus is transmitted during childhood or adolescence via a urine to oral or respiratory route (1, 4, 5). In immunocompromised individuals, JCV causes PML, a neurodegenerative disease induced by active replication of the virus in oligodendrocytes, the myelin-producing glial cells of the brain. Upon lytic destruction of these cells by the virus, large areas of demyelination occur in the central nervous system, often resulting in severe disability and death within six months of the initial symptoms. JCV also infects a second glial cell type, the astrocyte, however, contrary to the destructive outcome induced by the virus in oligodendrocytes, these cells
survive and resemble tumor cells found in glioblastoma patients. As a polyoma (“many tumors”) virus, JCV undergoes a complete infection cycle in permissive cells (e.g. oligodendrocytes), but may transform non-permissive cells, which cannot support viral replication, into tumor cells. The oncogenic activity of JCV can be demonstrated experimentally, either by transforming certain cell types in culture or by inducing tumors in inoculated rodents and non human primates (1, 6, 7). In the last decade, the JCV genome has been detected in certain human tumors, including colon and brain cancers. The ability of JCV to induce transformation in cell culture or tumor formation in vivo requires the production of its five tumor proteins, which interact with key cellular proteins, thereby interfering with their function and altering important cellular processes (1, 7). Results of the current study presented in Chapters 2 and 3, describe the interaction of the major JCV tumor protein, T Antigen (TAg), with components of the cell’s degradation machinery. These findings are relevant to our understanding of the oncogenic and pathogenic potential of this human virus.

**JCV Genome**

The JCV genome is a circular, double-stranded DNA of approximately 5000 base pairs in length (Figure 1-1). Its DNA is divided into three regions: the regulatory region (RR) and the early and late coding regions. The early and late genes are transcribed in opposite directions from different strands of the genome. Transcription of the late region of the genome results in two messenger RNAs (mRNA) that encode the late capsid or structural proteins VP1, VP2, VP3, and a fourth protein named Agnoprotein (1, 6, 8).
This latter protein influences viral transcription and replication, and affects cellular processes essential to the virus life cycle, including cell cycle progression and DNA repair (9, 10). Transcription of the early region yields a single precursor mRNA that is processed into five transcripts. These transcripts encode the proteins TAg, small t antigen (tAg), and three splice variants T′165, T′136 and T′135 (Figure 1-1). All five early proteins share the first 81 amino acids at their N-termini, and TAg and the three T' proteins also share the following 51 amino acids. Each protein has an unique C-terminus except for TAg and T′165, which terminate with the same 33 amino acids. Because of the extensive overlap of sequences, it has been suggested that the five tumor proteins would exhibit similar functions (1, 11). The RR of JCV consists of 425 base pairs that include the promoter/enhancer sequences for transcription, the early and late transcription start sites and the origin of DNA replication. During a lytic infection, transcription of the early region occurs first and expression of the early regulatory proteins initiates a series of events required for virus propagation, including driving the infected cell into S phase, mediating DNA replication and stimulating transcription of the late genes (1, 12).

In non-permissive cells, however, only the early region of the viral genome is expressed; DNA replication and late gene expression do not occur and virus is not produced. Under these conditions the episomal viral DNA may become integrated into the cellular genome, an initial step in oncogenic transformation (1, 12).
Figure 1-1. JCV Genome.

The inner circle represents the double stranded JCV genome. The early and late regions are expressed early and late in an infection, respectively, and the regulatory region (RR) consists of transcription and DNA replication signals. Bars indicate the encoded early and late proteins, and the direction of transcription of mRNAs is shown by the arrows. The discontinuous lines represent the introns of the early, unspliced mRNA.

JCV early tumor proteins

The five JCV tumor proteins, TAg, tAg, T′165, T′136 and T′135, contribute to lytic infection and to cellular transformation. Their interactions with several cellular proteins
lead to the alteration of critical cellular functions, including cell cycle regulation, proliferation and apoptosis. Structurally, TAg and the three T` proteins contain J and LXCXE domains at their N-termini, which represent binding sites for the cellular chaperone Hsc70 and the Rb family of tumor suppressors (pRb, p107 and p130), respectively (1, 11, 13). The Rb proteins control early stages of the cell cycle, making them key regulators of cellular proliferation. The Rb proteins are found in their active unphosphorylated state during G0/G1 phase of the cell cycle, and they bind the E2F transcription factors to inhibit the transcription of genes involved in S-phase progression. Phosphorylation by cyclin-dependent kinases (cdks) leads to the inactivation of the Rb proteins, promoting the release of the transcription factors and progression onto S phase (14). Because polyomaviruses require the cells’ DNA replication machinery to amplify their own genomes, they must be capable of driving non-dividing cells into S phase. A model explaining the ability of polyomavirus tumor proteins to promote the transition from the G0/G1 to the S phase of the cell has been proposed (Figure 1-2). The viral proteins are thought to activate E2F function by binding to the unphosphorylated form of the Rb proteins through their LXCXE domain. The J domain of the T proteins then recruits the cellular chaperone Hsc70 and stimulates its ATPase activity, thereby altering the conformation of the Rb-E2F complex, freeing E2F from the Rb partner, and activating the transcription of genes essential for cell cycle progression (Figure 1-2) (15, 16).

Besides the common domains found in all five early proteins (Figure 1-3), each tumor protein possesses distinct regions in its sequence that likely specify unique
functions. Understanding the functions attributed to these unique regions is essential to broadening our understanding of the oncogenic and pathogenic properties of JCV.

**Large T antigen**

The JCV TAg is a 688 amino acid (aa), highly phosphorylated, multifunctional protein that plays major roles in viral replication and cellular transformation. TAg initiates the replication of the JCV genome by specifically binding to a pentanucleotide sequence called TAg binding site II (BSII) found at the center of the origin of DNA replication. Subsequently, TAg oligomerizes into a double hexamer to facilitate the unwinding of the DNA and recruitment of the cellular replication machinery. Once the elongation phase starts, TAg utilizes its helicase and ATPase activities to promote bidirectional DNA replication (1).

In an in-vitro replication system only TAg is required to promote DNA replication. In cell culture, however, JCV DNA replication efficiency decreases in the absence of the T's and tAg, indicating these four tumor proteins are also required for TAg-mediated JCV DNA replication in vivo (11, 17, 18).
Figure 1-2. Model describing the mechanism by which Polyomaviruses drive cells into S phase.

TAg interacts with the underphosphorylated Rb-E2F complex via its LXCXE domain through the A and B pockets of Rb and recruits Hsc70 through its J domain. The latter domain activates the ATPase function of Hsc70 resulting in a conformational change in the TAg-pRb-E2F complex and the release of E2F. E2F then activates genes involved in S phase progression. The letter “k” represents the Hsc70 binding site within Rb (15, 16).

TAg disrupts, activates or substitutes for numerous cellular activities. It displays enzymatic functions such as helicase and ATPase activity, but many TAg functions are mediated through protein-protein interactions involving specific motifs within the amino acid sequence. Some of these interactions are required for DNA replication and transcription and include cellular DNA polymerase α, topoisomerase 1, the transcription factors YB-1 Pur-α and Tst-1, and the viral Agnoprotein (1, 12). Other interactions have been identified that contribute to TAg’s transforming activity and involve the Rb family of tumor suppressors, Hsc70, p53, β-catenin and IRS-1 (1). It is expected that some or all of these latter TAg targets are also critical to the viral life cycle.
**TAg-p53 interaction**

Polyomavirus TAg s possess a bi-partite domain through which the viral protein interacts with p53. p53 is a transcription factor that responds to cellular stresses by transcribing essential genes required to induce cell cycle arrest, DNA repair and/or apoptosis. This tumor suppressor is unstable, and in most non-tumor cells is detected at low levels. In unstressed cells, p53 is mainly regulated via degradation through the E3 ubiquitin ligase MDM2. Upon activation of a variety of stress signals, MDM2 is inactivated by translational modifications leading to stabilized p53. p53 then mediates the transcription of numerous genes, including the cyclin kinase inhibitor p21 that promote cell cycle arrest in G1 or induce apoptosis (19). During a polyomavirus infection, TAg expression inactivates Rb proteins, freeing E2F to transcribe its target genes, including ARF. ARF binds to and inactivates MDM2, which in turn increases the stability and levels of p53; but is inactivated by TAg binding (20). Thus, to drive quiescent cells into S phase and to prevent apoptosis DNA tumor viruses must interact with and inactivate both, p53 and Rb (1, 21).

**β-catenin-TAg interaction**

β-catenin is a transcription factor essential to the Wnt signaling pathway that regulates developmental control and adult cell homeostasis. Two pools of β-catenin can be identified within the cell: β-catenin anchored to the plasma membrane through E-cadherins and alpha-catenins, and cytoplasmic β-catenin, which is found in an Axin complex composed of the scaffolding protein axin, casein kinase (CK1), glycogen kinase
3 (GSK3) and the tumor suppressor adenomatous polyposis coli (APC). In unstimulated cells in the absence of Wnt, cytoplasmic β-catenin is sequentially phosphorylated at the N-terminus by CK1 and GSK3 leading to the recognition, ubiquitination and proteasomal degradation of β-catenin by the E3 ubiquitin ligase beta-transducin repeat containing proteins (β-TrCP) (22). Upon activation of the Wnt signaling pathway, phosphorylation of β-catenin is inhibited, leading to increased stability and accumulation of the protein, and translocation to the nucleus, where it activates the transcription of genes required for cell proliferation (22, 23). The regulation of cytosolic β-catenin is an essential step in the controlled functioning of the Wnt signaling cascade. Deregulation of this pathway results in tumorigenesis, and its alteration accounts for more than 90% of human colorectal cancers (23, 24).

JCV TAg has been reported to bind β-catenin, resulting in β-catenin’s stabilization, translocation into the nucleus and increased transactivation activity (25, 26). TAg also modulates β-catenin’s stability and subcellular localization through Rac1 activation (27). Rac 1 is a plasma membrane-bound, small GTPase of the RAS superfamily that is involved in cell growth, motility and cytoskeletal reorganization (28). JCV has been associated with colorectal cancers (25, 29, 30, 31), and it is known that the Wnt signaling pathway is aberrant in these neoplasmas (23). It is possible that TAg’s interaction with β-catenin contributes to the oncogenic potential of JCV.
**IRS-1-TAg interaction**

The insulin receptor substrate-1 (IRS-1), a key component of the insulin growth factor-1 (IGF-1) signaling pathway, transduces signals from the insulin receptor, eliciting responses that influence metabolic, proliferative and anti-apoptotic signals. IRS-1 has been demonstrated to have transforming potential in culture, and high levels of this ligand have been detected in various human neoplasias including medulloblastomas, hepatocellular and pancreatic carcinomas, pointing towards a potential involvement of IRS-1 in human carcinogenesis (32, 33, 34).

Khalili and co-workers (33, 34) discovered that JCV TAg binds IRS-1, thereby promoting the nuclear translocation of this cytoplasmic ligand. They also observed that transformation of murine cells by JCV correlated with high levels of IRS-1 expression (33, 34). Recent studies from the same group have shown that TAg, coupled with IRS-1 and Rad51, is capable of inhibiting homologous recombination-directed DNA repair (HRR), leading to an accumulation of mutations and speculation that these events contribute to JCV-induced transformation (35).

**Small t antigen (tAg)**

Very little is known about the roles of JCV tAg in cellular transformation and the viral life cycle. tAg is an 172 aa tumor protein that shares its first 81 aa with TAg; The C-terminal 91 aa are unique and are predicted to encode novel functions (1, 36). Using the better studied SV40 tAg as a guide, the JCV tAg C-terminal sequences have recently been shown to bind the Ser/Thr protein phosphatase PP2A through cysteine-containing
motifs (37, 18). PP2A is a heterotrimeric enzyme consisting of scaffolding A subunits, a catalytic C subunit, and a multiple regulatory B subunits. It is one of the four major cellular phosphatases involved in the negative regulation of cellular proliferation and division (38). SV40 studies indicate that tAg binds to the PP2A AC core enzyme and inhibits the phosphatase activity by displacing a regulatory B subunit. This, in turn, maintains PP2A substrates, such as PKC and AKT, in an active phosphorylated state and leads to the hyper-activation of pathways involved in proliferation and survival (38, 39). It is noteworthy that, while SV40 tAg is not needed for TAg-mediated transformation of rodent cells, it is required for transformation of human cells (40). Recent studies in our lab show that a JCV tAg null mutant was unable to replicate, highlighting the importance of this tumor protein in viral DNA replication (18). In addition, SV40 tAg promotes cell cycle progression. Mammalian cells expressing tAg enter S phase at a much higher rate than naive cells, an effect attributed to the high levels of the G1/S cyclins and cyclin dependent kinases cyclin B, E and cyclin-E-dependent kinase 2 (cdk2) present in the former cells (36). In my initial sequence-scanning studies I identified two LXCXE motifs in the unique region of JCV tAg, one of them is also present in BKV but absent in the SV40, WUV, KIV and MCV tAgs. Consequently, JCV tAg interacts with members of the retinoblastoma tumor suppressor family members (18). These data emphasize the importance of tAg in JCV replication and transformation (These results are described in Appendix A).
T’\textsubscript{165}, T’\textsubscript{136} and T’\textsubscript{135}

The discovery of the TAg isoforms, T’\textsubscript{165}, T’\textsubscript{135} and T’\textsubscript{136} immediately led to a debate about whether or not these proteins were functional (11). Although their primary structures are nearly identical, T’\textsubscript{135} and T’\textsubscript{136} do exhibit some differences in function and expression profiles. First, both proteins bind to p130 similarly, but appear to interact differently with p107 (41). Bollag et al. observed that T’\textsubscript{135} efficiently binds to the unphosphorylated form of p107. In contrast, the T’\textsubscript{136} interaction with p107 was difficult to detect, although the levels of phosphorylated and unphosphorylated forms of p107 in T’\textsubscript{136}-expressing Rat2 cells were reduced relative to the p107 levels detected in T’\textsubscript{135} expressing cells (41). Second, the pattern of T’ mRNA splicing varies in JCV lytically-infected versus transformed cells. In JCV transformed cells, T’\textsubscript{136} mRNA expression is much higher than that of T’\textsubscript{165} or T’\textsubscript{135} mRNAs, whereas in lytically-infected cells, the levels of the T’ mRNAs are similar at late times in the infection (11, Kartikeyan and Frisque, unpublished data). Although variations in the expression profiles of the T’ transcripts are not functional differences, these altered profiles suggest the two T’ proteins may display different functions during the viral life cycle.

The unique C-termini of T’\textsubscript{135} and T’\textsubscript{136} consist of three and four amino acids, respectively. Sequence analysis revealed that the C-terminus of T’\textsubscript{135} (KKASC) resembles a consensus Protein Kinase A or G (PKA/PKG) phosphorylation site: [R/K](2) - x - [S/T](5), and that of T’\textsubscript{136} (KGRF) appears to carry a peroxisome targeting sequence type 1 (PTS1) signal recognized by the cytoplasmic peroxisome receptor Peroxin 5 (Pex5) (Figure 1-2) (42). Pex5 is an essential receptor involved in transporting proteins.
into the peroxisomes, and it is needed for this organelle’s function and biogenesis (42). Peroxisomal disorders in which one or more peroxins, including Pex5, are defective, are associated with demyelination of the CNS (43, 44). Kassmann and her group generated a conditional Pex5-deficient mouse in which they exclusively targeted the oligodendrocytes. They showed that the lack of peroxisomes leads to demyelination and induces neuroinflammation and infiltration of CD8+ T cells into the damaged brain. These results suggest a neuroprotective function of peroxisomes in oligodendrocytes (45). JCV infection induces lytic destruction of human oligodendrocytes and demyelination of the CNS (46). Although interaction between Pex5 and T´ has not been confirmed, fatty acids analysis profiles of JCV transfected cells showed increased very long chain fatty acids (VLCFA) levels, and low amounts of short chain fatty acids, when compared with untransfected cells (Reviriego-Mendoza and Frisque, unpublished data). A high ratio of VLCFA to short chain fatty acids could indicate that the catabolism of VLCFA is impaired in cells expressing the tumor proteins. Accumulation of VLCFA in cells results, in part, due to peroxisomal disorders, and is linked to demyelination of the CNS (43, 44). While demyelination is readily explained by loss of oligodendrocyte during a lytic infection, it is possible that other mechanisms, including T´ targeting of Pex5 and disruption of peroxisomes, contribute to JCV-induced demyelination in PML patients.
Figure 1-3. Structure and Functional Domains of JCV Tumor Proteins.

All five proteins share the first 81 amino acids at their N-termini and have unique C-termini except for TAg and T'\textsubscript{165}, which terminate with the same 33 amino acids. The sequences of the five T proteins are predicted to contain specific motifs that confer upon the virus the ability to interact with cellular proteins to manipulate the cell. The interactions are discussed in the text. The figure is not drawn to scale.

The ubiquitin proteasome system

The ubiquitin proteasome pathway causes the selective turnover of cellular proteins through a series of steps mainly involving three enzymes: the E1 ligase, which activates the ubiquitin molecule in an ATP-dependent manner, the E2 enzyme that transfers the ubiquitin to a specific E3 ligase, and the E3 ligase, which, alone or in complex, transfers the ubiquitin to the protein targeted for degradation (47, 48). The specificity for ubiquitination of a particular substrate depends upon the E3 ligases. There
are hundreds of E3 enzymes and these have been grouped into 2 different classes depending on whether they possess a HECT or the Ring-finger domain. The two families of E3 ligases differ in that E3 ligases with a HECT domain form an intermediate with ubiquitin through a thioester bond prior to transferring the molecule to the target protein, while E3 ligases with a Ring-finger (Cullin-RING) domain transfer the ubiquitin molecule directly to the targeted substrate (49). The first HECT E3 ligase to be discovered was the E6 associated protein (E6AP), which was bound to the human papillomavirus (HPV) E6 tumor protein (50). The most abundant E3 ligases belong to the Cullin-RING E3 ubiquitin ligase class, and these enzymes form a complex composed of the RING domain-containing protein, RBX1 (RING Box-1) or Roc1 (regulator of Cullin-1), which links the E2 ligase to the substrate, a cullin protein that acts as a scaffold (Cul1 though 4, and Cul7), and one or more subunit(s) that serve as adaptor proteins or substrate recognition factors. The adaptor and substrate recognition factor will vary depending on whether they assemble with Cul1 or Cul2/5 (51, 52). The best studied complex is the Cul-1 based ligases, known as Skp-Cul1-F-box protein (SCFx), where x represents one of many F-box proteins. Complex formation is initiated by Cul1, which binds to SKP (S-phase kinase-associated protein), and the F-box protein. Over 70 human F-box proteins have been identified and each of them targets a specific set of substrates (53, 54).
Table 1-1. Association of DNA tumor viruses with cellular degradation machineries.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Protein</th>
<th>Effect on/by ubiquitin-proteasome pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV</td>
<td>E6</td>
<td>Serves as an E3 ligase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ubiquitinated by an E3 ligase</td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td>Substrate recognition factor of Cul2-based E3 ligases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ubiquitinated by SCF&lt;sup&gt;Skp2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ubiquitinated by SOCS1-containing E3 ligases</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>E4orf6/E1B55K</td>
<td>Substrate recognition factor of Cul5-based E3 ligases</td>
</tr>
<tr>
<td></td>
<td>E1B55K</td>
<td>Ubiquitinated by an E3 ligase</td>
</tr>
<tr>
<td></td>
<td>E4orf4</td>
<td>Inhibits APC E3 ligase</td>
</tr>
<tr>
<td>SV40</td>
<td>TAg</td>
<td>Inhibits Cul7-based E3 ligases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibits the SCF&lt;sup&gt;Fbw7&lt;/sup&gt; E3 ligase</td>
</tr>
</tbody>
</table>

(Adapted from 55)

Viruses interfere with the destruction machinery of the cell

Proteasomal degradation pathways are required for the proper regulation of cellular functions, including cell cycle regulation, differentiation and proliferation (56). There is increasing evidence that viruses exploit the degradation machinery of the cell to maintain an optimal cellular environment that favors their survival and ensures their replication (Table 1.1) (55,57). Welcker and Clurman (58) described a phosphorylation-dependent interaction of SV40 TAg with the F-box protein, Fbw7, the substrate recognition subunit of the SCF<sup>Fbw7</sup> ubiquitin ligase complex. This interaction decreases the turnover of at least one known SCF<sup>Fbw7</sup> substrate, cyclin E, resulting in enhanced
activity of cyclinE-Cdk2 kinase (58). Although the significance of this interaction is still uncertain, it is thought that the interference with the SCF$^{Fbw7}$ contributes to the lytic and transforming abilities of this tumor virus due to its effects on cyclin E, c-myc, c-jun and Notch. SV40 TAg has also been found to interact with Cul7, a subunit of a SCF-like E3 ligase complex (59, 60). Cul7 interacts with Fbwx8, Roc1 and Skp1 to target the ubiquitination and degradation of cyclin D1 and IRS-1 (61, 62). TAg mutations that abolish Cul7 binding result in a transformation-defective virus (59, 60).

Tumor proteins of the human papillomaviruses, HPV-16 and -18, facilitate proteasomal degradation of cellular tumor suppressor proteins. The E6 protein of these high risk HPV types recruits the cellular ubiquitin ligase, E6-associated protein (E6AP) (63). The E6-E6AP complex interacts with p53 to promote the polyubiquitination and degradation of this tumor suppressor. This event reveals a mechanism whereby a viral protein facilitates the recognition of a new non-natural target substrate by an ubiquitin ligase complex. A second HPV tumor protein, E7, targets Rb and recruits the ubiquitin conjugating enzyme, E2-25K, resulting in the degradation of this second tumor suppressor (63, 64).

Other viruses have been found to use the cellular degradation machinery to control their own replication. The Bovine Papilloma Virus (BPV) protein E1 replicative helicase, is unstable, and its turnover is controlled by the anaphase promoting complex (APC) E3 ligase. E1 contains a phosphodegron that is present in the natural substrates of the APC complex. Inhibition of the APC E3 ligase was found to stabilize the E1 protein and, consequently, increase the replication of the virus. Thus, controlled degradation of
E1 by the APC E3 ligase maintains a constant and low BPV genome copy number to favor latent infection (65).

The F-box protein, βTrCP, is a cellular target of HIV (66, 67, 68, 69). The HIV Vpu protein acts as pseudo-substrate that binds βTrCP and brings the degradation machinery in close proximity to substrates that include the CD4 receptor. This bridging action of Vpu results in recognition of CD4 by βTrCP and the proteasomal destruction of the T cell receptor (66). The CD4 sequence does not contain the βTrCP consensus motif, suggesting that Vpu promotes βTrCP recognition and degradation of a new substrate. In contrast, the Vpu-βTrCP1 interaction inhibits the degradation of some authentic βTrCP1 substrates, such as β-catenin, ATF4, IκB and CdC25A (67, 68, 69). Further, Em1, yet another βTrCP1 substrate, remains unaffected by the Vpu-βTrCP1 interaction. These findings indicate that Vpu directs βTrCP1 to multiple substrates that may be differentially affected by each interaction (69).

The functions of the F-box protein beta-transducin repeat containing proteins (βTrCP) and their role in mammalian cells

βTrCPs are F-box proteins and the substrate recognition factors of the SCF complex. Two essential domains are present in βTrCPs; the F box domain, which consist of a 42-48 aa motif located in the N-terminal region, and seven WD40 repeats at the C-terminus. βTrCP’s F-box domain links the protein to Skp1, a component of the SCF complex. The WD40 repeats are involved in recognizing the specific substrate that will be targeted for ubiquitination and eventual proteasomal degradation (Figure 1-4). There
are two βTrCP proteins, βTrCP1 and βTrCP2 (also known as HOS), which exhibit 86% amino acid identity and only differ in their N-terminal sequences (70, 71). Both βTrCPs recognize their substrates through a conserved destruction motif, DSGX_{2-4}S; both serines within the domain must be phosphorylated for binding to occur (72) (Figure 1-4). βTrCP promotes the controlled degradation of a diverse set of cellular proteins involved in cell cycle regulation, signal transduction and apoptosis, suggesting that βTrCP could possess both tumor suppressive and oncogenic activities (73) (Table 1-1, Table 1-3). The first substrates found to be targeted for proteasomal degradation by βTrCP were β-catenin and the inhibitor of nuclear factor kappa B-alpha (IκB) (74). Both substrates are targeted by βTrCP1 and βTrCP2. NFκB remains inactive in the cytoplasm in the presence of IκB in unstimulated cells. Upon activation of the NFκB pathway, IκB is phosphorylated at serines 32 and 36 within the destruction motif, bound by βTrCP, ubiquitinatinated and degraded in the proteasome. These events lead to the release of active NFκB and its translocation to the nucleus, where it transcribes multiple genes, including those involved in proliferation, immune response and stress (4).

The regulation of cytosolic β-catenin is an essential step in the control of the Wnt signaling cascade. β-catenin is controlled by βTrCP in a manner that is similar to that described for IκB. Degradation of β-catenin requires phosphorylation at serine 45 by CK1α, and serine 33, serine 37 and threonine 41 by GSK3-β. Phosphorylated β-catenin is recognized by βTrCP and targeted for ubiquitination and proteasomal degradation (22).
Failure to control β-catenin degradation leads to its stability and translocation into the nucleus, where it activates the transcription of proto-oncogenes, including cyclin D and c-myc (24).

Other targets regulated by βTrCP are proteins tightly involved in the cell cycle, making βTrCP a key player in the S and G2 DNA damage checkpoint pathways. βTrCP promotes cell cycle arrest following DNA damage by attenuating CDK1 activity through degradation of Cdc25A. Additionally, βTrCP1 restores cell cycle progression and entry into mitosis by facilitating the degradation of claspin and WEE1 (75, 76). It also indirectly regulates cell cycle progression through the controlled turnover of β-catenin and the NFκB inhibitor IκB, since both proteins regulate the activity of cyclin D1 (77).
The βTrCP structure includes two well-conserved domains: the F-box (F) and the WD40 (WD) repeats domains. βTrCP recognizes the substrates through the WD repeats, and binds to the rest of the degradation complex (Skp1, Cul1, and Roc1) through the F-box domain. Phosphorylation of the destruction motif within the substrate (DSpGX2-4Sp) is a requirement for the proper recognition by βTrCP. Several substrates are listed, together with the kinase responsible for phosphorylating the destruction motif. The recruitment of both the substrate and the degradation machinery promotes ubiquitination subsequent to proteasomal degradation of the targeted protein.

Redundancy within the targeted substrates is observed between the paralogues βTrCP1 and βTrCP2. For instance, both proteins target β-catenin and IκB for ubiquitination and proteasomal degradation (70), although some differences in the two proteins have been recognized. For example, βTrCP2, but not βTrCP1, is essential for the ubiquitination, degradation and down-regulation of the interferon-α receptor (IFNAR1) (78) and the prolactin receptor (PRLR) (79).
The ability of βTrCP1 and βTrCP2 to target a vast number of substrates implicates these two E3 ligases in several pathways involved in cell proliferation and apoptosis. Consequently, impaired turnover of βTrCP substrates due to aberrations affecting the βTrCP genes have been detected in various cancers (73) (Table 1.3).

<table>
<thead>
<tr>
<th>Function</th>
<th>Reported substrates</th>
<th>Full Name</th>
<th>Degron in humans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>REST</td>
<td>repressor element 1 (RE1)-silencing transcription factor</td>
<td>DEGIHS/STDSG</td>
</tr>
<tr>
<td></td>
<td>ATF4</td>
<td>activating transcription factor 4</td>
<td>DSGCMS</td>
</tr>
<tr>
<td></td>
<td>Snail</td>
<td></td>
<td>DSGKGS</td>
</tr>
<tr>
<td></td>
<td>p63</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>STAT1</td>
<td>signal transducer and activator of transcription 1</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>p100</td>
<td></td>
<td>DASYGS</td>
</tr>
<tr>
<td></td>
<td>p105</td>
<td></td>
<td>DSGVETS</td>
</tr>
<tr>
<td></td>
<td>β-catenin</td>
<td></td>
<td>DSGIHS</td>
</tr>
<tr>
<td>Transcription factors</td>
<td>kBa</td>
<td>inhibitor of NFkB-a</td>
<td>DSGLDS</td>
</tr>
<tr>
<td></td>
<td>kβB</td>
<td>inhibitor of NFkB-b</td>
<td>DSGLGS</td>
</tr>
<tr>
<td></td>
<td>kβc</td>
<td>inhibitor of NFkB-e</td>
<td>DGSIES</td>
</tr>
<tr>
<td></td>
<td>PRL-R</td>
<td>Prolactin receptor</td>
<td>DSGRGS</td>
</tr>
<tr>
<td></td>
<td>IFNR</td>
<td>interferon receptor</td>
<td>DSGNYS</td>
</tr>
<tr>
<td></td>
<td>DLG</td>
<td>discs large tumor suppressor</td>
<td>DSGLPS</td>
</tr>
<tr>
<td></td>
<td>IFNR</td>
<td>interferon receptor</td>
<td>TSGCSS</td>
</tr>
<tr>
<td></td>
<td>PER1</td>
<td>period homologue 1</td>
<td>SSGYGS</td>
</tr>
<tr>
<td></td>
<td>PER2</td>
<td>period homologue 2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PC2</td>
<td>protein polycystin2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>GHR</td>
<td>growth hormone receptor</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>WEE1</td>
<td></td>
<td>DEAFQE/EEGFGS</td>
</tr>
<tr>
<td></td>
<td>CDC25A</td>
<td>cell division cycle 25A</td>
<td>STDAG</td>
</tr>
<tr>
<td></td>
<td>CDC25B</td>
<td>cell division cycle 25B</td>
<td>DDGFVD/DSGFLDS</td>
</tr>
<tr>
<td></td>
<td>Claspin</td>
<td></td>
<td>DSGQGS</td>
</tr>
<tr>
<td></td>
<td>EM1</td>
<td>also known as F-box protein 5</td>
<td>DSGYSS</td>
</tr>
<tr>
<td></td>
<td>Bora</td>
<td></td>
<td>PLK1 activator</td>
</tr>
<tr>
<td></td>
<td>MCL1</td>
<td>myeloid cell leukemia 1</td>
<td>DGSLPS</td>
</tr>
<tr>
<td></td>
<td>Pro-caspase 3</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>PDCD4</td>
<td>programmed cell death 4</td>
<td>DSGRGS</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translation</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND denotes not determined. (Adapted from 73)
Table 1-3. Cancers associated with βTrCP deregulation.

<table>
<thead>
<tr>
<th>Human cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cancers displaying high β-TrCP levels</strong></td>
</tr>
<tr>
<td>Breast cancer</td>
</tr>
<tr>
<td>Colon cancer</td>
</tr>
<tr>
<td>Hepatoblastoma</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
</tr>
<tr>
<td>Melanoma</td>
</tr>
</tbody>
</table>

β-TrCP: β-transducin repeat-containing protein. (Adapted from 73).

**Achievements**

The ability of DNA tumor viruses to interfere with cellular functions ensures their survival and propagation. JCV’s five tumor proteins interact with cellular factors known to contribute to cellular transformation and the lytic cycle of the virus (13). Identifying the cellular targets affected by these viral proteins is critical to understanding the oncogenic and pathogenic mechanisms of JCV. During the course of my studies, I have identified domains within the primary sequence of tAg and TAg that will further our understanding of the functions of these tumor proteins. Only two papers have been published describing JCV tAg function, and most of the field’s information regarding tAg has relied upon studies with the corresponding SV40 protein. Through primary sequence analysis of JCV tAg using the Eukaryotic Linear Motif program (ELM), I identified 2
potential LXCXE domains. This domain is found in proteins that interact with the Rb family; it is present in the shared N-terminal sequences of JCV TAg and the three T’ proteins. However, in tAg, the two potential domains are found within its unique C-terminal sequence. The first of these two domains is found in both JCV and BKV tAgs, but not in SV40, while the second motif is only found in JCV tAg. Binding analyses confirmed that tAg interacts with the two Rb family members p130 and p107, suggesting potential novel contributions to cellular behavior by the JCV tAg (18) (Appendix A).

The main focus of my thesis research is based on the discovery of the first example of a JCV tumor protein interacting with the ubiquitin proteasome machinery of the cell. TAg, the most well studied JCV protein, has the ability to interact with a variety of cellular proteins, making it the pivotal player in the virus life cycle. Through primary sequence scanning, I uncovered a phosphodegron at its unique C-terminus that I predicted would be recognized by the E3 ligase, βTrCP. My work describes the characterization and functional importance of this interaction (Chapters 2, 3).

Finally, together with another graduate student in our laboratory, Ramya Kartikeyan, I collaborated with scientists at Biogen Idec. in the discovery of a drug to halt the propagation of JCV in primary human fetal glial (PHFG) cells (Appendix B). The compound, mefloquine, is an antimalarial drug that interferes with viral replication by an unknown mechanism (80). A clinical trial to determine the efficacy of this drug in HIV patients with PML is currently ongoing.

The study presented in this thesis describes new interactions between the JCV tumor proteins and cellular factors. The characterization of the events described herein
contributes to our understanding of the pathogenic potential of this ubiquitous human virus.
CHAPTER 2

JC VIRUS LARGE T ANTIGEN INTERACTS WITH THE F-BOX PROTEIN BETA-TRANSUDUCIN REPEAT CONTAINING PROTEIN 1 AND 2 (βTrCP1 AND 2) AND LOCALIZES IN THE CYTOPLASM
ABSTRACT

Lytic infection of permissive cells and transformation of cultured non-permissive cells by JCV require five tumor proteins, which interact with factors regulating critical cellular processes. I demonstrate that JCV large T antigen (TAg) binds the F-box protein, β-transducin-repeat containing protein-1 and 2 (βTrCP1/2). Phosphatase treatment suggests that these interactions are phosphorylation dependent and occur through a destruction motif at the C-terminus of TAg (639-DSGHGSS-645), which corresponds to the consensus recognition site for βTrCP (DSPGX2-4Sp). Site-directed mutagenesis within the destruction motif revealed that serine 640 is required for binding. The recognition of this motif by βTrCP does not affect TAg’s stability, suggesting that TAg may act as a pseudo-substrate. Immunofluorescence experiments show that βTrCP1 and TAg co-localize in the nucleus and cytoplasm, while only cytoplasmic staining is detected for βTrCP2 and TAg. The interaction promotes cytoplasmic localization of TAg and co-localization studies using the dominant negative form of βTrCP1, βTrCPΔF, reveal that a functional SCF complex is essential for the cytoplasmic translocation of TAg and βTrCP1. In this study I have, for the first time, linked JCV TAg to a cellular degradation complex, specifically SCFβTrCP. Proteasomal degradation is essential for the regulation of cellular functions, including cell cycle progression, differentiation and proliferation. Interference with the cellular proteasomal pathway highlights a possible oncogenic and pathogenic mechanism for this human pathogen.
INTRODUCTION

JCV large T antigen (TAg) is a highly phosphorylated multifunctional protein that plays a major role in viral DNA replication and cellular transformation. TAg orchestrates viral DNA replication through its ability to bind the origin of replication of the genome and to promote unwinding of the viral DNA via its helicase activity. These events lead to the recruitment of cellular factors required for DNA replication. The TAg sequence contains numerous domains and functional motifs that enable the protein to interact with several cellular proteins including DNA polymerase α, topoisomerase 1, Hsc70, members of the retinoblastoma family, p53, β-catenin and IRS-1. One outcome of the interaction of TAg with cellular proteins is to drive cells into S-phase, leading to viral DNA synthesis in permissive cells or cellular transformation in non-permissive cells (1, 18). Viruses have evolved to deregulate cellular processes including cell cycle progression, signal transduction, DNA damage repair and apoptosis to prepare a favorable environment for viral replication and propagation. Recently, attention has been focused upon the ability of viruses to interfere with protein destruction machinery of the cell (55). The ubiquitin proteasome system is involved in the selective turnover of key cellular proteins including oncoproteins, transcription factors, cellular receptors and tumor suppressors (56). The specificity of ubiquitination of a particular substrate depends upon the E3 ligases. There are hundreds of E3 enzymes that have been grouped into two different classes depending on whether they possess a HECT or the Ring-finger domain. The SCF (Skp1-Cullin-F-box) E3 ligases fall within the latter group, and consist of a complex formed by the linker protein Skp1, the scaffold protein Cul1, the Rbx1/Roc1/Hrt1 RING domain protein and
the substrate recognition factor called the F-box protein. Over 70 human F-box proteins that have been identified, and each of them exhibits specificity for targeted substrates (72). Each F-box protein recognizes its substrates by some form of translational modification, with phosphorylation being the most common means of recognition. The F-box proteins beta-transducin-repeat containing proteins βTrCP1 and βTrCP2 (also known as HOS), are components of the Skp1–Cul1–F-box protein (SCF) E3 ubiquitin ligase complex. These two proteins recognize most substrates via their phosphodegron consensus sequence, DpSGX2_4pS. Phosphorylation of the serine residues in this motif is required for βTrCP binding and for subsequent linkage to the ubiquitination machinery (72). Several cellular proteins involved in cell cycle regulation, signal transduction and apoptosis, including Cdc25A and B, Wee1, PDCD4, IFNAR1, β-catenin and p53, are targeted by βTrCP1 and βTrCP2 for proteasomal degradation (81, 82, 83, 84, 78, 23, 85). β-catenin and p53 have also been reported to interact with JCV TAg (20, 25, 26).

βTrCP1 and βTrCP2 exhibit 86% amino acid identity, and only differ in their N-terminal sequences (70, 71). These two proteins target β-catenin and IκB for ubiquitination and proteasomal degradation, thus demonstrating an overlap of function (70). Nonetheless, some differences in the two proteins have been recognized. For example, βTrCP2, but not βTrCP1, is essential for the ubiquitination, degradation and down-regulation of the interferon-α receptor (IFNAR1) and the prolactin receptor (PRLR) (78, 79). The targeting of different substrates by the two F-box proteins may be attributed, in part, to differences in their subcellular localization; βTrCP1 resides primarily in the nucleus while βTrCP2 is found in the cytoplasm (86, 87).
I have identified a potential phosphodegron at the C-terminus of JCV TAg (amino acids 639-645: DSGHGSS). I show that TAg directly interacts with βTrCP1 and βTrCP2 through this newly identified destruction motif, and that this interaction requires phosphorylated TAg and TAg residue serine 640. I analyzed whether TAg and the βTrCP proteins co-localized in the cell using immunofluorescence staining, and my data reveal that the TAg-βTrCP interaction promotes cytoplasmic localization of TAg, and requires a functional SCF complex. Additionally, I suggest that the components of the SCFβTrCP1 degradation complex, Skp1, Cul1 and Roc1, are required for the nucleo-cytoplasmic shuttling of βTrCP1.
MATERIALS AND METHODS

DNA constructs

The pCMV-JCV_E vector, which expresses the JCV early coding region, and the pCMV-T^+/T^-/T^- vector, which expresses TAg only, were described earlier (41, 18). The single mutants pCMV-JCV_E(S640A), pCMV-JCV_E(S644A) and pCMV-JCV_E(S645A) and the double mutants pCMV-JCV_E(S640A-S644A), pCMV-JCV_E(S640A-S645A) and pCMV-JCV_E(S644A-S645A) were created by PCR-based site-directed mutagenesis with primers identified in Table 1. The pDEST27, pGST-βTrCP1 and pGST-βTrCPΔF constructs were gifts from Dr. Wade Harper (88). The pCS2MT and pCS2MT-myc-β-catenin vectors were kindly provided by Dr. Arnold Levine (89).

Cell culture

U87MG cells were grown in minimum essential Eagle’s medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2% sodium pyruvate, 1% essential amino acids, 2mM L-glutamine, penicillin G (100U/ml) and streptomycin (100μg/ml). The human embryonic kidney cell lines 293 and 293T, PHFG cells and U2OS cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS, L-glutamine, penicillin G (100U/ml) and streptomycin (100μg/ml). PHFG and U87MG cells were incubated at 37°C in 10% CO₂, and 293, 293T and U2OS cells were incubated at 37°C in 5% CO₂.
Table 2-1. Site directed mutagenesis and RT-PCR primers.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.JCV_E S640AFwd</td>
<td>5′-CTGAAGCAGAAGACGCTGGACATGGATCAA-3′</td>
<td>2766-2727</td>
</tr>
<tr>
<td>2.JCV_E S640ARev</td>
<td>5′ CTTGATCCATGTCAGCTCTGTGTGGTTCAG-3′</td>
<td></td>
</tr>
<tr>
<td>3.JCV_E S644AFwd</td>
<td>5′GACTCTGGACATGGAGCAAGCAGCAGGATCA-3′</td>
<td>2745-2715</td>
</tr>
<tr>
<td>4.JCV_E S644ARev</td>
<td>5′- GGGATGTCAGTGCTTGATCCATGTCCAGAGTC-3′</td>
<td></td>
</tr>
<tr>
<td>5.JCV_E S645AFwd</td>
<td>5′-CTCTGACATGGATCAGGTCAGGCACACTGAAATACA-3′</td>
<td>2743-2711</td>
</tr>
<tr>
<td>6.JCV_E S645ARev</td>
<td>5′- GATTGATCCATGTCAGCTTGCCATGTCCAGAG-3′</td>
<td></td>
</tr>
<tr>
<td>7.JCV_E S640A-S644AFwd</td>
<td>5′-GACGCTGGACATGGAGCAAGCAGGATCCAC-3′</td>
<td>2745-2715</td>
</tr>
<tr>
<td>8.JCV_E S640A-S644ARev</td>
<td>5′-GCGATGTCAGTGCTTGATCCATGTCCAGAGGTC-3′</td>
<td></td>
</tr>
<tr>
<td>9.JCV_E S640A-S645AFwd</td>
<td>5′-CTGAAGCAGAAGACGCTGGACATGGATCAG-3′</td>
<td>2766-2727</td>
</tr>
<tr>
<td>10.JCV_E S640A-S645ARev</td>
<td>5′-CTGATCCATGTCAGCTCTGTGTGGTTCAG-3′</td>
<td></td>
</tr>
<tr>
<td>11.JCV_E S644A-S645AFwd</td>
<td>5′-GACTCTGGACATGGAGCAAGCAGGACACTGAAATCAC-3′</td>
<td>2745-2715</td>
</tr>
<tr>
<td>12.JCV_E S644A-S645ARev</td>
<td>5′-GGAATGTCAGTGCTTGCCATGTCCAGAGGTC-3′</td>
<td></td>
</tr>
</tbody>
</table>

Single point mutations were introduced into the TAg destruction motif (primers 1-6) using the pCMV-JCV_E construct. Serines 640 (primers 1, 2), 644 (primers 3, 4) and 645 (primers 5, 6) were changed to alanine (altered nucleotides are underlined). Double point mutations (primers 7 to 12) were performed by changing serines 644 (primers 7, 8), 640 (primers 9, 10) and 644 (primers 11, 12) to alanine. The single point mutant, pCMV-JCV_E(S640A), was used as a template to create the double mutant pCMV-JCV_E(S640A-S644A), and the single mutant pCMV-JCV_E(S645A) construct was used as a template to create the double mutants pCMV-JCV_E(S640A-S645A) and pCMV-JCV_E(S644A-S645A). These altered nucleotides are in bold and pre-existing mutations are underlined. Nucleotide numbers are based upon the JCV(Mad1) sequence (8).
Transfections

To determine if βTrCP and JCV TAg interact, and to analyze the subcellular localization of TAg and the GST-βTrCP proteins, transient transfections and co-transfections were carried out with lipofectamine 2000 following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Briefly, $5 \times 10^5$ of U87MG cells were plated on 60mm tissue culture plates in MEM supplemented with 10% fetal bovine serum (FBS), 2% sodium pyruvate, 1% essential amino acids, 2mM L-glutamine, penicillin G (100U/ml) and streptomycin (100μg/ml), and transfected 24 hours later with 1μg of each of the DNA constructs. After 24 hours post transfection (p. t.), medium was changed and cells were grown for 72 hours prior to cell lysis and analysis.

Antibodies

Monoclonal anti-T antibodies PAb962, PAb2000, PAb2001, PAb2003, PAb2024 and PAb2030 (referred in the text as cocktail of antibodies) (90, 91, 92) were used to detect JCV tumor proteins and PAb416 and PAb419 (93) were used to detect SV40 T proteins. GST-tagged proteins were detected with rabbit anti-GST antibody (1:5000) (Sigma-Aldrich, St. Louis, MO). Protein bands were visualized with secondary antibodies conjugated to either alkaline phosphatase (Sigma) or horseradish peroxidase (Cell Signaling Technology, Danvers, MA).
Cell extracts

Extracts of U87MG cells used to analyze the TAg-βTrCP interaction were prepared as follows: cells were washed twice with cold STE buffer (100mM NaCl, 10mM Tris-Cl pH 8.0, 1mM EDTA), and then were lysed with cold EBC lysis buffer (50mM Tris-HCl, pH 8.0; 120mM NaCl; 0.5% NP-40) containing the protease inhibitors leupeptin (10μg/ml), aprotinin (10μg/ml), pefabloc (1mM) and E-64 (10μg/ml), and the phosphatase inhibitors β-glycerophosphate (25mM), NaF (5mM) and NaVaO₄ (1mM) (Sigma) for 20 min while rocking at 4°C. Cell extracts were obtained 72 hours p.t. for binding analyses.

Immunoprecipitation, GST pull down experiments and Western blots analysis

Detection of T proteins was performed by immunoprecipitation (IP), by adding PAb962 (for JCV TAg) or PAb416 and PAb419 (for SV40 TAg) to the cell extracts, and incubating at 4°C with rocking for 1 hour. Subsequently, the immunocomplexes were collected with 15μl StaphA protein and electrophoresed in a 15% SDS-polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane and incubated with a cocktail of anti-T antibodies. The protein bands were visualized by adding a secondary antibody conjugated to alkaline phosphatase (41).

GST pull down assays to detect GST-tagged βTrCP1, GST-βTrCP2 and βTrCPΔF, and to analyze βTrCP-TAg interactions were described elsewhere (88). Briefly, 15μl of packed GSH agarose beads were added to the cell extracts and incubated overnight at 4°C while gently rocking. Next, beads were washed three times with lysis
buffer by centrifuging at 2000 rpm for 40 seconds in a microcentrifuge at 4°C. The samples were denatured in 2X Laemmli buffer and proteins were separated either in a 15% or 10% SDS-polyacrylamide gel to detect bound TAg or the GST-tagged βTrCP proteins, respectively. After transferring the proteins to a nitrocellulose membrane, a cocktail of anti-T antibodies was used to detect bound TAg; anti-GST antibody was used to detect the GST-tagged proteins. TAg and the GST-tagged βTrCP proteins were visualized with anti-mouse or anti-rabbit horseradish peroxidase, respectively.

**Phosphatase treatment**

λ-phosphatase treatment of 293 cell extracts was performed according to the manufacturer’s instructions (New England Biolabs, Ipswich, MA). 293 cells transfected with pCMV-JCV\textsubscript{E} were lysed 72 hours p.t. with EBC lysis buffer supplemented with protease inhibitors (2μg/ml leupeptin, 2μg/ml E-64, 1μg/ml aprotinin, 0.25mM pefabloc). Extracts were treated with λ-phosphatase for 25 min at 30°C. The reaction was terminated by adding 10mM Na\textsubscript{3}VO\textsubscript{4} and 50mM NaF.

**Immunofluorescence staining**

U87MG cells (3.5x10\textsuperscript{5}) were seeded onto coverslips and transfected with pCMV-T\textsuperscript{+}/t\textsuperscript{-}/T\textsuperscript{-} and the GST-tagged βTrCP constructs. At 48 hours p.t., the cells were fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.02% Triton X100 for 5 min. Cells were incubated with blocking solution (10% heat-inactivated goat serum)
(Millipore, Billerica, MA) for 45 min. Subsequently, cells were incubated with a cocktail of anti-T antibodies (PAb962, PAb2001, PAb2003, PAb 2024 and PAb 2030; 1:250) for 1 hour. Cells were washed with PBS and incubated for 30 min with goat anti-mouse Alexa Fluor 594 (1:1000) (Invitrogen). Cells were washed with PBS and incubated with blocking solution for 45 min prior to incubation with mouse anti-GST antibody (1:2000). Goat anti-mouse Alexa Fluor 488 (1:1000) (Invitrogen) was used as a secondary antibody. Double and single immunostainings were performed on cells that did not express TAg or GST-βTrCP1 (negative controls; data not shown). Immunostained cells were viewed under a confocal microscope (Olympus FV1000 Laser Scanning Confocal Microscope, Inverted Olympus IX-81, Cytometry Facility, Huck Institutes of the Life Sciences, Penn State University), using the FV10-ASW version 1.7 analyzing software. Sequential scans were used for all images.

**RT-PCR**

RNA was extracted from U87MG cells 42 hours p.t. using Trizol according to the manufacturer’s manual (Invitrogen). Preparation of cDNA from isolated RNA was carried out using the RevertAid first strand cDNA synthesis kit (Fermentas). The GST tag fragments were amplified using GoTaq PCR Core System I (Promega) with the primers GST-Fwd-5’CAACCCACTCGACTTCTTTTGGAA-3’ and GST-Rev-5’-GTCAGGATGGTTACATGATCACC-3’.
RESULTS

JCV TAg interacts with βTrCP1

The multifunctional JCV TAg induces transformation of cells by interacting with key cellular proteins involved in cell cycle regulation and signal transduction. I identified a potential βTrCP binding domain at the C-terminus of TAg (amino acids 639-645: DSGHGSS). To determine whether TAg interacts with βTrCP1, I performed GST pull down experiments with extracts of U87MG cells co-transfected with pCMV-JCV or pCMV-T+/t/T−, and plasmids expressing either wild type (WT) GST-βTrCP1 or mutant GST-βTrCPΔF. βTrCPΔF, a dominant negative form of βTrCP1, is missing the F-box domain required for interaction with components of the SCF complex (88). Because βTrCP1 targets its substrates for ubiquitination and degradation in the proteasome, I examined the integrity of the viral tumor proteins in the presence of GST-βTrCP1 (Figure 2-1A). The TAg expression level did not vary detectably in the presence of βTrCP1, suggesting that the stability of TAg is not altered in the presence of the F-box protein. I also analyzed the expression levels of the GST-tagged βTrCP1 and βTrCPΔF, and noticed that the level of the mutant protein was consistently higher than that of WT βTrCP1 (Figure 2-1B). In addition, protein bands ranging in size from 26 to 34 kDa and recognized by the anti-GST antibody were detected on the WB. These small peptides may result from partial degradation of GST-βTrCP1, and deletion of the F-box domain might confer increased stability to the βTrCPΔF protein. The GST pull down assay
Figure 2-1C) indicated that TAg binds both βTrCP1 and βTrCPΔF in the presence or absence of the other 4 early tumor proteins. Because TAg interacts with βTrCPΔF, which is unable to interact with the other components of the SCF complex, I conclude the interaction between TAg and the F-box protein is direct and does not occur through other members of the E3 ubiquitin ligase complex.

**SV40 TAg does not interact with βTrCP1**

The JCV and SV40 TAg sequences share 72% sequence identity (6), therefore I compared the primary sequence of SV40 TAg with that of JCV TAg to determine if SV40 TAg also possesses a βTrCP1 binding site. The C-terminus of SV40 TAg contains the related sequence DSGHETG (amino acids 646-662). The threonine at position 661 corresponds to serine 644 in the JCV TAg (Figure 2-2). Bora, an essential mitotic protein involved in spindle formation, is a known βTrCP1 target; it also has a threonine replacing the second serine of the binding motif (94).
Figure 2-1. JCV TAg interacts with βTrCP1.

Extracts from U87MG cells co-transfected with JCVE or TAg, and GST, GST-βTrCP1 or GST-βTrCPΔF (GST-ΔF) plasmids were subjected to IP or GST pull down analyses. (A) Cell extracts (50μg) were immunoprecipitated with anti-T antibody (PAb962) and the viral proteins were detected using an anti-T antibody cocktail. (B) Protein extracts (500μg) were analyzed for the expression of GST, GST-βTrCP1 and GST-βTrCPΔF by GST pull down and detection with an anti-GST antibody. (C) The interactions of TAg with βTrCP1 and βTrCPΔF were detected by performing GST pull downs of cell extracts (500μg) and immunoblotting with an anti-T antibody cocktail (lanes 1, 4-9). Cell extracts (30μg) containing either TAg or all five JCV early proteins (JCVE) were immunoprecipitated.
293T cells express SV40 early proteins and have been used in a number βTrCP1 studies (95, 74, 96, 84, 88). If SV40 TAg did bind βTrCP1, such an interaction would likely influence interpretations of data obtained in earlier studies. I performed GST pull down experiments with extracts from 293T cells transfected with the GST-βTrCP1 or GST-βTrCPΔF expressing constructs, or with extracts from the parental 293 cells co-transfected with pCMV-JCV\textsubscript{E} and either the GST-βTrCP1 or GST-βTrCPΔF plasmid. WB analyses demonstrated the presence of JCV and SV40 expressed in 293 and 293T cells, respectively (Figure 2-3A), and of GST-βTrCP proteins expressed in both cell lines (Figure 2-3B). The GST pull down analyses indicated that the SV40 TAg in 293T cells

<table>
<thead>
<tr>
<th>βTrCP1 consensus: DpSGX\textsubscript{2-4}pS</th>
</tr>
</thead>
<tbody>
<tr>
<td>JCV : 639–DSGHGSS–645</td>
</tr>
<tr>
<td>SV40 : 656–DSGHETG–662</td>
</tr>
</tbody>
</table>

**Figure 2-2. SV40 and JCV TAg sequence comparison of the destruction motif.** Sequence alignment of the SV40 and JCV TAg region containing the phosphodegron. The amino acid sequence in JCV TAg destruction motif spanning from amino acid 639 to 645 corresponds to the SV40 TAg region from amino acid 656 to 662. The phosphodegron is conserved between the two proteins, only differing in that SV40 TAg contains a threonine residue instead of a serine, as described by the consensus sequence (highlighted in red).
does not interact with βTrCP1 or βTrCPΔF, whereas JCV TAg binds both proteins in 293 cells (Figure 2-3C). These experiments were also performed in Cos-7 cells, an African green monkey kidney line transformed by an origin-defective mutant of SV40 (97), and again no binding was detected (data not shown).
Figure 2-3. SV40 TAg does not interact with βTrCP1.

Extracts of 293 and 293T cells expressing GST, GST-βTrCP1 or GST-βTrCPΔF (GST-ΔF), and either JCV or SV40 early proteins, respectively, were subjected to IP or GST pull down analyses. (a) Extracts (50µg) of 293T cells or 293 cells transfected with the JCV-E-expressing vector were immunoprecipitated with a mixture of PAb416 and PAb419 (anti-SV40 T) antibodies or PAb962 and immunoblotted with a mixture of anti-T antibodies that recognize both JCV and SV40 tumor proteins. (b) GST pull down assays were performed on extracts (500µg) of 293 and 293T cells, and GST, GST-βTrCP1 and GST-βTrCPΔF were detected by WB using anti-GST antibody. (c) Extracts (500µg) of 293 and 293T cells were examined for interactions of JCV and SV40 TAg with GST-βTrCP1 and GST-βTrCPΔF using GST pull down analyses, and immunoblotting with a mixture of anti-T antibodies (lanes 1, 4-11). Levels of JCV and SV40 TAg in the cell lines were examined by IP of cell extracts (15µg) using anti-JCV or –SV40 TAg antibodies and WB with the mixture of anti-T antibodies (lanes 2, 3). Small proteins recognized by the anti-GST antibody are marked with two asterisks.
**Phosphorylation is required for the TAg-βTrCP1 interaction**

βTrCP1 recognizes its target proteins through a phosphodegron with the consensus sequence DSpGX2-4Sp, where both serine residues are phosphorylated (72). The JCV TAg domain predicted to interact with βTrCP1, (DSGHGSS), contains three potentially phosphorylatable serine residues. To examine if phosphorylation is required for βTrCP1-TAg binding, I transfected 293 cells with pCMV-JCV, and 293T cells with either GST-βTrCP1 or GST-βTrCPΔF.

Since the endogenous SV40 TAg in 293T cells does not interact with βTrCP1 (Figure 2-3), I chose these cells to express GST-βTrCP1 and GST-βTrCPΔF. The expression plasmids for the GST-tagged proteins contain the SV40 origin of replication, therefore, their transfection into TAg-producing cells leads to amplification of the vector and high levels of βTrCP1 and βTrCPΔF expression. Phosphatase treatment of 293 cell extracts containing the JCV tumor proteins likely resulted in the dephosphorylation of the T proteins based on the visibly enhanced mobility of the T’ proteins on the blot (Figure 2-4A). These 293 cell extracts, with or without phosphatase treatment, were combined with extracts from 293T cells expressing GST-βTrCP1 or GST-βTrCPΔF (Figure 2-4B), and subjected to GST pull down analyses. Phosphatase treatment of the extracts containing the T proteins greatly reduced the interaction of TAg with βTrCP1 and βTrCPΔF (Figure 2-4C), indicating that phosphorylation of TAg may be required for the interaction between the viral and cellular proteins.
Figure 2-4. Phosphatase treatment abolishes the TAg-βTrCP1 interaction.

293 cells were transfected with pCMV-JCVE, and 293T cells were transfected with vectors expressing GST, GST-βTrCP1 or GST-βTrCPΔF (GST-ΔF). Extracts of 293 cells expressing the T proteins were either treated 72 hours p. t. with λ phosphatase (λPP) or left untreated. (A) 293 cell extracts (50μg) expressing the T proteins were either treated with λ phosphatase or left untreated and then immunoprecipitated with an anti-T antibody. T proteins were detected by WB with a cocktail of anti-T antibodies. (B) Cell extracts (600μg) were subjected to GST-pull down analysis and the GST-tagged proteins in 293T cells were detected with anti-GST antibody. (C) λ phosphatase treated or untreated 293 cell extracts (600μg) containing the T proteins were mixed and incubated with extracts from 293T cells (600μg) expressing GST, GST-βTrCP1 or GST-βTrCPΔF. Binding analyses of TAg to GST-βTrCP1 and GST βTrCPΔF were performed by the GST pull down method, and bound TAg was detected with a cocktail of anti-T antibodies. Phosphatase treatment abolished the TAg-βTrCP interaction.

Serine 640 in the JCV TAg is essential for binding βTrCP1

The two serines in the destruction motif of known βTrCP1 substrates must be phosphorylated for recognition by the F-box protein (98). To identify the serine
residue(s) in the JCV TAg destruction motif required for binding βTrCP1, I substituted each serine, either singly (Figure 2-5) or in combination (Figure 2-6), with alanine (e.g., Ser640 to Ala; S640A) and examined the ability of the mutant TAgs to interact with βTrCP1. U87MG cells were co-transfected with the GST-βTrCP1 or GST-βTrCPΔF expression plasmids and pCMV-JCV_E constructs either expressing WT or mutant TAgs. Equivalent expression levels of the WT and mutant T proteins in U87MG cells suggest the mutations do not affect the stability of any of the viral tumor proteins (Figure 2-5A, Figure 2-5B, Figure 2-6A, Figure 2-6B).

GST pull down analyses indicated that mutation of serine 640 abolishes the interaction of TAg with βTrCP1 and βTrCPΔF, while mutations of serines 644 and 645 do not (Figure 2-5D; Figure 2-6D). I did note; however, that mutant S644A consistently exhibited slightly reduced binding to βTrCP1 and βTrCPΔF (Figure 2-5D). These data indicate that serine 640, and to a lesser extent, serine 644, are involved in the interaction between TAg and βTrCP1.
Figure 2-5. Amino acids serine 640 and 644 influence TAg binding to βTrCP1.

pCMV-JCVE constructs carrying TAg point mutations S640A, S644A or S645A and GST-βTrCP1 or GST-βTrCPΔF (GST-ΔF) were co-transfected into U87MG cells, and extracts were collected 72 hours p. t. for IP and GST pull down analyses. (a) Expression levels of the wild type and single mutant T antigens were analyzed using extracts (50µg) immunoprecipitated with PAb962. The T proteins in cells expressing GST-βTrCP1, (a) or GST-βTrCPΔF, (b) were detected with a cocktail of anti-T antibodies. (c) GST pull down analysis of cell extracts (600µg) was performed to analyze the expression levels of GST, GST-βTrCP1 and GST-βTrCPΔF. The GST-tagged proteins were detected with α-GST antibody. (d) A GST pull down assay was performed on cell extracts (600µg) and wild type or mutant TAgS bound to GST-βTrCP1 and GST-βTrCPΔF were detected by WB with a cocktail of anti-T antibodies. Mutation of serine 640 of JCV TAg abolishes the TAg-βTrCP interaction. Small proteins recognized by the anti-GST antibody are marked with two asterisks.
Figure 2-6. TAg double mutants that include the S640A mutation are impaired in βTrCP1 binding.

pCMV-JCVE constructs carrying TAg point mutations S640A-S644A, S640A-S645A or S644A-S645A were co-transfected into U87MG cells with GST, GST-βTrCP1 or GST-βTrCPΔF (GST-ΔF), and extracts were collected 72 hours p. t. for IP and GST pull down analyses as described in the legend to Figure 4. Expression levels of the wild type and double mutant T antigens were determined in cells expressing GST-βTrCP1 (A), or GST-βTrCPΔF (GST-ΔF) (B), and expression levels of GST, GST-βTrCP1 and GST-βTrCPΔF are shown in (C). Constructs containing a mutation at serine 640 of JCV TAg greatly reduced the TAg-βTrCP interaction (lanes 5-7, 10-12) (D). Small proteins recognized by the anti-GST antibody are marked with two asterisks.
**TAg interacts with βTrCP2**

βTrCP2 shares 86% amino acid identity with βTrCP1, and both proteins recognize the phosphodegron, found in several common substrates, that contains the sequence DSpGX2-4Sp. However, the two proteins do exhibit functional differences, including the recognition of unique cellular targets and different subcellular localization patterns (78, 79). βTrCP1 is mainly found in the nucleus, whereas βTrCP2 localizes to the cytoplasm, suggesting that βTrCP1 promotes degradation of nuclear substrates, and βTrCP2 targets cytoplasmic substrates. Since some cellular targets, such as β-catenin or IκB, as well as the HIV viral protein Vpu, are known to interact with both βTrCP1 and βTrCP2 (70, 99), I determined whether TAg binds βTrCP2 by performing GST pull down analyses on cell extracts expressing GST βTrCP2 and either WT or mutant TAg. WB analyses demonstrating the expression levels of the T proteins (Figure 2-7A), and the GST-tagged βTrCP1 and βTrCP2 (Figure 2-7B) are shown. The GST pull down analysis confirmed that TAg interacts with βTrCP2 (Figure 2-7C, lane 6), and that serine 640 of TAg is required for this binding (Figure 2-7C, lane 8).
Figure 2-7 JCV TAg interacts with βTrCP2.

U87MG cells were co-transfected with pCMV-JCVE or the pCMV-JCVE construct carrying the TAg point mutation S640A, and GST, GST-βTrCP1 or GST-βTrCP2 plasmids. Cell extracts were prepared 72 hours post-transfection for IP and GST pull down analyses. (A) Cell extracts (50μg) were immunoprecipitated with anti-T antibody (PAb962) and viral proteins were visualized by WB, using an anti-T antibody cocktail. (B) Extracts (500g) were analyzed for the expression of GST, GST-βTrCP1 and GST-βTrCP2 using the GST pull down assay followed by WB with an anti-GST antibody. (C) The interactions of WT and mutant TAg with βTrCP2 were detected by performing GST pull downs of cell extracts (500μg) and immunoblotting with an anti-T antibody cocktail (lanes 1, 4-8). Cell extracts (30μg) containing WT TAg and mutant TAg were
immunoprecipitated with PAb962 followed by WB with an anti-T antibody cocktail (lanes 2, 3). Mock-transfected U87MG cells were used as a control (Con; lane 1). Small proteins recognized by the anti-GST antibody are marked with two asterisks.

**TAg co-localizes with βTrCP in the cytoplasm and depends on the ability of βTrCP to interact with the rest of the degradation complex**

βTrCP1 and polyomavirus TAg s are found predominantly in the nucleus, although a small percentage of these proteins are detected in the cytoplasm (100, 86).

To determine if TAg and βTrCP1 co-localize in the cell, double immunofluorescence staining was performed. U87MG cells were co-transfected with pCMV-T+/t/-T- and the GST-βTrCP1 plasmid. Cells were stained with antibodies directed against the T protein or against GST, and the subcellular localization of the viral or GST-tagged protein was analyzed by confocal microscopy. As expected, nuclear fluorescence predominated in cells expressing JCV TAg in the absence of GST-βTrCP1 (Figure 2-8A) and in cells expressing GST-βTrCP1 in the absence of TAg (Figure 2-8B). Some cytoplasmic staining could be detected in the latter case. When TAg and GST-βTrCP1 were co-expressed, the majority of cells exhibited TAg staining in both the nucleus and cytoplasm, and the two proteins co-localized in the cytoplasm (Figure 2-8C). Less frequently, some cells were observed to contain βTrCP1 in the nucleus, while co-localization again occurred in the cytoplasm (Figure 2-8D). A smaller number of cells showed co-localization in both cellular compartments (Figure 2-8E) or almost solely in the nucleus (Figure 2-8F).
Figure 2-8. TAg and βTrCP1 co-localize mainly in the cytoplasm.

(A to F) U87MG cells were transfected with 1μg of plasmid encoding TAg and GST-βTrCP1. 48 hours p. t. cells were fixed and immunostained with α-T and α-GST antibodies as described in Materials and Methods. Cells expressing TAg only show nuclear localization A). GST-βTrCP1, when expressed alone, is found in both cellular compartments, mainly in the nucleus. B). When TAg is co-expressed with GST-βTrCP1, co-localization of both proteins takes place in the cytoplasm in the majority of the cells observed (>100) C). Strong nuclear staining for either GST-βTrCP1 or TAg was also
observed C) and D). In a small number of cells, co-localization occurred in both compartments with the same intensity E) Fewer cells showed co-localization only in the nucleus f). The Pearson’s Coefficient varied between 0.97 and 0.99 for all the images.

Contrary to βTrCP1, βTrCP2 is mainly a cytoplasmic protein (86). I analyzed if TAg and βTrCP2 co-localize in the cell. Cytoplasmic fluorescence was detected exclusively in cells expressing GST-βTrCP2 in the absence of TAg as reported 86 (Figure 2-9B). When TAg and βTrCP2 were expressed together, co-localization was exclusively cytoplasmic, and TAg staining was observed in both nuclear and cytoplasmic compartments (Figure 2-9C).

Similarly, I analyzed the subcellular co-localization of TAg in the presence of βTrCPΔF, which lacks the F box domain. Deletion of the F-box prevents the interaction of βTrCP with the components of the degradation complex Skp1, Cul1 and Roc1. While still capable of interacting with its target proteins, βTrCPΔF can no longer degrade them. As expected, βTrCPΔF localizes in the nucleus when expressed independently (96) (Figure 2-9D). Interestingly, when TAg and βTrCPΔF were co-expressed, the two proteins co-localized only in the cell nucleus, and no cytoplasmic fluorescence was detected for TAg (Figure 2-9E), suggesting that the cytoplasmic localization of TAg depends on βTrCP and βTrCP’s ability to interact with other members of the SCF complex. This data also suggests βTrCP must interact with members of the SCF complex to induce its cytoplasmic translocation. Similar results were obtained in three independent experiments in which more than 100 individual cells were examined each time.
Figure 2-9. Co-localization of TAg and βTrCP.

U87MG cells were transfected with 1μg of plasmid encoding TAg and/or GST-βTrCP2 or GST-βTrCPΔF. Cells were fixed and immunostained with anti-T and anti-GST antibodies 48 hours post-transfection. (A) Cells expressing TAg only show nuclear localization. (B) βTrCP2 localizes in the cytoplasm. (C) Co-expression of TAg with βTrCP2 promotes relocalization of TAg to the cytoplasm. (D) βTrCPΔF localizes mainly in the nucleus when expressed alone. (E) When co-expressed, TAg and βTrCPΔF co-localize in the nucleus, and no cytoplasmic TAg staining is detected. Quantitative assessment of co-localization resulted in a Pearson’s Coefficient varying between 0.97 and 0.99 for all the images.
**JCV early tumor proteins elevate the levels of the exogenously-expressed GST-tagged proteins**

While analyzing the interaction between TAg and β-TrCP1, we noticed that high levels of expression of the GST-tagged βTrCP1 and βTrCPΔF proteins only occurred when JCV early proteins were co-expressed in the cells. This same observation was noted with the construct that only expresses the GST tag. These observations suggested that the JCV early proteins transactivate the CMV promoter within the GST expression vector. To test this possibility, U87MG cells were transfected with each JCV early protein: TAg, tAg, T′_{165}, T′_{136} or T′_{135} (Figure 2-10A, B), and co-transfected with βTrCP1 (Figure 2-10A), βTrCPΔF (Figure 2-10B) or GST-only vector. The levels of expression of the GST-tagged proteins were compared in extracts with or without T proteins. Each of the T proteins increase the levels of the GST-tagged proteins, as well as the GST-tag alone when compared with extracts from cells transfected with the GST-tagged proteins only (Figure 2-10C, D).

To determine if the increase in GST protein levels was due to the ability of T antigen to transactivate the CMV promoter, RT-PCR was performed to assess whether GST and GST-βTrCP1 mRNA levels correlated with the protein levels. The protein levels in U87MG cells co-transfected with CMV-JCV_{E} and GST or GST-βTrCP1 were detected by WB (Figure 2-11A, B). As expected, cells that were expressing the T proteins and the GST constructs showed increased levels of GST and GST-βTrCP1 when compared to cells that only expressed the GST constructs (Figure 2-11B, compare lanes 2 and 3, 4 and 5). Analysis of the mRNA levels by RT-PCR show slight or no elevation in the levels of mRNA in the presence of the tumor proteins (Figure 2-11C, D), indicating
that transactivation of the CMV promoter is not the cause of the elevated protein levels observed.

Figure 2-10. Expression levels of GST-\(\beta\)TrCP1 and GST-\(\beta\)TrCPΔF in the presence of the T proteins.

U87MG cells were co-transfected with pCMV-JCVE, pCMV-T+/t-\(\gamma\)-/T', pCMV-T+/t+/T', CMV-T'165, CMV-T'136, or CMV-T'135, and GST, GST-\(\beta\)TrCP1 or GST-\(\beta\)TrCPΔF (GST-\(\Delta\)F) plasmids. Cell extracts were prepared 72 hours p. t. for IP and GST pull down analyses. Expression levels of the single T proteins were determined in cells expressing GST-\(\beta\)TrCP1 (A), or GST-\(\beta\)TrCPΔF (GST-\(\Delta\)F). Cell extracts (50μg) were immunoprecipitated with anti-T antibody (PAb962) and viral proteins were visualized by WB, using an anti-T antibody cocktail. (B) Extracts (500g) were analyzed for the expression of GST, GST-\(\beta\)TrCP1 (C), and GST-\(\beta\)TrCPΔF (GST-\(\Delta\)F) (D) using the GST pull down assay followed by WB with an anti-GST antibody.
Figure 2-11. Analysis of the mRNA levels of GST and GST-βTrCP in the presence or absence of the T proteins.

A) mRNA levels of GST and GST-tagged βTrCP1 in U87MG cells transfected with pCMV-JCVE or pCR3 empty vector and GST or GST-βTrCP1 (top panel). Detection of GAPDH mRNA was used as a control (bottom panel). The graph represents the amount of GST and GST-βTrCP1 mRNA present in the presence or absence of the T proteins, relative to the GAPDH mRNA.
CHAPTER 3
MUTATION OF THE TAg DESTRUCTION MOTIF AFFECTS
VIRAL DNA REPLICATION AND p53 LEVELS BUT NOT β-
CATENIN LEVELS
ABSTRACT

Viral replication and transformation of cells in culture by JCV involves the actions of five viral tumor proteins, which interact with cellular factors regulating critical cellular processes. JCV TAg has a destruction motif that directs its interaction with the substrate recognition factors βTrCP1 and βTrCP2, which are involved in targeting cellular proteins for ubiquitination and proteasomal degradation. βTrCP targets include proteins critical to regulating signal transduction pathways and cell cycle progression. I examined the ability of wild type and mutant forms of JCV TAg to influence βTrCP’s activity towards two of its substrates, β-catenin and p53. I was unable to demonstrate that TAg elevates β-catenin levels as previously reported, and the mutant TAg, which is unable to bind βTrCP, also had no detectable effect on β-catenin stability. I did, however, observe that the wild type and mutant TAgS differentially influence p53 levels. Cells expressing mutant TAg, yielded higher levels of p53 than did cells expressing the wild type TAg. However, control experiments revealed that βTrCP is not responsible for the observed differences in p53 levels. DNA replication assays showed that mutants altered within the TAg destruction motif exhibit defects at late times of the viral replication cycle, highlighting a possible role for βTrCP in JCV replication and propagation.
INTRODUCTION

I have described an interaction between JCV TAg and beta-transducin repeat-containing protein (βTrCP), the substrate recognition molecule of the proteasome degradation complex SCF$^{βTrCP}$ ubiquitin ligase. βTrCP is involved in the recognition and subsequent degradation of cellular proteins that play pivotal roles in cell cycle regulation and signal transduction pathways, events that are tightly associated with viral oncogenic behavior in vivo and in vitro. Several studies have linked βTrCP activity to human cancers, including colorectal cancers, hepatocarcinomas and melanomas (101, 102, 103, 104). βTrCP is thought to contribute to the oncogenic process through its ability to regulate a variety of substrates that include proto-oncoproteins, such as β-catenin and NFκB, and tumor suppressor proteins, like p53 and REST (4, 23, 85, 88). Additionally, βTrCP involvement in cell cycle regulatory mechanisms links this substrate recognition factor to tumor formation. For instance, βTrCP1 is a key player in the S and G2 DNA damage checkpoint pathways, and it promotes cell cycle arrest following DNA damage through degradation of Cdc25A. βTrCP1 also has the ability to restore cell cycle progression and entry into mitosis by degrading the mitotic entry regulators clasin and WEE1 (75). Other targets regulated by βTrCP include IκB, Emi1, ATF4 and PDCD4 (4, 105, 106, 84).

DNA tumor viruses rely upon their host’s replication machinery to amplify their genomes. Viral interference with the function of key cellular proteins is essential to the virus life cycle. Furthermore, several viral-cellular protein interactions have been described that mediate virally-induced transformation. Two of the cellular proteins
targeted by JCV TAg are substrates for βTrCP proteasomal degradation, β-catenin and p53 (25, 26, 20). JCV TAg’s interactions with these two substrates contribute to the transforming potential and replication capabilities of the virus (1, 20, 107). It has been reported that TAg binds β-catenin resulting in β-catenin’s stabilization, translocation into the nucleus and increased transactivation activity (25, 26). TAg also modulates β-catenin stability and subcellular localization through Rac1 activation (27).

On the other hand, the interaction of TAg with p53 inhibits this tumor suppressor’s transcriptional activity and increases its stability, in part, by preventing its degradation via the E3 ubiquitin ligase, MDM2 (20). Given the importance of βTrCP in the regulation of several crucial cellular events that are vital to a successful viral life cycle, as well as the fact that JCV TAg interacts with βTrCP, I thus analyzed the effects this interaction might have on p53 and β-catenin levels. I also examined the consequences that mutations in the destruction motif of TAg might have on JCV DNA replication.

In this study, I asked whether the TAg-βTrCP interaction was responsible, in part, for the ability of TAg to alter β-catenin stability (25, 26, 27) However, under my experimental conditions, I was unable to demonstrate that WT TAg significantly alters the levels of endogenous or exogenous β-catenin as was previously reported. Similarly, mutations within the TAg destruction motif did not affect β-catenin levels, suggesting that TAg does not regulate β-catenin concentrations through an interaction with βTrCP. I did observe that the endogenous p53 levels in cells expressing a TAg mutant deficient in βTrCP binding was higher than that found in cells expressing wild type TAg, or no TAg. However, experiments performed using the dominant negative mutant βTrCPΔF or the proteasome inhibitor, MG132, suggest that βTrCP is not involved in this elevation of p53
levels. Finally, I determined that mutations in the destruction motif of TAg at serine 640 and, to a lesser extent, serine 644 and 645 are detrimental to JCV DNA replication.
MATERIALS AND METHODS

DNA constructs

The pCMV-JCV_E vector expresses the JCV early coding region and was described earlier (41, 18). The single mutants pCMV-JCV_E(S640A), pCMV-JCV_E(S644A) and pCMV-JCV_E(S645A) and the double mutants pCMV-JCV_E(S640A-S644A), pCMV-JCV_E(S640A-S645A) and pCMV-JCV_E(S644A-S645A) were created by PCR-based site-directed mutagenesis with primers identified in Table 1 (Chapter 2).

The pJCV(Mad1)BS construct contains the JCV variant Mad1 genome cloned into the Bluescript vector and was described earlier (17). The single mutants pJCV(Mad1)BS-S640A, pJCV(Mad1)BS-S644A and pJCV(Mad1)BS-S645A were created by isolating a Bpu10I-PF1MI restriction enzyme fragment containing the destruction motif mutation in pCMV-JCV_E(S640A), pCMV-JCV_E(S644A) and pCMV-JCV_E(S645A). Subsequently, these fragments were exchanged for the corresponding WT fragment in the pJCV(Mad1)BS vector by restriction enzyme digestion and ligation. Prior to transfection of cells in the replication assays experiments, WT and mutant JCV(Mad1) genomes were excised from the Bluescript vector by EcoRI digestion, purified and recircularized by ligation.

The pGST-βTrCPΔF constructs were gifts from Dr. Wade Harper (88). The pCS2MT and pCS2MT-myc-β-catenin vectors were kindly provided by Dr. Arnold Levine (89).
Cell culture

U87MG cells were grown in minimum essential Eagle’s medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2% sodium pyruvate, 1% essential amino acids, 2mM L-glutamine, penicillin G (100U/ml) and streptomycin (100μg/ml). PHFG, RKO and U2OS cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS, L-glutamine, penicillin G (100U/ml) and streptomycin (100μg/ml). HC116 cells were grown in McCoy medium supplemented with 10% FBS, L-glutamine, penicillin G (100U/ml) and streptomycin (100μg/ml). PHFG and U87MG cells were incubated at 37°C in 10% CO₂, and RKO, HC116 and U2OS cells were incubated at 37°C in 5% CO₂.

Transfections and cell extracts

Transient transfections and co-transfections for the immunoprecipitation and Western blots analyses were carried out with lipofectamine 2000 following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Briefly, 5x10⁵ cells were plated on 60mm tissue culture plates, and transfected 24 hours after seeding with 8μg of each of the DNA constructs. Cell extracts were prepared as previously described (Bollag, et al 2006). Prior to lysing, cells were washed twice with cold STE buffer (100mM NaCl, 10mM Tris-Cl pH 8.0, 1mM EDTA). Subsequently, cells were lysed with cold EBC buffer (50mM Tris-HCl, pH 8.0; 120mM NaCl; 0.5% NP-40) containing the protease inhibitors leupeptin (10μg/ml), aproatinin (10μg/ml), pefabloc (1mM) and E-64 (10μg/ml), and the phosphatase inhibitors β-glycerophosphate (25mM), NaF (5mM) and NaVaO₄
(1mM) (Sigma) for 20 min while rocking at 4°C. Cell extracts were prepared 48 hours p.t.

**Antibodies**

Monoclonal anti-T antibodies PAb962, PAb2000, PAb2001, PAb2003, PAb2024 and PAb2030 (90, 91, 92) were used to detect JCV tumor proteins. PAb416 was used to analyze the interaction of TAg with p53 and β-catenin (93). GST-tagged βTrCPΔF and β-actin were detected with rabbit anti-GST antibody (1:5000) and anti-β-actin (1:5000) (Sigma-Aldrich, St. Louis, MO), respectively. Rabbit anti-p53 (1:300) was used to detect endogenous p53, and mouse anti-β-catenin (E5) (1:250) was used to detect myc-tagged and endogenous β-catenin (Santa Cruz Biotechnologies, Santa Cruz, CA). Protein bands were visualized with secondary antibodies conjugated to either alkaline phosphatase (Sigma) or horseradish peroxidase (Cell Signaling Technology, Danvers, MA).

**Immunoprecipitation and Western blot experiments**

The detection of the T proteins by immunoprecipitation (IP) involved the addition of PAb962 to the cell extracts, and incubation at 4°C while rocking for 1 hour. Subsequently, the immunocomplexes were collected with StaphA protein and electrophoresed in a 15% SDS-polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane and incubated with a cocktail of anti-T antibodies. The protein
bands were visualized by adding a secondary antibody conjugated to alkaline phosphatase (41).

Analysis of the interaction of TAg with endogenous p53 or β-catenin was performed by IP. PAb416 was added to the cell extracts, and the mixture was incubated at 4°C while rocking overnight. Immunocomplexes were collected with 10µl of packed protein A/G sepharose beads (Santa Cruz Biotechnologies, Santa Cruz, CA) for 4 hours at 4°C with gentle rocking, and denatured proteins were electrophoresed in a 10% or 7.5% SDS-polyacrylamide gel for p53 and β-catenin, respectively. The separated proteins were transferred to a nitrocellulose membrane and incubated with anti-p53 or anti-β-catenin antibodies. The proteins were detected by incubating the membranes with a secondary antibody conjugated to horseradish peroxidase.

Expression levels of endogenous p53 and β-catenin, and exogenous myc-tagged β-catenin were detected by WB. Membranes were incubated with anti-p53 or anti-β-catenin primary antibodies. The proteins were visualized by incubating the membranes with a secondary antibody conjugated to horseradish peroxidase. The blots were stripped using the Blot Restore Membrane Rejuvination kit following the manufacturer’s directions (Millipore), and reprobed with either an anti T-protein cocktail (PAb962, PAb2000, PAb2001, PAb2003, PAb2024 and PAb2030), anti-GST or anti β-actin, for the detection of the T proteins, GST-βTrCPΔF or β-actin, respectively.
MG132 treatment

To determine if proteasome degradation was involved in TAg’s effect on p53 levels, 5x10^5 U2OS cells were plated in 60mm plates and transfected with 5μg of either WT CMV-JCV_E or pCMV-JCV_E(S640A) for 48 hours. Cells were then treated with 10 μM MG132 in DMSO or left untreated 4 hours prior to lysis of cells and collection of the extracts. The levels of endogenous p53 were determined by Western blot.

Dpn1 Replication Assay

Analysis of the replication phenotypes of the JCV(Mad1)-S640A, JCV(Mad1)-S644A and JCV(Mad1)-S645A mutants was performed in PHFG cells. Briefly, PHFG cells were seeded in 60mm plates and grown for 48 hours in DMEM + 10% FBS (supplemented with L-glutamine, penicillin G (100U/ml) and streptomycin (100μg/ml)). Transfection of cells with JCV(Mad1)-S640A, JCV(Mad1)-S644A and JCV(Mad1)-S645A DNA was carried out using the DEAE-dextran method. Prior to transfection, cells were washed twice with warm DMEM. Cells in each plate were then treated with 200ng of circularized viral DNA diluted in 1ml of a 50:50 solution consisting of 500μl of DMEM and 500μl DEAE-dextran reagent (50 mM Tris, pH 7.5; 0.2% DEAE-dextran, molecular weight 500,000; and DMEM). Cells were incubated with the transfection reagent for 2 hours at 37°C with gentle rocking every 15 minutes. Subsequently, the DNA-DEAE dextran solution was washed off the plates with warm DMEM and cells were grown in DMEM with 10% FBS. The medium was changed 24 hours p. t. to
DMEM + 3% FBS. Viral DNA was extracted from the cells at days 0, 5, 10 and 14 p. t. using the Hirt method (108).

To perform the DpnI replication assay, Hirt-extracted DNA was digested with EcoRI (to linearize the viral DNA) and DpnI (to digest the methylated input DNA) for 3 hours at 37°C. The linearized DNA was electrophoresed on a 0.8% agarose gel at 20 volts overnight. Separated DNA was transferred to a Hybond-N+ nylon membrane (Amersham) using a Rapid Downward Transfer System and an alkaline transfer protocol (Schleicher and Schuell, Amersham), and then crosslinked to the membrane using a UV-crosslinker (Ultra-LUM) for 1.5 minutes at 254nm.

The membrane was incubated with a linear JCV DNA probe radioabeled with [α-32P] dCTP using the Prime-a-Gene kit and following the manufacturer’s instructions (Promega). The linear 5kbp bands representing replicated JCV DNA were visualized using the Typhoon PhosphorImager, and band intensities were quantified using the ImageQuant 5.2 software (Molecular Dynamics).
RESULTS

Wild type and mutant TAg s do not alter the levels of the βTrCP substrate β-catenin

β-catenin is one of several cellular proteins targeted for degradation by SCF$^{βTrCP}$, and its recognition as a substrate requires phosphorylation of serines 33 and 37 residing within the phosphodegron recognized by βTrCP (22). TAg has been reported to interact with β-catenin and to promote its stabilization (26). I hypothesized that this TAg function might be mediated through its interaction with βTrCP. U87MG cells were transfected with pCMV-JCV$_E$ or pCMV-JCV$_E$ constructs encoding the S640A, S644A or S645A mutant TAg s, and TAg and β-catenin levels were examined by WB analysis (Figure 3-1A, B). I observed, as predicted, that endogenous β-catenin levels in cells expressing the S640A mutant TAg were similar to those found in untransfected cells (Figure 3-1B, lane 3 vs. 1). Contrary to reports in the literature, I did not observe a significant elevation of β-catenin in cells producing WT TAg (Figure 3-1B, lane 2).

The experiment was repeated in PHFG cells and the human colon cancer cell line RKO. In RKO cells, loss of expression of E-cadherins prevents the accumulation of β-catenin in the membrane resulting in a reduction of β-catenin levels (109, 110). I reasoned that if TAg enhances β-catenin stability, the change would be most easily detected in cells initially containing low amounts of β-catenin. The results obtained in both PHFG and RKO cells were similar to those observed with U87MG cells, i.e. TAg expression did not elevate β-catenin levels (Figure 3-2).
Figure 3-1. TAg does not alter β-catenin levels.

U87MG cells were transfected with 8μg of the wild type pCMV-JCVE plasmid (JCV E), or with constructs expressing mutant T proteins altered at serine 640, 644 or 645. Cell extracts were analyzed at 48 hours p.t. (A) Expression of T proteins in U87MG cells was detected by IP of cell extracts (50μg) with PAb962, and immunoblotting with a cocktail of anti-T antibodies. (B) Cell extracts (30μg) were subjected to WB, and endogenous β-catenin was detected with an anti-β-catenin antibody. Lane 1 shows the endogenous levels of β-catenin in cells transfected with pCR3 vector as a control. Endogenous β-catenin levels in the presence of WT or mutant TAg correpond to lanes 2 through 5. The blots were stripped and reprobed for β-actin with anti-β-actin antibody as a loading control. Relative differences in β-catenin levels between extracts were determined by normalizing β-catenin values to actin levels using the ImageJ program. Based upon these calculations, a value of 1.0 was arbitrarily assigned to JCVE–transfected cells. The relative β-catenin levels observed in cells expressing the S640A was 0.9 (B, lane 3).
Figure 3-2. Levels of endogenous β-catenin in the presence of WT or mutant TAg in RKO and PHFG cells.

RKO and PHFG cells were transfected with the wild type pCMV-JCVE plasmid, or with constructs expressing mutant T proteins altered at serine 640, 644 or 645. Cell extracts were analyzed at 48 hours p. t. Expression of T proteins in RKO cells (A) and PHFG cells (C) were detected by IP of cell extracts (50μg) with PAb962, and immunoblotting with a cocktail of anti-T antibodies. Cell extracts (30μg) were subjected to WB, and endogenous β-catenin in RKO cells (B) and PHFG cells (D) was detected with an anti-β-catenin antibody. Lane 1 shows the endogenous levels of β-catenin in RKO (B) and PHFG (D) cells transfected with pCR3 vector as a control. Endogenous β-catenin levels in the presence of WT or mutant TAg correspond to lanes 2 through 5 (B, D). The blots were stripped and reprobed for β-actin with anti-β-actin antibody as a loading control (B, D). Relative differences in β-catenin levels between extracts (B, D) were determined by normalizing β-catenin values to actin levels using ImageJ. Based upon these calculations, a value of 1.0 was arbitrarily assigned to JCVE–transfected cells. The relative β-catenin levels observed in RKO and PHFG cells expressing the S640A vs. WT TAg was 1 (B, lane 3) and 0.7 (D, lane 3), respectively.
In earlier studies demonstrating elevated β-catenin levels in TAg-expressing cells, experiments were carried out with exogenously expressed myc-tagged β-catenin (26, 27). To investigate whether differences between my findings and those previously published were due to changes in experimental conditions, I repeated my work using the myc-tagged β-catenin construct (89). U87MG cells were co-transfected with the pCMV-JCV<sub>E</sub> construct and the myc-β-catenin vector. The expression of WT and mutant T antigens were detected by IP/WB (Figure 3-3A). WB detection of exogenous and endogenous β-catenin indicated that the levels of myc-tagged β-catenin were not altered in the presence of the JCV early tumor proteins; the levels of endogenous β-catenin also remained unchanged (Figure 3-3 B). These data fail to support my prediction that JCV TAg stabilizes β-catenin by interfering with βTrCP-induced degradation, since i) the inability of mutant TAg to bind βTrCP does not result in decreased levels of β-catenin relative to that measured using WT TAg and ii) I was unable to confirm the earlier report that TAg stabilizes β-catenin (26), an observation upon which my hypothesis was formulated.

Similar results were obtained in the presence of WT and mutant TAg after synchronizing the cells by serum starvation and then analyzing β-catenin levels in cell extracts collected at 24 or 72 hours p. t.

**Mutations of serine 640 and 644 increase the levels of endogenous p53**

The tumor suppressor, p53, is another cellular substrate of βTrCP1. Upon phosphorylation of serine 366 and possibly serine 362 by IKK2, p53 is recognized by SCFβTrCP1 and targeted for ubiquitination and proteasomal degradation (85). JCV TAg
binds and stabilizes p53 (1), and I hypothesized that TAg’s stabilization function, in part, involves its interaction with, and inhibition of, βTrCP1. To test this possibility, PHFG cells were transfected with the pCMV-JCV_E constructs encoding WT or mutant TAg s. Expression of the T proteins (Fig. 3.4A) and endogenous p53 (Fig. 3.4B) was examined by WB. As expected, endogenous p53 levels were elevated in the presence of WT TAg (Fig. 3.4B, compare lanes 1, 2). However, contrary to my prediction, the endogenous levels of p53 were even higher in cells transfected with the S640A TAg mutant (Fig. 3.4B, compare lanes 2, 3). Higher levels of p53 were also observed in cells expressing the S644A mutant TAg (Fig. 3.4B, compare lanes 2, 4), which like the S640A TAg, exhibits reduced binding to βTrCP1 (Fig. 3.4B). The level of p53 detected in cells expressing the S645A TAg mutant was similar to that found in cells expressing WT TAg (Fig. 3.4B, compare lanes 2, 5).
Figure 3-3. TAg does not affect the exogenous myc-β-catenin levels.

U87MG cells were transfected with 8μg of the wild type pCMV-JCVE plasmid, with mutant T protein altered at serine 640 and with a construct expressing myc-tagged β-catenin. Cell extracts were analyzed at 48 hours p.t. (A) Expression of T proteins in U87MG cells were detected by IP of cell extracts (50μg) with PAb962, and immunoblotting with a cocktail of anti-T antibodies. (B) Cell extracts (30μg) were subjected to WB, and endogenous β-catenin and myc-β-catenin were detected with an anti-β-catenin antibody. Lane 1 shows the endogenous levels of β-catenin in cells transfected with pCR3 vector as a control. Endogenous β-catenin and myc-β-catenin levels in the presence of WT or mutant TAg correspond to lanes 2 through 4. The blots were stripped and reprobed for β-actin with anti-β-actin antibody as a loading control. Relative differences in endogenous and exogenous β-catenin levels between extracts were determined by normalizing β-catenin and myc-β-catenin values to actin levels using the ImageJ program. Based upon these calculations, a value of 1.0 was arbitrarily assigned to JCVE–transfected cells (lane 3).
To confirm these observations, I repeated my experiments using the human osteosarcoma-derived cell line U2OS. This cell line contains basal levels of constitutively active IKK2, the kinase required to phosphorylate p53 for the subsequent recognition by βTrCP1 (85). Expression of WT and mutant TAgS (Figure 3-4C), and endogenous p53 (Figure 3-4D) were analyzed by WB. As in PHFG cells, a modest elevation of p53 levels was consistently observed in U2OS cells producing the S640A TAg mutant as compared to p53 amounts in cells expressing WT TAg (Figure 3-4D). Importantly, TAg binding to p53 was not impaired by the mutation at serine 640 (Figure 3-4E).
Figure 3-4. Mutations of serines 640 and 644 of TAg alters p53 levels.

PHFG and U2OS cells were transfected with the wild type pCMV-JCVE plasmid, or with constructs expressing mutant T proteins altered at serine 640, 644 or 645. Cell extracts were analyzed at 48 hours p. t. Expression of T proteins in PHFG cells (A) and U2OS cells (C) were detected by IP of cell extracts (50μg) with PAb962, and immunoblotting with a cocktail of anti-T antibodies. Cell extracts (30μg) were subjected to WB, and endogenous p53 in PHFG cells (B) and U2OS cells (D) was detected with an anti-p53 antibody. Lane 1 shows the endogenous levels of p53 in PHFG (B) and U2OS (D) cells transfected with pCR3 vector as a control. Endogenous p53 levels in the presence of WT or mutant TAg correspond to lanes 2 through 5 (B, D). The blots were stripped and reprobed for β-actin with anti-β-actin antibody as a loading control (B, D). Relative differences in p53 levels between extracts (B, D) were determined by normalizing p53 values to actin levels using ImageJ. Based upon these calculations, a value of 1.0 was arbitrarily assigned to JCVE–transfected cells (D). The relative p53 levels observed in PHFG and U2OS cells expressing the S640A vs. WT TAg was 1.7 (B, lane 3) and 1.5 (D, lane 3), respectively. The graph (B, right panel) represents the mean p53 levels, relative to mock, from three independent experiments in PHFG cells. A value of 1 was assigned
to mock. The standard deviation from these data are indicated with the vertical error bars (B, right panel). The p values were calculated relative to mock and relative to WT, and were less than 0.05. The levels of p53 in U2OS cells were detected only once (D). Extracts (450µg) of PHFG cells transfected with the JCVE-expressing vector or with a construct expressing TAg with the S640A mutation were immunoprecipitated with PAb416 (E, lanes 3, 4) or IgG as a negative control (E, lanes 5, 6), and bound p53 was detected by immunoblotting with an anti-p53 antibody. Extracts (30µg) of cells expressing either WT or mutant TAg were subjected to WB analysis to detect the endogenous levels of p53 (E, lanes 1, 2).

To elucidate whether the observed effect on p53 levels in the presence of mutant TAgS was related to TAg’s ability to interact with βTrCP1, I transiently co-transfected PHFG cells with either WT TAg or TAg carrying the S640A mutation and the construct expressing the dominant negative form of βTrCP, βTrCPΔF. βTrCPΔF lacks the F-box domain, preventing the protein from interacting with the rest of the degradation complex. Thus, while still being able to interact with a particular substrate, βTrCPΔF cannot induce the ubiquitination and proteasomal degradation of the targeted protein (88). Based on this principle, I would expect that TAg bound to βTrCPΔF would not promote the degradation of p53, and cells would contain higher levels of p53 in the presence of WT TAg that would be equivalent to those detected in cells expressing the TAg mutant. The expression of WT and mutant T antigens was detected by IP/WB (Figure 3-5A), and the expression of GST-βTrCPΔF was detected by WB (Figure 3-5B, middle panel). The results indicate that the expression of GST-βTrCPΔF in the presence of WT TAg did not elevate p53 levels to those detected in cells co-transfected with βTrCPΔF and mutant TAg (Figure 3-5B, top panel, compare lanes 3, 4 and 5, 6), suggesting that βTrCP1 is not responsible for the increased levels of p53 observed in the presence of mutant TAg.
Figure 3-5. Expression of βTrCPΔF in the presence of TAg does not affect p53 levels.

PHFG cells were transfected with 8μg of the pCMV-JCVE or the pCMV-JCVE(S640A) plasmid, and either with or without a construct expressing GST-βTrCPΔF. Cell extracts were analyzed at 48 hours p.t. (A) Expression of T proteins in PHFG cells were detected by IP of cell extracts (50μg) with PAb962, and immunoblotting with a cocktail of anti-T antibodies. (B) Cell extracts (30μg) were subjected to WB, and endogenous p53 was detected with an anti-p53 antibody. Lane 1, 3 and 5 show the endogenous levels of p53 in cells transfected with pCR3 vector (control), pCMV-JCVE and pCMV-JCVE(S640A), respectively. The levels of p53 in the absence (B, lane 2) or presence of WT and mutant
TAG (B, lanes 4, 6, respectively) and GST-βTrCPΔF are shown. The blot was stripped and reprobed for GST with an anti-GST antibody to detect the presence of GST-βTrCPΔF, and for β-actin with anti-β-actin antibody as a loading control. (C) U2OS cells transfected with pCR3 (lanes 1, 2), WT-TAg (lanes 3, 4) or S640A-TAg (lanes 5, 6) were treated with 10µM of MG132 (lanes 2, 4, 6) for 4 hours or mock treated with DMSO (lanes 1, 3, 5) prior to cell lysis, and levels of p53 in 30µg of extracts were analyzed by WB using an anti-p53 antibody. The blot was stripped and reprobed with an anti-T antibody and an anti β-actin antibody to detect the levels of the T protein (middle panel) and β-actin (lower panel) as a loading control, respectively. Relative differences in endogenous p53 levels between extracts were determined by normalizing p53 to actin levels using the ImageJ program. Based upon these calculations, a value of 1.0 was arbitrarily assigned to JCVE–transfected cells.

It is important to note that in cells transfected with GST-βTrCPΔF in the absence of TAg, levels of p53 are not altered relative to cells that do not express βTrCPΔF (Figure 3-5B, top panel, compare lanes 1, 2). In the one previous report describing the effects of βTrCP1 on p53 levels, the experimental conditions required treatment with doxorubicin, a DNA damaging agent that induces high levels of stable p53 (85). The levels of p53 in the presence of GST-βTrCPΔF may remain similar to those found in untransfected cells due to the lack of a stressor that promotes the increased levels of p53. Additionally, it was shown by Xia and co-workers (85) that proteasomal degradation of p53 by the SCFβTrCP1 complex requires active IKK2, and it is possible that levels of active IKK2 in PHFG cells are too low to phosphorylate p53 within the phosphodegron and therefore p53 is not recognized by βTrCP. Finally, it is plausible that the reported ability of βTrCP to degrade p53 is much lower than that caused by other ubiquitin ligases, such as MDM2, and thus its activity is masked in my experiments.

Although I did not find βTrCP to play a major role in regulating p53 stability, I used the proteasome inhibitor MG132 to confirm whether the increased level of p53 detected in the presence of mutant TAg was due to the failure of TAg to degrade p53 in a
proteasome-dependent manner. If TAg were interfering with the proteasome degradation machinery, I would expect that MG132 treatment of cells expressing WT TAg would increase p53 levels to those detected in cells expressing mutant TAg. Thus, I transiently transfected U2OS cells with either WT or mutant S640A TAg and, prior to lysing, treated the cells with MG132 or left them untreated (Figure 3-5C). As previously observed, the levels of p53 detected in cells expressing WT TAg were elevated when compared to mock-transfected cells (Figure 3-5C, compare lanes 1, 3), and the levels of p53 in cells expressing TAg carrying the S640A mutation were higher than those found in cells expressing WT TAg (Figure 3-5C, compare lanes 3, 5). As expected, endogenous p53 in mock-transfected cells increased upon treatment with MG132 (Figure 3-5C, compare lanes 1, 2). However, contrary to my expectations, the levels of p53 from cells transfected with WT TAg remained unchanged after treatment with MG132 (Figure 3-5C, compare lanes 3, 4). The levels of endogenous p53 in MG132-treated cells expressing mutant TAg were comparable to those found in untreated cells (Figure 3-5C, compare lanes 5, 6). Thus, based on these results, it is probable that TAg does not stabilize p53 by inhibition of a proteasome degradation pathway.

**Mutation of serine 640 within the TAg destruction motif inhibits JCV DNA replication.**

DNA viruses require cellular machinery to replicate their genomes, produce infectious virions and spread to neighboring cells, and TAg mediates these processes through its interactions with a variety of cellular proteins (1). βTrCP contributes to the regulation of cell cycle progression and DNA damage response pathways by controlling
the turnover of key players such as Cdc25A/B, Wee1 and Claspin (87). I determined whether mutations in the destruction motif, which reduce TAg’s interaction with βTrCP, affected viral DNA replication activity. I transfected PHFG cells with JCV(Mad1), JCV(Mad1)-S640A, JCV(Mad1)-S644A and JCV(Mad1)-S645A genomes and examined viral DNA replication activity at days 0, 5, 10 and 14 p. t. using the DpnI replication assay (Figure 3-6). Quantitation of the WT and mutant replicating DNAs revealed that mutations at serine 640 and 645 of TAg negatively affect JCV replication (Fig 3.6, compare lanes 3, 4 with lanes 5,6 and 9,10), while replication of the mutant S644A was similar to that of WT (Fig 3.6, compare lanes 3, 4 with lanes 7, 8). Reduced replication activity was most apparent late in infection (days 10, 14 p. t.). I also observed that JCV(Mad1)-S645A consistently exhibited a slightly enhanced replication potential at day 5 p. t. when compared to WT JCV(Mad1) (Figure 3-6, compare lanes 3, 4 with 9, 10).

Similar results were obtained in three independent experiments, and their relative DNA replication activity values are shown (Table 3-1A, B, C). Table 3-1D represents the average values of the three independent replication activity assay results.
Figure 3-6. Mutations at serines 640 and 645 of TAg negatively affects JCV DNA replication.

PHFG cells were transfected in duplicate with 200ng of JCV(Mad1) (lanes 3 and 4), JCV(Mad1)-S640A (lanes 5, 6), JCV(Mad1)-S644A (lanes 7, 8) or JCV(Mad1)-S645A (lanes 9, 10). DNA from the duplicate independent samples was extracted at days 0, 5, 10 and 14 p. t. using the Hirt protocol (Hirt, 1967), digested with DpnI and EcoRI and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a nitrocellulose membrane and hybridized with a [α-32P] labeled JCV DNA probe. The replicated DNA (arrows) was visualized with a Typhoon PhosphorImager and bands were quantified with the ImageQuant 5.2 Software. Lanes 1 and 11 (M) show the marker, consisting of 1ng of linear JCV (5130 base pairs). Lane 2 represents untransfected cells used as a control (C).
Table 3-1. JCV relative DNA replication activity in PHFG cells.

The relative replication activity from three independent experiments, experiment 1, 2 and 3, is listed in tables A, B and C, respectively. The average value of the DNA replication activity of WT and mutant JCV(Mad1) genomes from three independent experiments (Table A, B and C) was calculated (Table D). A value of 1 was arbitrarily assigned to WT JCV(Mad1).
CHAPTER 4

DISCUSSION
My thesis work reveals a new activity for JCV TAg that links this tumor protein to the SCF$^\beta$TrCP\$ destruction machinery. Interestingly, a potential $\beta$TrCP recognition domain is also present in the TAgS of the human BKV and MCV polyomaviruses (amino acids 640-646; amino acids 811-817, respectively), but not in two other recently discovered human polyomaviruses, KIV and WUV (Figure 4-1).

$\beta$TrCP consensus: DpSGX$_{2-4}$pS

**JCV**: 639–DSGHGSS–645

**BKV**: 640–DSGHGSS–646

**MCV**: 811–DSGTFSQ–817

*Figure 4-1. Sequence alignment of JCV, BKV and MCV large T antigen.*

The sequence of the JCV, BKV and MCV TAg region containing the phosphodegron is shown. The amino acid sequence in JCV TAg destruction motif spanning amino acids 639 to 645 corresponds to the BKV TAg region from amino acid 640 to 646, and to the MCV TAg region from amino acid 811 to 817. The two serines that influence binding of TAg with $\beta$TrCP are highlighted in red.

An important characteristic common to small DNA viruses is the promotion of DNA replication through the deregulation of the cell cycle and the induction of S phase. These viruses interfere with cellular processes that are under tight control and that regulate cellular function and survival. Two cellular tumor suppressors, p53 and Rb, which are intimately involved in cell cycle control, are frequently targeted by DNA tumor viruses for ubiquitination and proteasomal degradation (20). Some viral proteins inhibit E3 ubiquitin ligases. For example, SV40 TAg protein interferes with the E3 ubiquitin
ligase Fbw7, the recognition factor of the SCF<sup>Fbw7</sup> degradation complex, resulting in elevated cyclin E levels and increased transformation capabilities (58). HPV E5, a protein produced by oncogenic human papillomaviruses, inhibits the E3 ligase c-Cbl, thus preventing the degradation of the epidermal growth factor receptor (EGFR) and enhancing growth factor signal transduction (111). Further, HPV E2 protein disrupts the anaphase promoting complex (APC), an E3 ubiquitin ligase that controls progression of a cell through M- and G1-phases. Inhibition of APC by E2 induces chromosomal instability in cells infected with the high risk HPVs (112). Some viral proteins are themselves targets of the cellular ubiquitination machinery. For instance, the BPV E1 protein is targeted for degradation by the APC complex, an event that leads to the control of the viral load within an infected cell (65). HPV E7 protein has a short half life as a result of protein degradation by Skp2, the F-box protein of the SCF<sup>Skp2</sup> degradation complex (113). Interestingly, E7 stability is also controlled by its interaction with the deubiquitinating enzyme USP11, leading to reduced ubiquitination and thus, inhibiting degradation of this viral protein. This event results in enhanced E7-induced Rb degradation and increased expression of Bcl-2, thereby contributing to HPV transforming ability (114). These two opposing mechanisms of controlling E7 stability through the cellular proteasome point towards a system where the regulated, temporal degradation of this tumor protein may promote virus survival within an infected cell.

While certain viral survival strategies imply the timely, controlled ubiquitin-dependent degradation of some of their proteins, other viral proteins interact with and utilize E3 ligases, resulting in the degradation of specific cellular proteins, as well as redirecting the E3 ligase to new substrates. Examples include the E6 protein of high risk
HPV-16 and -18 viruses, which recruits E6AP to promote the ubiquitination and degradation of p53, a non-natural substrate for E6AP (63), or the HPV E7 tumor protein, that interacts with the ubiquitin conjugating enzyme E2-25K to degrade Rb (64).

Reports that link viruses to the cellular proteasome degradation machinery suggest a common mechanism that provides viruses with an advantage in controlling their replication, propagation and survival (55). In this study I found that JCV TAg, but not the related SV40 TAg, binds to βTrCP1 and βTrCP2, and that this interaction is phosphorylation dependent. Binding requires a destruction motif within the C-terminus of TAg that includes a serine residue at position 640. A second serine at position 644 appears to make a small contribution to TAg-βTrCP1 binding. The observation that just one serine within the destruction motif is required for βTrCP binding highlights a difference between TAg and other known βTrCP substrates for which two phosphorylated serines are required (98). In addition, JCV TAg stability was not altered detectably by its interaction with the F-box protein, suggesting TAg acts as a pseudo-substrate (66), bringing βTrCP in close proximity to potential target proteins without being degraded itself.

An interesting observation made in this study related to the differences in expression of the GST-tagged βTrCP constructs expressed alone or when co-expressed with the T proteins. High levels of expression of the GST tag control and the GST-tagged βTrCP1 and βTrCPΔF proteins only occurred when JCV early proteins were co-expressed in the cells, suggesting that the JCV early proteins transactivate the CMV promoter within the GST expression vector; polyomavirus T proteins are known to activate promoters recognized by RNA polymerase II (115). However, mRNA analyses...
showed only slight or no elevation of the GST mRNAs in the presence of the T proteins, excluding transactivation of the CMV promoter as the cause of elevated GST-tagged protein levels. Protein expression varies during the cell cycle, and it is speculated that some viruses utilize the cellular protein synthesis machinery to manipulate protein production important to the virus life cycle \(116\). Thus, effects on mRNA translation could be a mechanism by which JCV T proteins alter the levels of the GST-tagged proteins.

The immunofluorescence data presented herein indicate that TAg and βTrCP co-localize when expressed in the same cell. The polyomavirus TAgS have a strong nuclear localization signal (NLS) and are predominantly found in the nucleus; only a small amount of TAg is found in the cytoplasm \(100\). Subcellular localization of βTrCP1 and βTrCP2 has been detected in the nucleus and cytoplasm, respectively \(70, 86\), and my results support this observation. Upon overexpression of βTrCP in cells, TAg staining became readily apparent in the cytoplasm. Additionally, while βTrCP2 remains cytoplasmic in the presence of TAg, significant βTrCP1 staining is redirected from the nucleus to the cytoplasm, where the cellular and viral proteins co-localize. The relocalization of TAg and βTrCP1 to the cytoplasm might be substrate-dependant. Nucleo-cytoplasmic shuttling of βTrCP1 is regulated by the protein’s nuclear binding to the pseudo-substrate, hnRNP-U. High affinity βTrCP1 substrates compete with hnRNP-U for binding, leading to the release of active βTrCP1 that may then target nuclear or cytoplasmic targets for subsequent degradation \(86, 87\). The co-localization of TAg and βTrCP1 in the cytoplasmic compartment might indicate that TAg successfully competes with hnRNP-U in the nucleus for βTrCP1 binding, resulting in the cytoplasmic
localization of the two proteins and subsequent degradation of a targeted substrate (Figure 4-2).

F-box proteins, including βTrCP, are characterized by the presence of two conserved regions; the WD repeats, which are essential for recognition of the targeted substrates, and the F-box domain, which enables βTrCP to interact with Skp1, Cul1 and Roc1 (87). I detected βTrCPΔF, as well as βTrCP1, in the nuclear compartment as already reported (96). My immunofluorescence data indicate that TAg and βTrCPΔF co-localize exclusively in the nucleus. It is noteworthy that no cytoplasmic relocalization of TAg was detected in cells expressing βTrCPΔF. The SCF complex members Cul1, Skp1 and Roc1 are detected in both the nucleus and cytoplasm, and it is suggested that their recruitment to the nucleus occurs upon activation of the SCF complex (117, 118, 119). My results suggest that the cytoplasmic localization of TAg in the presence of βTrCP requires a functional SCFβTrCP complex. This observation, together with the finding demonstrating cytoplasmic relocalization of βTrCP1 and TAg, point to the possibility that the cytoplasmic shuttling of the proteins requires βTrCP to interact, not only with its substrates as already proposed (86) but also with the other members of the degradation complex (Figure 4-2).
Figure 4.2. Diagram depicting the hypothesized nucleo-cytoplasmic shuttling of βTrCP1 and TAg.

A proposed mechanism for the nucleo-cytoplasmic shuttling of TAg and βTrCP is described. A) hnRNP-U, which acts as a pseudosubstrate, binds to and keeps βTrCP1 in the nucleus. TAg, bound to a substrate, will compete with hnRNP-U for binding to βTrCP1. Once bound to βTrCP1, the TAg-SCFβTrCP complex translocates to the cytoplasm where ubiquitination and degradation of the targeted substrate take place. B) Upon deletion of the F-box domain of βTrCP1, TAg still competes with hnRNP-U for binding to βTrCP1. However, the inability of βTrCP1 to interact with the rest of the
degradation complex in the nucleus restricts the shuttling of βTrCP, as well as TAg, to the cytoplasm.

TAG has been reported to stabilize β-catenin, a known βTrCP substrate (26, 27, 22), therefore I investigated whether TAg binding to βTrCP influences β-catenin stability. As previously reported (26), an interaction between TAg and endogenous β-catenin was detected (data not shown). However, only minor differences in the levels of β-catenin were detected in cells expressing WT vs. the mutant S640A TAg. I am unable to explain the discrepancy between the results obtained in this experiment and those previously reported (26, 27); however, the data presented in this study indicate that, if TAg does stabilize this critical component of the Wnt pathway, it is not due to its ability to block βTrCP function.

Human papillomaviruses and adenoviruses produce tumor proteins that target p53 for degradation (120, 63), whereas polyomaviruses have been thought to differ in this regard, inducing higher, but inactive levels of p53 through the action of TAg. JCV TAg interacts with and increases the levels of the tumor suppressor p53, albeit less efficiently than its SV40 counterpart (121). SV40 TAg binds p53’s DNA binding domain and blocks its transcriptional activity, leading to a variety of outcomes including the inhibition of p53 growth-arrest and proapoptotic activities (20). Because βTrCP1 targets p53 for proteasomal degradation (85), I again asked whether JCV TAg interferes with βTrCP1 activity to increase the stability of one of the substrates of this F-box protein. As expected, p53 levels were increased in TAg-expressing cells. However, even higher levels of p53 were detected in cells expressing the mutant S640A or S644A TAg. Although I found serine 644 made only a minor contribution to βTrCP1 binding by TAg,
these data suggest serine 644, like serine 640, influences p53 levels. The differences in p53 levels in the presence of WT versus mutant TAg were observed in permissive primary human glial cells as well as in the U2OS tumor cell line, which contains low levels of p53. In addition, U2OS cells contain basal levels of constitutively active IKK2, a kinase that is required for the phosphorylation of p53 and its recognition by βTrCP1 (85). However experiments overexpressing the dominant negative form of βTrCP1, βTrCPΔF, suggested that βTrCP1 is not involved in the increased levels of p53 observed in the presence of mutant TAg. The previous work describing the effects of βTrCP1 on p53 stability by Xia and co-workers (85) was performed in MDM2−/−-p53−/−-IKK2−/− mouse embryonic fibroblasts cells (MEF), into which IKK2 and p53 were introduced exogenously. Additionally, all the experiments carried out by this group involved treatment of cells with the DNA damaging agent doxorubicin, which induces high levels of stable, active p53 (85). The differences in experimental settings used in the previous study and the ones used in my work may explain the negative results I obtained. Moreover, it is possible that the ability of βTrCP to degrade p53 is much lower than that caused by other ubiquitin ligases, such as MDM2, and thus its activity is masked in my experiments. Additionally, the MG132 treatment of cells expressing WT TAg did not increase p53 levels to those observed with mutant TAg, pointing towards a mechanism whereby JCV TAg prevents an increase in p53 levels independent of the proteasomal degradation machinery. Nonetheless, these results do not exclude the possibility that the MG132 treatment, together with TAg expression, elevated p53 to saturated levels, thus making the differences in p53 levels in cells transfected with different TAg more subtle
to interpret. Additional experiments designed to pursue these observations are described in Chapter 5.

DNA viruses use the cellular machinery to replicate their genomes and produce virus particles that are capable of infecting neighboring cells, and JCV TAg mediates these processes through its interactions with a variety of cellular proteins. Such interactions promote transition of the cell to S-phase, creating an optimal environment for viral replication. Further, βTrCP is involved in the regulation of the cell cycle progression and DNA damage response pathways (105, 122, 83). Thus, I analyzed whether mutations within TAg’s destruction motif that alter the TAg-βTrCP interaction, affected JCV DNA replication. Viral DNA replication analysis revealed that mutations at serines 640, 645 and, to a lesser extent, 644, negatively affected JCV DNA replication activity, and that the effect was greater late in infection (days 10 and 14 p. t.). Interestingly, even though mutation of serine 645 did not have an obvious effect on the interaction of TAg with βTrCP, it caused a dramatic decrease in JCV DNA replication. Although not required for binding, it is possible that serine 645 contributes to the proper assembly of βTrCP and the SCF complex to TAg and, consequently, the proper targeting of a cellular substrate or inhibition of βTrCP function. Alternatively, the replication defect of the S645A mutant may not result from an alteration to the TAg-βTrCP interaction. The C-terminal region of TAg, including the destruction motif, contains several potential phosphorylation sites, some of which are predicted to influence viral DNA replication (123). Thus, it is possible that mutation of one serine residue within the phosphodegron could alter a priming phosphorylation site that affects a cascade of phosphorylation events within the TAg sequence that would lead to a defect in viral replication.
The effects of the S640A, S645A and, to a lesser extent, S644A mutations on viral DNA replication were most notable late in the infection cycle. In addition to their influence upon the G1/S phase transition of the cell cycle, polyomaviruses prevent mitosis by triggering the DNA damage check point pathway to accumulate cells in S and G2 phase. This event promotes additional rounds of cellular and viral DNA replication (116, 124). Recent studies demonstrated that JCV TAg induces G2 arrest as a result of the activation of the ATM and ATR checkpoint pathways, thereby promoting viral replication in permissive cells (125). βTrCP is a key player in the S and G2 DNA damage checkpoint pathways, and it promotes cell cycle arrest following DNA damage by attenuating CDK1 activity through degradation of Cdc25A. Additionally, βTrCP has been shown to restore cell cycle progression and entry into mitosis by facilitating the degradation of claspin and WEE1 (75). Consequently, one might speculate that the TAg-βTrCP interaction contributes to these cell cycle events, thus promoting viral DNA replication. In this case, TAg could be forcing the degradation of CDC25A by bringing βTrCP in close proximity to this substrate. On the other hand, TAg may inhibit βTrCP-dependent proteasomal degradation of Wee1 and/or claspin, thus leading to cell cycle arrest in G2 phase.

Alternatively, mutations within TAg’s destruction motif could affect late gene transcription, and/or interfere with viral assembly. Late gene transcription follows early gene transcription and DNA replication, and occurs as early as day 3 p. i. in permissive cells; the structural proteins are detected by day 5 p. i. (126, 127). Expression of the JCV late transcription unit requires a functional TAg (128). It is possible that mutation of the serine residues within TAg’s destruction motif affects its transactivation activity on the
late promoter, either directly, or indirectly by affecting a required cellular transcription factor. It is important to note that the mutations in the phosphodegron may also affect a region of TAg called the host range domain (HR), which is involved in viral capsid assembly (129,130). Deletions within the C-terminus of SV40 TAg interfere with the incorporation of viral DNA into virions, leading to a reduction in infectious viral particle formation and subsequent propagation. Such an effect would explain the reduction in JCV DNA replication activity observed in the presented study.

I have identified for the first time a link between JCV TAg function and a mechanism of proteasome degradation in the cell. The interaction between JCV TAg and cellular βTrCP requires TAg phosphorylation and a destruction motif at the C-terminus of this viral protein. Cellular localization of TAg and βTrCP are influenced by this interaction. Further, mutations to the βTrCP binding site in TAg, prevent efficient viral DNA replication. The two βTrCP substrates investigated in this study, p53 and β-catenin, have been identified as important targets in the process of TAg-induced cellular transformation. Although TAg’s interaction with βTrCP has not been shown to influence these two substrates, other potential βTrCP targets remain to be explored. Given the pathogenic and oncogenic potential of JCV, and the increasing evidence for the involvement of ubiquitin proteasomal pathways in cell growth and transformation (Frescas and Pagano, 2008; Fuchs et al., 2004), it will be important to identify the cellular proteins affected by the JCV TAg-βTrCP interaction.
CHAPTER 5

FUTURE EXPERIMENTS
As a result of my studies characterizing the interaction of TAg with βTrCP and the resulting potential downstream effects, new questions relative to our understanding of JCV became apparent. In this chapter I have suggested additional experiments that might be pursued by my laboratory to address these new questions.

**Analysis of the interaction of SV40 TAg with βTrCP1 and βTrCP2**

The JCV and SV40 TAg sequences share 72% sequence identity (6). I compared the primary sequence of SV40 TAg with that of JCV TAg to determine if SV40 TAg also possesses a βTrCP binding site. The C-terminus of SV40 TAg contains the related sequence DSGHETG (amino acids 646-662); the threonine at position 661 corresponds to serine 644 in the JCV TAg. A serine within the destruction domain could be replaced by a threonine and still be recognized by βTrCP, as seen in one βTrCP substrate, Bora (94). Thus, I analyzed whether SV40 TAg interacts with βTrCP1 in 293T cells and Cos-7 cells. The results demonstrated that SV40 TAg does not interact with βTrCP1 in the cell lines used in this experiment (Chapter 2). However, these cell lines have been transformed with an origin-defective mutant of SV40. The long term expression of the simian virus T proteins in these immortalized cell lines may have created an environment that altered a potential TAg-βTrCP interaction. Thus, I could not rule out the possibility that SV40 TAg interacts with βTrCP in cells transiently expressing the SV40 protein.
Experimental approach

To reexamine whether SV40 TAg interacts with βTrCP, one could perform a transient co-transfection of CV-1 cells, an African green monkey kidney line, with constructs expressing the SV40 TAg and GST-tagged βTrCP proteins. The experiment could also be performed in human cells that do not stably express SV40 TAg, such as 293-HEK cells or U87MG cells. GST pull down analyses performed on extracts from these transiently transfected cells would clarify whether SV40 TAg interacts with βTrCP.

Analysis of the phosphorylation status of wild type and mutant TAg by mass spectrometry (MS).

βTrCP recognizes its target through a phosphodegron with the consensus sequence DSpGX2-4Sp, where both serine residues are phosphorylated (72). The JCV TAg domain predicted to interact with βTrCP1, (DSGHGSS), contains three potentially phosphorylatable serine residues. Phosphatase treatment of cell extracts expressing TAg greatly reduced its interaction with βTrCP, demonstrating the requirement for a modified TAg in the binding event. By using the NetPhos 2.0 server to scan TAg’s primary sequence, several potentially phosphorylatable residues were identified, including serines 640, 644 and 645. These residues yielded high scores for predicted phosphorylation sites (0.93, 0.89, 0.98, respectively; a value of 1 is the highest score, and residues with scores above 0.5 are predicted to be phosphorylated). I would suggest that our group confirm that the serines within TAg’s destruction motif are phosphorylated.
Experimental approach:

To elucidate if the serines within TAg’s destruction motif are phosphorylated, the first approach would be to analyze WT TAg. The viral protein can be directly isolated from mammalian cells transiently transfected with pCMV-JCV_{E}. Proteins in lysates can be separated by SDS-PAGE and the band corresponding to TAg excised for mass spectrometry analyses. Additionally, one could analyze the phosphorylation status of TAGs carrying the point mutations at serines 640, 644 or 645 as a comparison control, as well as to detect if the mutations within the destruction motif have altered the phosphorylation of residues around the phosphodegron and within the N-terminus of TAg. The C-terminal region of TAg contains several potentially phosphorylatable residues (123), and as discussed in chapter 4, it is possible that the mutation of one serine residue within the phosphodegron could alter a priming phosphorylation site that affects a cascade of phosphorylation events within the TAg sequence. This phenomenon has been observed for SV40 TAg, where mutations of serine 677 at the C-terminus affect the phosphorylation status of serines 120 and 123 within the N-terminus of the protein (131). To detect the phosphorylation status of mutant TAg{s, mammalian cells could be transiently transfected with pCMV-JCV_{E} constructs carrying TAg point mutations S640A, S644A or S645A, and isolated mutant TAg{s then analyzed using electron capture dissociation (ECD) combined with Fourier transform ion cyclotron resonance (FTICR) mass spectrometry, where the whole protein can be studied at once giving a more comprehensive view of TAg’s phosphorylation status (132).
Analysis of the effects of the TAg destruction motif mutant on p53 levels.

JCV TAg binds and stabilizes p53 (reviewed in 1, 20), and I hypothesized that TAg’s stabilization function, in part, involves its interaction with, and inhibition of, βTrCP1. Contrary to what I predicted, cells expressing TAg with mutations at serines 640 and 644 within the destruction motif contained higher levels of p53 when compared to cells expressing WT TAg. The results suggest the possibility that TAg could, in fact, be re-directing βTrCP1 to p53 to promote the degradation of this tumor suppressor. However, the control experiments I carried out to verify that the differences observed were due to the ability of TAg to interact with βTrCP1 failed to support my hypothesis. Thus, I recommend that the lab continues to investigate whether βTrCP is or not responsible for the changes in p53 levels observed in the presence of WT versus mutant TAg.

Experimental approach

My hypothesis was based on one study reporting that p53 stability was influenced by βTrCP1 (Xia, et al., 2009). It should be noted that some experimental conditions in this study differed with those used in my work. A key difference in the Xia et al. report (85) was that the analysis of the proteasomal degradation of p53 by βTrCP1 was performed in MDM2−/−- IKK2−/−-p53+/− MEF cells in which p53, βTrCP and IKK2 were added exogenously. By using a cell line lacking the endogenously-expressed proteins of interest, and deficient in MDM2, it might have been easier for Xia and co-workers to manipulate the factors that influence p53 stability, thereby uncovering smaller
contributions that could be attributed to βTrCP. Additionally, their experimental protocol included treatment with doxorubicin, a DNA damaging agent that induces the expression of p53. Thus, I have not definitely ruled out the possibility that TAg alters p53 levels through its interaction with βTrCP. Future experiments could re-examine this question by analyzing p53 levels in the presence of WT or mutant TAg under the conditions employed by Xia and co-workers (85). Additionally, one could examine the effects of WT and mutant TAg proteins on the levels of p53 in cells transfected with an siRNA that recognizes βTrCP1 and βTrCP2 mRNAs. If TAg directs βTrCP towards p53 to promote its degradation, knocking down βTrCP expression would reduce or abolish the ability of TAg to degrade p53. These experimental approaches would be relevant to the question of whether the observed effects on p53 levels are related to TAg’s interaction with βTrCP.

**Investigation of the effect of the TAg-βTrCP interaction on specific βTrCP natural substrates**

JCV TAg interferes with the functions of cellular proteins that are directly or indirectly linked to pathways and events controlled, in part, by βTrCP proteasomal degradation. These substrates include the inhibitor of the NFκB pathway IκB, the translation inhibitor and tumor suppressor PDCD4, the tumor suppressor RE1-silencing transcription factor (REST), and the cell cycle regulators CDC25A/B, claspin, Wee1 and Emi1 (73). It is predicted that TAg binding to βTrCP alters the degradation of βTrCP substrates, either by blocking or enhancing βTrCP function. It is important to identify
such substrates to confirm this new TAg function and to understand its role on JCV-host cell interactions.

**IκB:**

NFκB is a cellular transcription factor involved in innate immune responses to pathogens and regulation of cell proliferation and survival. In unstimulated cells, NFκB is detected in the cytoplasm, complexed with members of the family of NFκB inhibitors (IκB). Upon activation of the NFκB pathway, IκBs are phosphorylated at serines within the destruction motif, which will then be recognized by βTrCP, promoting the ubiquitination and degradation of these inhibitors. These events result in the release of active NFκB and its translocation to the nucleus, where it activates transcription of multiple genes (4). The NFκB pathway is frequently targeted by viruses, which either promote the activation of NFκB or inhibit its function (133). Studies of JCV transcriptional activity reveal that the NFκB pathway is involved in the stimulation of JCV early and late gene transcription. JCV promotes the activation of NFκB, resulting in its translocation to the nucleus and subsequent activation of the early and late promoters of JCV (127). It is plausible to suggest that JCV TAg promotes the degradation of IκB by directing βTrCP to this cellular substrate, thus freeing NFκB to participate in the transcription of the JCV coding regions.
**CDC25A/B, WEE1 and clasin:**

βTrCP is involved in the regulation of the S and G2 DNA-damage response pathway. Upon cellular stress, ATM and ATR activate the checkpoint kinases-1 and -2 (CHK1 and 2), respectively, which results in the phosphorylation and βTrCP-dependent degradation of CDC25A. Once DNA is repaired, βTrCP restores cell cycle progression by facilitating the degradation of clasin and WEE1 (75). JCV TAg has recently been shown to induce G2 arrest as a result of the activation of the ATM and ATR checkpoint pathways. This function enhances replication of the virus in permissive cells (125). The JCV TAg-βTrCP interaction might contribute to G2 arrest by interfering with the degradation of one or more of these cell cycle regulators. One possible scenario would be that TAg promotes the degradation of CDC25A by bringing βTrCP in close proximity to this substrate. Concurrently (or alternatively), TAg might inhibit the proteasomal degradation of Wee1 and/or clasin by inhibiting βTrCP. Each event would contribute to cell cycle arrest in G2 phase.

**PDCD4:**

PDCD4 inhibits protein translation by binding to the eukaryotic translation initiator factor 4 (eIF4A). During cell proliferation, the protein kinase S6K1 phosphorylates PDCD4 which is then recognized by βTrCP1. The subsequent proteasomal degradation of PDCD4 is essential to the initiation of protein translation and cell growth (84). One reason viruses arrest cells in G2 is to increase viral protein synthesis (116). The ability of JCV TAg to promote degradation of PDCD4 through
binding to βTrCP might, therefore, be a mechanism to enhance viral translation and replication.

Interestingly, it has been demonstrated that PDCD4 inhibits the transcriptional activity of AP-1 family of transcription factors. AP-1 proteins stimulate transcription from the JCV early and late promoters, and AP-1 levels and transcriptional activities peak late in the infection cycle (day 10 p. i.) (134). The findings that i) AP-1 proteins activate JCV transcription, ii) their functions are enhanced late in infection, and iii) mutations within the βTrCP destruction motif of TAg have a negative effect on JCV DNA replication in the late stages of infection, point towards the possibility that TAg regulates the availability of these transcription factors by promoting the degradation of PDCD4 through its interaction with βTrCP.

**REST:**

The tumor suppressor REST is a master regulator of neuronal genes and neuronal programs in non-neuronal cells. In embryonic stem cells, REST actively represses a myriad of genes that promote differentiation of cells into mature neurons. In neuronal progenitor cells however, REST is targeted for proteasomal degradation by βTrCP, permitting the transcription of REST target genes and leading to neuronal cell differentiation (135, 88). A recent study has demonstrated that REST not only is involved in neuronal cell differentiation, but also in glial cell lineage formation, including oligodendrocyte maturation and myelination (136). Differentiation of mammalian cells is, as a general rule, coupled with cell cycle arrest in G1 phase (137). DNA tumor viruses induce cell cycle progression to sustain their own DNA replication and survival. Thus,
cellular differentiation could be detrimental to the viral life cycle, and it would be an advantage to a viral pathogen to interfere with this process. Studies by Tretiakova and co-workers (138) revealed that JCV is capable of preventing differentiation of the bipotential CG-4 cell line, a rat cell that can differentiate into either oligodendrocytes or type II astrocytes. One might speculate that JCV prevents differentiation of progenitor glial cells by inhibiting the degradation of REST through the interaction of TAg and βTrCP. By inhibiting differentiation through REST degradation, the viral DNA replication program would be favored.

**Experimental approach:**

Given the potential of the four cellular proteins listed above to influence the JCV life cycle, I would propose that future work begin with these proteins when searching for βTrCP substrates targeted by TAg. It is important to reiterate that a TAg-βTrCP complex could either promote or inhibit the degradation of a particular substrate.

WB analyses could be used to analyze the effects of the TAg-βTrCP interaction on IκB, CDC25A, Wee1, PDCD4 and REST. Protein levels could be assessed in extracts of PHFG cells transfected with DNAs representing the genomes of JCV(Mad1) or JCV(Mad1) carrying mutations within TAg’s destruction motif. As noted above, at least two outcomes are possible: TAg could either promote βTrCP’s degradation of a substrate, or it could inhibit βTrCP function, thus increasing the stability of the cellular protein under study. Using WB analyses, mutations within TAg’s destruction motif would reveal whether the interaction increases or decreases the levels of a specific substrate.
To evaluate if TAg’s ability to interact with βTrCP is responsible for the degradation of a substrate, transiently co-transfections of PHFG cells with either JCV(Mad1) or mutant JCV(Mad1) genomes and the construct expressing either WT βTrCP or the dominant negative form of βTrCP, βTrCPΔF, could be performed. βTrCPΔF lacks the F-box domain, preventing the protein from interacting with the rest of the degradation complex. Thus, while still being able to interact with a particular substrate, βTrCPΔF cannot induce the ubiquitination and proteasomal degradation of the targeted protein. Based on this principle, I would expect that TAg bound to βTrCPΔF would not promote the degradation of the substrate in question, and cells would express substrate, in the presence of WT TAg, at a level equivalent to that detected in cells expressing the TAg mutant.

Additionally, the use of siRNA to knock down βTrCP1 and βTrCP2 could allow one to determine whether the effects observed in the presence of WT or mutant forms of TAg are dependent on its interaction with βTrCP. By knocking down the production of βTrCP1 and βTrCP2, WT TAg could not promote a βTrCP-dependent degradation of the targeted proteins, thus resulting in an accumulation of the substrate in question.

**Identification of new substrates affected by the TAg-βTrCP interaction**

It is likely that additional βTrCP substrates will be identified in cells and some of these might be affected by TAg-βTrCP binding. Additionally, it is possible that TAg directs βTrCP to non-natural βTrCP substrates as observed for the HIV protein Vpu and the CD4 receptor (66). Detection of new substrates affected by the TAg-βTrCP
interaction might be crucial to our understanding of the JCV life cycle; however, it is expected that identification of such substrates would represent a significant investment of time. Thus, as a first step, a proteomic-based approach might offer an advantage to detecting potential targets not known to be related to TAg nor to βTrCP.

**Experimental approach**

New substrates affected by a TAg-βTrCP interaction could be detected using a proteomics-based approach involving two-dimensional difference gel electrophoresis (2D-DIGE) and subsequent mass spectrometry analysis (139). This technique analyzes protein abundance simultaneously in up to three different samples labeled with three fluorescent dyes. Once all proteins from three different samples are separated on a 2-dimensional (2D) gel, the gel is scanned to detect each dye using its own excitation wavelength. By using this technique, one could detect changes in protein abundance within differing samples. If the objective is to detect potential targets affected by the TAg-βTrCP interaction, the three samples to be scanned would be derived from extracts of i) untransfected PHFG cells (mock sample), ii) cells transfected with the WT JCV(Mad1) genome (WT sample) and iii) cells transfected with the JCV(Mad1)-S640A mutant genome (mutant sample). Labeled proteins within the three different labeled samples will generate a peak, when excited by the particular wavelength that will correlate with the amount of that protein in each sample. Differences in protein abundance will be determined for each sample, paying most attention to those that show distinct patterns when comparing WT versus mutant samples. Proteins whose abundance
has been altered within the WT sample when compared with the mutant sample will be identified by mass spectrometry. The ability to analyze three samples within the same 2D gel is an advantage over traditional 2D gel analyses, since variations resulting from the separation of proteins in three different gels will be reduced. Once candidate proteins are identified, differences in their levels of expression could be confirmed by WB with specific antibodies using extracts of PHFG cells transfected with JCV(Mad1) and JCV(Mad1)-S640A genomes. This technique has been successfully employed to identify proteins targeted for degradation by adenovirus (140).

**Examination of the effects of the TAg-βTrCP interaction on JCV DNA replication.**

Experiments presented in this work demonstrate that mutations within the destruction motif of TAg negatively interfere with JCV DNA replication. This effect is most apparent at a time just prior to the release of infectious virions from a cell (day 10 p. t.). Polyomavirus TAg induces cell cycle progression in order to replicate the viral DNA. Viruses have evolved to use the activation of the DNA checkpoint pathway to their advantage. Preventing mitosis by triggering the DNA damage checkpoint pathway, leads to an accumulation of cells in S and G2 phase, thereby promoting additional rounds of replication of cellular and viral genomes (124). A recent study reported that JCV TAg induces the DNA damage checkpoint pathway to arrest cells in G2 phase and to enhance JCV DNA replication (125). Interestingly, knockdown of Wee1 in JCV-infected cells prevents TAg-mediated G2 phase arrest, resulting in a decrease in viral DNA replication. Wee1 is a Ser/Thr protein kinase that inhibits entry into mitosis from G2 phase upon
activation of the DNA damage checkpoint pathway, thus lengthening the G2 phase of the cell cycle. Wee1 is regulated by its phosphorylation by the M-phase kinases polo-like kinase1 (Plk1) and Cdc2, thereby promoting the recognition of Wee1 by βTrCP and its subsequent proteasomal degradation. This event is essential for the progression of the cell cycle, specifically the onset of M-phase (83).

Based on the data observed in the JCV DNA replication experiments presented in this work (Chapter 3) and upon evidence that TAg promotes cell cycle arrest in G2 phase, it is attractive to speculate that TAg could prevent entry into mitosis by interacting with βTrCP and inhibiting the degradation of Wee1. This outcome could explain the decrease in DNA replication activity observed in cells transfected with a JCV(Mad1) genome carrying mutations within TAg’s destruction motif.

**Experimental approach:**

I predict that mutations within TAg’s destruction motif interfere with viral DNA replication because TAg cannot block βTrCP-dependent degradation of Wee1 and thus fails to arrest cells in G2 phase. To address this issue, cell cycle analysis would be conducted in PHFG cells transfected with either WT JCV(Mad1), JCV(Mad1)-S640A, JCV(Mad1)-S644A or JCV(Mad1)-S645A using flow cytometric analysis. This approach would indicate whether cells transfected with mutant JCV(Mad1) genomes fail to arrest in G2 phase. Additionally, G2 phase could be monitored by measuring the levels of cyclin B1, a G2 phase marker, by WB analysis using extracts of cells transfected with WT and mutant JCV(Mad1) genomes.
My replication studies presented in chapter 3 suggest that mutations within TAg’s destruction motif have a large negative effect on DNA replication at day 10 p.i., a time that coincides with infectious viral particle production and release. It is possible that mutations within TAg’s destruction motif interfere with virus assembly, rather than with failure to arrest cells in G2 phase. The C-terminal region of polyomavirus T'ags, called the host range domain, is involved in viral capsid assembly (I30). Deletions within the C-terminus of SV40 TAg interfere with the assembly process by preventing the incorporation of viral DNA into virions, leading to a reduction in numbers of infectious viral particles and in their subsequent propagation. Since the negative effects on JCV DNA replication caused by mutant TAg occur late in infection, one could speculate that host range mutations at the C-terminus interfere with viral capsid assembly or viral DNA packaging. To investigate these possibilities, the ability of WT and mutant JCV(Mad1) to assemble virions in transfected permissive cells can be analyzed by observing infected cells in the electron microscope, and determine the percentage of empty (no packaged DNA) versus complete virions within a cell. Additionally, a viral particle release assay can be performed to analyze whether virion formation is completed. PHFG cells can be transfected with WT JCV(Mad1), JCV(Mad1)-S640A, JCV(Mad1)-S644A and JCV(Mad1)-S645A genomes and the supernatant subjected to IP to collect viral capsid proteins. The immunoprecipitates would be examined both by WB to detect the levels of VP1 protein and by Southern blotting to quantify the amount of encapsidated viral DNA (I41). If mutant virus fails to properly encapsidate DNA, I would expect to see similar amounts of VP1 protein but less DNA in the mutant virions.
REFERENCES


80. Brickelmaier, M., Lugovskoy, A., Kartikeyan, R., Reviriego-Mendoza, M. M.,
Allaire, N., Simon, K., Frisque, R. J., Gorelik, L. (2009) Identification and
characterization of mefloquine efficacy against JC virus in vitro. *Antimicrob Agents

undescribed nonphosphorylated destruction motif in Cdc25A and Cdc25B

82. Busino, L., Donzelli, M., Chiesa, M., Guardavaccaro, D., Ganoth, D., Dorrello, N.

83. Watanabe, N., Arai, H., Nishihara, Y., Taniguchi, M., Watanabe, N., Hunter, T.,

84. Dorrello, N. V., Peschiaroli, A., Guardavaccaro, D., Colburn, N. H., Sherman, N. E.,

Phosphorylation of p53 by IkappaB kinase 2 promotes its degradation by beta-TrCP.


106. Lassot, I., Ségueral, E., Berlioz-Torrent, C., Durand, H., Groussin, L., Hai, T.,
Benarous, R., Margottin-Goguet, F. (2001) ATF4 degradation relies on a
phosphorylation-dependent interaction with the SCF(betaTrCP) ubiquitin ligase.
*Mol Cell Biol* 21(6), 2192-2202.

107. Maginnis, M. S., Atwood, W. J. (2009) JC virus: an oncogenic virus in animals and

108. Hirt, B. (1967) Selective extraction of polyoma DNA from infected mouse cell

109. Buck, E., Eyzaguirre, A., Barr, S., Thompson, S., Sennello, R., Young, D., Iwata, K.
by epithelial-mesenchymal transition or mutation limits sensitivity to epidermal

110. Major, M. B., Camp, N. D., Berndt, J. D., Yi, X., Goldenberg, S. J., Hubbert, C.,
Biechele, T. L., Gingras, AC., Zheng, N., Maccoss, M. J., Angers, S., Moon, R. T.
(2007) Wilms tumor suppressor WTX negatively regulates WNT/beta-catenin

disrupts the c-Cbl-EGFR interaction and EGFR ubiquitination in human foreskin
keratinocytes. *Oncogene* 24(15), 2585-2588.


APPENDIX A:

SMALL T ANTIGEN OF JC VIRUS LOCALIZES IN THE NUCLEUS AND CYTOPLASM AND INTERACTS WITH THE RB FAMILY PROTEINS p130 AND p107
JCV tAg shares 78% homology with BKV tAg, and 67% homology with SV40 tAg (1). The C-terminal half of each tAg represents the least conserved sequences among the polyomavirus tAgs; these amino acids are unique to tAg and do not overlap with other viral T proteins. Most predictions relating to JCV tAg functions are based upon SV40 tAg research. However, because the JCV and SV40 tAgs do differ at their C-termini, it is possible that tAg contributes to differences observed in the transformation and viral DNA replication activities of these two viruses. Like SV40 tAg, JCV tAg interacts with PP2A in-vitro and in-vivo through cysteine-rich motifs in the carboxy portion of tAg (2, 3). On the other hand, recent studies in our laboratory have revealed that unlike SV40 tAg, JCV tAg contributes significantly to viral DNA replication in vivo (3). JCV tAg null and substitution mutants that exhibit impaired PP2A binding, display replication-defective phenotypes.

Polyomavirus tAgs have not been reported to interact with members of the Rb family of tumor suppressor proteins. Previously our laboratory identified a 20kDa band in a CoIP/WB experiment that interacted with p130 (4) and was recognized by an anti-T monoclonal antibody. We had suggested that this band, even though it had the appropriate molecular weight of tAg, was unlikely to represent this tumor protein because the tAg sequence was not known to contain a LXCXE Rb-binding motif (4) While analyzing the primary sequence of JCV tAg using the eukaryotic linear motif software (ELM), I detected two potential LXCXE domains within the unique, C-terminal portion of the protein, at amino acids 92-97 and 155-159 (Figure A-1).
Figure A-1 Protein sequence alignment of JCV, BKV and SV40 tAg sequences harboring LXCXE domains.

Alignment of tAg sequences including amino acids 87 to 101 (A), and amino acids 149 to 163 (B) for JCV and BKV tAg. The corresponding sequences in SV40 tAg are amino acids 89 to 103 (A), and amino acids 151 to 165 (B). The first potential LXCXE domain (highlighted in red and underlined) is found in JCV and BKV tAg, but not in SV40 tAg (A), whereas the second LXCXE domain is only present in JCV tAg (B). The asterisks identify a proline that contributes to the SV40 tAg-PP2A interaction (P99), and a cysteine in the center of the second LXCXE domain unique to JCV tAg (C157).

Figure A-1A

<table>
<thead>
<tr>
<th>Sequence</th>
<th>87-101</th>
<th>149-163</th>
</tr>
</thead>
<tbody>
<tr>
<td>JCV-tAg</td>
<td>PPNSDTLYC EWPC</td>
<td>DLTQEALHC WEKVLG</td>
</tr>
<tr>
<td>BKV-tAg</td>
<td>PLCPDTLYC EWPI</td>
<td>DLTEETLQWWVOIG</td>
</tr>
<tr>
<td>SV40-tAg</td>
<td>NPGVDAMYCKQWPEC</td>
<td>DLCEGTLLWCDIG</td>
</tr>
</tbody>
</table>

Comparison of JCV, BKV and SV40 tAg sequences revealed that SV40 tAg does not have an LXCXE domain, and that the first LXCXE motif in JCV tAg is also found in the BKV sequence (Figure A-1). Thus, I asked whether JCV tAg interacted with the Rb proteins p130 and p107. Extracts were prepared from G418-selected 3T3 cells expressing various combinations of JCV early proteins, including lines expressing tAg mutants. The cysteine at residue 157 of tAg (Figure A-1B, asterisk) was changed to alanine by site-directed mutagenesis to disrupt the second LXCXE motif (C157A). The proline at position 99 of tAg (Figure A-1A, asterisk), which is located at a residue adjacent to the
first LXCXE motif and which targets a portion of tAg’s PP2A-binding site, was mutated to alanine (P99A). Extracts were immunoprecipitated with anti-p130 or anti-p107 antibodies. Complexes were electrophoresed in an 18% SDS-polyacrylamide gel and immunoprecipitated proteins were detected by WB using a cocktail of anti-T monoclonal antibodies. The results show that WT tAg interacts with both p130 (Figure A-2A, lanes 4, 6) and p107 (Figure A-2B, lanes 4, 6). The C157A mutation, which disrupts the second potential LXCXE domain, also interacted with both p130 and p107 (Figure A-2A, B, lane 8), suggesting that the second LXCXE motif is not required for binding to the Rb proteins. As expected, the mutant P99A tAg also bound p130 and p107 (Figure A-2A, B, lane 10). These results suggest that the first LXCXE domain may be responsible for the interaction between tAg and the Rb proteins, or that the two motifs are functionally redundant and the presence of just one LXCXE motif is sufficient for Rb binding. The JCV early proteins, including tAg, contain a J domain within the shared N-terminal sequences, thus, it is plausible that the two LXCXE motifs in tAg cooperate to release E2F transcription factors, bound to the Rb tumor suppressors, thereby contributing to regulation of cell cycle progression. In an effort to determine if one or both motifs are required for the Rb interaction to occur, mutational analysis of the LXCXE domains of tAg is continuing.
Figure A-2: JCV tAg interacts with the Rb proteins p130 and p107.

(A) JCV tAg interacts with p130. Protein extracts prepared from stably transfected 3T3 cell lines expressing all 5 early proteins (SR: T^+ / t^+ / T^-), tAg only (SR: T^- / t^+ / T^-), or TAg, the 3 T^-proteins and one of the two t Ag mutants (SR: T^+ / tP99A^- / T^-; SR: T^- / tC157A^- / T^-) were immunoprecipitated with Pab962 (lanes 1, 3, 5, 7, 9) or anti-p130 antibodies (lanes 2, 4, 6, 8, 10). The proteins were separated on an 18% SDS-polyacrylamide gel and WB analysis was performed using a cocktail of antibodies that recognize the JCV early tumor proteins (PAb962, 2000, 2001, 2003, 2023, 2024, 2030, 901 and 416).

(B) JCV tAg interacts with p107. Protein extracts from these same 3T3 cell lines were immunoprecipitated with PAb962 (lanes 1, 3, 5, 7, 9) or anti-p107 antibodies (lanes 2, 4, 6, 8, 10), and the immunoprecipitated proteins were separated on a 18% SDS-polyacrylamide. The tumor proteins were detected by WB using the cocktail of anti-T monoclonal antibodies.

PP2A is known to interact with and promote the dephosphorylation of members of the Rb family (5, 6, 7, 8). Dephosphorylation of p130 by PP2A occurs at amino acids serine 1080 and threonine 1097, adjacent to the nuclear localization signal (NLS).

Dephosphorylation promotes nuclear localization of p130 where this tumor suppressor arrests cell cycle progression (8). Similarly, PP2A has been shown to interact with and dephosphorylate p107, leading to cell cycle arrest (5, 6, 9). JCV tAg interacts with both PP2A and the tumor suppressors p107 and p130 (3). Based on SV40 studies, JCV tAg is
expected to inhibit the dephosphorylation of these tumor suppressors, promoting progression of the cell cycle and preventing cellular differentiation (10). Consequently, one could speculate that JCV tAg, through its interactions with PP2A, contributes to transformation and the lytic cycle of JCV. Interestingly, the other four JCV viral proteins promote, possibly through interactions with protein phosphatases (11), the dephosphorylation of the Rb family proteins, although some hyperphosphorylated forms remain in an asynchronous cell population. Hypo- and hyperphosphorylated forms of p130 and p107 are detected throughout the cell cycle, and it is speculated that the different phosphorylated forms of these tumor suppressors might function by activating transcription of genes and initiating DNA replication by delivering the cycE/cdk2 activity to DNA (12). It is possible that the JCV T proteins may promote a controlled balance of hyperphosphorylated and unphosphorylated Rb proteins to achieve proper temporal regulation of the cell cycle that is optimal for the virus replication. Mutations within the LXCXE domains of tAg in combination with a mutation that inhibits tAg-PP2A binding could reveal whether the interaction between tAg and the Rb proteins occurs through PP2A. Likewise, determining the levels of cellular p130 and p107 in the presence of WT or mutant tAgs in synchronized cells could reveal whether tAg affects the phosphorylation status of p130 and p107 through its binding to PP2A.

JCV tAg is predicted to be a cytoplasmic protein. To confirm this prediction, I performed immunofluorescence studies in U87MG cells transiently transfected with pCMV-JCVtAg, a construct that expresses JCV tAg only (described in Chapter 2). Mock-transfected cells were stained for tAg as a negative control. The results revealed that
some cells showed cytoplasmic staining only, while both cytoplasmic and nuclear tAg staining are detected in the majority of cells (Figure A-3).

SV40 tAg has been detected in both cellular compartments (13). A NLS or a nuclear export signal (NES) have not been detected in the primary sequence of tAg. Nonetheless, the shuttling of small proteins between the nucleus and cytoplasm of takes place by diffusion through the nuclear pores; only proteins of a molecular weight greater than 40-60kDa require a NLS and NES for transport. Thus, tAg, a 20kDa protein, is likely to freely move back and forth between cytoplasm and nucleus. Alternatively, proteins lacking a NLS and NES can be translocated to the nucleus by binding to macromolecules that do contain these signals (14, 15). Both p130 and p107 shuttle between the nucleus and cytoplasm (7, 8, 16), and it is possible that tAg movement depends upon its interaction with these tumor suppressors. Immunofluorescence analysis using an LXCXE mutant tAg that abolishes the tAg-p130/p107 interaction could be used to test this possibility.

If tAg shuttling between cytoplasm and nucleus does involve Rb family proteins, one could speculate that the subcellular localization of tAg would be cell cycle dependent. Nucleo-cytoplasmic shuttling of p130 and p107 depends on the stage of the cell cycle. Although different phosphorylated forms of these two tumor suppressors can be detected in the nucleus and cytoplasm throughout all phases of the cell cycle, p107 nuclear localization peaks at S phase, while p130 nuclear abundance occurs in G1 (12, 17, 18). Thus, it would be interesting to perform immunofluorescence analyses to detect the subcellular localization of WT and mutant tAgs with respect to p130/p107 and/or PP2A in synchronized cells.
Figure A-3. tAg localizes in the cytoplasm and in the nucleus.

U87MG cells were transiently transfected with 1 ug of pCMV-tAg plasmid expressing tAg only. Cells were fixed and immunostained with a cocktail of anti-T antibodies 72 hours p. t. as described earlier (Chapter 2). A) U87MG cells transiently transfected with pCR3 empty vector were immunostained with anti-T antibodies and the secondary antibody anti-mouse Alexa Fluor 488 as a negative control. B) U87MG cells showing tAg localization in the cytoplasm only (B), as well as in both cellular compartments (C).
References:


APPENDIX B:
IDENTIFICATION AND CHARACTERIZATION OF MEFLOQUINE
EFFICACY AGAINST JC VIRUS IN VITRO

I contributed to this work by analyzing the effects of mefloquine on JCV DNA replication in naïve PHFG cells.

Margot Brickelmaier¹, Alexey Lugovskoy¹, Ramya Kartikeyan², Marta M. Reviriego-Mendoza², Norm Allaire¹, Kenneth Simon¹, Richard J. Frisque² and Leonid Gorelik¹

¹ Biogen IDEC Inc., 14 Cambridge Center, Cambridge, Massachusetts 02142 and ² Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802, USA.

Corresponding Author: Leonid Gorelik, Ph.D. tel: (617) 679-3297 email: Leonid.gorelik@biogenidec.com
Progressive multifocal leukoencephalopathy (PML) is a rare but frequently fatal disease caused by uncontrolled replication of JC polyomavirus in the brain of some immunocompromised individuals. Currently, no effective antiviral treatment for this disease has been identified. Therefore, it is critical that the search for therapeutics that target JCV directly be continued. As a first step in the identification of such therapy, we screened the SPECTRUM collection of 2000 approved drugs and biologically active molecules for anti-JCV activity. An in vitro infection assay was used to look for drugs that inhibit infection of cells in culture. We identified a number of different drugs and compounds with significant anti-JCV activity at micromolar concentrations that lacked cellular toxicity. Of the compounds with anti-JCV activity, only mefloquine, an antimalarial agent, has been reported to show sufficiently high CNS penetration such that it would be predicted to achieve efficacious concentrations in the brain. Therefore, our attention was focused upon this drug. Additional in vitro experiments demonstrated that mefloquine inhibits the viral infection rate of three different JCV isolates, JCV(Mad1), JCV(Mad4) and JCV(M1/SVEA), and does so in three different cell types, transformed human glial cells (SVG-A), primary human fetal glial (PHFG) cells and primary human astrocytes. Using qPCR to quantify the number of viral copies in cultured cells, we have also shown that mefloquine inhibits viral DNA replication. Finally, mefloquine does not block viral cell entry; rather it inhibits viral replication in cells post viral entry. Although no animal model of PML or JCV infection is available to test mefloquine in vivo, our in vitro results, combined with the published biodistribution literature, suggest that mefloquine could be an effective PML therap
INTRODUCTION

Progressive Multifocal Leukoencephalopathy (PML) is a progressive, usually fatal, demyelinating disease caused by JC virus (JCV) infection and destruction of oligodendrocytes in multiple brain foci of susceptible individuals. JCV is a double-stranded DNA Polyomavirus that is believed to cause asymptomatic infections in 65-90% of the human population as judged by the presence of virus-specific antibodies (35). There is a persistent viral shedding in the urine of 20-40% of individuals (35), which together with observed viral presence in kidney tubular epithelial cells (32, 49), indicates JCV establishes a persistent and chronic infection in a large fraction of the human population. Despite this high infection rate and viral prevalence, PML is a rare disease that almost exclusively afflicts individuals who are immunocompromised due to genetic factors, human immunodeficiency virus (HIV) infection, hematological malignancies or immunosuppressive therapies (8, 14). Currently there are no approved or proven therapies for PML. Although a number of preclinical reports and case studies suggested potential anti-PML effects of antiviral and antineoplastic drugs such as cytarabine, cidofovir and topotecan, larger case-controlled studies failed to establish efficacy of these drugs (1, 21, 27, 28, 31, 39). To date, the most effective intervention for treatment of PML is reconstitution of the patient’s immune system. Thus, the introduction of highly active antiretroviral therapy (HAART) was the single most significant development, reducing mortality from PML in HIV-positive individuals from 90% to 50-70% (8, 10, 16-18). Similarly, a reduction in the drug regiment of PML patients undergoing immunosuppressive therapy may halt the worsening of clinical symptoms (20, 54). However, an immune reconstitution approach is not
possible or successful in all patients. Therefore, it is imperative that the search for therapeutics targeting JCV directly be continued. To identify drugs with anti-JCV activity, we screened a commercially available collection of approved drugs and bioactive compounds in an *in vitro* JCV infection assay. As a primary screen, we monitored inhibition of the viral infection rate of SVG-A cells (38) exposed to JCV(M1/SVEΔ), a modified form of JCV (57). Infection rate was measured as the percent of cells expressing the viral capsid protein VP1. Out of 2000 compounds in the SPECTRUM collection, 14 were found to reduce the number of infected cells by ≥50% at concentrations ≤20 μM (IC₅₀<20 μM). Since PML is a result of uncontrolled viral replication in the CNS, it is critical that potential therapeutic agents cross the blood-brain barrier in a sufficient concentration to be effective. Based on the published literature of the 14 drug candidates identified with *in vitro* anti-JCV activity, only mefloquine, an antimalarial agent, appears to exhibit a level of CNS penetration that could be expected to achieve *in vitro*-derived efficacious concentrations in humans (33, 47). Our experiments characterizing mefloquine anti-JCV activity of mefloquine, together with available published data, suggest that the efficacy of mefloquine treatment should be examined in PML.

**MATERIALS AND METHODS**

Source and Identity of Potential Therapeutic Agents

The Spectrum Collection (MicroSource Discovery Inc., Groton, CT) consists of ~1000 bioactive compounds and natural products plus ~1000 Food and Drug Administration (FDA)-approved drugs that are defined according to name designations set
forth in the USP Dictionary of USAN and International Drug Names (2005, US Pharmacopeia). An alphabetical listing of the compounds is available at http://www.msdisccovery.com/spectrum.html. The compounds are supplied as 10 mM solutions in dimethyl sulfoxide (DMSO). In our follow-up experiments, mefloquine and all other drugs were purchased from Sigma (Sigma-Aldrich, St Louis, MO) and 100 mM stock solutions were prepared in DMSO. Two mefloquine enantiomers were separated from a commercial mefloquine sample by Chiral Technologies (Chiral Technologies, West Chester, PA) using chiral HPLC on a CHIRALPAK IA column. Mefloquine analogs were purchased from BioBlocks (BioBlocks, Inc., San Diego, CA.). Cidofovir was from Gilead as a 0.238M aqueous stock solution (Vistide®)

Propagation and Identity of Cultured Cells

SVG-A cells (gift from Walter Atwood), established by transforming human fetal glial cells with an origin-defective SV40 mutant (Major, Miller et al. 1985), were cultured in 1X Eagle Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 4 mM L-glutamine (Mediatech, Inc.). Viral infection was performed in the same medium supplemented with 2% heat-inactivated FBS.

Human astrocytes were isolated from fetal cerebral cortex (ScienCell Research Laboratories, Carlsbad, CA) and cultured in proprietary astrocyte growth medium (ScienCell Research Laboratories). Viral infection of astrocytes was performed in this medium. Cultures of PHFG cells were prepared from fetal brain tissue using a modification of an earlier protocol (44). Briefly, tissue was received in glial cell medium (DMEM supplemented with 3% FBS, 7% bovine calf serum, 100U/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamicin, 2.5µg/ml fungizone). After removal of
meninges and blood vessels, tissue was washed twice in 10-30 ml glial cell medium without gentamicin and containing 200µg/ml amikacin (Sigma) and then pelleted in a clinical centrifuge (3 minutes, 1120 rpm). Tissue was transferred to a sterile Petri dish containing 10-20 ml medium, and tissue fragments were reduced in size by expressing them through a 10-ml syringe (without needle) and a 40-mesh screen in a tissue-dissociation device (Sigma). Cells derived from this procedure were seeded on 100-ml tissue culture plates in glial cell medium using a volume of 0.5 ml of cells per plate. After one week, if the astrocyte layer was confluent, cultures were placed in maintenance medium (DMEM supplemented with 3% FBS, penicillin and streptomycin). If the astrocyte layer was not confluent, cultures were placed in glial cell medium without amikacin. Two to three weeks after seeding, the cells were prepared for freezing. Briefly, cell cultures were incubated overnight in DMEM containing 10% FBS. The next day the heterogeneous population of cells containing astrocytes and small round cells clustered on top of the astrocyte layer was washed with saline A. After aspiration, saline A was added again to each plate, and the cells were incubated 3 minutes at 37°C. Trypsin (in saline A) was added, and cells were incubated for 4 minutes at 37°C. DMEM containing 10% FBS was added and cells were gently collected in 50-ml conical tubes. The cell suspension was centrifuged, the pellet washed once with DMEM containing 10% FBS and cells were then pelleted again. Cells were suspended in Freezing Medium (DMEM, 10% FBS, 10% DMSO) and stored in liquid nitrogen until they were used for JCV(Mad1) infectivity assays.
**JCV Isolates**

The hybrid virus, JCV(M1/SVEΔ) (gift from Walter Atwood), was constructed by first inserting SV40 regulatory sequences into the JCV(Mad1) transcriptional control region to create the hybrid genome, JCV(M1/SVE) (57). Transfection of SVG-A cells with this DNA yielded JCV(M1/SVEΔ) virus that is comprised of JCV-SV40 enhancer signals linked to the JCV(Mad1) genome. To produce purified preparations of virus, SVG-A cells were plated at 50% confluence and infected with a 1:50 dilution of the JCV(M1/SVEΔ) virus for 1 hour at 37°C (25, 36). Cells were cultured for 3 weeks, and then scraped from the flasks, pooled, including cells detached during prior medium changes, and pelleted. The cell pellet was resuspended in 20 ml of supernatant and disrupted in a microfluidizer (Microfluidics, Inc.). Deoxycholate was added to cell lysates at a final concentration of 0.25% and incubated at 37°C for 30 minutes. The virus-containing supernatant was centrifuged at 10,000 RPM for 30 minutes in a SA600 rotor and aliquoted and stored at -80°C. The JCV(Mad4) isolate (46) was obtained from the American Type Tissue Collection (Manassas, VA), and the prototype JCV(Mad1) isolate (24, 45) was a gift from Duard Walker.

**Detection Antibodies**

PAb597 (gift from Walter Atwood; (19)), a mouse monoclonal directed against the SV40 major capsid protein VP1, cross-reacts with JCV VP1 (4) and was used with an Alexa-Fluor 488- labeled goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) to visualize JCV-infected cells by indirect immunofluorescence. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). Neutralizing anti-JCV rabbit antisera was a gift from Walter Atwood (3).
**JCV Infectivity Assay**

SVG-A cells were seeded at 2,000 cells/ well/ 0.075 ml of culture media in flat-bottom 96-well plates (Corning). Compounds to be tested for antiviral activity were prepared the next day in assay medium (1X MEM with 2% heat-inactivated FBS, 4 mM L-glutamine). A master viral plate was prepared by mixing equal volumes of [2X] compound and [2X] diluted virus to yield the final working concentrations of compound and virus. The plate containing cells was gently inverted and shaken to remove medium. From the master plate, 0.035 ml of compound plus virus was added to designated wells. The cells were incubated with the compound-virus mixture for 60 minutes in a humidified, 37°C incubator containing 5 % CO₂. Medium containing the final concentration of the drug was added to designated wells to bring the final volume to 0.1 ml/well. After incubating the plates for 3 days, cells were washed once with 1X PBS, and fixed in 2% paraformaldehyde/1X PBS for 30 minutes at room temperature. The fixative was removed and the cells were permeabilized with 0.5% Triton X100 in PBS for 30 minutes. Cells infected with JCV(M1/SVEΔ) were visualized by staining with 0.05 ml of PAb597 (2 μg/ml in 1X PBS) for 60 minutes at 37°C. Following a wash step with 1X PBS, an Alexa-Fluor 488- labeled goat anti-mouse secondary antibody (1:100 dilution in 1X PBS) and DAPI (1 μg/ml, 0.05 ml/well) were added to the cells for 30 minutes at 37°C. Cells were washed with 1X PBS and field images of each well were acquired and analyzed using a Cellomics ArrayScan (Thermo Scientific Inc, USA) and Target Activation software.

The infectivity assay was also performed in human astrocytes or in PHFG cells with the following modifications. Human astrocytes were seeded at 4,000 cells/ well/ 0.075 ml of culture media in flat-bottom 96-well plates. Culture medium was used as the
virus diluent, and the duration of the infection was 6 to 10 days. PHFG cells (~1.2 x 10^5) were seeded onto 35mm plates containing 2.0 ml DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine. The cells were exposed 48 hours later to 5 hemagglutinating units (HAU) of JCV(Mad1) diluted in DMEM, 0.5% heat-inactivated FBS, and 2.5µM, 5µM, 7.5µM or 10µM of mefloquine in 0.01% DMSO. Three independent sets of experiments were performed, each with two samples at each time point. Cells not exposed to mefloquine were infected with 1 or 5 HAU of JCV(Mad1) and served as controls to permit determination of mefloquine’s inhibitory activity and to confirm that infected cell numbers increased proportionally with a five-fold increase in virus inoculum. Virus was allowed to adsorb to cells for 3 hours at 37ºC, and then DMEM supplemented with 3% heat-inactivated FBS and the appropriate amount of mefloquine was added. Medium was replaced 5 days post infection, and DNA was extracted from the cells at days 7 and 10 post infection.

**DNA Extraction and Sample Preparation**

DNA was extracted from cells using QIAamp 96 blood kit (Qiagen, Inc.) with optional RNase A treatment. DNA was quantitated using Quant-iT dsDNA high sensitivity assay according to the manufacturer’s recommendations (Molecular Probes Inc., Eugene, OR). Purified DNA was stored at –20ºC until use. **Real Time qPCR Assay** Taqman forward and reverse primers and MGB probes were designed to recognize conserved JCV early coding sequences using Primer Express v3.0 (Applied Biosystems, Foster City, CA). Sequence of the forward primer: AGGCAGCAAGCAATGAATCC, reverse primer: ATGGCAATGCTGTATTAGAGCAA and 6FAM-labeled probe:
CCACCCAGCCATAT. To create a copy number standard curve for absolute quantification, we linearized pUC19 plasmid containing the JCV genome with SmaI. Quadruplicate PCR reactions were run in a 384-well optical plate (Applied Biosystems, Foster City, CA). Real time reactions were performed in a 7900HT (Applied Biosystems, Foster City, CA) thermal cycler under the following conditions: 50°C for 2 minutes, 95°C 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds with 900nM forward and reverse primers, 200nM Taqman probe, and 1X Universal master mix (Applied Biosystems, Foster City, CA). JCV copy number was determined for each experimental sample by comparison to the JCV plasmid standard curve using Sequence Detection Software (Applied Biosystems, Foster City, CA), and normalization to the total DNA extracted from a sample. Zero copies of JCV DNA were detected in a non-infected negative control. P values were calculated using a Student t test.

_Determination of Rates of Viral Inhibition_

We determined the percent of cells infected following exposure to JCV by dividing the number of immunofluorescent cells by the total number of nucleated cells, multiplied by 100%: \( \% \text{ JCV}^+ \text{ cells} = \frac{\text{total } \# \text{ VP1}^+ \text{ cells}}{\text{total } \# \text{ DAPI}^+ \text{ cells}} \times 100\% \). The percent of viral inhibition by a compound was calculated on the basis of percent JCV\(^+\) cells rather than the number of VP1\(^+\) cells: \( \% \text{ JCV Inhibition} = \frac{1 - (\% \text{ JCV}^+ \text{ cells with a compound} - \% \text{ JCV}^+ \text{ cells in the negative control})}{(\% \text{ JCV}^+ \text{ cells in the positive control} - \% \text{ JCV}^+ \text{ cells in the negative control})} \times 100\% \), where the positive control represents cells infected with virus in the absence of any compound and the negative control denotes cells not
infected with virus. The numbers of JCV+ cells in the negative control samples quantitated by Cellomics ArrayScan were always <1% of the number of JCV+ cells in the positive control. The percent of JCV DNA replication inhibition was calculated as (1 - (JCV DNA copy# in cells treated with a compound/ JCV DNA copy# in the positive control) *100 %.

Zero copies of the JCV genome were detected in negative control samples. For high throughput screening of compounds, we calculated Z-factor=1-3*( (σp+σn )/|μp-μn| ; the mean (μ) and standard deviation (σ) of both the positive (p) and negative (n) controls (60). Intraplate intragroup coefficient of variance (CV) was always below 20% and Z’>0.5. IC50 values were calculated using Prism software (GraphPad Software Inc., La Jolla, CA).

**Molecular modeling and in silico screening.**

The program ROCS version 2.3.1 (Openeye Scientific Software, Santa Fe, NM, USA) was used to compare three-dimensional shapes of diverse compound classes and to perform virtual screening against clinical compounds extracted from the MDL Drug Data Report database (Symyx Technologies, Inc., CA 95051). The program OMEGA2 version 2.3.0 (Openeye Scientific Software, Santa Fe, NM, USA) was used to generate multiple low-energy conformations of the compounds. The 3D-enumerated conformers were compared based on the implicit Mills-Dean atom chemical forcefield scheme in conjunction with the shape-base matching algorithm of program ROCS version 2.3.1 (Openeye Scientific Software, Santa Fe, NM, USA). The best scoring overlays were visualized in the PyMOL program version 1.0.0b15 (DeLano Scientific LLC, , Palo Alto, CA, USA).
RESULTS

Primary Screen: JCV Infectivity Assay

To identify drugs with anti-JCV activity, we screened a commercially available collection of approximately 2000 approved drugs and bioactive compounds, called the SPECTRUM collection, for anti-JCV activity in an in vitro viral infectivity assay (48). As a primary screen, we monitored inhibition of the viral infection rate in a human fetal astroglial cell line (SVG-A) infected with JCV(M1/SVEΔ), a modified form of JCV. SVG-A cells (38) were chosen for the primary screening assay, as they represent one of the few available cell lines permissive for JCV replication, and JCV(M1/SVEΔ) virus was chosen because its infection of SVG-A cells results in an accelerated rate of viral replication that permits earlier detection of infected cells (3 vs. 6-10 days post infection) relative to that observed with other cell types and JCV isolates. The JCV(M1/SVEΔ) genome includes the coding sequences of the prototype JCV(Mad1) virus isolated from a PML patient (24, 45) linked to hybrid JCV-SV40 non-coding regulatory sequences (57). This rearrangement of transcriptional signals extends the species and cell-type 5 host range of the virus.

To facilitate screening of the SPECTRUM collection, we adapted the JCV infectivity assay (48) to a 96-well format and employed a Cellomix ArrayScan high content imager to measure JCV replication. In this system, infected cells can be detected by immunofluorescent staining with antibodies that recognize the JCV capsid protein, VP1. Total numbers of cells in culture were visualized via staining with the DNA stain, DAPI (Fig. 1A). The ArrayScan® allowed us to simultaneously identify and count each
DAPI- and VP1-positive event in the assay well, routinely counting 300-800 VP1+ and 8,000-16,000 DAPI+ events per well, thus minimizing variability due to non-uniform cell growth pattern and/or intrawell viral spread. Using this format, 4-7% of all cells express JCV VP1 72 hours post-infection, and the number of infected cells (i.e. VP1+ cells) at the end of the culture period was proportional to the number of infectious viral particles used to infect the cell culture (Fig. 1B). In each experiment, the highest viral dilution that yields maximum infectivity was used. Using neutralizing rabbit anti-JCV serum as a positive control for inhibiting infectivity, we showed that the assay responds to viral inhibition in a predictive fashion (Fig. 1C).

We initially tested compounds for antiviral activity at single dose (10 μM) and noticed that some of the compounds that inhibited the number of virally-infected cells (i.e. VP1+ cells) to a great extent also dramatically reduced the total number of cells (i.e. DAPI+ events), suggesting the occurrence of cytotoxic/cytostatic effects. To determine whether a particular compound had decreased the number of virally-infected cells because of its antiviral effect, we calculated percent viral inhibition using infection rate (i.e. % JCV+ cells), rather than total number of JCV-infected cells (i.e. VP1+ cells). With this system, we observed that treatment with cidofovir, a drug that has been tested clinically for efficacy against PML (21, 27, 39), inhibited the number of infected cells and the total number of cells in culture to the same degree, indicating the percent inhibition of infection rate (i.e. %JCV+ cells) was not significant. (Fig. 1D). Similar effects were noted for other drugs reported to have cytotoxic effects, e.g. mytomycin C and cytarabine (data not shown). Therefore, we determined antiviral activity of each compound by calculating viral
inhibition on the basis of percent of JCV$^+$ cells, rather than absolute number of JCV$^+$ cells per group.

Drug screening and selection

A number of drugs and compounds were identified that inhibited JCV infection rate by $\geq 20\%$ without causing significant cell toxicity ($<20\%$ total cell number inhibition; Fig. 2). We chose 20% as a cut off for the first pass screening because the CV value of our assay was consistently $<20\%$; 67 compounds fulfilled this criterion (Supplementary Table S1). These compounds were subsequently tested in the same assay using several different concentrations to further evaluate their therapeutic potential. Based upon the dose response data, 14 drugs proved to be effective, demonstrating $\geq 50\%$ inhibition of virally-infected cells (IC$_{50} < 20\, \mu\text{M}$) without inducing significant cell toxicity ($<20\%$ total cell number inhibition; Table I). The compounds that had reduced total cell number by $\geq 80\%$ were retested at lower concentrations, but none were identified that demonstrated clear anti-JCV effect without concomitant cytotoxic/cytostatic effect (Fig. 2). We chose to proceed only with the drugs that did not reduce total cell numbers to diminish the chance of confounding an antiviral effect with a cytotoxic/cytostatic effect. JCV actively replicates and destroys oligodendrocytes in the CNS of PML patients. Therefore, it is crucial to the success of any potential PML therapy that the candidate drug be capable of achieving an efficacious concentration in the brain. Unfortunately, many drugs are incapable of penetrating the blood-brain barrier (BBB). Based upon a review of the literature (Table I), only 1 of the 14 compounds with anti-JCV activity, mefloquine, has been shown to accumulate in the brains of treated patients at the level of its in vitro efficacious
concentration (Fig. 3A; IC₅₀=3.9 ± 2.1 μM). A postmortem brain analysis of people taking mefloquine prior to their deaths measured 35-50 nmol of drug per gram of brain tissue, or approximately 35-50 μM (33, 47). Since potentially efficacious doses of mefloquine could be achieved in the brains of patients receiving approved doses of the drug, subsequent experiments focused upon characterizing the anti-JCV activity of mefloquine.

**Characterization of Mefloquine Activity in Primary Cell Culture**

To further characterize the effect of mefloquine on JCV activity, experiments were performed to evaluate whether the JCV inhibitory effect of mefloquine was dependent on the cell line used in the primary screen. The SVG-A cell line has been propagated *in vitro* for many generations and was transformed by an SV40 large T antigen that can enhance JCV replication. To evaluate whether mefloquine is capable of inhibiting viral replication in cells more closely resembling the JCV target cell in the human brain, experiments were performed in primary glial cells. *In vitro* infection of homogeneous cultures of primary oligodendrocytes, a primary JCV target in PML brain, has not been established, therefore we employed human fetal astrocytes to test the ability of mefloquine to inhibit viral infection in a primary cell culture. In these cells, mefloquine inhibits JCV infection with essentially the same efficacy as it inhibits viral infection in SVG-A cells (Fig. 3B). These data suggest that the anti-JCV effect of mefloquine occurs in a more relevant cellular background.

**Characterization of Mefloquine Effects on Natural JCV Isolates**
Experiments were performed to demonstrate that the inhibitory effect of mefloquine is not limited to the JCV(M1/SVEΔ) construct used in the primary screening assay. JCV(M1/SVEΔ) virus had been used because of its faster growth kinetics and ease of preparation relative to in vivo isolates of JCV, however, transcription of its JCV genes is not regulated by an authentic JCV enhancer element. To ensure that mefloquine’s antiviral activity is not limited to this modified virus, we tested mefloquine’s ability to inhibit JCV(Mad4), an in vivo isolate from a PML patient (46). Based upon the results of five independent experiments, mefloquine inhibited JCV(Mad4) infection of SVG-A cells with the same efficacy as it did the JCV(M1/SVEΔ) infection (Fig. 3C), demonstrating that mefloquine has an antiviral effect on a known pathogenic form of JCV.

Characterization of Mefloquine’s Effect on JCV DNA Replication

To better understand mefloquine’s mechanism of action and address whether this drug inhibits viral DNA replication as opposed to a later step in the viral life-cycle, a qPCR assay was employed to measure viral DNA level in treated and untreated cells. Results presented in Figure 3D indicate that the percent of JCV DNA replication inhibition by mefloquine closely parallels the percent of infection rate inhibition, suggesting mefloquine inhibits infection rate at one of the steps involved in viral DNA replication, and not VP1 protein expression. We sought to extend this observation to a JCV(Mad1) infection of the naïve cells most commonly used to propagate the virus in culture, PHFG cells. At days 7 and 10 post infection, mefloquine inhibited JCV(Mad1) DNA replication in these cells with nearly the same efficacy (IC₅₀~5 μM) as it inhibited JCV(M1/SVEΔ) DNA replication in SVG-A cells (Fig. 3E).
**Effect of Mefloquine on Established JCV Infection**

Our experiments indicated that mefloquine inhibited JCV infection when added to cells at the same time as the virus, but it was not clear from those experiments whether mefloquine inhibited viral entry of the cells or a later step in the viral life-cycle. Once PML is diagnosed, many cells have already been infected, and a preferred drug candidate for PML treatment should demonstrate an ability to inhibit an ongoing viral replication cycle. To investigate this possibility, mefloquine was added to cells at the time of infection or 3 or 24 hours post infection and the antiviral activity was measured. Mefloquine effectively inhibited JCV infection under all three conditions (Fig. 3F). Since most of the virus enters the cells within 1 hour, and all of the virus has entered by 24 hours post infection in our assay (adding JCV neutralizing anti-serum 24 hours after virus addition was completely ineffective at blocking viral infection), our results suggest that mefloquine would effectively inhibit viral replication in cells already infected with JCV.

**Lack of Inhibitory Effect of CSF on Anti-JCV Effect of Mefloquine.**

While 98% of mefloquine in plasma is reported to be protein bound, high tissue/plasma ratios have been described (41). It is not clear how mefloquine’s high protein binding might affect its anti-JCV activity, therefore we tested the drug’s efficacy in the presence of increasing concentration of human cerebral spinal fluid (CSF) to mimic conditions encountered by the drug in the brain. To this end we added CSF at final concentrations of 2%, 10% or 20% over a range of mefloquine concentrations and
calculated the IC$_{50}$ for viral inhibition. As shown in Figure 4, the IC$_{50}$ values were consistent across all levels of CSF added to the culture (up to 20%).

*Characterization of Antiviral Effects of Mefloquine Enantiomers and Analogs*

Mefloquine is a racemic mixture of (11R,12S) and (11S, 12R) enantiomers of (2,8-Bis- trifluoromethyl-quinolin-4-yl)-piperidin-2-yl-methanol hydrochloride (Fig. 5A,B). While there is a minimal difference between the activities of the enantiomers against malaria (5, 13), the (R,S) enantiomer has a much more potent (~1000-fold) antagonistic activity against the A2a adenosine receptor (58). The two enantiomers also display different pharmacokinetics and brain penetration properties (6, 11). To better understand the anti-JCV effects of the two components of the marketed mefloquine racemate, each enantiomer was separated from the racemate using chiral chromatography and tested in the JCV inhibition assay. Both enantiomers were found to have similar efficacy in inhibiting JCV, with (R,S)-mefloquine only 2-fold more active (2.7 vs.4.5μM) than (S,R)-mefloquine and less than 2-fold more active than a racemate (2.7 vs. 4.0μM) (Fig. 5A,B). Based on this result and on the reported brain concentration for mefloquine enantiomers (6), we conclude that an efficacious concentration of mefloquine would be achieved in the brain of PML patients taking the drug. Furthermore, the lack of a significant difference in the anti-JCV activities of the enantiomers suggests that this effect is not mediated by the A2a receptor. To further explore the structure-activity relationship (SAR) for mefloquine, we acquired and tested other mefloquine analogs. Specifically, we tested a racemic mixture of (11S, 12S) and (11R, 12R) enantiomers of mefloquine (Fig. 5C) and of the pyridine analog of mefloquine, (2,8- Bis-trifluoromethyl-quinolin-4-yl)-pyridin-2-yl-
methanol (Fig. 5D). Activity of *threo* (R*, R*)-mefloquine (Fig. 5C) was almost the same as the activity of *erythro* (R*, S*)-mefloquine (4.6 vs. 4.5 μM) indicating that some degree of flexibility exists in the chiral centers at the positions 11 and 12 of the mefloquine molecule required for inhibition of JCV replication. On the other hand, when the piperidin moiety was replaced with the pyridine as in (2,8-Bis-trifluoromethyl- quinolin-4-yl)-pyridin-2-yl-methanol (Fig. 5D), anti-JCV activity was dramatically reduced. These results indicate that the chirality at position 12 is crucial for target inhibition, although the exact direction of hydrogen at that chiral center is less crucial for that activity, possibly due to some symmetry of the molecular target of mefloquine.

*Modeling Studies Suggest Shape Similarities Among Chemically Diverse JCV Inhibitors.*

To gain insights into the potential mechanism of action or molecular target of mefloquine in the inhibition of JCV replication, we analyzed the chemical classes of drugs with inhibitory activity (Table S1) and found that arylantranilic and arylalkanoic acid NSAIDS (Table 1, Fig. 6) represented the most frequent class of inhibitors. This observation suggests that a common structural motif is shared by at least some JCV inhibitors. To explore structural relationships among chemically diverse JCV inhibitors, we compared their three-dimensional shapes, while assuming that molecules have similar shape if their volumes overlay well. Conversely, any volume mismatch would represent a measure of dissimilarity. Such shape- and chemical feature-based comparisons (52) indicate that mefloquine, mfenamic acid, and indomethacin can occupy the same conformational space (Fig. 7). We then extended this analysis to compounds in clinical
testing reported in the MDL Drug Data Report database and discovered that mefloquine overlaid well with several nucleoside analogues, such as 8-chloroadenosine 3',5'-monophosphate (Fig. 7) and 3-deazaadenosine (not shown). These latter two compounds inhibited the infection rate of JCV(M1/SVEΔ) in SVG-A cells with efficacies similar to that observed with mefloquine (Fig. 7 and not shown). Taken together, these data suggest that chemically-diverse JCV inhibitors may have a common mechanism of viral inhibition and act, in part, as mimetics of nucleoside analogues.

**DISCUSSION**

PML is a devastating neurodegenerative viral disease that affects some immunosuppressed individuals, including 4-5 % HIV+ patients with AIDS and those undergoing immunosuppressive therapies (reviewed in (8)). Reconstitution of a patient’s immune system, either via HAART therapy for HIV+ individuals or via moderating immunosuppressive therapies where possible for others, is the only treatment option available today for managing this disease. Although different drugs have been tested as potential treatments for PML, all have failed to demonstrate clinical efficacy, thus keeping the search for drugs with anti-JCV activity a high priority. We report here identification of a number of drugs with anti-JCV activity via *in vitro* screening of a commercially available collection of approved drugs and bioactive compounds.

Cytarabine, Cidofovir and IFN-alpha have all been reported to exert anti-JCV activity *in vitro*, but they do not offer protection or improvement for PML patients over that observed with placebo (reviewed in (7)). It is possible that specific pharmacological properties of these drugs *in vitro and in vivo* are responsible for discrepancies between
results obtained in *in vitro* infectivity assays vs. *in vivo* clinical trials. Cytarabine has a short half life, and although it may reach a concentration of ~4μM in the CSF (37), animal studies suggest it does not cross the BBB to accumulate in the brain parenchyma in an amount required to inhibit JCV replication (30). Well known toxicities of cytarabine (i.e. bone marrow suppression, nephrotoxicity) do not allow it to be administered frequently and for an extended period of time to compensate for its unfavorable pharmacokinetic properties. Cidofovir was chosen as a treatment for PML based on its activity against murine polyomavirus and SV40 polyomavirus (2). However, cidofovir displays little (31), or no (Fig. 1D) anti-JCV activity *in vitro* at the doses achievable in plasma of treated patients (10μM); brain biodistribution data does not appear to be available for this drug. Data addressing the ability of IFN-α to cross the BBB is also lacking, but based on the size of the interferon molecule and its short half-life in blood, one would not predict for it to accumulate in the brain in significant amounts (53). Because JCV infects and replicates in cells throughout the entire white matter of the brain, an effective drug candidate for PML must be able to cross the BBB and accumulate throughout the entire brain parenchyma at a dose sufficient to suppress JCV proliferation.

Our initial screening identified several drugs with anti-JCV activity (Table 1), but only mefloquine has been shown to accumulate to therapeutically relevant levels in brain tissue of people receiving clinically-approved doses. This drug crosses the BBB and accumulates in the brain at concentrations that, when tested in cultured cells, lead to the inhibition of JCV infection (33, 47). The drug is likely to accumulate in the brain parenchyma at a concentration much higher than in the plasma because of its long plasma half-life, lipophilicity (LogP=2.47) and inhibition of MDR-1, a multi-drug resistance
protein responsible for the efflux of drugs out of the brain (47, 50). It was suggested recently that 5HT2A receptor blockers might be potential drug candidates for the treatment of PML based upon their ability to obstruct binding of the JCV capsid to its purported cellular receptor (23, 43). This observation remains controversial as a second group failed to detect anti-JCV activity of 5HT2A blockers (15), and we failed to uncover such activity for more than 20 drugs in the SPECTRUM collection belonging to this class of inhibitors (data not shown). Even in those studies reporting antiviral activity of 5HT2A blockers, the inhibitory mechanism involves a block to JCV cell entry, not a block to viral proliferation in cells already infected with JCV (43). Since a great many glial cells are already infected once a diagnosis of PML is made, the best strategy for prevention of further brain damage and for treatment would be to inhibit viral replication that has already been established. We report here that mefloquine reduces JCV replication by acting at a step subsequent to viral entry into the cell. We have employed qPCR to quantify the number of viral genome copies in infected PHFG and SVG-A cultures, and we have used this approach to demonstrate that mefloquine inhibits JCV DNA replication. Although mefloquine was discovered more than 30 years ago, its molecular target(s) in malaria patients has not been identified. Overall very few molecular targets of mefloquine have been identified, so it is not surprising that the precise molecular mechanism by which mefloquine interferes with JCV DNA replication remains unknown. (11S, 12R)-mefloquine is a specific and high-affinity inhibitor of the adenosine A2a receptor (58). Still, the A2a receptor does not appear to be a relevant target for JCV inhibition because while (11R, 12S) mefloquine is ~1000-fold more active than the (11S, 12R) enantiomer against A2aR, it is only 2-fold more active than the other enantiomer as a JCV inhibitor.
Furthermore, we tested a number of specific adenosine receptor inhibitors, and none of them were found to effectively inhibit JCV infection (data not shown). In our search for a common motif among compounds in the SPECTRUM collection having anti-JCV activity, we noted that N-arylanthranilic and arylalkanoic acid NSAIDS are disproportionately represented as a class. Although many of these molecules inhibit COX-1 and -2 activity as well as prostaglandin synthesis, such mechanisms do not seem relevant to their anti-JCV activity, as other NSAIDS in the library from a different molecular class, e.g. arylpropionic acid ibuprofen or flurbiprofen, do not display anti-JCV activity despite their COX inhibitory activity.

It is intriguing that mefloquine three dimensional confirmation fits into the shapes of arylanthranilic and arylalkanoic acid NSAIDS (Fig. 7), suggesting that while these compounds belong to different chemical classes, all may share a common molecular target. A search for other molecules with 3D structures similar to mefloquine and mefenamic acid revealed several adenosine analogs (e.g. 3-deazaadenosine and 8-chloroadenosine 3',5'-monophosphate) with anti-JCV activity. One might speculate that these inhibitors bind an ATP- or nucleotide-binding pocket of a molecule crucial for viral replication and disrupt its function. Since these drugs do not show cytotoxic effects at doses exhibiting anti-JCV effects, it is possible they directly inhibit T Antigen, the JCV-encoded replication protein, rather than the cellular DNA replication machinery required for viral replication. The conserved T Antigen of Polyomaviruses is a hexameric helicase that hydrolyzes ATP and forms an ATP-dependent replication complex at the AT-rich viral origin of replication (42, 59). Direct biochemical experiments will be required to investigate whether mefloquine targets this multifunctional viral protein. In summary,
mefloquine inhibits the replication of three different JCV isolates in three different cell types. Furthermore, mefloquine inhibits viral replication in cells previously infected with JCV. Finally, mefloquine accumulates in brain tissue at levels >6-fold above its IC$_{50}$ concentration. Although no animal model is available to test the ability of mefloquine to inhibit JCV in vivo, our in vitro data coupled with published biodistribution data for this drug, suggest that mefloquine could represent an effective therapeutic agent in the treatment of PML.

Currently, a controlled randomized clinical trial is underway to determine if mefloquine provides clinical efficacy in viral inhibition and protection from neurological damage in PML patients.

**ACKNOWLEDGMENTS**

We are grateful to Drs. Walter Atwood (Brown University, RI) and Duard Walker (Madison, WI) for their generosity in providing valuable reagents, and to Drs. Atwood and Brigitte Bollag (Penn State University) for assistance in establishing the JCV infectivity assay and the protocol for propagating PHFG cells, respectively. We wish to thank Ted Lin and Dr. Kevin Guckian (both Biogen Idec) for their help with compound verification. We appreciate editorial help and discussions from Drs. Susan Goelz, Petra Duda and Debra Kinch.
REFERENCES


Dussaix, and J. F. Delfraissy. 1999. Prolonged survival without neurological 
improvement in patients with AIDS-related progressive multifocal leukoencephalopathy 

29. Glazko, A. J. 1966. Experimental observations on flufenamic, mefenamic and 

delivery to rat brain by intravenous, intrathecal, intraventricular and intraparenchymal 

1998. Failure of cytarabine in progressive multifocal leukoencephalopathy associated with 
human immunodeficiency virus infection. AIDS Clinical Trials Group 243 Team. N Engl 

Polyomavirus- associated nephropathy in renal transplantation: critical issues of screening 


Pharmacokinetic studies of 13-cis-retinoic acid in pediatric patients with neuroblastoma 


FIGURES

Figure 1. Detection and measurement of JCV infection.

(A) SVG-A cells infected with JCV(M1/SVEΔ) were fixed and stained 3 days post infection with murine monoclonal antibodies specific for VP1 protein (green staining). Total cells present in the culture were visualized with DAPI DNA nuclear staining (blue). The picture was taken with a Cellomics ArrayScan® camera at x200 magnification. (B) The number of infected cells (i.e. VP1+ cells) per group is plotted against the dilution factor of the viral stock used to infect the cells (mean ±SD, n=2; blue line). Total number of cells (yellow bars) is similar for all groups. Cells were infected in the presence of various dilutions of JCV neutralizing antiserum (C) or cidofovir (D). Cells were fixed and stained at 3 days post infection, and the total numbers of VP1+ and DAPI+ events per treatment group were determined using Cellomics ArrayScan®. Data are presented as percent inhibition relative to no drug control of number of JCV+ cells ( ), number of total cells ( ) or number of JCV+ cells normalized by total cell number, % JCV+ cells.
Figure 2. Flow chart for SPECTRUM collection screening. Primary screening employed the SVG-A cell line and the virus JCV(M1/SVEΔ), and the assay was performed as described for Figure 1. * IC\textsubscript{50} - inhibition of % JCV-infected cells by 50% TC\textsubscript{50} - inhibition of total cell numbers by 50%.
Figure 3. Characterization of mefloquine anti-JCV effect.

A-B: To further characterize anti-JCV effect of mefloquine in different cell types and against different JCV isolates, viral infections were performed over the range of mefloquine concentrations in (A) SVG-A cells with JCV(M1/SVEΔ) (n=12); (B) in primary human fetal astrocytes with JCV(M1/SVEΔ) or (C) in SVG-A cells with JCV(Mad4) (n=5). D,E: To characterize effect of mefloquine on inhibition of JCV DNA replication, viral T antigen DNA was quantified in the presence of various drug concentrations using qPCR in (D) SVG-A cells infected with JCV(M1/SVEΔ). Inhibition of JCV DNA copy number and inhibition of %JCV+ cells (±) were measured in replicate plates. (E) Using the same qPCR assay, mefloquine’s ability to inhibit JCV(Mad1) DNA replication in PFHG cells was measured over a range of drug concentrations at days 7 and 10 post infection. The graph represents the average % JCV DNA inhibition for 3 independent experiments with duplicate samples per time point. (F) Effect of the delay of mefloquine addition was measured in cultures of primary human fetal astrocytes infected with JCV(M1/SVEΔ). Cells were exposed to various concentrations of mefloquine at the same time as virus addition (z) or at 3 hours, or 24 hours after virus addition. Ten days after infection with virus, cells were fixed and stained and the number of virally-infected cells was determined. Calculation of % JCV inhibition is described in Materials and Methods. Inhibition of total cell numbers (i.e. DAPI+ events) was less than 20% for all drugs concentrations plotted. Unless noted, only one representative graph is shown, but the IC50 is calculated as an average of all experiments.
Figure 4: Human CSF does not interfere with mefloquine’s anti-JCV activity.

SVG-A cells were infected with JCV(M1/SVEΔ) over a range of mefloquine concentrations in the presence of 2% (z), 10% (□) or 20% (S) human CSF. Three days later cells were fixed and stained and the total numbers of VP1+ cells and DAPI+ events per treatment group were determined using a Cellomics ArrayScan®. Results from one representative experiment (total of 2 independent experiments) are shown. Calculation of % JCV Inhibition is described in Materials and Methods.
Figure 5. Anti-JCV activity of various forms of mefloquine.

(A) (R, S)-mefloquine and (B) (S, R)-mefloquine enantiomers separated from a mefloquine drug racemate via chiral chromatography, (C) racemate of (S, S)- and (R,R)-enantiomers of mefloquine or (D) (2,8-Bis-trifluoromethyl-quinolin-4-yl)-pyridin-2-yl-methanol were added to SVG-A cells simultaneously with JCV(M1/SVEΔ). Cells were fixed and stained at 3 days post infection and total numbers of VP1+ cells and DAPI+ events per treatment group were determined using a Cellomics ArrayScan®. One representative experiment out of a total of six (A and B) or two (C and D) performed, is shown. IC_{50} values are a mean of all experiments performed. *10μM was the highest concentration tested; #TI- Therapeutic Index (TC_{50}/IC_{50}).
Figure 6. SAR of the arylantranilic and arylalkanoic acid JCV inhibitors.

Viral inhibition was measured using an infectivity assay with SVG-A cells and the JCV(M1/SVEΔ) virus. The IC₅₀ data represent averages calculated from 2 or more experiments and the therapeutic index (TC₅₀/IC₅₀) was >3 for all compounds shown.
Figure 7. Shape similarly among chemically-diverse JCV inhibitors.

The shape- and chemical feature-based comparisons of mefloquine (shown in magenta), mefenamic acid (shown in yellow), indomethacin (shown in gray) and 8-chloroadenosine 3',5'-monophosphate (shown in green). The overlays were achieved with program ROCS and visualized in PyMOL software.
<table>
<thead>
<tr>
<th>Inhibitor name</th>
<th>Therapeutic use</th>
<th>Statusabcdef</th>
<th>TC_{50} (μM)</th>
<th>EC_{50} (μM)</th>
<th>Conc (μM)def</th>
<th>Reference(s) for pharmacokinetic data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotretinone</td>
<td>Antineoplastic</td>
<td>USP, INN, BAN</td>
<td>&gt;40</td>
<td>4.4</td>
<td>~7.3&quot;</td>
<td>24</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>Antimarial</td>
<td>USAN, INN, BAN</td>
<td>16.1</td>
<td>4.0</td>
<td>30-50</td>
<td>6.0</td>
</tr>
<tr>
<td>Diclofenac sodium</td>
<td>Anti-inflammatory</td>
<td>USP, JAN</td>
<td>30.5</td>
<td>8.3</td>
<td>2.7&quot;</td>
<td>8.0</td>
</tr>
<tr>
<td>Diltiazem hydrochloride</td>
<td>Ca channel blocker</td>
<td>USP, INN, BAN, JAN</td>
<td>&gt;40</td>
<td>8.5</td>
<td>~1.1&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>Antibacterial</td>
<td>USAN, INN, BAN</td>
<td>&gt;40</td>
<td>8.6</td>
<td>1.9</td>
<td>19-171</td>
</tr>
<tr>
<td>Miconazole nitrate</td>
<td>Antifungal</td>
<td>USP, JAN</td>
<td>22.9</td>
<td>8.6</td>
<td>NA&quot;</td>
<td>0.024</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>Anti-inflammatory</td>
<td>USP, INN, BAN, JAN</td>
<td>&gt;40</td>
<td>10.9</td>
<td>2.4&quot;</td>
<td>40-80</td>
</tr>
<tr>
<td>Flunixin meglumine</td>
<td>Anti-inflammatory</td>
<td>USP, veterinary</td>
<td>&gt;40</td>
<td>16.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propanil</td>
<td>NA</td>
<td>NA</td>
<td>&gt;40</td>
<td>7.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydroabietamide</td>
<td>NA</td>
<td>NA</td>
<td>&gt;40</td>
<td>13.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffractic acid</td>
<td>NA</td>
<td>NA</td>
<td>&gt;40</td>
<td>14.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harmame</td>
<td>NA</td>
<td>NA</td>
<td>&gt;40</td>
<td>14.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthone</td>
<td>NA</td>
<td>NA</td>
<td>&gt;40</td>
<td>16.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methoxyvone</td>
<td>NA</td>
<td>NA</td>
<td>&gt;40</td>
<td>17.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The compounds selected had anti-JCV activity (EC_{50}) at ≤20 μM and a therapeutic index (TC_{50}/EC_{50}) of >2.

# USP, United States Pharmacopeia; INN, International Nonproprietary Name; BAN, British Approved Name; USAN, U.S. Approved Name; JAN, Japanese Approved Name.

" TC_{50} inhibition of total cell numbers by 50%.

f The highest concentration achieved in the brain or plasma/serum, as reported in the literature.

g No data for humans are available; the data are based on the data reported from animal studies.

/NA not available.
APPENDIX C: SOURCES OF CONTENT

Portions of this thesis, including text and figures were from the following publications:


VITA

MARTA MARIA REVIRIEGO-MENDOZA

EDUCATION

- **Ph. D. Biochemistry, Microbiology and Molecular Biology (2005-2011)**
  The Pennsylvania State University, USA

  University of Leeds, UK

AWARDS AND HONORS

- The Pennsylvania State University Graduate Research Exhibition, Third Place, 2010.

- Outstanding Althouse Teaching Assistant Award, BMB Department, 2005-2006.


PUBLICATIONS

