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Signaling pathways and polyomavirus oncoproteins: Importance in malignant transformation

Review Article

Martyn K. White and Kamel Khalili

Center for Neurovirology and Cancer Biology, College of Science and Technology, Temple University, Philadelphia, PA 19122

***Correspondence**: Dr. Martyn K. White, Center for Neurovirology and Cancer Biology, College of Science and Technology, Temple University, Bio-Life Sciences Building, Room 238, 1900 North 12th Street, MS 015-96, Philadelphia, PA 19122; Tel: 215-204-0620; Fax: 215-204-0679; Email: martyn.white@temple.edu

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Abbreviations: acquired immunodeficiency syndrome, (AIDS); bcl-2 homology type 3, (BH3); BK virus, (BKV); central nervous system, (CNS); human JC virus, (JCV); Institute of Medicine, (IOM); insulin receptor substrate 1, (IRS-1); mitogen-activated protein kinase, (MAPK); non-polyposis colorectal carcinoma, (HNPCC); origin of DNA replication, (ORI); progressive multifocal leukoencephalopathy, (PML); protein phosphatase 2A, (PP2A); RNA polymerase I, (Pol I); Simian vacuolating virus 40, (SV40); small t-antigen, (t-Ag); stress-activated protein kinase, (SAPK); T-antigen, (T-Ag); upstream binding factor, (UBF)

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Summary

Treatment of a particular cancer, whether by gene therapy or by small molecule inhibitors, is predicated upon a knowledge of the signal transduction pathways that became dysregulated as the tumor developed and cells underwent malignant transformation. Usually cancer is thought of as a disease that progresses through the gradual accumulation of multiple successive genetic "hits" leading from normal cells to fully malignant metastatic tumors in a fashion such as that described for the colorectal adenoma-carcinoma sequence. These hits include mutations that activate oncogenes, knockouts of anti-oncogenes (tumor suppressors) and events that increase mutation rates or destabilize the genome. Polyomaviruses are small DNA viruses that encode proteins that promote cell transformation in culture, induce tumors in experimental animals and have been found in association with some human cancers. The small genome size of the polyomaviruses means that a few proteins must perform many tasks. There is evidence that these multifunctional viral proteins can provide several "hits" at once to cells and thus circumvent certain steps that are necessary for malignant transformation during non-viral tumorigenesis. In this review, the biological and pathological properties of polyomavirus proteins are discussed. The relevance of these is underscored by the growing evidence for the involvement of polyomaviruses in some human cancers. Polyomavirus gene-specific therapeutic strategies might be considered in these cancers since ablation of the function of viral oncoproteins would be expected to reverse multiple molecular events involved in malignant transformation.

I. Introduction

Cancer occurs when a normal cell accrues enough genetic changes to be able to grow continuously and kill the host. A great many of these mutations have been reported and can be grouped into three broad categories. Oncogenes are mutated versions of the cellular genes that drive cell growth, such that cell proliferation is promoted inappropriately. These include growth factor receptors (Baserga, 1994), plasma membrane molecular switches such as Ras (Vojtek and Der, 1998; Sears and Nevins, 2002), protein and lipid kinases that are part of signal transduction pathways in the cytoplasm such as Raf/MEK/Erk (White, 2004) and PI3K/Akt (Blalock et al, 2003), apoptotic regulators (White and McCubrey, 2001) and transcription factors (Sears and Nevins, 2002). Mutations in the Ras and Raf proto-oncogenes are very common in human cancers. (Barbacid, 1987; Davies et al, 2002; Chan et al, 2003).

A second category of cancer-associated genetic change involves the tumor suppressor genes, also known as anti-oncogenes. Tumor suppressors are negative regulators of cell growth and mutations that inactivate them are associated with cancer. The first tumor suppressor gene to be identified was p53. The p53 protein is malfunctional in most human cancers (Vogelstein et al, 2000). p53 is the central regulator of a network of

signaling that is turned on when cells are stressed or damaged. The p53 network inhibits cell proliferation and promotes apoptosis under these circumstances. This serves to eliminate damaged cells and thus provides a brake on tumor development explaining why p53 is so often mutated in cancer cells (Vogelstein et al, 2000). Another important tumor suppressor gene is the retinoblastoma gene product pRb (Sears and Nevins, 2002). pRb is the central supervisor of cell cycle progression. It is regulated by phosphorylation and sequesters the transcription factor E2F that is responsible for activating the genes whose products are necessary for cells to enter S-phase (Sears and Nevins, 2002).

A third category of cancer-associated genetic change does not affect cell growth *per se* but involves impairment of DNA repair. This leads to a mutator phenotype that predisposes cells to incur mutations at a faster than normal rate. Since these mutations may occur in oncogenes or tumor suppressor genes, the mutator phenotype predisposes cells to malignant progression. Three intracellular mechanisms are involved in DNA damage repair that lead to a mutator phenotype: nucleotide excision repair, base excision repair and mismatch repair. DNA repair genes can become mutated in human cancer and a number of hereditary cancer predisposition syndromes are characterized by defects in a particular DNA repair gene. These include hereditary non-polyposis colorectal carcinoma (HNPCC), Bloom syndrome, ataxia-telangiectasia, and Fanconi anemia (Charames and Bapat, 2003). For example, HNPCC is associated with a defect in a DNA mismatch repair gene (Bronner et al, 1994; Papadopoulos et al, 1994).

Cancer is a multi-step process involving changes of the three types outlined above. A good example of this is the colorectal adenoma-carcinoma sequence (Fearon and Vogelstein, 1990). The genetic changes during the progression of an intestinal polyp to an adenoma and then to a carcinoma have been elucidated in detail. It is likely that such a model is applicable to other cancers. However, in this article, we will make the case that tumorigenesis evoked by the polyomaviruses may be an exception. By expression of highly multifunctional proteins, polyomaviruses can deliver many hits at once and interestingly these hits fall into all of the three categories. Indeed it has been shown that the polyomavirus simian virus 40 large-T oncoprotein can effect the direct tumorigenic conversion of normal human diploid epithelial and fibroblast cells when expressed ectopically together with H-ras and telomerase (Hahn et al, 1999).

II. Polyomaviruses

Polyomaviruses are a genus of non-enveloped DNA viruses with icosahedral capsids containing small, circular, double-stranded DNA genomes. As the name polyoma implies, they are able to induce a variety of different tumors when they are inoculated into newborn mice. They also transform cells in culture (Cole, 1996). Discussion is limited to the three polyomaviruses that are of possible clinical relevance: SV40, JC virus and BK virus. Simian vacuolating virus 40 (SV40) was discovered as a contaminant in the monkey kidney cell cultures that were used to grow the poliovirus for the vaccinations of millions of people between 1955-1963 (Sweet et al, 1960). In 1971, two human polyomaviruses were discovered. JC virus was isolated from the brain of a patient suffering from progressive mutifocal leucoencephalopathy (Padgett et al, 1971). BK virus was isolated from the urine of a kidney allograft recipient with chronic pyelonephritis and advanced renal failure (Gardner et al, 1971). JCV is widespread throughout the human population with more than 80% of adults exhibiting antibodies specific for JCV. Infection is thought to occur during early childhood and is usually subclinical. However under immunosuppressive conditions, *e.g.*, in patients with acquired immunodeficiency syndrome (AIDS), JCV can emerge from latency and cause progressive multifocal leukoencephalopathy (PML) (Gordon and Khalili, 1998; Safak and Khalili 1998). BKV is also widespread in the human population and primary infection occurs during childhood and is usually subclinical. BKV can reactivate from latency under conditions of immunocompromization. Kidney transplant recipients who receive highly immunosuppressive drugs may develop BKV-associated nephropathy, which is a leading cause of allograft failure (Hirsch and Steiger, 2003).

SV40, JC and BK viruses are very similar to each other with respect to size (~5.2 Kb), genome organization and DNA sequence. The circular genome of these polyomaviruses contains two regions of approximately equal size known as the early and late transcription units. Transcription of both units is initiated from a common regulatory region at the origin of DNA replication (ORI) with early transcription proceeding in a counterclockwise direction. Late transcription proceeds from ori in the opposite direction and goes around the genome in a clockwise direction (Cole, 1996). The late region encodes the capsid structural proteins VP1-3 that are encoded by alternatively spliced mRNAs derived from the same primary late transcript and a small regulatory protein known as Agnoprotein. The early region encodes the alternatively spliced transforming proteins: large T-antigen (T-Ag) and small t-antigen (t-Ag). These proteins are important in promoting transformation of cells in culture and oncogenesis *in vivo*.

The infection of cells by polyomaviruses is initiated by the binding of the virion to a receptor on the outside of the cell membrane. Polyomavirus capsids undergo endocytosis and are transported to the nucleus where the viral DNA is uncoated and transcription of the early region begins. The primary transcript from the early region is alternatively spiced to give two mRNAs that encode large T-antigen (T-Ag) and small t-antigen t-Ag. T-Ag is a large nuclear phosphoprotein and is an essential factor for viral DNA replication. It binds to the viral origin of replication region (ORI) where it promotes unwinding of the double helix and recruitment of cellular proteins that are required for DNA synthesis including DNA polymerase-α and replication protein A (Cole, 1996). Polyomaviruses rely on cellular enzymes and cofactors for DNA replication and these proteins are expressed in S-phase. Another major function of T-Ag is to modulate cellular signaling

pathways to induce cells to enter S-phase and this accounts for the ability of T-Ag to transform cells. These activities include stimulating the cell cycle by binding and inactivation of the tumor suppressor proteins p53 and pRb. Functions of T-Ag will be discussed in detail below.

As viral replication proceeds, the late genes begin to be expressed. T-Ag acts to stimulate transcription from the late promoter and repress transcription from the early promoter. The gene products of the late region are the capsid proteins VP1, VP2 and VP3, which assemble with the replicated viral DNA to form virions, which are released upon cell lysis. A small protein known as Agnoprotein is encoded near the 5' end of the late region. This protein is 62-71 amino acids in length and is produced late in the infectious cycle although it is not incorporated into virions (Cole, 1996).

In this review, the biological and pathological properties of each of the polyomavirus non-capsid proteins are discussed, together with the relevance of these mechanisms to the pathogenesis of human cancers.

III. Polyomavirus proteins

A. Large-T antigen (T-Ag)

Large T-Ag is a remarkable protein. Not only does it interact with the polyomavirus DNA at the origin of replication where it recruits cellular factors for DNA replication and unwinds the two DNA strands, but it also interacts with a plethora of cellular proteins that are responsible for driving cells into S-phase and mediates cellular transformation. These interactions will be discussed in this section. The T-Ag proteins of SV40, JC and BK are 708, 688, and 695 amino acid residues in size, respectively, and share 70-80% sequence identity. Most of the early work on T-Ag focused on SV40 and it is not always certain to what extent information about SV40 is applicable to JC and BK. Where comparative data are available, they will be presented.

T-Ag is mainly localized to the nucleus with a small amount being found at the plasma membrane. Indeed SV40 T-Ag was the first protein for which a nuclear localization signal was characterized (Kalderon et al, 1984; Lanford and Butel, 1984). T-Ag has multiple post-translational modifications including phosphorylation, O-glycosylation, acylation, adenylation, poly-ADP-ribosylation and N-terminal acetylation (Cole, 1996). The functions of T-Ag can be divided into two categories: regulatory events at the viral origin of DNA replication and interaction with cellular proteins that are involved in cell cycle progression.

SV40 T-Ag recognizes and binds to several pentameric sequences at the viral origin where it has a central role in the initiation of DNA replication and also promotes late region transcription and represses early region transcription. Another activity of T-Ag is to unwind the DNA strands at the viral origin. This DNA helicase activity is coupled to T-Ag ATP-ase activity. T-Ag recruits replication protein A and DNA polymerase-α and DNA synthesis is initiated (Cole, 1996).

Since polyomaviruses rely on host proteins for replication, they require cells to enter the S-phase of the cell cycle at which time these cellular factors are produced. A number of mechanisms have evolved whereby this is achieved and some of these involve interaction of T-Ag with key tumor suppressor proteins. These interactions are key events in the malignant transformation of cells by polyomaviruses. One such key regulatory protein is pRb.

1. The pRb protein and its family members

The Rb family of so-called pocket proteins negatively regulates progression from G0 through G1 to S-phase. pRb, p107 and p130 possess a binding pocket for members of the E2F family of transcription factors that control expression of S-phase-related genes, e.g., cyclin A, cyclin E, DNA polymerase-α, ribonucleotide reductase, thymidine kinase, c-*fos*, c-*myc* etc (Helt and Galloway, 2003). Inactivation of pRB family members by T-Ag binding leads to increased E2F activity and bypass of pocket protein-mediated G1 arrest. Binding of T-Ag to pRb family members involves the T-Ag pRb-binding domain (residues 101-118 on SV40 T-Ag) and the T-Ag J-domain (see below). This leads to hypophosphorylation of the pocket protein (Helt and Galloway, 2003). JC and BK viral T-Ags also possess conserved pRb-binding and J-domains and are able to bind to and inactivate pRb (Dyson et al, 1990; Krynska et al, 1997). The pRb-binding domain of polyomavirus T-Ag is required for transforming activity (Helt and Galloway, 2003).

2. p53

p53 protein is a tumor suppressor encoded by a gene whose disruption is associated with ~50-55% of all human cancers. p53 protein acts as a checkpoint in the cell cycle; either preventing or initiating programmed cell death. When DNA damage occurs in a cell or when cell proliferation is activated inappropriately, e.g., by an oncogene, p53 inhibits cell cycle progression and can activate programmed cell death. This serves to eliminate damaged or transformed cells and is an important defense against cancer. DNA damage activates p53 through the protein kinases ATM and Chk2 while oncogenic transformation acts through a different protein $p14^{ARF}$. The release of E2F from pRb by T-Ag activates $p14^{ARF}$ transcription which interferes with MDM2 (a negative regulator of p53) leading to stabilization of p53 that would slow the cell cycle and viral replication. However, polyomavirus T-Ags bind to and inactivate p53 and thus prevent inhibition of the cell cycle or apoptosis. This is important for providing an optimal cell environment for viral replication and packaging during polyomavirus lytic infection and also facilitates transformation in non-permissive cells (Levine, 1997; Vogelstein et al, 2000). Interaction of T-Ag with p53 has been demonstrated for SV40 T-Ag (Pipas and Levine, 2001) and for human JC virus (JCV) and BK virus (BKV) (Krynska et al 1997; Bollag et al, 1989). p53 is a transcription factor that upregulates proteins that are inhibitory for cell cycle progression, e.g. p21Waf1/Cip1 which inhibits the kinase activities if cyclin E/cdk2 and cyclin D/cdk4-6 (el-Deiry et

al, 1993). T-Ag binding to p53 blocks the ability of p53 to bind to DNA (Ali and DeCaprio, 2001).

3. The p300 protein and its family members

Polyomavirus T-antigen binds to members of the p300/CBP/p400 family of transcriptional co-activators. These proteins are histone acetylases (Ogryzko et al, 1996) and interact with a variety of cellular regulatory proteins including p53, mdm2 and NFκB p65 subunit (Ali and DeCaprio, 2001). Knockout mice lacking a CBP allele develop hematological malignancies indicating a tumor suppressor function (Kung et al, 2000). The roles of these proteins in the regulation of cell growth and transformation have been reviewed recently (Goodman and Smolik, 2000). The binding of T-Ag to p300 and p400 is mediated by the C-terminus of T-antigen and mutations that abrogate p53 binding also abrogate p300 and p400 binding (Lill et al, 1997a). p300 is a co-activator of transcription from p53-dependent promoters such as p21Waf1/Cip1 and bax and this is disrupted by T-Ag (Lill et al, 1997b). All studies on T-Ag interaction with p300 family members have been done with SV40.

4. IRS-1

JCV T-Ag has been shown to directly bind to insulin receptor substrate 1 (IRS-1) and cause it to be translocated to the nucleus. Fusion proteins between IRS-1 and green fluorescent protein that usually localize mainly in the cytoplasm, are found in the nucleus in cells that express JCV T-Ag (Lassak et al, 2002). A dominant negative mutant of IRS-1 inhibited growth and survival of JCV T-Ag–transformed cells in anchorage-independent culture conditions demonstrating the importance of this interaction in cell transformation (Lassak et al, 2002). There is growing evidence that the interaction of JCV T-Ag with IRS-1 contributes to the process of malignant transformation in childhood medulloblastomas and this has been reviewed recently (Khalili et al, 2003a). The consequences of IRS-1 nuclear translocation are still being characterized but one possibility is that it may be important in Rad51 trafficking and homologous recombination-directed DNA repair (Trojanek et al, 2003). There is also evidence that interaction with IRS-1 is involved in transformation by SV40 T-Ag (Fei et al, 1995 and Prisco et al, 2002) but a role for IRS-1 in BKV T-Ag transformation has not been explored.

5. β-Catenin

JCV T-Ag has also been shown to directly bind to β-catenin (Enam et al, 2002; Gan and Khalili, 2004). The interaction of T-Ag with β-catenin occurs through the central domain of T-antigen spanning residues 82-628, and the C-terminus of β-catenin located between amino acids 695 and 781. The association of T-Ag with β-catenin increases the level of β-catenin in the cell due to increased protein stability (Gan and Khalili, 2004). This interaction causes β-catenin to translocate to the nucleus where it enhances expression of genes such as c-myc and cyclin D1. These observations ascribe a new mechanism for the deregulation of the Wnt pathway through stabilization of β-catenin. These signaling events may be important in certain human cancers that are associated with JCV such as medulloblastoma and colon cancer (Gan et al, 2001; Coyle-Rink et al, 2002; Enam et al, 2002).

6. TATA-binding protein-associated factors (TAFs)

Ribosomal RNA synthesis by RNA polymerase I (Pol I) is tightly associated with cell growth and proliferation. T-Ag of SV40 is able to upregulate the rate of Pol I transcription by directly binding to the Pol I transcription factor SL1 (Zhai et al, 1997). The 538 amino-terminal domain of T-Ag is necessary for SL1 binding. Human rRNA synthesis by Pol I requires at least two auxiliary factors, upstream binding factor (UBF) and SL1 (TATA-binding protein/TAF(I) complex). UBF is a DNA binding protein that binds to the ribosomal DNA promoter. The carboxy-terminal region of UBF is necessary for transcription activation and is extensively phosphorylated. SL1 is recruited to the promoter is mediated by specific protein interactions with UBF. UBF phosphorylation plays a critical role in the regulation of the recruitment of SL1 (Tuan et al, 1999). UBF is phosphorylated by a kinase activity that is strongly associated with T-Ag and the carboxy-terminal activation domain of UBF is required for the phosphorylation to occur. T-Ag-induced UBF phosphorylation promotes the formation of a stable UBF-SL1 complex, i.e., the large T antigen-associated kinase appears to mediate the stimulation of Pol I transcription (Zhai and Comai, 1999).

T-Ag can also stimulate Pol II promoters by associating with the transcription factor TFIID (Damania and Alwine, 1996), and Pol III promoters through interaction with the TBP-containing Pol III transcription factor human TFIIB-related factor (Damiana et al, 1998).

7. p185/p193

Kohrman and Imperiale reported a protein with an apparent molecular weight of 185 kD that was specifically co-immunoprecipitated with SV40 T-Ag (Kohrman and Imperiale, 1992). Binding to this protein mapped to the N-terminal 121 amino acid residues of T-Ag. This may be the same protein as that described by Tsai et al, (2000) which has an apparent molecular weight of 193 kD. p193 co-immunoprecipitates with T-Ag and binds to its N-terminus. Cloning and sequencing of p193 revealed that it had a bcl-2 homology type 3 (BH3) domain suggesting a role in the regulation of apoptosis. Expression of p193 in NIH-3T3 cells promoted apoptosis whereas mutants of p193 lacking the BH3 domain did not. p193 localizes to the cytoplasm of transfected cells. Apoptosis induced by p193 is antagonized by co-expression of SV40 T-Ag which resulted in the cytoplasmic localization of both proteins. This is evidence for a p53-independent mechanism of apoptosis suppression by T-Ag.

8. Other proteins

SV40 T-Ag binds to DNA polymerase-α (Gannon and Lane, 1987; Dornreiter et al, 1990) and replication protein A (Melendy and Stillman, 1983) and these interactions are

essential for SV40 DNA replication as discussed above. SV40 T-Ag binds to the transcription factors AP-1 (Kim et al, 2003), AP-2 (Mitchell et al, 1987) and the heat shock protein hsp73 (Sawai and Butel, 1989). Recently it has been reported that SV40 T-Ag targets the spindle assembly checkpoint protein Bub-1 (Cotsiki et al, 2004). This interaction suggests a mechanism for the ability of T-Ag to cause chromosomal aberrations and aneuploidy (see below).

9. Chaperone activity of T-Ag

There is sequence similarity between the N-terminus of T-Ag and the J-class of chaperonins (Sullivan and Pipas, 2002). Domain-swapping experiments have shown that the N-terminus of T-Ag can functionally substitute for the J-domain of *E. coli* DnaJ in a bacteriophage λ growth assay (Kelley and Georgopoulos, 1997). J-proteins are co-chaperonins for the DnaK (Hsp70) family of proteins that undergo a global conformational change upon J-protein binding. This stimulates hsp70 ATP-ase activity and is involved in binding of protein substrates (Sullivan and Pipas, 2002). The N-terminus of T-Ag functions as a J-protein in assays *in vitro*, e.g., stimulation of bovine Hsp70 ATP-ase activity (Srinivasan et al, 1997). The J-domain is essential for many of the functions of T-Ag including DNA replication. In addition to the Rb-binding domain (amino acid residues 101-118), the J-domain of T-Ag is required in *cis* for binding to the Rb family of proteins described in Section III.A.1. It is thought that the J-domain may recruit Hsp70 to the Rb-E2F complex where the action of the Hsp70-mediated ATP hydrolysis liberates E2F. The role of the J-domain in other aspects of transformation is complex and has been reviewed recently (Sullivan and Pipas, 2002). Although much remains to be elucidated about the exact biophysical nature of the interactions of T-Ag and cellular proteins, it seems likely that the chaperonin functionality of T-Ag will turn out be an important determinant in the ability of T-Ag to bind to its plethora of cellular targets.

10. Genetic instability induced by T-Ag

As well as being able to transform cells by virtue of interacting with cellular signaling proteins, polyomavirus T-Ag has a mutagenic effect on cellular DNA and induces karyotypic instability. It seems likely that secondary mutations induced by T-Ag contribute to the tumorigenicity of infected cells. Theile and Grabowski reported increases in spontaneous mutation frequencies up to 100-fold when cultured cells were infected with BKV. The variant strain BKV-IR, which does not express small t-Ag, was also mutagenic. Besides cultured cells, BKV was also shown to mutate human peripheral blood lymphocytes. The mutagenicity of BKV in these studies was comparable to that observed for JCV and SV40 (Theile and Grabowski, 1991). Human fibroblasts transfected with plasmid containing the BKV early region exhibited cytogenetic damage including deletions and translocations. This damage preceded morphological transformation (Trabanelli et al, 1998). Ray et al constructed a plasmid containing SV40 large T-Ag driven by the Rous sarcoma virus promoter but lacking small t-Ag and the SV40 origin. When this was transfected into human fibroblasts, 99% of T-Ag-positive clones exhibited numerical or structural chromosome aberrations. These changes were evident before the transformation indicators of the clones were positive and continued throughout neoplastic progression (Ray et al, 1990, 1992). Chromosome changes have been reported in two glioblastoma cell lines that express SV40 and BKV large T-Ags (Tognon et al, 1996). Antibody titres to BKV and JCV have been correlated with the occurrence of “rogue cells” – lymphocytes with multiple chromosome aberrations (Lazutka et al, 1996, Neel et al, 1996).

In the case of SV40, recent research has revealed a possible molecular mechanism for the induction of chromosomal instability. It is known that karyotypic changes are induced by large T-Ag but not small t-Ag (Stewart and Bacchetti, 1991) and this function maps to the N-terminal 147 amino acid residues of T-Ag (Woods et al, 1994). Using the yeast two-hybrid system with the N-terminus of SV40 T-Ag as bait, it has recently been shown that T-Ag binds to the mitotic checkpoint protein Bub-1 (Cotsiki et al, 2004). T-Ag is known to disrupt the mitotic checkpoint and attenuate radiation-induced mitotic delay (Chang et al, 1997) suggesting that this is the mechanism of T-Ag induction of chromosome aberrations and aneuploidy.

B. Small-t

The second of the two proteins encoded by the early region of primate polyomaviruses is small t-antigen (t-Ag). t-Ag is 174, 172 and 172 amino acid residues in length for SV40, JCV and BKV respectively. The N-terminal 82 amino acids are the same as the N-terminus of T-Ag but the C-terminus is a unique domain that is incorporated by alternative splicing of the early region primary transcript. The J-domain (Section III.A.9) is contained within the N-terminus which is highly conserved between the three primate polyomaviruses (82-89% amino acid sequence identity) while the C-terminus is less conserved (56-69%). Published research on primate polyomavirus small-t has concentrated almost exclusively on SV40. t-Ag is found in both the nucleus and the cytoplasm (Ellman et al, 1984). Deletion mutants of SV40 t-Ag are able to replicate (at least in some cells) indicating that it is not essential for viral lytic infection (Shenk et al, 1976). However t-Ag augments viral DNA replication (Cicala et al, 1994) and enhances transformation (Chang et al, 1984). t-Ag has a mitogenic role in SV40 transformation of cells (Sleigh et al, 1978; Martin et al, 1979). Cellular proteins of 36 and 63 kd that associate with SV40 small t antigen were shown to be the catalytic (C) and structural (A) subunits of protein phosphatase 2A (PP2A)(Pallas et al, 1990). Phosphatases are the negative regulators of the growth-promoting protein kinase signal transduction pathways. PP2A is the major serine/threonine-specific protein phosphatase of eukaryotic cells (Cohen, 1997). By inactivating the negative regulator PP2A, t-Ag is able to activate several pathways that promote cell proliferation including the mitogen-activated protein kinase (MAPK) pathway

(Sontag et al, 1993), stress-activated protein kinase (SAPK) pathway (Watanabe et al 1996) and PKC-ζ/NF-κB (Sontag et al, 1997). PP2A is a key regulator with multiple functions in cellular signaling that are due to the presence of an assortment of holoenzymes. Each holoenzeyme contains a conserved AC dimer with an array of different regulatory (B) subunits. The specificity of PP2A is conferred by regulation of its endogenous subunit composition. Distinct classes of B subunits bind to AC to give a wide variety of ABC heterotrimers with differing substrate specificities (Sontag, 2001). Small t-Ag is also able to bind to the AC dimer and this interaction occurs via the t-Ag unique C-terminal domain that possesses two cysteine cluster motifs that are conserved in all polyomaviruses (Pipas, 1992). The association of t-Ag with AC displaces the cellular regulatory B subunit and results in the inhibition of phosphatase activity (Yang et al, 1991). Thus small t-Ag is an oncoprotein that stimulates multiple growth promoting pathways.

C. Truncated T-Ag variants

In the case of the early regions of SV40 and JCV, splice variants can occur that yield truncated protein consisting of the N-terminal portion of T-Ag. SV40 can express a 135 amino acid residue protein all of which correspond to T-Ag N-terminal sequence except for the last 4 which come from a different reading frame (Zerrahn et al, 1993). Similarly JCV can produce 3 truncated variants of T-Ag: T'135, T'136, and T'165 which are generated via an alternative splicing mechanism (Trowbridge and Frisque, 1995). Unlike small-t, these variants do not possess a unique domain not found in large T-Ag and so their importance is unclear. However they do have the T-Ag J-domain and pRb binding site and it has been reported that the JCV T and T' proteins differentially interact with members of the pRB family of proteins (Bollag et al, 2000).

D. Agnoprotein

The late region of polyomaviruses encodes the viral capsid proteins VP1, VP2, and VP3 and a small regulatory protein known as Agnoprotein encoded near the 5' end of the primary late transcript. Agnoprotein is 62, 71 and 66 amino acid residues in length for SV40, JCV and BKV respectively (Cole, 1996). Agnoprotein is produced late in the infectious cycle although it is not incorporated into virions (Cole, 1996; Jay et al, 1981). The predominant intracellular localization of Agnoprotein is in the cytoplasm and especially the perinuclear region in association with the outer nuclear membrane for all three primate polyomaviruses (Nomura et al, 1983; Rinaldo et al, 1998; Okada et al, 2001). A small amount Agnoprotein is also found in the nucleus in the case of JCV and SV40 (Nomura et al, 1983; Okada et al, 2001) but not in the case of BKV (Rinaldo et al, 1998). Interestingly BKV Agnoprotein is phosphorylated and associates with three cellular proteins of unknown function (Rinaldo et al, 1998). Agnoprotein has a role in the lytic cycle since SV40 mutants in which the agnogene is deleted produce virions more slowly than wild-type virus (Ng et al, 1985). Agnoprotein has regulatory roles in viral transcription, translation as well as in virion assembly and maturation and this has been reviewed recently (Safak and Khalili, 2003). JCV Agnoprotein can interact with the large T-antigen and downregulates viral gene expression and DNA replication (Safak et al, 2001). It also interacts with YB-1, a cellular transcription factor that contributes to JCV gene expression in glial cells, and negatively regulates YB-1-mediated JCV gene transcription (Safak and Khalili, 2001; Safak et al, 2002).

At least in the case of JCV, Agnoprotein binds directly to p53 (Darbinyan et al, 2002). JCV Agnoprotein dysregulates cell cycle progression when expressed in the absence of other viral proteins (Darbinyan et al, 2002). Constitutive expression of JCV Agnoprotein causes cells to accumulate at the G2/M stage of the cell cycle with a decline in cyclins A and B-associated kinase activity. Agnoprotein augments the activity of the p21/WAF-1 promoter and the level of p21/WAF-1. Activation of p21/WAF-1 gene expression is mediated, at least in part, through cooperation with p53 (Darbinyan et al, 2002). Agnoprotein also binds to the Ku70 DNA repair protein and impairs double-strand break repair and interferes with DNA damage-induced cell cycle regulation (Unpublished Data).

Thus Agnoprotein, like T-Ag, has multiple roles both in the polyomavirus life cycle and in interacting with cellular proteins to disturb normal cellular functions.

IV. Polyomaviruses and human cancer

Three lines of evidence suggest that the polyomaviruses JCV, BKV and SV40 have a role in neoplasia. The viruses are able transform cells growing in culture. The hallmark phenotypic changes of cell transformation include growth to high saturation density, focus formation, growth in soft agar, serum-independence, morphological changes, elevated glucose uptake, plasminogen activator production etc. In other words the polyomaviruses endow upon normal cells properties that are associated with malignancy. SV40 is able transform a wide variety of rodent and human cell types (Saenz-Robles et al, 2001). JCV is able to transform cells in culture although not as efficiently as SV40. JCV can transform human fetal glial cells and primary hamster brain cells. The transforming ability of JCV appears to be limited to cells of neural origin and this property maps to the viral regulatory region at the origin of replication (Del Valle et al, 2001a). In the case of BKV, transformation is not efficient, often abortive and features of the transformed phenotype are not fully displayed. However a fully transformed phenotype can be achieved in co-operation with other oncogenes such as adenovirus E1A, c-rasA or c-myc (Corallini et al, 2001).

Secondly polyomaviruses are able to induce tumors in laboratory animals either when the virus is injected or when viral genes are introduced into transgenic mice. Soon after its discovery, the oncogenic potential of SV40 was demonstrated in that it was able to induce sarcoma

formation after subcutaneous injection into hamsters (Eddy et al, 1962). Hamsters injected intravenously with SV40 develop leukemia, lymphoma, and osteosarcoma (Diamandopoulos et al, 1972) whereas hamsters injected intrapleurally develop mesothelioma (Cicala et al, 1993). Transgenic mice bearing the SV40 T-Ag develop characteristic brain tumors localized to the choroid plexus (Brinster et al, 1984). Many studies have established the highly oncogenic potential of JCV in laboratory animals, e.g., JCV induces multiple types of brain tumors when injected into the brains of newborn Golden Syrian hamsters. JCV is the only polyomavirus that induces tumors in nonhuman primates. These animal studies have been reviewed recently (Del Valle et al, 2001a; Khalili et al, 2003b). Transgenic mice with the JCV T-Ag gene can exhibit adrenal neuroblastomas (Small et al, 1986), tumors of primitive neuroectodermal origin (Franks et al, 1986) and can develop tumors that arise from the pituitary gland (Gordon et al, 2000). BKV virus is highly oncogenic in newborn rodents. The efficiency of tumorigenesis by BKV in hamsters is dependent upon the route of injection. BKV is only weakly oncogenic when injected subcutaneously but gives a high incidence of tumors when injected intracerebrally or intravenously. The types of tumors induced include ependymoma, neuroblastoma, pineal gland tumors, pancreatic islet tumors, fibrosarcoma and osteosarcoma. This suggests that BKV has a tropism for certain cell types (Corallini et al, 2001). Transgenic mice containing the BKV early region developed primary hepatocellular carcinomas and renal tumors (Small et al, 1986). Lymphomas have also been reported in BKV-transgenic mice (Dalrymple and Beemon, 1990).

Thirdly the presence of polyomavirus DNA sequences and viral oncoprotein expression has been found to be associated with some cancers. Perhaps the strongest evidence for a role in human cancer has been presented for JCV. The first indication that this is the case came from reports of brain tumors being found in patients with PML, reviewed in (Del Valle et al, 2001a). JCV has also been associated with brain tumors in patients without PML. For example, Recnic et al were able to detect JCV DNA by PCR in tumor tissue from a patient with oligoastrocytoma. The identity of the amplified PCR product with JCV was confirmed by DNA sequencing. Moreover JCV RNA and T-Ag protein were detectable by primer extension analysis and Western blotting respectively indicating gene expression (Recnic et al, 1996). Del Valle et al examined 85 samples of tumors of glial origin for JCV DNA and T-Ag expression. It was found that 57-83% of tumors were positive depending on tumor type (Del Valle et al, 2001b). Krynska et al detected JCV DNA in 45% of pediatric medulloblastomas that were examined with 20% of them being positive for T-Ag expression (Krynska et al, 1999). In another study JCV Agnogene DNA was detected in 11 of 16 medulloblastoma samples and Agnoprotein expression in 11 of 20 samples. Since some of the Agnoprotein positive medulloblastoma samples were negative for concomitant T-Ag expression, Agnoprotein may have a role in the development of JCV-associated medulloblastoma (Del Valle et al, 2002). Many other studies that have employed PCR-mediated DNA amplification and/or immunocytochemistry of various brain samples provide support for an association of JCV with a wide variety of tumors of the central nervous system (CNS) and in other tumors such as colon cancer where JCV T-Ag disrupts beta-catenin signaling (Enam et al, 2002). The involvement of JCV in brain and non-brain tumors has recently been reviewed (Del Valle et al, 2001a). BKV has been detected in some tumors of the brain, pancreatic islet tumors, Kaposi's sarcoma and tumors of the urinary tract. These reports have been reviewed recently (Corallini A et al, 2001). Perhaps one of the most controversial issues in cancer research is the association of SV40 with human cancer. Because primary cultures of rhesus monkey kidney cells that were used to grow the poliovirus between 1955-1963 were contaminated with SV40, millions of people were exposed to SV40 inadvertently through poliovirus vaccinations. The first report of an association of SV40 with human cancer was made by Soriano et al, (1974). SV40 and SV40 T-Ag were detected in malignant melanoma metastases from a patient who had antibodies to viral capsid and T-Ag (Soriano et al, 1974). Since then there have been a large number of reports of the association of SV40 and human tumors and these have been reviewed recently (Arrington and Butel, 2001). Many of these reports have described the association of SV40 with mesothelioma, a rare but aggressive cancer of mesothelial cells. A major factor in the development of mesothelioma is thought to be exposure to asbestos. The frequent association of SV40 with mesothelioma has led to the hypothesis that the virus is a co-factor in the development of this malignancy. In cell culture studies, human mesothelial cells have been found to be non-permissive for SV40 replication and consequently to undergo cell transformation in response to SV40 infection. Asbestos was found to be a synergistic factor for transformation in these experiments (Bocchetta et al, 2000). It has also been reported that human mesothelial cells are permissive for BKV replication but refractory to infection by JCV. This may provide an explanation as to why SV40 is associated with mesothelioma rather than the more ubiquitous human polyomaviruses, JCV and BKV (Carbone et al, 2003). Despite the many reports of SV40 in mesothelioma samples, several studies have failed to detect SV40 sequences (Arrington and Butel, 2001). To address this technical issue, Testa et al conducted a multi-center study. SV40 DNA was detected by PCR using primers directed at two conserved regions and positive clones were verified by Southern blot and/or sequencing. Immunostaining of selected samples demonstrated SV40 T-Ag expression (Testa et al, 1998). SV40 has also been reported to be associated with some brain tumors (Arrington and Butel, 2001) and with non-Hodgkin lymphoma (Shivapurkar et al, 2002; Vilchez et al, 2002). Interestingly, SV40 has been found in association with pediatric tumors of the choroid plexus (Bergsagel et al, 1992). This is the type of tumor that is seen in transgenic mice containing the SV40 early region. However the association of SV40 with human cancer is still a highly contentious issue. It has been argued that the association of SV40 with human tumors is not causal but incidental.

Since SV40 expresses potent oncoproteins, as described above and is highly tumorigenic in cell cultures and animals, it would be surprising if SV40 was associated with tumors merely by chance. However there still remains a lack of clear epidemiological evidence. The Institute of Medicine (IOM) recently reviewed the evidence associating polio vaccines and SV40 with human tumors and concluded that the epidemiological studies are sufficiently flawed that the evidence was inadequate to conclude whether or not contaminated polio vaccine caused cancer (IOM report, 2002).

It remains to be stringently proven that they have a causal role in human neoplasia. Problems include the following. The polyomaviruses are ubiquitous in nature but the associated cancer is rare. The incubation period between infection and appearance of cancer is long. The initial viral infection is usually subclinical making it difficult to establish when it occurred. Environmental co-factors (*e.g.*, co-carcinogens) or host factors (*e.g.*, immune status) modulate pathogenesis. These considerations make it difficult to apply Koch's postulates to the polyomaviruses. However the evidence for a role is quite convincing. These viruses transform cells in culture and produce tumors in inoculated animals or transgenic mice with patterns consistent with their putative role in human tumorigenesis, as we have described. The presence of viral DNA and viral gene expression in some human tumors is established beyond doubt.

V. Conclusions

The three non-capsid proteins encoded by the primate polyomaviruses are highly multi-functional agents that disrupt cell signaling homeostasis. In the introduction, three types of intracellular changes were discussed that occur along the road to cancer, i.e., activation of oncogenes, inactivation of anti-oncogenes (tumor suppressors) and acquisition of a mutator phenotype. All three types of changes can be effected by these viral proteins. t-Ag is an *oncogene* that stimulates kinase signaling pathways as do the oncogenes v-src and v-raf, but t-Ag is able to alter many pathways at once since it inhibits a common phosphatase, PP2A. T-Ag incapacitates the *anti-oncogenes* pRb and p53. Inactivation of these proteins occurs in most if not all non-viral human cancers indicating their importance. T-Ag also acts as a *mutator* inducing genomic instability leading to the susceptibility for further deleterious events. Presumably the genes of the polyomaviruses evolved to allow the viruses to push resting cells into S-phase so that viral DNA can be replicated. A corollary of this deregulation of cell growth control is that the viruses can advance oncogenesis. The properties of the multifunctional polyomavirus proteins that have been described in this review reveal how multiple "hits" can be delivered to cells that can propel them down the multi-step road to malignancy.

JCV and BKV are widespread throughout the human population. The mechanism of human-to-human transmission of polyomaviruses has not been firmly established but the presence of infectious JCV in raw sewage suggests that ingestion of contaminated water or food could represent a possible portal of entrance of JCV into the human population (Bofill-Mas et al, 2001, 2003; Bofill-Mas and Girones, 2001). JCV DNA sequences are present in a high percentage of normal tissue samples taken from the upper and lower human gastrointestinal tract (Laghi et al, 1999; Ricciardiello et al, 2000, 2001). We recently reported the presence of JCV DNA sequences in 22 of 27 well-characterized epithelial malignant tumors of the large intestine (Enam et al, 2002). In 17 of the 27 samples, JCV T-antigen expression was detected and 12 of these 17 were also positive for Agnoprotein expression. Thus polyomaviruses are widespread but usually dormant, becoming active in certain malignancies.

It is possible that the expression of polyomavirus protein could be an early or a late step in tumorigenesis. On the one hand, it is possible that expression occurs at an early stage in tumor progression and that the genetic instability caused by T-antigen induces further pathological changes (Ricciardiello et al, 2003). Alternatively other early abnormal cellular changes may occur first that precede T-antigen and Agnoprotein expression. For example, conceivably there could be a mutation in the structure of, or a change in the level of expression of, one of the cellular transcription factors that bind to the control region of the polyomavirus that regulates the viral genes (Raj and Khalili, 1995). This could lead to a transcriptional activation of latent polyomaviral genomes during the course of tumorigenesis leading to the onset of production of viral oncoproteins such as T-antigen and subsequently to a more malignant phenotype.

The relatively high incidence of polyomavirus T-antigen involvement with certain malignancies indicates that molecular strategies for the disruption of the interaction of T-antigen with cellular proteins may represent a fruitful avenue for the development of new types of therapeutic interventions. For example, delivery of antisense transcripts to the early region of SV40 using an adenoviral gene therapy vector achieved significant growth inhibition and apoptosis of a T-antigen-expressing human mesothelioma cell line (Waheed et al, 1999; Schrump and Waheed, 2001). Further investigation of the utility of gene therapy agents that target the polyomavirus proteins is clearly warranted.

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