INTERPLAY BETWEEN JCV LARGE T-ANTIGEN AND CULLIN-7 IN BRAIN CANCER

A Dissertation
Submitted to
The Temple University Graduate Board

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in Partial Fullfillment
of the Requirements for the Degree
DOCTOR OF PHILOSOPHY

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by
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May, 2011

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ABSTRACT

A convincing body of evidence suggests that ubiquitination and the ubiquitin proteasome degradation pathway play a key role in neoplastic transformation. Ubiquitination, as post-translation modification, is involved both in functional regulation and degradation of specific cellular targets known as proto-oncogenes and tumor suppressors. Oncogenic viral proteins interact both with proto-oncoproteins and tumor suppressors leading to the modulation of their cellular function by several mechanisms including ubiquitination. Interestingly, viral oncoproteins themselves can also be regulated by this post-translation modification. Additionally, viruses can assemble their own E3 ligases or regulate the activity of cellular E3 ligases. E3 ligases, involved in the final step of the ubiquitination process, are the enzymes that provide the specificity for the interaction with target substrates by the means of a large number of proteins. Recent studies on the potential correlation between viral infection and oncogenesis, have addressed the emerging role of the ubiquitination system as a possible mediator for cancer transformation. In this scenario we hypothesized that JCV T-antigen may interfere with the ubiquitination system and we investigated a possible interaction between JCV T-antigen and the E3 ligase Cul7. To prove our hypothesis we performed co-immunoprecipitation and co-immunofluorescence experiments using the glioblastoma cell lines HJC12, U87MG and HJC5. Our results indicate that JCV T-antigen and Cul7 interact in the cytoplasmic compartment. In addition, JCV T-antigen stabilizes Cul7. These observations suggest that JCV T-antigen can modulate Cul7 E3 ligase activity leading to oncogenesis. Further study addressing the biological significance of this interaction will decipher the cellular

processes modulated by JCV T-antigen and Cul7 and will indicate new avenues for therapeutic intervention.

ACKNOWLEDGMENTS

To my sister Catherine with love

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CHAPTER 1

INTRODUCTION

The role of ubiquitination in human cancer virology

It is now widely accepted by the scientific community that viruses are not only the agents of infectious diseases but they are also involved in causing certain types of cancer, both in animals and humans. However, it must be emphasized that viruses generally act as initiating or promoting factors of the carcinogenic process, consistent with the principle that cancer development occurs not by a single event but rather by the accumulation of cooperating events. Additionally, generally many years pass between initial infection and tumor appearance. Although there has been a long debate regarding the correlation between viruses

and carcinogenesis over the last century, a small number of viruses are now accepted and recognized by cancer biologists to be involved in the development of human cancers. In fact, since the first human virus, the papillomavirus, has been linked to the development of cervical cancer, new viruses, both DNA and RNA, have been identified as potential human carcinogens. These include six viruses that belong to different virus families: Human Papilloma virus (HPV), Epstein-Barr virus (EBV), Hepatitis B virus, together with the most recent discovered Hepatitis C, Human T Lymphotropic virus type I (HTLV-1) and KSHV/HHV-8 virus. Presently, approximately 20% of human cancers have been linked to a viral etiology in predisposed individuals following an early primary infection event. In this respect, different vaccination strategies, either prophylactic or therapeutic, are under intense investigation in order to prevent and treat infections caused

by viruses with an oncogenic potential (Howley, 2006).

Interestingly, in this scenario, another family of viruses is now receiving a particular attention as a possible candidate in causing certain types of cancer. In particular, there is an emerging interest in the family of polyomaviruses, which includes the well known SV40, BK, and JC virus. SV40, which naturally infects the rhesus monkey, was inadvertently introduced into the human population as a contaminant of early poliovirus vaccines, whereas the BK and JC are natural human pathogens associated with infectious disease in the urinary tract and brain, respectively. DNA sequences of these three polyomaviruses, which are tumorigenic in experimental animals, have been detected in mesothelioma, osteosarcoma, non–Hodgkin lymphoma, brain tumors, and prostate cancer. In addition to SV40, BK and JC, three new human polyomaviruses have recently been isolated. These are KI polyomavirus (KI), WU polyomavirus (WU) and Merkel cell polyomavirus (MCV). Whereas MCV has been observed in samples from Merkel cell carcinomas (MCC), an aggressive and rare human skin cancer, no evidence exists for the presence of KI and WU viral genomes in any type of human cancer. Both KI and WI have been linked with high tract respiratory diseases and like for MCV, the potential association with malignant transformation is still speculative.

To complete the picture, additional virus families have been implicated in the onset of human cancers. These include adenoviruses, human endogenous retroviruses, human mammary tumor virus (HMTV)/Pogo virus, exonotropic murine leukemia virus-related virus, Torque teno virus and cytomegalovirus. Some studies report the association of these viruses with several different human malignancies such as glioma in the case of cytomegalovirus. However, there is no compelling evidence in support of this

hypothetical but intriguing possibility and further studies would be desirable to clarify their oncogenic potential.

Ubiquitination system

Ubiquitin is a highly conserved regulatory protein and it is ubiquitously expressed in eukaryotes (Shenoy, 2009). This small polypeptide consists of 76 amino acids, which include seven lysine residues at positions 6, 11, 27, 29, 33, 48, and 63. The attachment of ubiquitin monomers to the ε-amino group of a lysine residue of target proteins is a reversible post-translational modification called ubiquitination. This process, at the molecular level, requires the sequential action of three enzymatic events. The first step, ATP-dependent, involves the ubiquitin-activating enzyme, or E1, that activates and binds ubiquitin in a high-energy thioester bond. Ubiquitin is then transferred in a second thioester linkage to an ubiquitin conjugating enzyme, E2. In the final step E2, in concert with the action of an ubiquitin ligase or E3, catalyzes the transfer of ubiquitin to the substrate protein in a covalent bond between the C-terminal Gly75-Gly76 residues and the ε-amine of a lysine residue of the target protein. Although the most prominent function of ubiquitin is the labeling of target proteins for proteasomal degradation, a number of recent studies have shown that, beside this function, ubiquitination also controls the stability, function, and intracellular localization of a wide variety of proteins. The overall outcome of this molecular modification is the regulation of many biological processes, including gene transcription, cell-cycle progression, DNA repair, apoptosis, virus budding and receptor endocytosis. In this respect, substrates can be either mono-ubiquitinated or conjugated to a poly-ubiquitin chain that varies in length and linkage type. As expected, the different types of ubiquitin modifications are responsible for

distinct cellular physiological functions. The typical lysine 48-linked polyubiquitin chain labels proteins for recognition and proteasome degradation. During this process, ubiquitin is cleaved from the substrate by the action of deubiquitinating enzymes, which are associated with the proteasome. This allows the shredding of the targeted proteins and the recycling of ubiquitin itself. Ubiquitin, however, can also be degraded by the ubiquitin-proteasome system. In this regard, a recent study has shown that this polypeptide can be targeted for degradation both as monomer and as a part of its conjugated target. Additionally, ubiquitin monomer degradation requires ubiquitination of both internal lysine and N-terminal NH2 group being ubiquitination dependent on one other. Whereas ubiquitin when coniugated to substrates does not require a specific E3 ligase, monomeric ubiquitin must be specifically recognized. An E3 ligase for the ubiquitin substrate itself has not been yet identified. However, it is clear that ubiquitin turnover is tightly regulated (Shabek et al., 2007).

Ubiquitination associated with proteasome degradation represents the canonical function for this biologic modification. However, in the last decade, the importance of atypical chains conjugated through the other six lysines in the ubiquitin polypeptide has been revealed. Atypical chains can be of different types: homotypic, when the same lysine is sequentially used for conjugation or mixed linkage when different lysines are used to connect consecutive ubiquitin moieties. The most interested atypical homotypic chain is the lysine 63-linked type, that, differently from the 48-linked type, is independent of proteolysis and plays a role in kinase activity, DNA repair and in receptor endocytosis (Huang et al, 2006; Herman et al. 2007). Interestingly, recent studies suggest that the activity of E3 ligases, which are also polyubiquitinated for proteasome degradation, is

modulated through their modification by the attachment of atypical polyubiquitin chains. In this respect, it is generally accepted the post-translation modifications of enzymatic proteins leads to their activation.

Monoubiquitination consists in the attachment of one ubiquitin molecule to a single lysine residue. Monoubiquitination can occur at different lysine residues and when multiple sites are monoubiquitinated is called multi-monoubiquitination. This modification is reversible and it not linked to the proteasome degradation system. In fact, monoubiquitination has been shown to play a role in virus budding, histone regulation, nuclear export, DNA repair (Huang et al., 2006), receptor endocytosis and also in directing proteins toward the endosome-lysosome pathway (Woelk et al., 2007). A pivotal role in these functions seems to be played by a new class of proteins called ubiquitin-binding proteins or ubiquitin receptors which, through a non-covalent interaction with the monoubiquitinated substrates by means of specific ubiquitin binding domains, direct the biological activity functions of their targets (Huang et al. 2006). Generally, monoubiquitination requires the concerted activity of a hierarchical cascade of E1, E2 and E3 enzymes. However, it has also been reported that E2 enzymes can drive monoubiquitination without the support of an E3 ligases (Hochstrasser, 2007). An example of such a phenomenon is indeed represented by ubiquitin receptors, which are regulated by monoubiquitination. However, it has been pointed out that this type of ubiquitination differs in both mechanism and function from E3-mediated monoubiquitination (Holler et al., 2007).

E3 ubiquitin ligases consist of more than 500 hundred proteins in the human genome (Yen and Elledge, 2008). Different combinations of E2 and E3 are involved in the ubiquitination process and are thought to be responsible in defining substrate specificity (Pagano et al.). Because only a small number of E3 ligases substrates have been identified, the field is at present an intensive subject of research investigations. E3 ligases belong to two main families: the RING-finger type and the HECT-domain type. Additionally, RING E3 ligases have been further classified into the single-subunit RING-finger type and the multi-subunit RING-finger type (Cradozo and Pagano, 2004). Among the multi-subunit RING-finger type, the cullin-dependent ligases (CDLs) are the best characterized being central to the ubiquitin-mediated proteolysis of many important biological substrates. CDLs function with other molecules as multi-subunit complexes. They are characterized by two key components: a cullin (CUL) protein and a RING-finger protein ROC1 known also as Rbx or Hrt (Sarikas et al. 2008). Seven mammalian cullins have been identified so far: Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, and Cul7, that form different complexes depending on the type of adaptors and substrate recognition molecules that participate in the complex (Tsunematsu et al. 2006). Cullins share homology mostly in the cullin domain at their C-terminus, which interacts with the RING-finger protein.

Human Polyomaviruses

Polyomaviruses are a family of non-enveloped DNA viruses with icosahedral capsids that can infect multiple species such as humans, monkeys, rodents and birds (..). Human polyomaviruses include BK virus (BKV), JC virus (JC) and the most recent identified KI polyomavirus (KI), WU polyomavirus (WU) and Merkel cell polyomavirus (MCV).

BKV, the first discovered human polyomavirus, was isolated from the urine of a kidney allograft recipient with chronic pyelonephritis and advanced renal failure (Gardener et al., 1971) whereas JCV, discovered in the same year, was isolated from the brain of a patient affected by Hodgkin's lymphoma who developed progressive multifocal leukoencephalopathy (PML) (Padgett et al., 1971). The most recent human polyomaviruses KIV and WUV have been associated with acute respiratory infections and appear to be a new subfamily of human polyomaviruses being distantly related to BKV and JCV (Allender et al,. 2007; Gaynor et al., 2007). Differently, Merkel cell polyomavirus has been identified in Merkel cell carcinomas (MCC). In this respect, experimental evidence indicates that this virus is clonally integrated in tumor genomic DNA suggesting its role in early tumorogenesis (Feng et al., 2008).

Each polyomavirusus shows a very limitated host range and doesn't usually infect other species. However, in this scenario, the role of simian vacuolating virus 40 (SV40), as human pathogen is still an open question. SV40 is a very closely related phylogenetically with BKV and JCV as shown by their similarity in size, genomic organization and DNA sequences. Together with mouse polyoma virus, SV40 was the first polyomavirus to be described (Stewart et al., 1958; Sweet et al., 1960) and it has been used as simple model system for the study of basic eukaryotic molecular biology including DNA replication, transcription, malignant transformation and signal transduction (White et al, 2005). SV40, which naturally infects the rhesus macaque, was accidentally introduced in the human population as a contaminant of polio vaccine. Although there is not good serological evidence for SV40 in humans and it is largely accepted that monkeys are the natural host for this virus, some research groups have reported the presence of SV40

sequences in various human tumors (Garcia and Imperiale, 2003) which suggests a role of SV40 in the etiology of human diseases.

JC virus

JCV belongs to the Polyomavirinae family which consists of eight members: JCV, BKV, Simian viruses 40, Monkey B-lymphotropic papovavirus, mouse polyomavirus, bovine polyomavirus and avian polyomavirus (Stoner and Hubner, 2001).

JCV is a small human DNA tumor virus and the causative agent of the progressive multifocal leukoencephalopathy (PML). Patients affected by this fatal demylination disease of the brain show severe neurologic symptoms together with a highly compromised immune system. JCV was first identified in PML samples by electron microscopy in 1965. However, the limitation in collecting human samples for research purpose, followed by several unsuccessful attempts in establishing a suitable in vitro cell culture system, didn't allow the immediate further characterization of this virus. Finally, in 1971, JC virus was first isolated in culture in primary human fetal glial (PHFG) cells, indicating the restricted host cell range of this virus. In this respect, in fact, it has been shown that JCV infection occurs preferably in oligodendrocytes and at a lesser extent in astrocytes leading to cytolitic destruction of the myelin-producing glial cells and degeneration of the myelin sheath in the white matter of the brain. Some reports indicate that JCV can also infect neurons. However, other research groups have not been able to identify the virus genome in neuronal cell, suggesting that additional studies are required to confirm this finding.

JCV infection is widespread in the human population worldwide as shown by the high prevalence of antibodies in the human sera against JCV. In this respect, it is generally

accepted that the virus infects most people during the childhood and remains in a persistent but dormant state known as latency. In this context, JCV-induced PML develops only in the context of severe immunopression, such as AIDS, autoimmune diseases, agammaglobulinemia, lymphoma, and immunosuppressive drug agents. In this respect, it must be observed that if clearly immunosuppression plays a role in reactivation, not all immunosuppresed patients develops PML and that maybe polymorphism in the human population affects the immune system (Jiang et al., 2008).

Beside of being the opportunist etiologic agent of PML, JCV has been associated with central nervous system (CNS) and non-CNS tumors. In this scenario, the possible role of eukaryotic viruses in the development of cancer has been the subject of intense investigation for the past 50 years (Reiss et al., 2003). A few examples of viruses as clearly causative agents of human cancers are the human papilloma virus, the Epstein virus, and the human herpesvirus 8 among others. However, the role of the polyomaviruses and in particular of JCV regarding its ability to cause human tumors is not yet proven. On the other hand, based on the numerous studies that link JCV to cancer formation, its oncogenic potential cannot be rule out.

JCV viral genome consists of covalently bound, double-stranded, circular supercoiled DNA with an average size of 5kb (Croul et al., 2003). It can be divided into early and late coding sequences whose transcription is initiated in opposite directions from a common non-transcribed regulatory region composed of two 98 base pair repeats that encompasses the viral origin of DNA replication.

The viral early region is transcribed before DNA replication and encodes the alternatively spliced protein large T-antigen and small t-antigen. After DNA replication, the viral late

region is transcribed encoding the capsids protein VP1, VP2 and VP3 together with the small accessory protein known as Agnoprotein. Based on the sequence of the common non-coding regions whose transcriptional regulation involves cell-type specific transcription factors, several JCV strains have been identified and link either to PML or to different type of human tumors.

The transmission of the JCV within the human population is still unknown and the life cycle of the virus inside the human body poorly understood. The archetype form of JCV, which is excreted with the urine, seems to be the transmissible form of this virus. In this respect, the archetype JCV is found in the kidney of healthy human subjects and probably is able to replicate in people that shows a lower immune system activity leading to the virus secretion into the urine.

However, it must be pointed that the archetype JCV is not the form identified brain samples from patients affected by PML. In this respect, it has been reported that the JCV forms isolated in these sample, are characterized by rearrangements in the JCV non-coding control region (NCCR). The rearrangements include deletions, duplications and point mutations and these additional JCV forms have been designated as “PML-type”. Based on these findings, together with the observation that latent JCV has been found in other organs such as tonsils and B-lymphocytes, it has been suggested that the archetype form can reach the brain through bloods cells at which time NCCR rearrangements may occur.

Large T-antigen

JCV T-antigen has been characterized as a cellular tranforming agent through several cell

biology and biochemistry studies. Protein alignment shows that JCV large T-antigen shares 70% homology with SV40 large T-antigen.

Large T-antigen is a highly phosphorylated multifunctional protein that plays a role both in cellular transformation and in viral DNA replication. Its phosphorylation occurs in the nuclear compartment. T-Ag is an essential factor for viral DNA replication which requires the recruitment of proteins involved in DNA synthesis. T-antigen functions are linked to different structural domains and functional motifs. In this respect three domains have been linked to its oncogenic potential, the LXCXE domain, the J domain and the p53 binding domain. The first two domains are part of the N-terminus, whereas the p53 binding domain is localized on the C-terminus.

Cullin-7

Cullin family proteins are structurally related proteins that share a cullin domain, evolutionarily conserved, which is located on the C-terminus. Several members of this family are involved in the ubiquitin (Ub)-dependent proteolysis. Among them, Cullin 1 (Cul1), which represents the molecular scaffold of the SCF-ROC1 E3 ligase complex, has been shown to target a variety of substrates, such as those involved in the control of DNA replication, transcription, cell cycle transition, and signal transduction. As a molecular scaffold, Cul1 contains two distinct binding domains, the N- terminal and the C-terminal regions. The first region is responsible for binding to the adapter protein Skp1 (S-phase kinase associated protein), whereas the second region is responsible for the association with the ROC1 RING (Really Interesting New Gene) finger protein. The proteins responsible for substrate recognition belong to the large family of the F-box proteins. Depending on the target, a specific F-Box protein binds to the adapter protein Skp1 and

this complex, within the proximity of ROC1, recruits an E2-conjugating enzyme (Dias et al., 2002).

Cullin-7 (CUL7), also known as KIAA0076, p185, p193, has been recently identified as cullin-RING complex (Dias et al., 2002). It belongs to the cullin family of E3 ligases, which includes Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, and it is the seventh mammalian member of this group that has been identified to date. Cul7 organizes an SCF-like E3 complex composed of the WD40 repeat containing F-box protein Fbw8 (also termed Fbx29, Fbw6 or Fbxw8), the adapter protein Skp1 and the RING finger protein ROC1 also named Rbx1 or Hrt1 (Kohrman and Imperiale, 1992; Sarikas et al., 2008).

Initially Cul7 was isolated as a cellular protein bound to the SV40 large T-antigen (Kohrman and Imperiale 1992; Tsai et al., 2000). The first research group identified a protein of 185 kDa, which consistently immuno-precipitated with T-antigen, while optimizing the conditions for the detection of T-antigen-pRb complexes in rodent cells. In the attempt to characterize this new protein, they made several interesting observations. First, the showed that p185 is present in normal cells being a host protein and that its expression is independent of viral proteins functions. Second, they show the presence of p53 and p185 in the same T-antigen complexes. Finally, they indicated that the N-terminal 108 residues of T-antigen complexes with p185 suggesting the importance of this interaction in both transformation and transcriptional trans-activation.

The second group (Fields and colleagues) identified and cloned a 193 kDa SV40 large T-antigen binding protein from transgenic cardiac tumor cell lines and suggested that p193 could be a member of a family of apoptosis promoting proteins containing only the BH3 domains. In agreement with the previous group, they indicated that the N-terminus of

SV40 T-antigen binds to p193 and suggested that the interaction occurs in the cytoplasmic compartment of the cell. Additionally they indicated that these two proteins co-localized in the cytoplasm at the same point of the cell cycle, G1, when p193-induced cell death occurs. A recent work (Dias et al., 2002) identified Cul7 by spectrometry as a ROC1 interacting protein and characterized it as an E3 ligase. A later work (Ali et al., 2004) demonstrated that p185 and p193 T-antigen associated proteins are identical and that this protein is indeed Cul7. Cul7 assembles an SCF-like E3 ligase complex which, like Cul1, contained the adapter protein Skp1, ROC1 and the WD40 repeat-containing F-box protein Fbw8 (also named Fbx29, Fbxw8). Presently Fbx28 is the only F-box protein known to bind Cul7 via Skp1, suggesting that this protein is highly selective. In this regard, two cellular proteins have been shown being degradated by Cul7: cyclin D1 and insulin receptor substrate 1 (IRS-1). Cyclin D1 plays a very important role in the regulation of cell cycle progression and it is regulated at the post-translational level. Fbw8, which is the substrate recognition subunit of the Cul7 E3 ligase, is involved in the ubiquitination of cyclin D1, in a phosphorylation dependent manner. Fbw8 interacts also with IRS-1, which is a critical component of the signaling pathways downstream of the insulin and insulin-like growth factor 1. In this regard, Xu et al. have shown that Fbw8 interaction with IRS-1 leads to its degradation through the proteasome system.

Although it has been shown that Cul7 is involved mainly in proteolytic activities, it has been suggested the this E3 ligase may exert also non proteolytic effects. In this respect, it has been reported that Cul7 binding to p53 doesn't lead to its degradation. However, if Cul7 contributes to p53 dependent apoptosis is still an open question.

SV40 T-antigen has been reported being a binding partner of Cul7. Considering that T-antigen is not degraded by Cul7, it appears that T-antigen might neutralize a function of Cul7 that protects against cellular transformation. Additionally, it has been suggested that T-antigen could recruits Cul7 in order to re-direct its ligase function towards cellular proteins for viral propagation.

Although most of the studies on Cul7 suggest that this protein is involved in tumor suppression, other studies have shown that Cul7 could function as growth promoter. It is possible that this protein play a role both in promoting and suppressing roles in a context-dependent manner.

CHAPTER 2

MATERIALS AND METHODS

Plasmids and recombinant DNA

The following plasmids were used: pcDNA3-JCV large T-antigen and pcDNA3- JCV (ΔJ) large T-antigen (kindly provided by Dr. Kamel Khalili); pcDNA3-HA-Cul7 (kindly provided by Dr. James DeCaprio); pcDNA3-pRb2/p130 and the empty vector pcDNA3 (Claudio *et al*., 1994). All plasmids were propagated in and isolated from JM109 competent cells (Promega), using standard techniques.

Cell lines

The JCV-induced hamster astrocytoma cell lines HJC12 (Howard *et al*., 1998), HJC5 (Raj *et al*., 1995) and the human glioblastoma U-87MG cell line (ATCC, Bethesda, MD, USA) were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air in Dulbecco's Modified Eagle (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen).

Transfection

Transient transfection of sub-confluent 10-cm plates of cells were performed with Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol.

Antibodies

For immunoprecipitation and Western blot analysis the following antibodies were used: anti-SV40 T-antigen monoclonal antibody (clone PAb 416, Oncogene Science, Boston, MA, USA), anti-cullin7 polyclonal antibody (clone PAb 653 Bethyl Laboratories), anti-pRb2/p130 polyclonal antibody (C-20 Santa Cruz-317) anti-Skp1 p19 polyclonal antibody (H-163, Santa Cruz-7163), anti-ROC1 polyclonal antibody (ROC1 Ab-1

Thermo-Scientific), anti-Fbxw8 polyclonal antibody (A01, Abnova) and anti-beta-actin polyclonal antibody (ab 8227, Abcam).

Immunoprecipitation and Western blot

For immunoprecipitation cells were lysed in NP-40 buffer supplemented with a complete cocktail of inhibitors proteases (Roche) and centrifuged to remove cell debris. In order to reduce the background of non-specific proteins, cell lysates were first precleared with the control antibody, either normal mouse serum (NMS) or normal rabbit serum (NRS), and protein A-Sepharose beads (Pharmacia). The beads were subsequently removed by centrifugation and the precleared lysate when then reacted with primary antibody overnight at 4° C while rocking. Protein A-Sepharose beads (Pharmacia) were then added and the incubation continued for additional two hours. Immune complexes were then collected by centrifugation, washed four times (two times with NP-40 buffer and two times with PBS) and eluted in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer for 15 minutes while boiling. Samples were resolved on 7% SDS-PAGE gels. Fractionated proteins were then transferred on nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany by wet electrophoretic transfer according to Towbin *et al*. 1979 at 300-mA constant current and analyzed by Western blot. For western blotting, membranes were blocked in TBS-T buffer (10mM Tris-HCl pH 8.0, 150mM NaCl, 0.1% Tween-20) containing 5% no fat dry milk for 1 hour. Following blocking, the membranes were probed with the appropriate primary antibody in 5% no fat dry milk diluted in TBS-T overnight at 4°C. After extensive washing with TBS-T, blots were incubated with the appropriate secondary horseradish peroxidase-conjugated antibody (Amersham, Buckinghamshire, UK) diluted in 1:10,000

in TBS-T containing 5% no fat dry milk for 1 h at room temperature. After rinsing, signals were visualized by the ECL method according to the manufacturer's recommendations (Amersham).

Immunocytofluorescence

Immunofluoresce staining was peformed on U-87MG cells. Cells, grown on 2 x 2 cm^2 glasses, were fixed with 4% paraformaldeyde in PBS for 20 min at room temperature and permeabilized with 0.1% Triton X-100 (Fisher Scientific, Pittsburgh, PA) in PBS for 5 min at RT. Non-specific binding sites were blocked with PBS containing 8% bovine serum albumine (BSA; Sigma-Aldrich) for 1 h at room temperature. The cells were then incubated overnight at 4°C days with the appropriate primary antibody diluted in PBS with 1% BSA overnight at 4°C. Secondary antibodies, respectively anti-mouse IgG FITC-conjugated or anti-rabbit IgG Texas-Red-conjugated (Vector Laboratories, Burlingame, CA) were diluted 1:100 in PBS containing 1% BSA. Negative controls were performed by omitting the primari antibody. Nuclear staining was performed with DAPI 100 ng/ml (4',6'-diamino-2-phenylindole; Molecular Probes, Carlsbad, CA), 1 min at room temperature. After washing, glasses were mounted with Vectashield mounting medium (Vector Laboratories) and visualized with an Olympus IX81 fluorescence microscope (Olympus Microscopes, Center Valley, PA).

Colony formation assay

Approximately 5 x 10^4 (U-87MG) were plated in 60 mm plates and after 24h cell were transfected either with the pcDNA3-HA-Cul7 or with the empty vector pcDNA3 by the Lipofectamine 2000 (Invitrogen) method according to the manufacture's protocol. After 24h, transfected and untransfected cells were collected and seeded in a 100 mm plate

(approximately 5 x 10^5 cells) in complete medium (DMEM supplemented with 10% FBS) containing G-418 Sulfate (0.8mM) (Geneticin Liquid, Invitrogen). Cells were maintained for 3 weeks during which complete medium containing G-418 was changed every 48 hours. At the end of third week, cells were staining with 1% methylene blue for 5 minutes and the number of colonies was determined.

CHAPTER 3

RESULTS

Interaction between JCV large T-antigen and Cullin-7 in T-antigen positive glioblastoma cells

Previous studies from our and other laboratories have shown the importance of large T-antigen–host protein complexes in JCV cancer transformation. In this respect, a number of host cell proteins have been identified as targets of large JCV T-antigen, which include p53, pRb family proteins, beta-catenin and IRS-1. Cullin-7, also known as p185, has been reported to co-precipitate by SV40 large T-antigen (Khorman and Imperiale, 1992; Ali et al., 2004). JCV, like SV40, is a member of the polyomaviruses. Furthermore, several studies have shown that SV40 large T-antigen and JCV large T-antigen target similar host cell proteins. (reviewed in Reiss et al.) We were interested in analyzing the formation of a stable complex between JCV large T-antigen and Cullin-7. To this end, experiments were performed by transient transfection of JCV large T-antigen positive glioblastoma cells (HJC12) with a plasmid encoding human Cullin-7. The interaction was identified by method of co-immunoprecipitation and Western Blot. Immunoprecipitation of either T-antigen or Cullin-7 was performed by incubating desired antibodies the T-antigen positive hamster glioblastoma cell lysate. Results, as shown in Figure 1., show that when T-antigen was immunoprecipitated by anti-T-antigen antibody, Cul7 was co-precipitated from Cul7 transfected cells but not from Cu7 non-transfected cells, as revealed by Western Blot analysis. The reciprocal experiment, performed by using anti-Cullin-7 antibody, shows that, when Cul7 was immunoprecipitated by anti-Cul7 antibody, T-antigen was co-precipitated from Cul7 transfected cells, but not from Cu7

non-transfected cells. Results from this experiment have confirmed our hypothesis that a stable interaction between JCV large T-antigen and Cul7 occurs in the cells and that large T-antigen binds specifically to Cul7. The intensity of the co-precipitated proteins indicates that only a relative small portion interacts to each other in the cell (Figure 1).

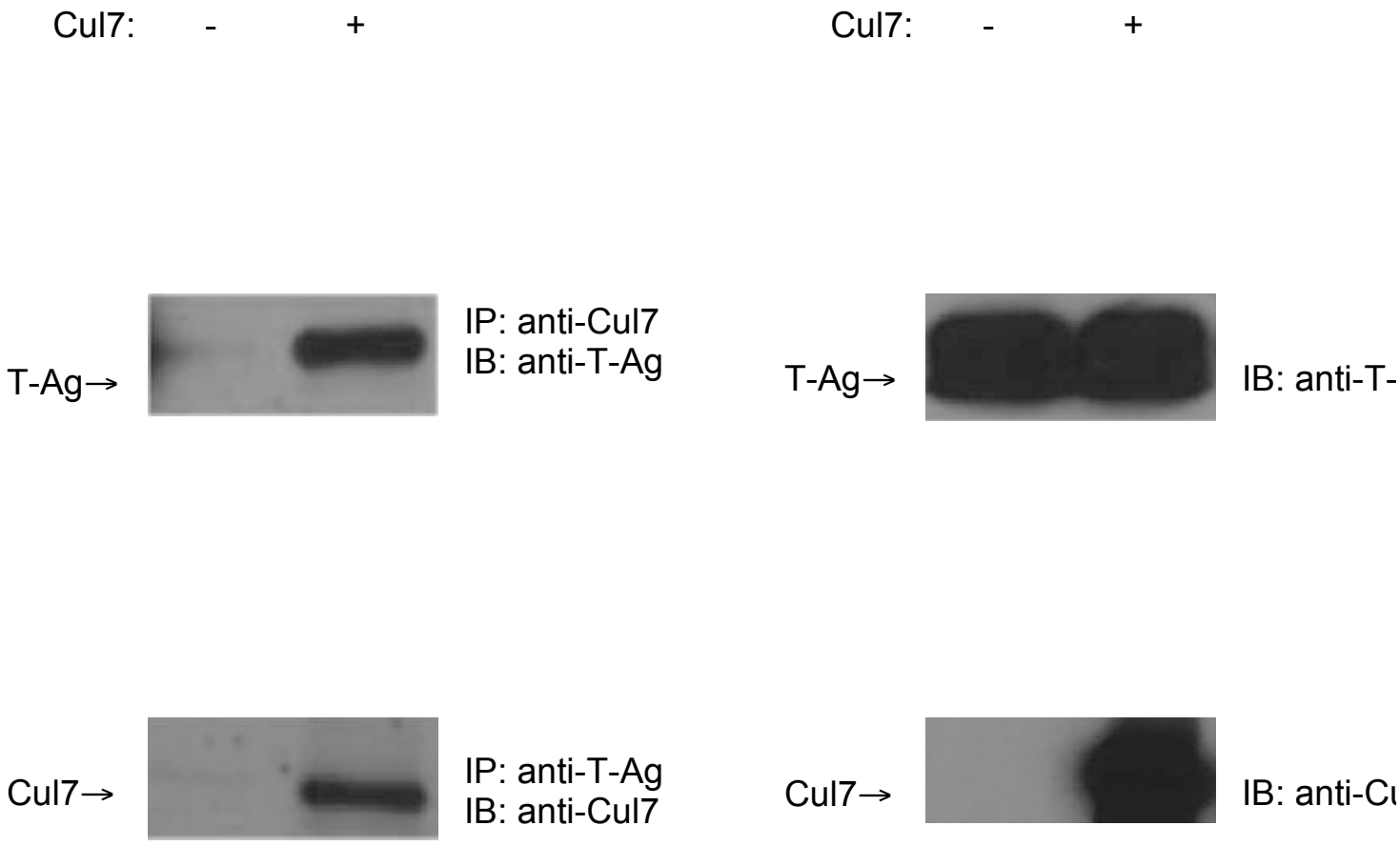


Figure 1. **Association of large T-antigen and Cul7 in vivo.** The JCV-induced hamster astrocytoma cell line HJC12 was transfected with a plasmid encoding HA-CUL7 by the lipofectamine method. Proteins extracts derived from these cells were prepared 24h after transfection and subjected to Western analysis to verify the expression of both Cul7 and large T-antigen. SDS-PAGE (8%) was performed and the expression of ectopic Cul7 or T-antigen was confirmed with anti-Cul7 or anti-T-antigen antibodies (right panel). Co-immunoprecipitation procedure was performed with a polyclonal anti-Cul7 or a monoclonal anti-T-antigen antibody followed by WB analysis. Proteins isolated from non-transfected cells were used as negative controls. Extracts were co-precipitated with a polyclonal anti-Cul7 or a monoclonal anti-T-antigen antibody. Western blot analysis of the precipitate confirmed the association of JCV large T-antigen with Cul7 (left panel).

Cul7 binding domain of JCV large T-antigen

Each polyomaviruses T-antigen, including JCV T-antigen contains at least three transforming domains which are: a bipartite C-terminal domain which binds and inactivate p53; the LXCXE domain which mediates binding to the retinoblastoma family; and the N-terminal 82 residues, encoded by the first exon and shared with small T-antigen. The N-terminus of T-antigen, which contains a HPD motif, resembles the J domain of the DnaJ (Hsp40) family of molecular chaperones and interacts with dnaK-type chaperones, such as the heat shock protein 70 (hsp70). This interaction stimulates the chaperone's ATPase activity, with the consequent modulation of their ability to bind and release protein complexes in the cell.

It has also been reported that the J domain together with the LXCXE motif is required to alter the phosphorylation state of the retinoblastoma family p107 and pRb2/p130. The same study has suggested that the J domain could participate in the regulation of protein stability through the proteasome-mediated degradation. In this scenario, considering the recent function of Cul7 as E3 ligase involved in protein degradation, we investigated if the J domain of T-antigen was required for this interaction. To this end, experiments were performed by transient transfection of human glioblastoma cells (U-87MG) with a plasmids encoding (ΔJ) large T-antigen and a plasmid encoding HA-Cul7 either alone or in combination, by the lipofectamine method. The interaction was identified by method of co-immunoprecipitation and Western Blot. Immunoprecipitation of (ΔJ) T-antigen was performed by incubating the desired antibody with (ΔJ) T-antigen positive hamster glioblastoma cell lysate. Results from this experiment show that when (ΔJ) T-antigen was immunoprecipitated by anti-T-antigen antibody, Cul7 was co-precipitated from Cul7

transfected cells but not from Cu7 non-transfected cells or (ΔJ) non-transfected cell, as revealed by Western Blot analysis (Figure 2).

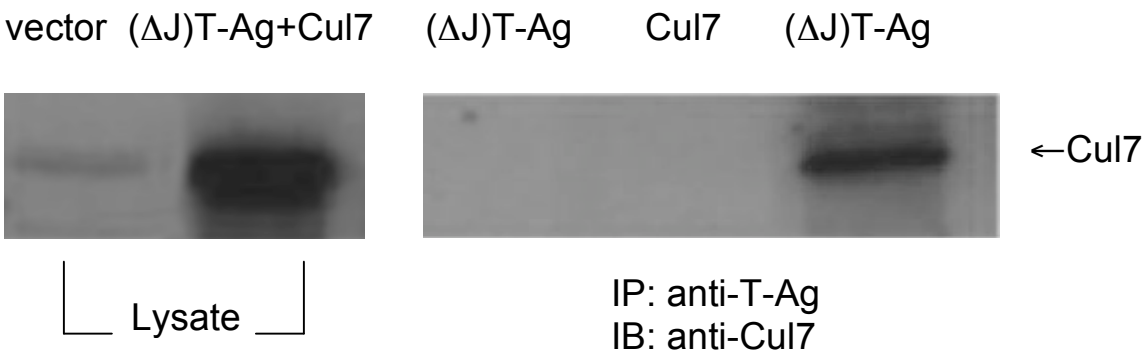
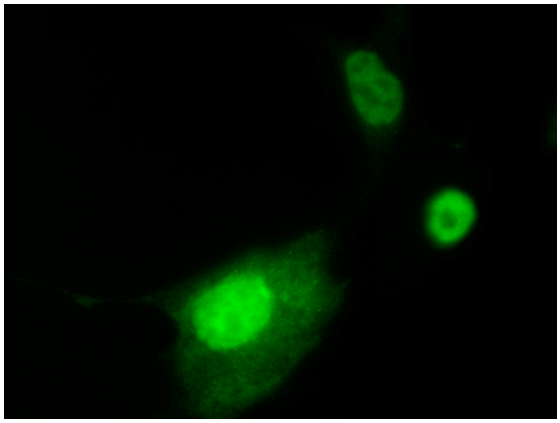


Figure 2**. Mapping the binding domain of Cullin-7 on JCV large T-Ag.** Human glioblastoma cells (U-87MG) were transfected with a plasmids encoding (ΔJ) large T-antigen and a plasmid encoding HA-Cul7 either alone or in combination by the lipofectamine method. Transfection was performed also using an empty vector as negative control. Proteins extracts (50 μg) derived from these cells were prepared 24h after transfection and subjected to Western analysis to verify the expression of both Cul7 and large T-antigen. SDS-PAGE (8%) was performed and the expression of ectopic Cul7 or T-antigen was confirmed with anti-Cul7 or anti-T-antigen antibodies (left panel). Co-immunoprecipitation procedure was performed with a monoclonal anti-T-antigen antibody followed by WB analysis. Extracts were co-precipitated with a monoclonal anti-T-antigen antibody. Western blot analysis of the precipitate confirmed the association of (ΔJ) large T-antigen with Cul7 (right panel).

Sub-cellular Co-localization of Cullin-7 and (ΔJ) T-antigen

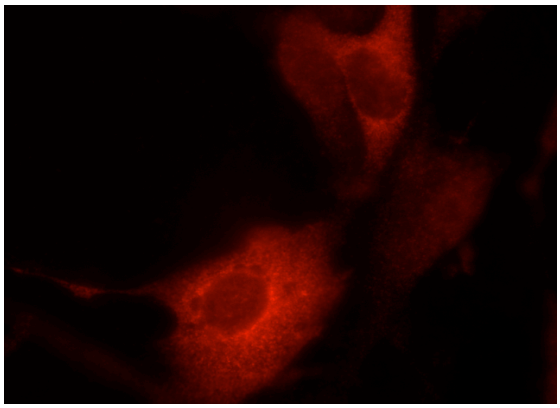
In order to study the sub-cellular co-localization of T-antigen and Cullin7, U-87MG cells were transient transfected with (ΔJ) T-antigen and Cullin7 expression plasmids and the protein cellular localization of (ΔJ) T-antigen and Cullin-7 was studied by the means of immunocytochemestry techniques. The transient transfection was preferred to the stable transfection in order to eliminate the possibility for the occurrence of a secondary mutation during the selection process for a stable clone. The results from these experiments show that (ΔJ) T-antigen localizes mostly in the nucleus. However, the presence of (ΔJ) T-antigen can also be detected in the cytoplasm (Figure 3). This finding is in agreement with studies that have described the same type of localization for the full length of the protein T-antigen. Conversely, Cullin-7 seems mostly cytoplasmic localized, showing a higher accumulation in the peri-nuclear area (Figure 3). No evidence of Cullin-7 in the nucleus was observed. Generally, in human cells, members of the cullin family proteins are distributed both in the cytoplasmic and nuclear compartments. Our results, showing the sub-cellular localization by indirect immuno-fluorescence, indicate that, even when ectopically over-expressed, Cul7, like its closest related PARC (Nikolaev et al., 2003), localized predominantly in the cytoplasm. Previous studies have shown that Cul7, unlike its binding partner T-antigen, is not actively shuttling between the nucleus and the cytoplasm. In fact, treatment of cells with leptomycin B (LMB), an inhibitor of the Crm-1 specific nuclear export pathway has not detectable effect on the cytoplasmic localization of Cul7 (Andrews et al., 2006). Additionally, it has been reported that ROC1 when over-expressed together with Cul7, it was localized mostly in the cytoplasm, suggesting that high level of Cul7 can interfere with the function of other

cullins by sequestering ROC1 in the cytoplasm. These observations are in agreement with our observations: the localization of Cul7 in the peri-nuclear may in fact indicate a role of Cul7 E3 ligase both in the regulation and degradation of proteins that indeed shuttle between the nucleus and the cytoplasm. Considering that Cul7 is expressed in the cytoplasm and (ΔJ) T-antigen is mainly localized in the nucleus, experiments were carried out to investigate if co-expression of Cul7 and (ΔJ) T-antigen could relocalize each other. Results show that both (ΔJ) T-antigen and Cullin-7 retain their respective localization and no significant change of sub-cellular localization was seen for either protein. Co-localization of (ΔJ) T-antigen and Cullin-7 seems to occur in the cytoplasm compartment, in the peri-nuclear area (Figure 3). The low relative abundance of (ΔJ) T-antigen in the cytoplasm compartment suggests that Cul7-T-antigen interaction may be transient. This observation is in agreement with the relative low level of Cul7-TAg interaction observed by co-immunoprecipitation (Figure 1).



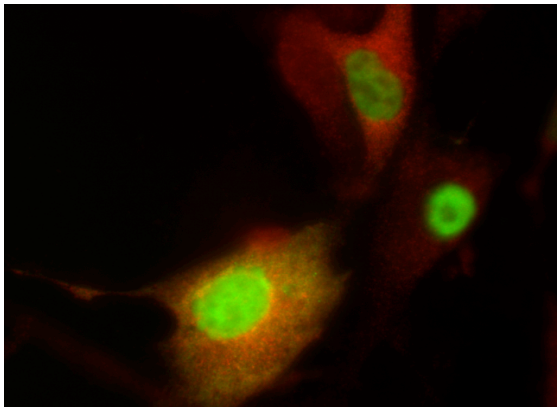
(ΔJ)T-Ag+Cu7

anti-T-Ag (mono)



(ΔJ)T-Ag+Cu7

anti-Cul7 (poly)



(ΔJ)T-Ag+Cu7

anti-T-Ag (mono) +Anti-Cul7(poly)

Figure 3. **Sub-cellular co-localization of Cullin-7 and (ΔJ) T-antigen.** The human astrocytic cell line U-87MG was transfected with plasmids encoding (ΔJ)T-antigen and HA- CUL7. Cells were fixed and were prepared for immuno-fluorescence microscopy using anti-T-antigen antibody (mono) and anti-Cul7 antibody (poly). Cells show a staining pattern that indicates that (ΔJ)T-antigen localizes both in the nucleus and in the cytoplasm compartments whereas Cul7 shows a cytoplasmic distribution. As shown, co-localization of (ΔJ)T-antigen and Cul7 occurs in the peri-nuclear region.

Large T-antigen and mutant T83-688 enhance Cul7 level

Cul7 is an E3 ligase whose main function is to ubiquitinate targeted proteins for degradation. In this respect, it has been shown that, Cul7 degradates cyclin D1 and IRS-1, through the proteasome system. Additionally, it has been reported that Cul7 can ubiquitinate targeted proteins without leading to their degradation. This post-translation modification has been shown to occur for p53 and probably represents a new type of protein activity regulation.

In order to decipher the biological significance of Cul7-T-antigen interaction several experiments were carried out. Based on the fact that an interaction between T-antigen and Cul7 occurs and that an E3 ligase for T-antigen has not been identified yet, the possibility that T-antigen could be a target for degradation by Cul7 was explored**.** Through the means of transient transfection, T-antigen was expressed alone or in combination with Cul7. Cell lysates were prepared and the same amount of proteins from each sample was resolved on a 10% SDS-PAGE followed by Western blot. Results from this experiments showed that the level of T-antigen doesn't change in the presence of Cul7, excluding the hypothesis that Cul7 could be the E3 ligase for T-antigen.

Recent studies on the interaction between β-catenin and T-antigen have showed that the T-antigen stabilizes β-catenin at the protein level. In this study, experiments were carried out to demostrate that when β-catenin and T-antigen are co-expressed in U-87MG, the protein level of β-catenin is higher when compared to its expression in the absence of T-antigen. Based on these observations, experiments were carried out to evaluate the possibility that T-antigen could stabilize Cul7. Similarly, U-87MG cells were transient transfected with plasmids encoding T-antigen and Cul7 and the level of Cul7 in the

presence and in absence of T-antigen was evaluated by SDS-PAGE followed by Western blot techniques. Results show that in the presence of T-antigen, the protein level of Cul7 is enhanced (Figure 4, left panel), which suggests that Cul7 is stabilized in the presence of T-antigen. It could be argued that the level of Cul7 increases due too gene expression regulation, However, since both T-antigen and Cul7 genes are under the same cytomegalovirus (CMV) promoter regulation this appear unrealistic.

To further understand how T-antigen enhances Cul7 expression, experiments were carried out to evaluate if the J domain of T-antigen is required for Cul7 stabilization. The J domain of T-antigen is localized at the N-terminus of T-antigen and includes the first 82 amino-acids. It has been reported by several studies that this domain is required for the oncogenic activity of T-antigen. Similarly, U-87MG cells were transient transfected with plasmids encoding (ΔJ) T-antigen and Cul7. Results show that, as for the full length of T-antigen, in the presence of (ΔJ) T-antigen the protein level of Cul7 is enhanced (Figure 4, right panel). This observation suggests that this domain is not required for the binding of T-antigen to Cul7. However, this doesn't rule out that the J domain is required for the biological activity of this cellular complex in the cell.

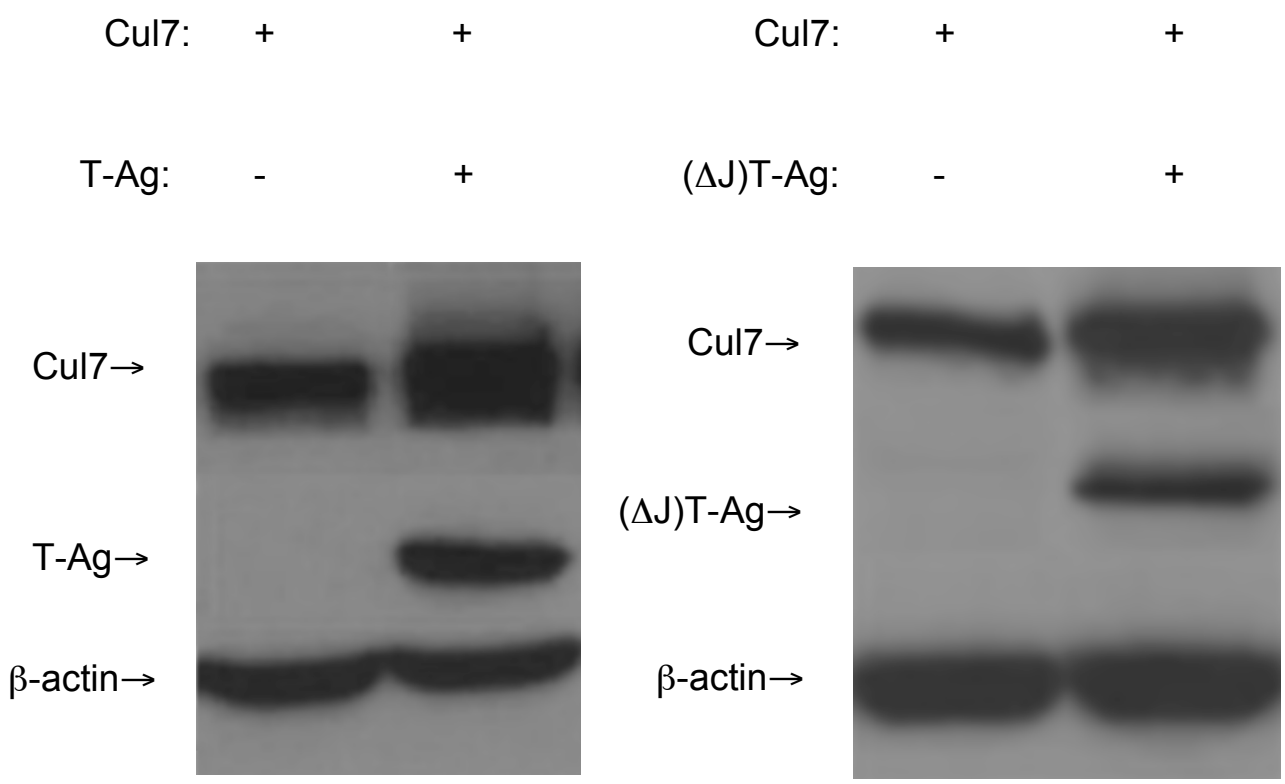


Figure 4. **Large T-antigen and mutant T83-688 enhance Cul7 level.** The human astrocytic glial cell line U87MG was transfected with a plasmid encoding HA-Cul7 alone or in combination either with a plasmid encoding large T-antigen or with a plasmid encoding the mutant T83-688 by the lipofectamine method. Proteins extracts derived from these cells were prepared 24h after transfection and subjected to Western analysis. SDS-PAGE (8%) was performed and the higher protein level of Cul7 in the presence of T-antigen or (ΔJ)T-antigen was confirmed with a polyclonal anti-Cul7 antibody. The expression of large T-antigen and mutant T83-688 was confirmed with a monoclonal anti-T-antigen antibody. The expression of beta-actin was used as loading control.

Interaction between large T-antigen and pRb2/p130

Together with pRb/105 and p107, pRb2/p130 forms the Rb family proteins, which regulate cell cycle and cell growth. Several studies have reported pRb2/p130 as a target of JCV T-antigen which, through inactivation of this and other tumor suppressors, is able to mediate cancer transformation. The interaction between Cul7 and JCV T-antigen occurs in the absence of the J domain of T-antigen suggesting that other regions of T-antigen are involved in the binding to Cul7. The pRb binding region of T-antigen is known as LxCxE motif (residues 103 to 107). Experiments were carried out to evaluate the possibility that Cul7 could compete with pRb2/p130 for binding to T-antigen through the LxCxE motif. Transfection and co-immunopreciptation assays were performed. The JCV-induced hamster astrocytoma cell line HJC5 was transfected with a plasmid encoding HA-CUL7 alone or in combination with a plasmid encoding pRb2/p130. Results shows that expression of Cul7 doesn't affect the level of the interaction between T-antigen and pRb2/130 (figure 5.) suggesting the possibility that T-antigen could be in complex with Cul7 and pRb2/p130 in the cytoplasmic compartment of the cell.

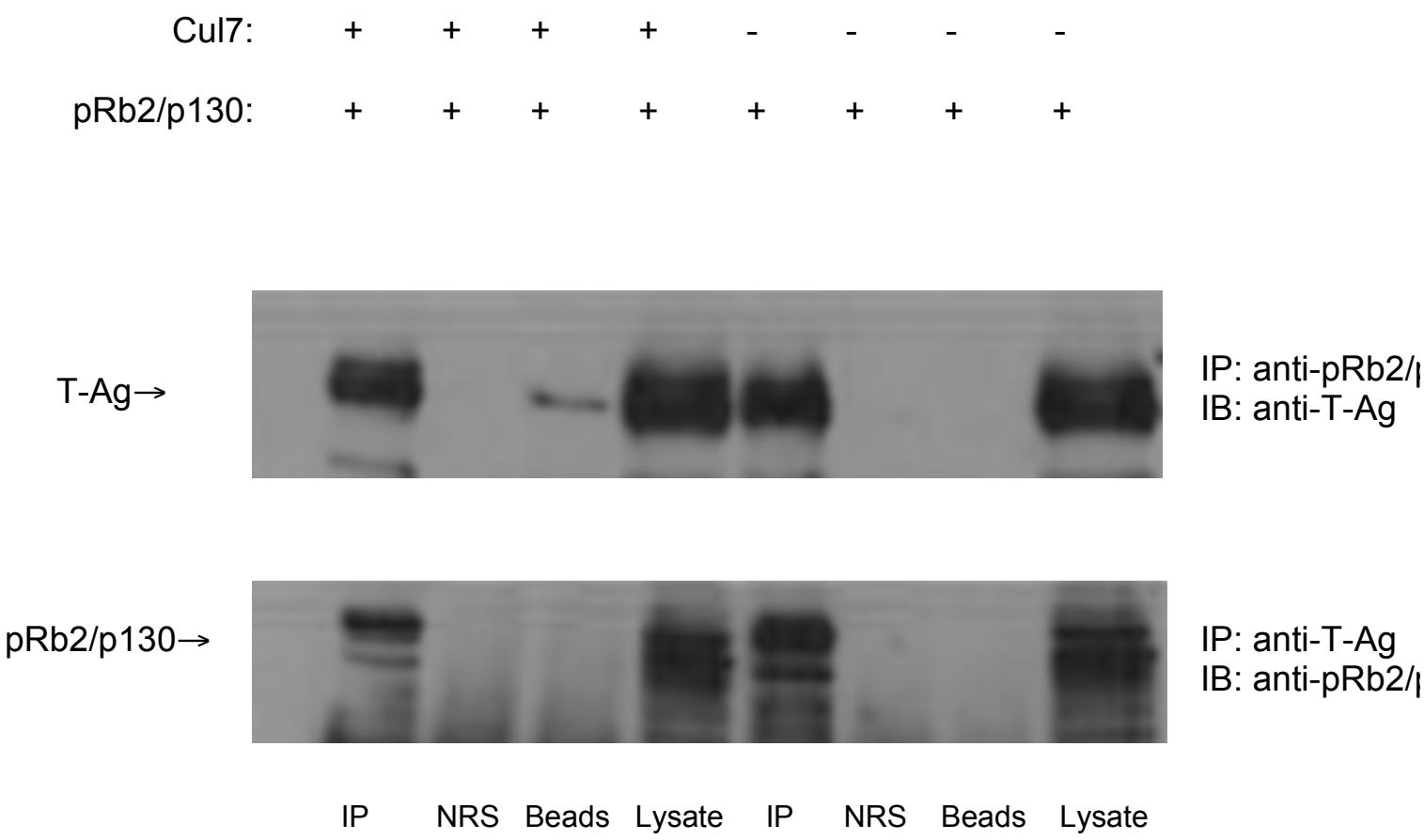


Figure 5. **Interaction between large T-antigen and pRb2/p130.** The JCV-induced hamster astrocytoma cell line HJC5 was transfected with a plasmid encoding HA-CUL7 alone or in combination with a plasmid encoding pRb2/p130 by the lipofectamine method. Proteins extracts derived from these cells were prepared 24h after transfection and subjected to Western analysis to verify the expression of Cul7 and pRb2/p130. SDS-PAGE (8%) was performed and the expression of Cul7 and pRb2/p130 was confirmed with polyclonal anti-Cul7 or anti-pRb2/p130 antibodies. Co-immunoprecipitation procedure was performed with a polyclonal anti-pRb2/p130 or with a monoclonal anti-T-antigen antibody followed by WB analysis. Extracts were co-precipitated with a polyclonal anti-pRb2/p130 or with a monoclonal anti-T-antigen antibody. Western blot analysis of the precipitate confirmed that JCV large T-antigen and pRb2/p130 in the presence of Cul7.

Co-immunoprecipitation between Cul7 and pRb2/p130 in T-Ag positive cells

Cul1 has been reported to be the E3 ligase for pRb2/p130 leading to its degradation through the proteasome system. Cul1 and Cul7 share a high homology leading to the speculation that these E3 ligases could share the same targets but being active in different cellular compartments. In this respect, it is possible that Cul1 could degrade /modify targeted proteins in the nucleus while Cul7 being functionally active in the cytoplasm. In order to test the hypothesis that Cul7 could be an E3 ligase for pRb2/p130, experiments were performed in order to establish an interaction between Cul7 and pRb2/p130. The JCV-induced hamster astrocytoma cell line HJC5 was transfected with a plasmid encoding HA-CUL7 alone or in combination with a plasmid encoding pRb2/p130. Cell lysates were prepared and co-immunoprccipitation assay, followed by Western Blot analysis, was performed. Results from these experiments show that Cul7 and pRb2/p130 don't interact in the presence of T-antigen. This result, however, doesn't rule out the possibility that Cul7 could interact with pRb2/p130 in the absence of the viral oncoprotein.

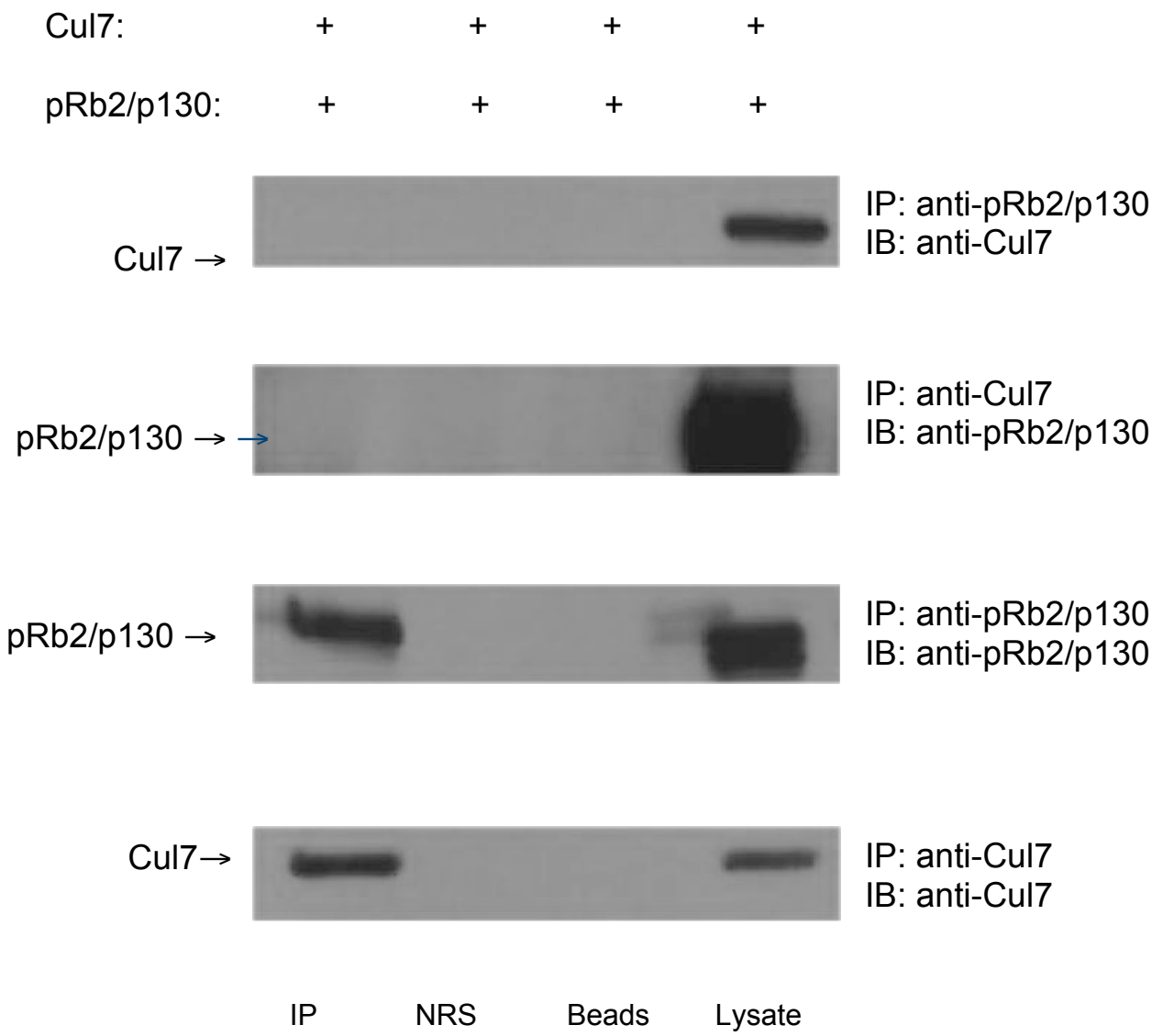


Figure 6. **Co-immunoprecipitation between Cul7 and pRb2/p130 in T-Ag positive cells.** The JCV-induced hamster astrocytoma cell line HJC5 was transfected with plasmids encoding HA-CUL7 and pRb2/p130 by the lipofectamine method. Proteins extracts derived from these cells were prepared 24h after transfection and subjected to Western analysis to verify the expression of Cul7 and pRb2/p130. SDS-PAGE (8%) was performed and the expression of Cul7 and pRb2/p130 was confirmed with polyclonal anti-Cul7 or anti-pRb2/p130 antibodies. Co-immunoprecipitation procedure was performed with polyclonal anti-Cul7 or anti-pRb2/p130 antibodies Extracts were co-precipitated with a polyclonal anti-Cul7 antibody or with a polyclonal anti-pRb2/p130 antibody. Western blot analysis of the precipitate confirmed that Cul7 doesn't associate with pRb2/p130 in the presence of JCV large T-antigen.

Cul7 E3 ligase complex in U-87MG cells

Cul7 E3 ligase complex includes the scaffold protein Cul7, the adapter protein Skp1, the FBox protein Fbx29 and ROC1. To better understand the role of Cul7 as an E3 ligase, experiments were done in order to evaluate the level of expression of Cul7 E3 complex in U-87MG cells. Cells were tranfected with plasmids encoding Cul7, Skp1, Fbx29 and ROC1. Cell lysate of transfected and non-transfected cells were prepared and the same amount of proteins from each sample was resolved on a 10% SDS-PAGE followed by Western blot. Results from this experiments show that while ROC1 and Skp1 are expressed in U-87MG cells, the level of Cul7 and Fbx29 is very low suggesting that both Cul7 and Fbx29 could have a role in cell growth control (Figure 7).

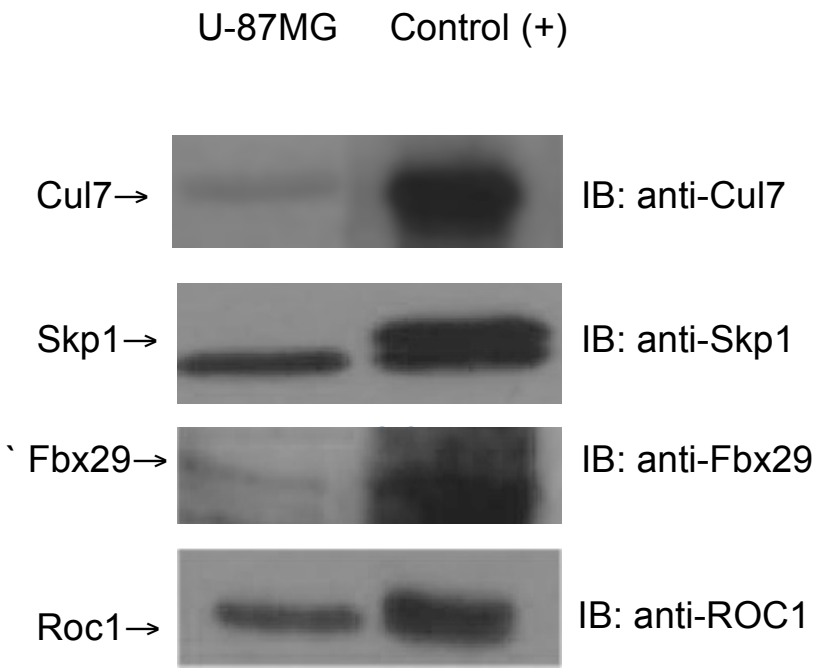


Figure 7. **Cul7 E3 ligase complex in U-87MG cells.**

	Number of colonies	Inhibition *
Control	88	0
Cul7	40	54%

*The percent (%) inhibition represents the average of three independent experiments

Figure 8. **Inhibition of cell growth in U-87MG cells.**

CHAPTER 4

DISCUSSION

Study of T-Antigen cellular transformation has led to the discovery of different key growth control pathways. In this respect, p53 was discovered as a co-precipitating protein. Additionally, when the regions of T-antigen that bind to pRb family proteins and p53 were mutated, it was discovered that these two tumor suppressors play a key role in cancer tranformation. The main finding of this study is the association of Cullin-7 and JCV Large T-antigen in an *in vitro* system such as U-87MG cells. Furthermore, large T-antigen is the first viral oncoprotein that has been showed to bind to with Cul7.

The J domain of T-antigen is required for cancer transformation. This study indicates that this domain is not necessary for the binding between Cul7 and T-antigen. Additionally, it seems that T-antigen stabilizes Cul7 suggesting a regulation of Cul7 by T-antigen and not viceversa. Cul7 has been proposed to be a tumor suppressor protein. In the context of T-antigen cancer transformation, it is possible that T-antigen blocks its activity by the inhibition of its E3 ligase activity. On the other hand, it also possible that T-antigen can redirect the Cul7 activity toward new cellular targets in favor of T-antigen cancer transformation.

CHAPTER 5

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