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Review

The large tumor antigen: A "Swiss Army knife" protein possessing the functions required for the polyomavirus life cycle

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ABSTRACT

The SV40 large tumor antigen (L-Tag) is involved in the replication and cell transformation processes that take place during the polyomavirus life cycle. The ability of the L-Tag to interact with and to inactivate the tumor suppressor proteins p53 and pRb, makes this polyfunctional protein an interesting target in the search for compounds with antiviral and/or antiproliferative activities designed for the management of polyomavirus-associated diseases. The severe diseases caused by polyomaviruses, mainly in immunocompromised hosts, and the absence of licensed treatments, make the discovery of new antipolyomavirus drugs urgent.

Parallels can be made between the SV40 L-Tag and the human papillomavirus (HPV) oncoproteins (E6 and E7) as they are also able to deregulate the cell cycle in order to promote cell transformation and its maintenance. In this review, a presentation of the SV40 L-Tag characteristics, regarding viral replication and cellular transformation, will show how similar these two processes are between the polyoma- and papillomavirus families. Insights at the molecular level will highlight similarities in the binding of polyoma- and papillomavirus replicative helicases to the viral DNA and in their disruptions of the p53 and pRb tumor suppressor proteins.

Contents

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1. Introduction

Polyomaviruses are circular double-stranded DNA viruses that are responsible for severe diseases in the immunocompromised host, such as HIV-infected patients and transplant recipients (Table 1). Among the family *Polyomaviridae*, the simian vacuolating virus 40 (SV40), the BK polyomavirus (BKPyV) (Gardner et al., 1971), the JC polyomavirus (JCPyV) (Padgett et al., 1971), and the African green monkey-derived lymphotropic polyomavirus (LPyV) (Brade et al., 1981; Kean et al., 2009; Takemoto and Segawa, 1983; Viscidi and Clayman, 2006; zur Hausen and Gissmann, 1979) have been widely studied during the past four decades. In the case of SV40, the nonhuman primate virus was introduced into the human population when people were inoculated with contaminated polio vaccines prepared in SV40-positive Vero cells (Ferber, 2002).

Recently, new members of the *Polyomaviridae* have been discovered, bringing to ten the total number of human polyomaviruses: KI polyomavirus (KIPyV) (Allander et al., 2007), WU polyomavirus (WUPyV) (Gaynor et al., 2007), Merkel cell polyomavirus (MCPyV) (Feng et al., 2008), HPyV6 and HPyV7 (Schowalter et al., 2010a), *Trichodyplasia spinulosa*-associated virus (TSPyV) (van der Meijden et al., 2010), HPyV9 (Scuda et al., 2011a) and HPyV10 (Buck et al., 2012). These new viruses are possible threats to immunocompromised populations. Thus, MCPyV and TSPyV are linked or directly associated with rare but severe skin diseases, such as Merkel cell carcinoma (MCC) and *T. spinulosa*, respectively. WUPyV and KIPyV have been discovered in the respiratory tract and their association with respiratory illnesses cannot be excluded. It is worth noting that Siebrasse et al. (2012) have recently identified, in human stool samples, the Malawi polyomavirus (MWPyV) but there is no evidence that the MWPyV is able to infect humans. In parallel to the study of Siebrasse et al., Buck et al. (2012) discovered a new polyomavirus (human polyomavirus 10) from a patient suffering from the WHIM syndrome (warts, hypogammaglobulinemia, infections and myelokathexis). This polyomavirus shares 95–99% sequence identity with the MWPyV. Because knowledge concerning these two new viruses is not sufficient, they are not further discussed.

BKPyV reactivation occurs in transplant recipients, especially after kidney or bone marrow transplantation (BMT), leading to hemorrhagic cystitis (HC), nephritis and polyomavirus-associated nephropathy (PVAN). HC happens mostly in BMT patients, while PVAN can be triggered in kidney transplant recipients (Fioriti et al., 2005; Wiseman, 2009). If left untreated, PVAN can lead to severe complications such as graft loss (Dropulic and Jones, 2008; Leung et al., 2005; Ramos et al., 2002). In the case of JCPyV reactivation in AIDS patients, a neurodegenerative disease [i.e., progressive multifocal leukoencephalopathy (PML)], is triggered, leading to cognitive and motor impairements and a short life expectancy (<1 year) (Berger and Houff, 2006). Cases of PML have appeared in patients suffering from autoimmune diseases (such as multiple sclerosis, severe psoriasis or Crohn's disease) who were treated with humanized monoclonal antibodies such as natalizumab (Tysabri®) or Efalizumab (Raptiva®). Currently, in the USA, 30,000 patients who were treated or are on treatment with natalizumab are being monitored by Biogen Idec (the company that developed and manufactured the drug), following agreements with the U.S. Food and Drug Administration (FDA) (Major, 2010).

For the management of polyomavirus-associated diseases, many groups have focused their efforts on the search for drugs able to target one of the viral replication steps, at the *in vitro* level (Andrei et al., 1997; Bernhoff et al., 2008, 2010; Gosert et al., 2011; Lebeau et al., 2007; Seguin et al., 2012a,b; Sharma et al., 2011; Topalis et al., 2011) and the *in vivo* level (Kuypers, 2012, 2009, 2005; Naess et al., 2010). Until now, the use of the discovered drugs, including fluoroquinolones, nucleotide analogs or leflunomide, in the clinic remains controversial.

Polyomaviruses are able to induce cell transformation when they infect non-permissive cells. The transforming capacity of polyomaviruses was first demonstrated when the inoculation of SV40 DNA into Syrian hamsters led to the formation of tumors (Sol and van der Noordaa, 1977). In humans, the MCPyV genome, integrated into the host-cell DNA, has been found in 80% of Merkel cell carcinoma cases (Feng et al., 2008). Furthermore, the presence of an intact pRb binding domain of the L-Tag is needed to promote growth of Merkel cell carcinomas (Feng et al., 2008; Houben et al., 2010). Thus, there is strong evidence in favor of a virus-induced event in Merkel cell carcinogenesis. In *T. spinulosa*, cell alterations leading to skin disease (observed in transplant patients) are caused by TSPyV (Burns et al., 2011; Chen et al., 2011; Fischer et al., 2012; Haycox et al., 1999; Kazem et al., 2011; Lee et al., 2008; Matthews et al., 2011; Moens et al., 2011; Sadler et al., 2007; Tan and Busam 2011; van der Meijden et al., 2010, 2011).

Table 1
Diseases associated with polyomavirus infection in humans. As of today, BKPyV, JCPyV, MCPyV and TSPyV have been clearly linked to human diseases. Although it has not yet been established, the other polyomaviruses may also have a role in the etiology of several diseases, especially in the respiratory tract (in particular KIPyV and WUPyV).

Natural host	Virus	Associated diseases	References
Nonhuman primate	SV40	n.d. (etiology in human cancers?)	Ferber (2002); Mendoza et al. (1998); Mutti et al. (1998); Shah (2007, 1998, 2000)
	LPyV	n.d.	Crandall et al. (2006); Delbue et al. (2010); Kean et al. (2009)
Human	BKPyV	PyV-associated nephropathy, hemorrhagic cystitis, urethral stenosis	Bohl and Brennan (2007); Dropulic and Jones (2008); Pahari and Rees (2003); Ramos et al. (2002)
	JCPyV	Progressive multifocal leukoencephalopathy	Bag et al. (2010); Berger (2011); Dorries et al. (1994); Khalili and White (2006); Khalili et al. (2007); Korman et al. (2009); Major (2009, 2010); Razonable et al. (2001); Ryschkewitsch et al. (2010)
	WUPyV	n.d. (possible association with respiratory diseases)	Barzon et al. (2009); Han et al. (2007); Lin et al. (2008); Miller et al. (2009); Teramoto et al. (2011b); Zhuang et al. (2011)
	KIPyV	n.d. (possible association with respiratory diseases)	(Falcone et al. (2012); Hormozdi et al. (2010); Rao et al. (2011); Teramoto et al. 2011a)
	MCPyV	Merkel cell carcinoma	de Biase et al. (2012); Erovic et al. (2012); Haitz et al. (2012); Kaibuchi-Noda et al. (2011); Martel-Jantin et al. (2012); Mitteldorf et al. (2012); Schrama et al. (2012)
	HPyV6	n.d.	Schowalter et al. (2010b)
	HPyV7	n.d.	Schowalter et al. (2010b)
	TSPyV	*Trichodysplasia spinulosa*	Burns et al. (2011); Fischer et al. (2012); Kazem et al. (2011); Lee et al. (2008); Moens et al. (2011); Sadler et al. (2007); Tan and Busam (2011)
	HPyV9	n.d.	Sauvage et al. (2011); Scuda et al. (2011b); Trusch et al. (2012)
	HPyV10	Possibly involved in WHIM syndrom	Buck et al. (2012)

n.d., not demonstrated.

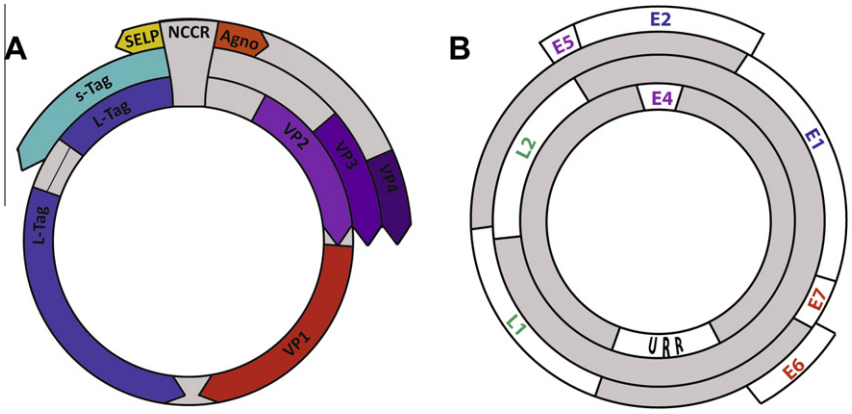

Fig. 1. Genome organization of polyomaviruses and papillomaviruses. (A) The polyomavirus genome is composed of three main regions, the 'early stage' genes [large (L-Tag) and small (s-Tag) Tag and the SV40 early leader protein (SELP)], the 'late stage' genes (VP1-VP4 and Agno) and a noncoding regulatory region (NCRR) that contains the origin of replication and is located between the SELP and AGNO genes. (B) Similar to PyVs, papillomaviruses express during the early stage of replication E1 and E2 proteins required for viral replication and E6 and E7 involved in dysregulation of the cell cycle. E4 participates also in viral DNA amplification and E5 harbors transforming properties. During the late stage of the lytic cycle, L1 and L2, the two structural proteins, are expressed for the production of new virions. An untranslated regulatory region (URR) allows orchestration of the viral life cycle.

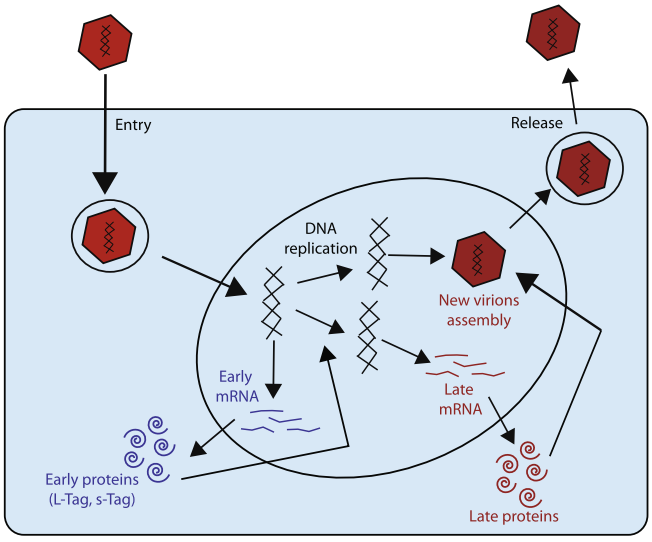

Fig. 2. Two-stage life cycle of the polyomaviruses. After entry, viral particles (red) pass through the cytoplasm to reach the nucleus, where the main processes of viral replication take place. The first step consists of expression of the early genes (blue), especially the large tumor antigen (L-Tag) which is necessary for replication of the viral genome. The following step permits the production of structural proteins (late stage, red) prior to the assembly of new virions and their release into the extracellular medium (adapted from Fields, 4th edition).

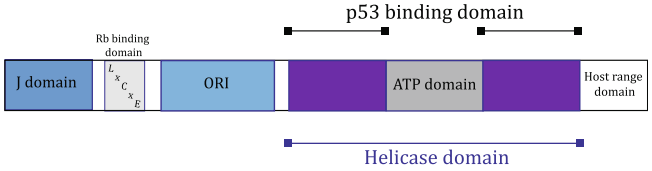

Fig. 3. Scheme of the functional domains of the large T antigen. The L-Tag is a polyfunctional protein that can bind a wide variety of cellular proteins. Its structure is composed of different functional domains that are critical for viral replication and cell transformation.

The genome organization is highly conserved among human polyomaviruses (Fig. 1A). The 5-kb circular DNA genome contains three different regions, composed of early genes, late genes and the non-coding control region (NCCR). During the early stage, two proteins are expressed: the large (L-Tag) and the small (s-Tag) tumor antigens. They have critical roles in the viral life cycle, especially in replicating the viral genome (Fig. 2). They are able to recruit the host cell machinery for the different steps in the replication process and to control the cell cycle, promoting cell proliferation. In the late stage, the structural proteins, i.e., VP1-VP4, are produced to form new virions that will be released into the extracellular space. This genomic organization is similar to that described for human papillomaviruses (HPV) (Fig. 1B), a family of viruses that induce hyperproliferation of epithelial cells. Members of this family are classified in low-risk and high-risk types, according to their capacity to induce malignancy. Oncogenic HPV types are responsible for 95% of cervical cancers, and are also involved in vulvar, vaginal, penile, anal and oropharyngeal cancers (Parkin, 2006). Indeed, HPVs express oncoproteins (E6 and E7) that can control the cell cycle, as well as a helicase (E1/E2) that allows replication of the genome (zur Hausen and de Villiers, 1994).

This review will discuss the basic properties of the L-Tag among the polyomaviruses and will make parallels with the E1 protein of the papillomavirus family, highlighting similarities and contrasts between the replicative helicase proteins of both viral families. A particular focus will be on potential drugs targeting these proteins that can be developed for the management of polyomavirus-related diseases.

2. L-Tag: a key player in polyomavirus replication

2.1. Structure and general features of the L-Tag

The polyfunctional protein L-Tag (Fig. 3) is expressed during the early stage of the viral life cycle and is involved in both viral replication and virus-induced cell transformation. The L-Tag is a large protein ranging in size from 641 to 817 residues among the human polyomaviruses (Table 2). It exhibits some post-translational modifications such as phosphorylation of threonines and serines at the amino- and carboxy-terminal parts (Scheidtmann, 1986; Scheidtmann et al., 1991; Schneider and Fanning, 1988; Swenson and Frisque, 1995; Swenson et al., 1996), as well as acetylation of residue Lys697 (in SV40) located in the host-range domain (Poulin and DeCaprio, 2006; Poulin et al., 2004; Shimazu et al., 2006), poly ADP-ribosylation (Goldman et al., 1981), glycosylation and acylation (Klockmann and Deppert, 1983a,b; Medina et al., 1998). Among human polyomaviruses, alternative splicing of the L-Tag mRNA has

Table 2
Features of the L-Tag among primate polyomaviruses. SV40, BKPyV, JCPyV and MCPyV present truncated L-Tags due to different splicing of the mRNA. In the case of JCPyV, three different truncated L-Tags have been identified: T′135, T′136 and T′165. For the newly discovered KIPyV, WUPyV and TSPyV, no data have been published concerning any truncated L-Tag.

Virus	Genome length (nt)	L-Tag length (nb. residues)	Truncated L-Tag length (*nb. residues*)	References
SV40	5,243	708	135/17kT	Zerrahn et al. (1993)
LPyV	5.270	697	–	Furuno et al. (1984)
BKPyV	5,153	695	136	Abend et al. (2009b)
JCPyV	5,130	688	135, 136, 165	Bollag et al. (2006, 2000)
WUPyV	5,229	648	n.d.	Gaynor et al. (2007)
KIPyV	5,040	641	n.d.	Allander et al. (2007)
MCPyV	5,387	817	258/57kT	Shuda et al. (2008)
HPyV6	4,926	669	n.d.	Schowalter et al. (2010b)
HPyV7	4,952	671	n.d.	Schowalter et al. (2010b)
TSPyV	5,232	697	n.d.	van der Meijden et al. (2010)
HPyV9	5,026	680	n.d.	Scuda et al. (2011a)
HPyV10	4,939	668	n.d.	Buck et al. (2012)

n.d., not detected.

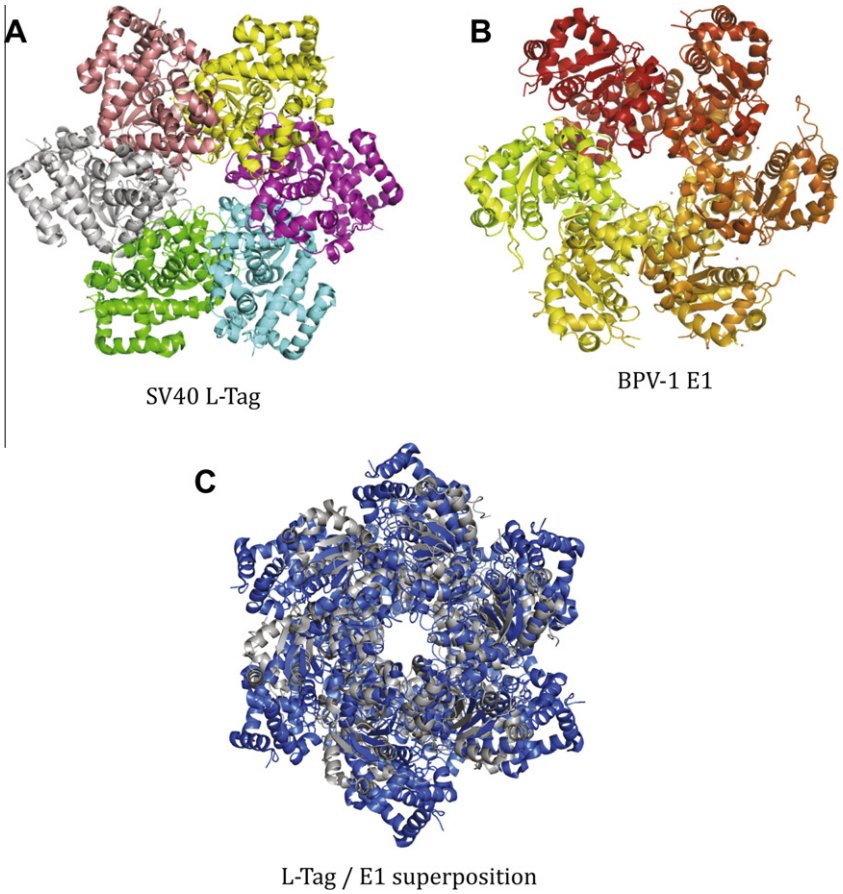

Fig. 4. SV40 L-Tag and BPV-1 E1 hexamer tridimensional structures (pdb code: 1SVM and 2V9P). (A) The SV40 L-Tag adopts a hexameric conformation to form a ring around the origin of replication of the polyomavirus DNA. Two hexamers are needed to unwind the circular double-stranded genome before the cellular DNA polymerase can begin to duplicate the viral DNA. (B) The papillomavirus E1 protein presents a quaternary structure similar to the L-Tag, involving the association of six monomers driven by ATP binding. Two hexamers are required around the viral DNA as well as ATP hydrolysis to trigger unwinding of the double-stranded genome. (C) Superimposition of the two hexamers, SV40 L-Tag (blue) and BPV E1 (grey) highlights the similarities between the two replicative helicases (picture generated using PyMol, Delano software, California, USA).

been described, resulting in a truncated viral protein that lacks the putative "helicase" domain but preserves the domain of interaction with the Retinoblastoma protein family. The truncated L-Tags are involved in cell transformation, but not in lytic replication of the virus. The presence of truncated L-Tag proteins has been demonstrated for SV40, BKPyV, JCPyV and MCPyV but not for the other human polyomaviruses (Table 2). The oligomerization state of the L-Tag required for the unwinding of the viral genome is the hexameric form, in a first step. A dodecamer assembly composed of two rings is then necessary for the helicase activity which starts at the origin of replication (ORI) of the viral DNA (Fig. 4A) (Wessel et al., 1992) (Valle et al., 2000) (Smelkova and Borowiec, 1997).

The E1 replicative helicase of the bovine papillomavirus type 1 (BPV-1) exhibits a similar hexameric quaternary structure. Super-

Table 3
Sequence identity of the SV40 L-Tag in comparison with MCPyV L-Tag, BPV-1 E1, HPV16 E1, E6 and E7. Amino acid identity ranges from 23% to 25% between SV40 LTag and homologous proteins in HPV16 that inactivate the tumor suppressor proteins p53 and pRB. Percentages have been calculated on the basis of the HPV16 protein length (649 residues for HPV16 E1, 98 residues for HPV16 E6 and 158 residues for HPV16 E7). Sequence alignments were made using clustalw (Blossom 16 algorithm).

	SV40 large T antigen			
	Identical residues	% Identity	Similar residues	% Similarity
BKPyV	521	72	120	17
JCPyV	509	71	121	17
KIPyV	334	46	194	30
WUPyV	357	49	173	27
MCPyV	292	34	190	23
HPyV6	281	39	200	30
HPyV7	284	38	215	32
TSPyV	302	39	200	28
HPyV9	288	38	191	28
BPV-1 E1	110	10	197	18
HPV16 E1	72	11	245	34
HPV16 E6	36	23	58	37
HPV16 E7	25	25	39	40

imposition of the L-Tag and E1 assemblies shows how close the machinery of DNA unwinding is between polyoma- and papillomaviruses (Fig. 4B and C). The SV40 L-Tag helicase domain shares 10% (110 residues) and 11% (72 residues) of sequence identity with BPV-1 and HPV16 E1, respectively (Table 3). However, a percentage of similarity of 18% (197 residues) and 34% (245 residues) with BPV-1 and HPV-16, respectively, indicate a conserved fold and the same degree of oligomerization of the replicative helicases in these two viral families.

2.2. Helicase domain/ATPase activity

The L-Tag contains an SF3 helicase domain that is critical for the unwinding of the circular double-stranded DNA of polyomaviruses prior to DNA replication (Fig. 3). The helicase domain is associated with an ORI binding domain (OBD) that recognizes a specific DNA sequence on the viral genome. The binding of ATP is required for the hexamerization of the "ring" (Fig. 4A) and the hydrolysis of ATP produces the energy required for origin DNA melting and replication fork unwinding. The conserved motif GX4GTK (or P-loop) is present in most of the ATP-binding proteins (Saraste et al., 1990). Two "rings" of L-Tags bind to the ORI site of the viral DNA and start to unwind the double-stranded genome in opposite directions (bidirectional replication). The binding of L-Tag at the ORI site is stimulated by the presence of ATP at the ATP binding domain (Borowiec and Hurwitz, 1988).

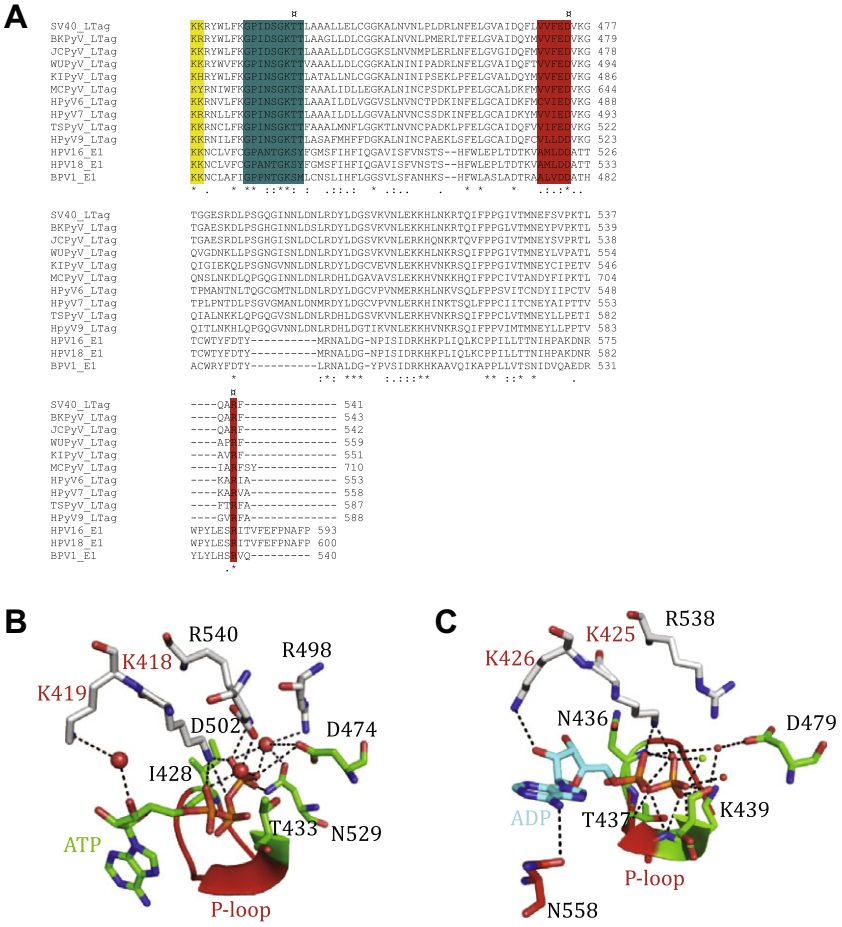

Fig. 5. ATP binding site of the SV40 L-Tag and BPV-1 E1 complexed to ATP or ADP. (A) Sequence alignment of the ATP binding domain of the L-Tag and E1 proteins form SV40, BKPyV, JCVPyV, WUPyV, KIPyV, MCPyV, HPyV6, HPyV7, TSPyV, BPV-1, HPV-16 and HPV-18. [*Fig. 6A and B generated using PyMol Delano software, (California, USA) and multiple sequence alignment was performed with Clustalw using a BOSSOM algorithm. Sequence identity is represented by an asterisk (*) and homology by single (.) or double dots(:)*]. (B) Close view of the ATP binding site where ATP (green) interacts with the cis-residues (green) from one monomer and the trans-residues (grey) from the second monomer of the L-Tag. The red spheres represent water molecules and the red loop is the GX_4GKT motif (Phosphate-loop or P-loop), characteristic of ATP binding proteins. The main interactions between the ATP molecule and the active site involve the α-, β- and γ-phosphates rather than the ribose and the nucleobase. (C) Close view of the BPV-1 E1 ATP site with the ADP represented in cyan and the p-loop in red. Important similarities between the two active sites are shown. The two successive critical lysines and the arginine are conserved as well as the p-loop motif (GX_4GKT/S).

Table 4
Kinetic parameters of SV40 L-Tag, BPV-1 E1, HPV-6 E1 and HPV-11 E1. The turnover number (k_{cat}), representing the amount of substrate metabolized per minute and the Michaelis–Menten constant (K_M), reflecting the concentration of substrate needed to reach 50% of the maximal enzymatic rate, make it possible to determine the catalytic efficiency of each replicative helicase as the ratio k_{cat}/K_M. Parameters calculated using a recombinant protein or crude extract are specified. Differences between SV40 L-Tag and BPV-1 E1 ATPase activities can be seen.

	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}.s^{-1}$)	References
SV40 L-Tag	1.8	5	6000	Clark et al. (1981);Clertant et al. (1984) (crude extract)
	0.72	20	600	Weiner and Bradley (1991) (Sf9 recombinant protein)
	20 (no ssDNA)	270	1234	Greenleaf et al. (2008) (recombinant protein)
	156 (+ ssDNA)	–[a]	–[a]	Greenleaf et al. (2008) (recombinant protein)
BPV-1 E1	18.3	12.1	2.5×10^4	Abbate et al. (2004) (recombinant protein)
	3	750	67	Santucci et al. 1995 (crude extract)
HPV-6 E1	6.0 ± 0.6	12 ± 2	8.3×10^3	White et al. (2001) (recombinant protein)
HPV-11 E1	16 ± 3	6.1 ± 0.7	4.37×10^4	White et al. (2001) (recombinant protein)

[a] Not mentioned in Greenleaf et al. if K_M and k_{cat}/K_M are affected by addition of ssDNA.

Several domains of the SV40 L-Tag have been crystallized and their structures solved, highlighting the importance of the ATPase domain (Gai et al., 2004) (Li et al., 2003), the ORI binding domain (Bochkareva et al., 2006; Meinke et al., 2007, 2011a,b) and the protein segments that allow interactions with p53 and the pRb tumor suppressor proteins (Lilyestrom et al., 2006) (Kim et al., 2001). The SV40 L-Tag and the E1 replicative helicase of BPV-1 share a similar folding, even though the sequence identity between the two viral proteins is as low as 10% (Sanders et al., 2007). SV40 L-Tag and BPV-1 E1 were used for the analysis of their respective ATP binding sites. Significant similarities emerged between the two active sites, since the phosphate-loop (P-loop) that binds the β- and γ-phosphates of an ATP molecule and the lysine motif (Lys-Lys) are conserved among the members of the two virus families (Fig. 5A). In the L-Tag, the lysine finger at positions 418–419 establishes hydrogen bonds (H-bonds) with the 2′-hydroxyl group of the ribose and the α- and β-phosphates, while in the BPV-1 E1 protein, K425-K426 bound the ribose moiety and the two phosphates. Among the polyomaviruses, the motif Lys-Lys may vary to Lys-X, with X being Arg, His or Tyr (Fig. 5A). All three amino acids are able to

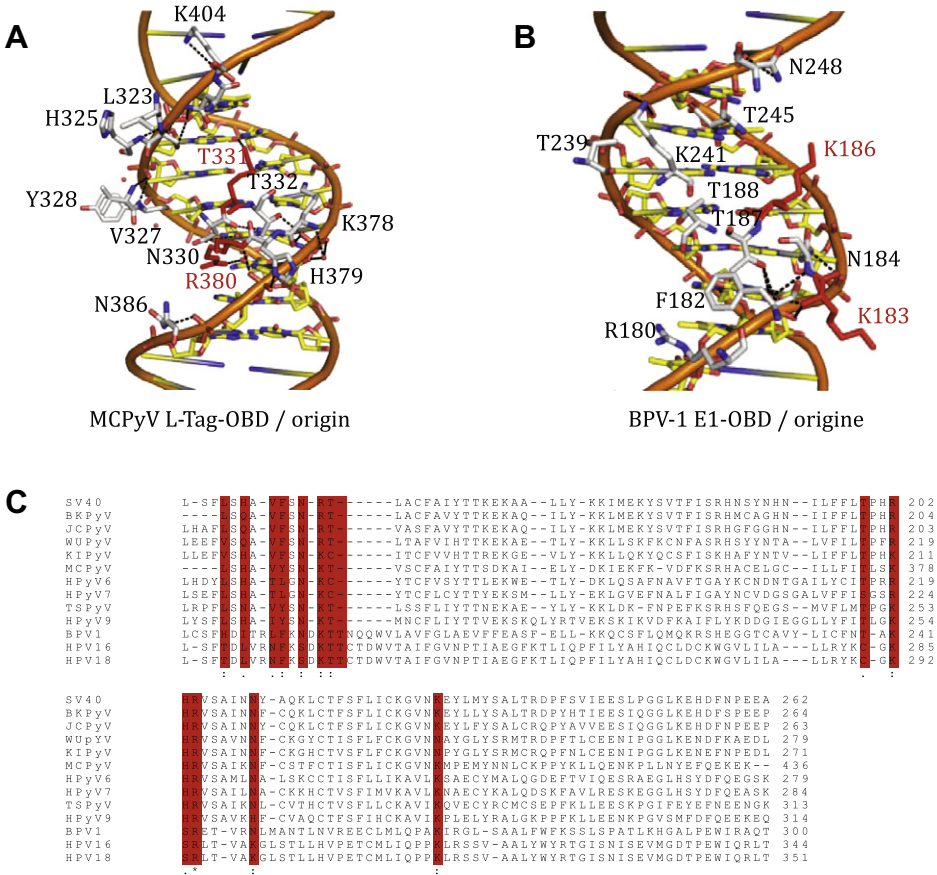

Fig. 6. Protein-DNA interaction of the (A) MCPyV L-Tag-OBD and (B) the BPV-1 E1-OBD at the ORI site of their respective viral DNAs. Residues (grey sticks) from a monomer of MCPyV L-Tag-OBD interacting with the ORI DNA sequence (yellow sticks). In red are represented the key residues Lys331 and Arg380 that recognize the repeat sequence 5′-GAGGC-3′. Important interactions with the phosphate backbone of the viral DNA are seen, by direct interactions (i.e., N386, Y328, V327, H325, and L323) or indirect interactions via water molecules (K378 and H379). In the E1-DNA binary complex, the interactions between the OBD residues and the DNA backbone are mostly Van der Waals interactions, with fewer hydrogen bonds compared to the L-Tag-DNA complex. (C) Sequence alignment of the OBD from SV40, BKPyV, JCPyV, WUPyV, KIPyV, MCPyV, HPyV6, HPyV7, TSPyV and HPyV9 L-Tag and the E1 from BPV1, HPV16 and HPV18 (Multiple sequence alignment performed using clustalw, blossom algorithm). In red are highlighted the residues involved in the ORI DNA binding. *Sequence identity is represented by an asterisk (∗) and homology by single (.) or double dots (:).*

establish H-bonds with water molecules involved in the binding of phosphate moieties in the ATP binding site (Fig. 5B and C). D474 and R540 (in SV40) are conserved among the human polyomaviruses and also in the BPV-1, HPV-16 and -18. The P-loops of L-Tag and E1 (^{426}GPIDSGKT^{433} and ^{433}GPPNTGKS^{440}, respectively) interact with the β- and γ-phosphates of the ATP via residues Ile428, Lys432 and Thr433 (L-Tag) and, Thr437, Lys439 and Ser440 (E1) (Fig. 5B and C).

Kinetic parameters of ATPase activities show turnover numbers (k_{cat}) of 18.30 min^{-1} and 0.72 min^{-1} and apparent affinities (K_M) of 12.1 μM and 20 μM, respectively, for BPV-1 E1 and SV40 L-Tag, using recombinant proteins (Abbate et al., 2004; Weiner and Bradley, 1991) (Table 4). A more recent study showed a K_M of 270 μM and a k_{cat} of 20 min^{-1} with the truncated SV40 L-Tag$^{(131–627)}$ expressed in *Escherichia coli*. Interestingly, addition of single-stranded DNA to the reaction mixture stimulates by 7.8-fold the ATPase activity of the SV40 L-Tag$^{(131–627)}$, increasing the turnover number to 156 min^{-1} (Table 4) (Greenleaf et al., 2008). However, the observed kinetic parameters are different when using crude extracts, as the turnover numbers are 3 min^{-1} (BPV-1) and 1.8 min^{-1} (L-Tag), and the affinities are 750 μM (BPV-1) and 5 μM (L-Tag) (Clark et al., 1983; Santucci et al., 1995) (Table 4). Although apparent affinities (K_M) for ATP were similar when assayed with recombinant proteins, the determined turnover numbers showed an important difference for BPV-1 E1, which catalyzed ATP hydrolysis 20 times faster than the SV40 L-Tag. This might reflect a more flexible E1 protein, in comparison with the SV40 L-Tag that has domains similar to the papillomavirus proteins E6 and E7, the pRb and p53 binding domains.

2.3. The ORI binding domain (OBD) recognizes specific motifs on the viral DNA

The OBD recognizes a particular sequence, the ORI site, located on the SV40 genome, composed of 3 different regions: a central palindrome containing 4 repeats of the sequence 5′-GAGGC-3′, an AT-rich element and an imperfect palindrome (Bochkareva et al., 2006; Dean et al., 1987; Deb and Tegtmeyer, 1987). The OBD region is largely composed of positively-charged and uncharged polar residues, allowing interactions with the negatively-charged backbone of the viral DNA. Arg154 and Arg204 in the L-Tag have important roles in the recognition of the 5′-GAGGC-3′ sequences in the viral DNA, by interacting with the first guanosine (Bochkareva et al., 2006).

The OBD of the MCPyV L-Tag was crystallized and its structure was solved in 2011 by A. Bohm's group, who reported the important role of Lys331 and Arg380, which are able to recognize the 5′-GAGGC-3′ repeat, but with a weaker affinity than that observed for the Arg154 and Arg204 in the SV40 ORI (Fig. 6A) (Harrison et al., 2011). SV40 and MCPyV L-Tags share 34% sequence identity (292 residues) and 23% of similarity (190 residues) (Table 3) and the OBD is not highly conserved. However, a high similarity exists among the residues involved in ORI recognition (Fig. 6C). In general, L-Tag sequence identity among the polyomaviruses ranges from 34% (MCPyV) to 72% (BKPyV and JCPyV) (Table 3).

In contrast to polyomavirus L-Tags, the papillomavirus E1 protein binds to the viral DNA at the ORI site, recognizing the motif 5′-ATTGTT-3′ via Van der Waals interactions rather than hydrogen bonds, even though the residues of the OBD active site are highly conserved. Fig. 6 shows the major residues of the protein-DNA complex of MCPyV and BPV-1 (Enemark et al., 2002). Arg180, Lys183, Lys186, Thr187 and Lys241 in the BPV-1 OBD are critical residues for the binding of the DNA to the E1 replicative helicase (Enemark et al., 2000) (Fig. 6B). A multiple sequence alignment of the polyomaviruses L-Tag and the BPV-1, HPV-16 and HPV-18 E1 proteins shows that the residues involved in DNA binding (i.e., the positively-charged Arg-Lys and the polar Asn, Thr, Ser and His) are systematically present (Fig. 6C).

Unlike the polyomavirus helicase L-Tag, which does not need any associated protein to target the ORI sequence of the viral genome, the papillomavirus E1 replicative helicases associate with the E2 protein for the initiation of viral DNA duplication. Indeed, E2 helps E1 to target the ORI sequence located in the viral genome and is critical for formation of the helicase complex (DNA-E1-E2) (Chiang et al., 1992; Mohr et al., 1990; Sedman and Stenlund 1995; Yang et al., 1991). E2 allows specific binding of E1 to the viral DNA, rather than nonspecific binding to the cellular genome (Abbate et al., 2004).

2.4. The helicase domain as a drug target for inhibition of polyomavirus replication

The highly conserved sequence identity and similarity of the ATP binding site observed in the L-Tags from different polyomaviruses make the L-Tag an interesting target in the search for anti-polyomavirus compounds. Several studies have focused on the discovery of drugs that target the ATPase domain of the polyomavirus replicative helicase. High-throughput screening of a library of compounds was used to discover hits leading to potential new antipolyomavirus drugs. Seguin and co-workers screened the National Institutes of Health Molecular Libraries Probe Centers Network library, composed of 306,015 compounds, for SV40 ATPase inhibitors. They found bisphenol A as a potential inhibitor of the ATPase activity of the L-Tag (IC_{50} = 41 μM) but due to the high toxicity of this compound, drug design studies were required to improve its antiviral activity and reduce cytotoxicity (Seguin et al., 2012a). A second study by Seguin and co-workers followed, based also on screening for modulators of L-Tag ATPase that inhibit SV40 and BKPyV replication (Seguin et al., 2012b). Bithionol and hexachlorophene were found as the best inhibitors from the tested library, with EC_{50}'s against SV40 of 2.2 and 3.2 μM, respectively.

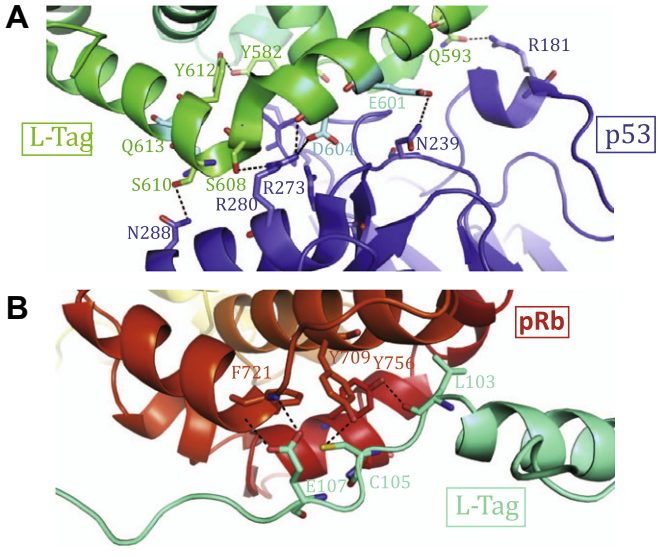

Fig. 7. Interactions between the L-Tag and tumor suppressor proteins p53 and pRb. (A) The L-Tag (in green) exhibits negatively-charged (Asp and Glu) and polar (Ser, Tyr, Gln) residues in order to mimick DNA negative polarity and to establish interactions with the positively-charged p53 (Arg and Asn) (blue). (B) The L-Tag is able to bind to retinoblastoma proteins (in red) via the highly conserved motif LxCxE. Phe721 backbone amino- and carboxyl moieties establish hydrogen bonds with E107. Hydroxyl groups of Y709 and Y756 interact with L103 and C105 via polar interactions. The pdb codes of the proteins structure used for the production of Fig. 7 are 2H1L (p53) and 1GH6 (pRb).

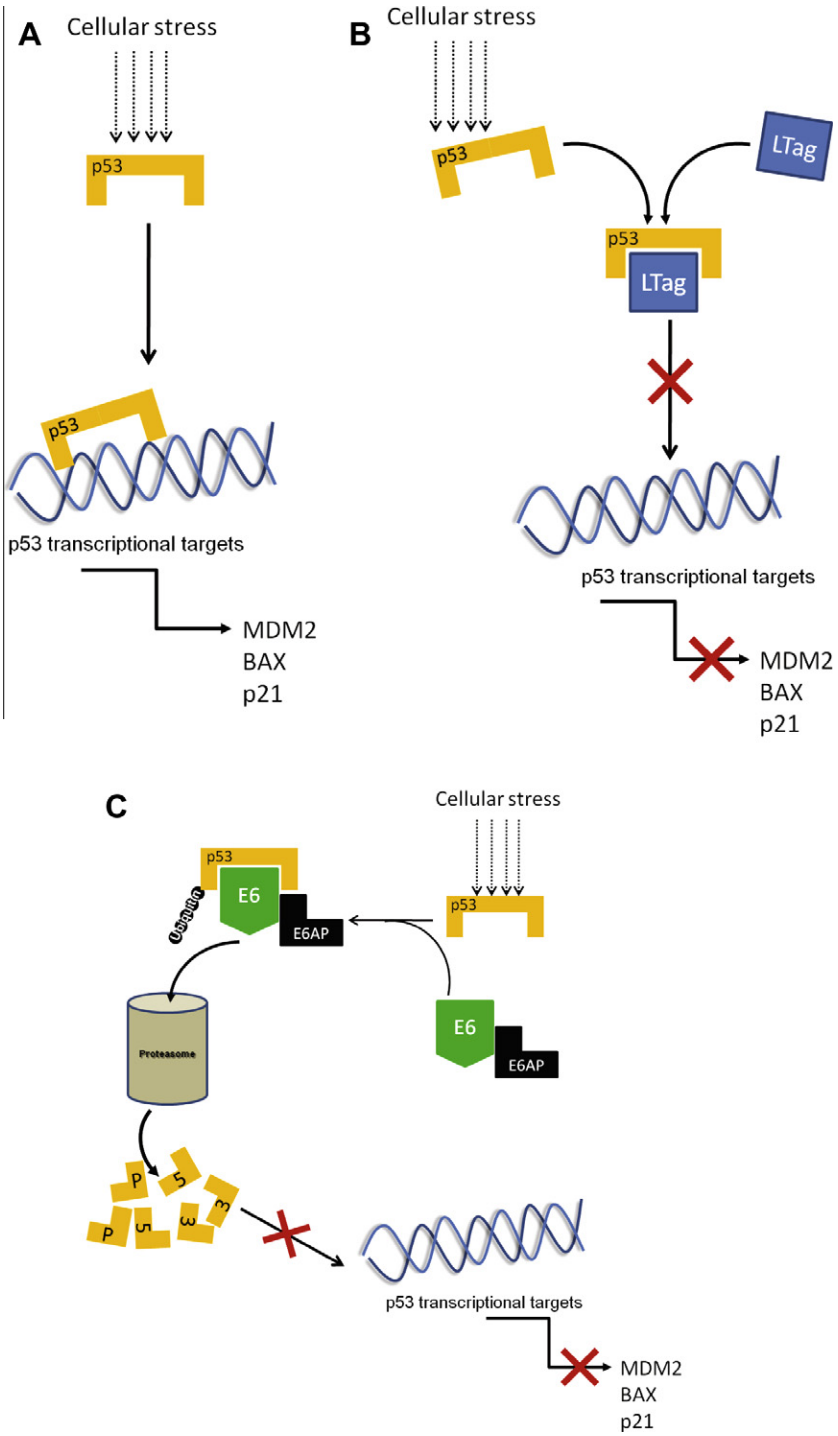

Fig. 8. In activation of p53 by SV40 LTag and HPV-16 E6. In an uninfected cell, the p53 pathway is activated in response to cellular stress in order to promote the transcription of genes involved in apoptosis or DNA repair (e.g., MDM2, BAX, p21) (A). The polyomavirus SV40 L-Tag inactivates p53 by direct interaction, preventing its binding to DNA (B), while the HPV E6 forms a ternary complex with the E6-associated protein (E6AP) and p53, in order to ubiquitinylate p53 (black filled circles) for proteasome-mediated degradation (C).

In 2009, Wright and colleagues reported that MAL2–11B, a heterocyclic compound, had an inhibitory effect against polyomaviruses *in vitro*, by impairing the activities of the J domain and the ATPase domain of the L-Tag (Wright et al., 2009). The concentration of MAL2–11B required to observe an inhibitory effect was greater than 100 μM. Hence, chemical modifications of this molecule should be envisaged to improve its selectivity. Similar studies have been performed in the search for papillomavirus helicase inhibitors. In 2005, Archambault's group described inhibition of the HPV-6 E1 enzymatic helicase activity by targeting the ATPase domain with derivatives of biphenylsulfonacetic acid (BPSA). These analogs of BPSA showed IC_{50} values in the nanomolar range against the HPV-6 E1 protein, but when assayed against HPV-11 and HPV-18 E1 proteins, the IC_{50}s were 375- to 2400-fold higher (White et al., 2005). Despite the low IC_{50}s observed with these different heterocyclic derivatives against the HPV-6 E1 protein, none of them seemed to be a good candidate for the development of an antipolyoma- or antipapillomavirus drug.

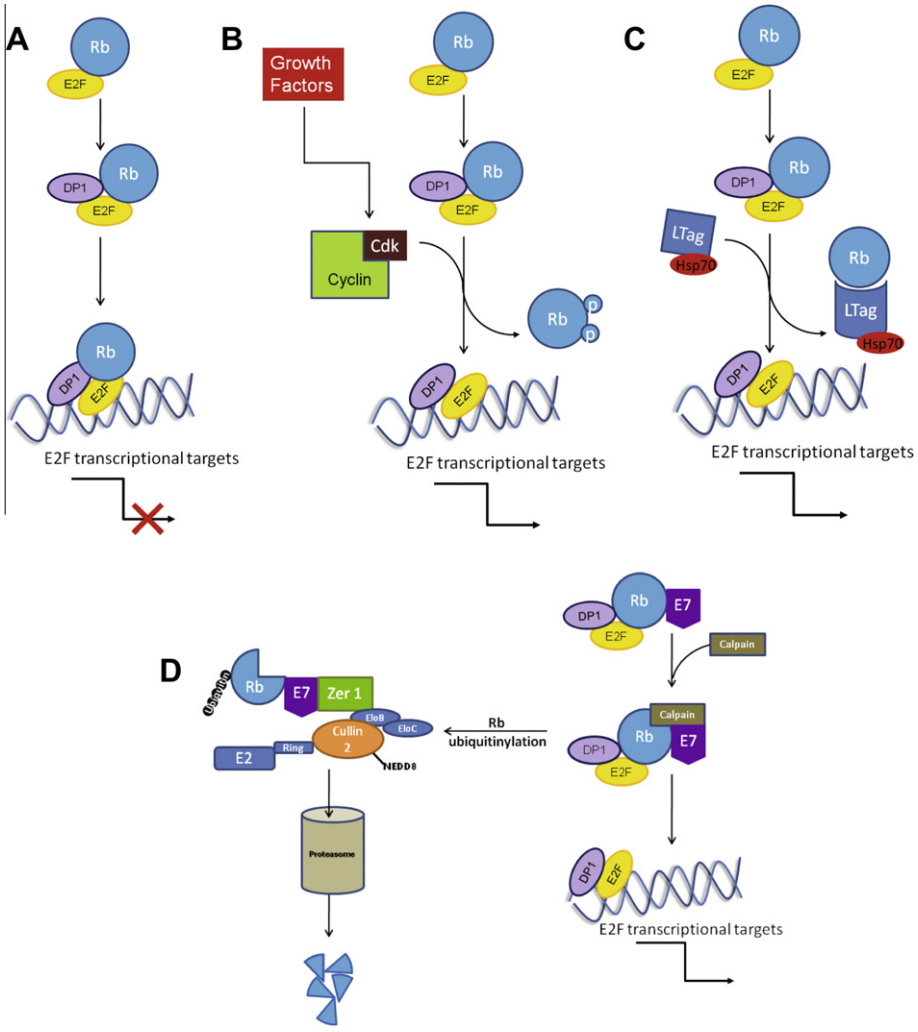

Fig. 9. Repression of Rb functions in SV40 and HPV-16 infected cells. (A) Without any mitose signal entry, E2F remains attached to Rb and transcription of targeted genes is repressed. (B) The cellular response to growth factor permits the activation of cycline-dependent kinases (Cdk) which can phosphorylate the Rb protein. The phosphorylation of Rb disrupts the DP1/E2F/Rb complex and allows binding of E2F on the DNA and activation of transcription of genes necessary for cell division. (C) In the case of an SV40-infected cell, the L-Tag binds to Rb, preventing the formation of the complex E2F/Rb and allowing the entry in mitosis. (D) In a HPV-16 infected cell, E7 triggers the cleavage of the N-terminal domain of Rb by a calpain, leading to Rb degradation (proteasomal pathway). The complex E2-Cullin2-ElonginB/C-ZER1-E7 mediates the ubiquitinylation of Rb before its degradation [adapted from White et al. (2012)]. E2F can then bind to the DNA, permitting transcription of the genes required for the G1/S transition.

3. Transformation capacity of the L-Tag

3.1. Why does the L-Tag highjack cell cycle control?

The control of cell proliferation is ensured by different check-points, its major role being the maintenance of integrity of the cellular genome. DNA damage can occur after a genotoxic stress, such as UV light or osmotic shock, and repair is crucial for the survival of the cell (Kastan, 2008). Cell cycle arrest allows the cell to repair DNA damage or trigger apoptosis (p53-dependent), to avoid the introduction of mutations into the cellular genome. Several viruses, including the polyoma- and papillomaviruses, are able to bypass cell cycle control check-points to induce cellular proliferation, through the interaction of their oncoproteins with tumor suppressor proteins (Lavia et al., 2003; Levine, 2009; Tommasino and Crawford, 1995; Vousden, 1995; Werness et al., 1990). The two major ways of inducing cellular proliferation are to suppress the activities of p53 and Rb proteins, through binding to L-Tag (Fig. 8).

The tumor suppressor protein p53 is activated by cellular stress (i.e., DNA damage) and stimulates the transcription of specific genes that code for p21 (cyclin dependent kinase inhibitor-1), MDM2 (p53 E3 ubiquitin ligase) and BAX (Bcl-2 associated X protein) (Fig. 8A). These proteins are important for the arrest of the cell cycle and repair of DNA damage, or alternatively, if the cell cannot repair the damage, to induce apoptosis (Chipuk et al., 2004; Lane, 1992). Mutations in p53, leading to a protein that is not able to bind to DNA, occur in a majority of mutated-p53-dependent cancers (Rivlin et al., 2011). In parallel, Rb proteins are able to form complexes with E2F and DP1 (E2F dimerization partner-1) that inhibit the transcription of a series of genes involved in cellular proliferation (Fig. 9A). Only after induction by growth factors, the complex cyclin/cyclin dependent kinase will phosphorylate the Rb protein and release the transcription factor E2F/DP1, allowing transcription of several genes that stimulate cellular proliferation (Fig. 9B) (Dyson, 1994a, 1998). The polyomaviruses and the papillomaviruses, by respectively expressing L-Tag and E6 and E7, are able to bypass the check-points, to induce cell division and proliferation, as well as to avoid apoptosis of virus-transformed cells (White et al., 2012).

3.2. From transformation to tumorigenesis: involvement of polyomaviruses in human cancers?

In the late 1970s, Sol and Van Der Noordaa showed the oncogenic ability of SV40 in Syrian hamsters (Sol and van der Noordaa, 1977). This biological process is possible in nonpermissive cells, where viral replication is not supported, in contrast to permissive cells, where the virus can replicate. The transformation function is driven by the L-Tag, because this viral protein can bind and inactivate the two major proteins regulating the cell cycle, the p53 and pRb tumor suppressor proteins. It has been shown that mutations in p53 are involved in 50% of human cancers (Hollstein et al., 1991; Ko and Prives, 1996; Levine et al., 1991). These mutations, responsible for molecular instability, impair the cellular functions of p53, especially DNA damage repair and induction of apoptosis by transcription of cellular genes coding for BAX, MDM2 and p21. By inactivating p53, the L-Tag prevents p53-dependent gene expression (Fig. 8). The expression of the L-Tag is required for the maintenance of transformation. However, the disruption of p53 and pRb functions is not sufficient for cellular transformation and its maintenance, suggesting that other processes are required (Pipas, 2009). SV40 has been investigated for its possible role in carcinogenesis in humans, especially in mesotheliomas and brain tumors (i.e., ependydoma, astrocytoma, meningioma, medulloblastoma and glioblastoma multiforme) (Shah, 1998;Testa and Giordano, 2001; Zhen et al., 2001, 1999). Up to now, there has been no clear evidence that SV40 is a causative agent of human cancers, at least in the initiation of carcinogenesis (Shah, 2007).

In contrast, there is strong evidence of the relationship between MCPyV and Merkel cell carcinoma (MCC) (Feng et al., 2008; Houben et al., 2011, 2010). Thus, it has been demonstrated that MCPyV L-Tag is required for the proliferation of MCC cells, and its truncated form (i.e., 57kT; Table 2), that bears the conserved LxCxE motif but not the helicase and p53-binding domains, was found in MCPyV-positive MCC cells (Houben et al., 2011, 2010; Shuda et al., 2008).

Abend et al., showed that BKPyV was able to express a truncated form of the L-Tag by alternative splicing, named truncated Tag, which has a size of 136 residues (17–20 kDa; Table 2) (Abend et al., 2009b). Inoculation of newborn hamsters with BKPyV resulted in the production of tumors at the injection site (Shah et al., 1975). Transgenic mice expressing the BKPyV LTag are subject to tumor appearance such as kidney carcinoma (Dalrymple and Beemon, 1990). *In vitro*, expression of BKPyV LTag can transform rodent cells and immortalize human cells (Abend et al., 2009a). Therefore, it is opportune to consider a link between BKPyV and oncogenicity in humans, but the conflicting reports on this subject do not allow one to draw a conclusion as to a causal effect (Abend et al., 2009a).

JCPyV produces alternative L-Tags, namely T'135, T'136 and T'165 that share the first 81 residues of the amino-terminal extremity (Bollag et al., 2006, 2000). These alternative L-Tags have been reported to have transforming capacity, since their different activities act synergistically to inactivate cell regulators such as pRb, p107 and p300. Expression of the JCPyV L-Tag in transgenic mice triggers different tumors in the brain or the pituitary gland (Franks et al., 1996; Gordon et al., 2000; Krynska et al., 1999). JCPyV L-Tag is also able to transfom bone marrow-derived mesenchymal stem cells, leading to tumor formation (Del Valle et al., 2010). To date, no truncated Tags have been described among the other human polyomaviruses, and no capacity to induce tumors in animals has been reported.

3.3. Role of the J domain and the LxCxE motif in cellular transformation

The J domain of the L-Tag is a heat-shock-protein-70 binding domain (Hsp70-BD) and is located in the amino-terminal extremity (residues 1–81) of the oncoprotein (Fig. 3). Hsp70 is activated after cellular stress, and its function is to participate in the folding and unfolding of proteins (De Maio, 1999). The HPDKGG motif in the L-Tag J domain is conserved among the polyomaviruses. This motif allows interaction between the two partners (i.e., L-Tag J domain and Hsp70), resulting in stimulation of the ATPase activity of Hsp70 (Tsai and Douglas, 1996). Mutations within this motif impair the recognition of Hsp70 by the L-Tag (Campbell et al., 1997). The L-Tag recruits Hsp70 in order to disrupt complexes involving p107 and p130, two proteins that belong to the Rb family (Sullivan et al., 2004, 2000) (Fig. 9C). These two tumor-suppressor proteins are able to inhibit the transactivation activity of the E2F transcription factor (Dyson, 1994b, 1998, 1989b). The inactivation of p107 and p130 by the L-Tag results in the entry of the cells into S phase and, therefore in the induction of cell proliferation (Lundberg and Weinberg, 1998) (Dyson et al., 1989a).

The role of the L-Tag is to bring the Hsp70 and Rb proteins, in the right orientation, close enough to release the transcription factor E2F/DP1 complex, a mechanism that depends on ATP hydrolysis (Sullivan et al., 2004, 2000). The J domain and the LxCxE motif are involved in important functions of the L-Tag for cellular transformation. Indeed, the LxCxE motif is the Rb-binding domain, conserved in all proteins able to interact with pRb, p107 and p130 (Berk, 2005; de Souza et al., 2010; Dyson et al., 1989b; Ferreon et al., 2009; Hume and Kalejta, 2009). The papillomavirus E7 oncoprotein does not possess a J domain, and the mechanism of E2F/pRb disruption by E7 is therefore slightly different than that of the L-Tag. The proposed mechanism involves inhibition of the association of E2F with Rb proteins by steric hindrance (Helt and Galloway, 2001). E7 interacts with pRb and hides the domain of interaction with E2F, preventing association of the two cellular proteins (Fig. 9D).

3.4. The p53 binding domain of the L-Tag

Residues 350–450 and 550–650 of the SV40 L-Tag form the domain of interaction with the p53 tumor-suppressor protein, but not with the homologues p63 and p73 (Kierstead and Tevethia, 1993; Murray-Zmijewski et al., 2006). The p63 and p73 proteins therefore may not be important for the transformation process, since they are not inactivated by the L-Tag. It is not totally clear if L-Tag inhibits the entire pool of p53 proteins, or only part of it. This interaction was quantified *in vitro* by Kühn et al., in 1999, using surface plasma resonance. Under conditions of 10 mM Mg^{2+}, the constant of dissociation (K_D) of the p53-LTag complex was 5.26×10^{-9} M (constant of association $K_A = 1.9 \times 10^8$ M^{-1}) (Kuhn et al., 1999). The nanomolar-range affinity between the oncoprotein L-Tag and p53 shows how strong is the interaction (similar to an antigen–antibody complex) and underlines how well the L-Tag is able to mimick DNA negative charges to inactivate p53 functions.

The interaction between p53 and L-Tag is also useful for other reasons, since p53 has other partners that can interact with the L-Tag in a p53-mediated process. This is the case with p300 and the CREB-binding protein (CBP), two proteins that bind to p53 and catalyze the acetylation of a lysine residue at position 697 in the SV40 L-Tag (Borger and DeCaprio 2006; Poulin et al., 2004). The role of the acetylation of K697 is unknown for the human polyomavirus, but for its murine counterpart, acetylation of the L-Tag is required to activate replication at the ORI site (Xie et al., 2002). The conservation of the acetylation site at the L-Tag carboxyl-terminal extremity among the human and nonhuman primate polyomaviruses (i.e., SV40, BKPyV and JCPyV) shows that an important function is activated through acetylation, but up to now no link has been made with viral replication. In MCPyV-positive MCC cells, the L-Tag is truncated and the 57kT protein does not harbor a complete p53-binding domain. Therefore, the MCPyV truncated L-Tag cannot inactivate p53 functions, but still contains the LxCxE motif

for interaction with Rb family proteins for cellular transformation. Similar to the polyomavirus L-Tag, the papillomavirus E6 protein interacts with p53 and induces its ubiquitinylation prior to cellular degradation (Fig. 8C).

3.5. Mechanism of cellular transformation: molecular insights

The SV40 L-Tag shares 23% and 25% sequence identity with HPV-16 E6 and E7, respectively. Regarding sequence similarity between the SV40 L-Tag and the HPV-16 oncoproteins, the number of similar residues is 58 (37%) with E6 and 39 (40%) with E7. Based on these similarities, a conserved folding pattern is expected (Table 3). In 2006, Lilyestrom and colleagues published the tridimensional structure of the SV40 L-Tag complexed to the p53 tumor-suppressor protein, showing that the viral oncoprotein interacts with its cellular partner by mimicking the negative charges of the DNA (Lilyestrom et al., 2006). Hydrogen bonds and dipolar interactions are established between the two partners, mainly involving Gln593, Glu601, Ser608, Asp604 and Ser610 of the SV40 L-Tag, and Arg181, Asn239, Arg280 and Asn288 from the p53 protein (Fig. 7A). It is worth noting that, although the L-Tag inactivates p53 functions, the viral oncoprotein is also inhibited by p53, since the functions of L-Tag are also affected by the formation of this binary complex. Only free L-Tag is able to unwind the viral DNA, which is necessary for viral replication. This inhibition is overcome by the production of large amounts of L-Tag, sufficient both to bind p53 and to remain in a free form and participate in viral DNA replication.

In 2001, Kim et al. (2001) solved the structure of the SV40 L-Tag pRb binding domain complexed to pRb. Fig. 7B depicts the interaction between the L-Tag and pRb and shows the established interactions between Tyr709, Phe721 and Tyr756 from pRb, with Leu103, Cys105 and Glu107 (conserved LxCxE motif) from the L-Tag. Through these interactions, the L-Tag is able to dissociate the DP1/E2F/Rb complex and allow the DP1/E2F complex to initiate the transcription of genes necessary for stimulation of cell proliferation (Fig. 9C). In a similar study, Lee et al. (1998) determined the structure of the binary complex pRb/E7 (from HPV-16) where the conserved LxCxE motif of E7 established interactions with pRb as follows: Tyr709 with Leu22, Tyr756 and Lys713 with Cys24, and Val725 with Glu26. In papillomavirus-positive cells, E7 induces the ubiquitination of pRb, leading to its degradation via the 26S proteasome (Fig. 9D) (White et al., 2012).

The tridimensional structure of the complex p53/E6AP/E6 has not yet been solved, but studies have identified the important residues involved in the interaction among these three partners (Nomine et al., 2006). Although both polyoma- and papillomaviruses impair p53 and pRb function, and their oncoproteins share a high percentage of sequence identity, the mechanism of inactivation of the tumor-suppressor proteins is relatively distinct. On the one hand, polyomaviruses disrupt tumor-suppressor protein activity by sequestration *via* direct interaction. On the other hand, papillomaviruses induce the degradation of p53 and pRb *via* the proteasome pathway through ubiquitinylation. Indeed, E6 interacts with p53 and initiates the formation of the ternary complex E6/p53/E6AP (E6 associated protein) (Fig. 8C). The E6AP is a ubiquitin ligase that promotes the ubiquitination of p53, in the presence of the E6 protein, prior to proteolysis (Talis et al., 1998). In a similar way, pRb inactivation is initiated by the disruption of the pRB/E2F complex triggered by the papillomavirus E7 oncoprotein, allowing ubiquitination of pRb via the cellular E2-ubiquitin ligase (Boyer et al., 1996) (Fig. 9D).

3.6. Small tumor antigen: an alternative product of the L-Tag gene

The s-Tag is a product of the L-Tag early gene that is generated *via* alternative splicing of the mRNA, resulting in a shorter protein that ranges from 174 residues in SV40 to 199 residues in TSPyV. The entire polyomavirus family is able to express this alternative form of the L-Tag, which has a role in viral replication and cellular transformation. Indeed, many studies have shown the role of s-Tag in utilization of the serine/threonine protein phosphatase 2A (PP2A) to activate pathways for cellular proliferation and survival (Hahn et al., 2002; Mungre et al., 1994; Sontag et al., 1993; Sontag and Sontag, 2006; Yu et al., 2001). The s-Tag/PP2A interaction deregulates pathways such as JNK/SPAK, MEK-ERK, PI3-kinase/Akt and PI3-kinase/PKC, facilitating proliferation of transformed cells (Arroyo and Hahn, 2005; Howe et al., 1998; Janssens and Goris, 2001; Sontag, 2001). The tridimensional structure of the SV40 s-Tag interacting with the murine PP2A has been published by Cho et al., showing the J domain, the unique domain of the s-Tag, as area of interaction (Cho et al., 2007).

MCPyV s-Tag is involved in optimal MCPyV replication in synergy with the L-Tag, similarly to JCPyV replication (Feng et al., 2011). Interestingly, the transformational activity of MCPyV s-Tag is not PP2A-dependent, as was shown for SV40, but occurs via a cap-dependent translation mechanism (Shuda et al., 2011).

4. Conclusion

Thanks to advances in molecular biology, new members of the polyomavirus family have been discovered during the past 5 years. The genome organization and the conserved features of their gene products, especially the L-Tag, make interesting targets for the study of this virus family. For example, understanding the mechanism of tumorigenesis due to MCPyV L-Tag may be helpful for the design of new strategies to manage human cancers in general, and against virus-induced cancers, particularly HPV-related cancers. The lack of drugs in the pharmacopeia for the treatment of polyomavirus-associated diseases also makes it urgent to validate new targets. In contrast to HPyV10, which might be related to WHIM syndrome, the newly discovered WUPyV, KIPyV, HPyV6, HPyV7 and HPyV9 are not associated with any specific disease. However, we cannot assume that these five polyomaviruses are not causative agents of dysfunction in the human population, in particular WUPyV and KIPyV, which have been detected in patients with respiratory tract diseases, or HPyV6 and -7, which form part of the human skin microbiome.

Because the L-Tag has a key role in all the major processes of the polyomavirus life cycle (replication and cellular transformation), it appears to be a promising drug target for management of diseases such as PML, BKPyV-induced nephropathy, Merkel cell carcinoma and *T. spinulosa*. This hypothesis is supported by the existence of well conserved L-Tag domains among the polyomaviruses, especially the ATP-binding site. Indeed, this specific domain has been selected in several *in vitro* studies as a target for antipolyomavirus compounds.

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