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# Agnoprotein of mammalian polyomaviruses

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ABSTRACT

Polyomaviruses are naked viruses with an icosahedral capsid that surrounds a circular double-stranded DNA molecule of about 5000 base-pairs. Their genome encodes at least five proteins: large and small tumor antigens and the capsid proteins VP1, VP2 and VP3. The tumor antigens are expressed during early stages of the viral life cycle and are implicated in the regulation of viral transcription and DNA replication, while the capsid proteins are produced later during infection. Members of the *Polyomaviridae* family have been isolated in birds (*Avipolyomavirus*) and mammals (*Orthopolyomavirus* and *Wukipolyomavirus*). Some mammalian polyomaviruses encode an additional protein, referred to as agnoprotein, which is a relatively small polypeptide that exerts multiple functions. This review discusses the structure, post-translational modifications, and functions of agnoprotein, and speculates why not all polyomaviruses express this protein.

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## Introduction

### *Discovery of polyomaviruses*

In 1953, Ludwig Gross accidently discovered the first polyomavirus as a contaminant of murine leukemia virus. He observed that mice inoculated with murine leukemia virus not only developed leukemia, but also adenocarcinomas of the parotid gland (Gross, 1953). Newborn mice injected with extracts of parotid gland cancer tissue developed a variety of solid tumors. Because of this property, the new infectious agent was originally named murine SE polyomavirus after the Greek for many (*poly*) for and cancer (*oma*) (Stewart et al., 1957).

In the following years several other polyomaviruses were isolated from birds and mammalians, including man (Table 1). So far, no polyomavirus infections have been recognized in fish, amphibians or reptiles, although polyomaviruses-like particles have been observed in these species, and the genome of a virus infecting the Japanese eel contains one open reading frame that shows homology to polyomavirus LT-ag (Essbauer and Ahne, 2001; Mizutani et al., 2011). Eight genuine human polyomaviruses (HPyV) have been described so far: BKPyV, JCPyV, KIPyV, WUPyV, Merkel cell polyomavirus (MCPyV), HPyV6, HPyV7, trichodysplasia spinulosa-associated polyomavirus (TSPyV), and HPyV9 (Allander et al., 2007; Gardner et al., 1971; Gaynor et al., 2007; Padgett et al., 1971; Feng et al., 2008; Schowalter et al., 2010; Scuda et al., 2011; van der Meijden et al., 2010). HPyVs seem to be harmless in immunocompetent individuals, but have been associated with nephropathy (BKPyV), PML (JCPyV), and trichodysplasia spinulosa (TSPyV) in immunocompromised patients and with Merkel cell carcinoma (MCPyV) (Brew et al., 2010; Hirsch and Steiger, 2003; Feng et al., 2008; van der Meijden et al., 2010).

### *The polyomavirus particle*

In the sixties, the monkey polyomavirus simian virus 40 (SV40) was isolated and became the first polyomavirus whose complete genome was sequenced (Fiers et al., 1978). Sequence analyses of other polyomavirus genomes confirmed that they all have a similar functional organization encoding the large (LT-ag) and small tumor antigen (st-ag) proteins and the capsid proteins VP1, VP2 and VP3. The virions lack an envelope, but contain an icosahedral capsid of approximately 40–45 nm in diameter that encloses a single copy of a ~5000 base-pair double stranded, circular DNA molecule (Imperiale and Major, 2007).

The viral genome is packed with cellular histones H2A, H2B, H3 and H4, except for the non-coding region, which contains the origin of replication and controls expression of the viral genes (Imperiale and Major, 2007). The early transcripts for LT- and st-ag are derived by alternative splicing of a common precursor. The resulting LT- and st-ag proteins are involved in viral DNA replication and transcription (Gjoerup and Chang, 2010; Moens et al., 2007). Two major late transcripts encode the capsid proteins; one of the transcripts is translated into VP1, while the other gives rise to VP2 and VP3 by using two different start codons (Imperiale and Major, 2007).

### *Agnoprotein*

Although all polyomavirus genomes contain open reading frames for the above-mentioned five proteins, several members

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**Table 1**
Polyomaviruses whose genomes have been completely sequenced.

| Polyomaviruses | Agnoprotein | aa[a] | Accession number | References |
|---|---|---|---|---|
| **Mammalia** | | | | |
| *Primates* | | | | |
| Human | | | | |
| BKV (WW strain) | + | 62 | NC_001538 | (Seif et al., 1979) |
| JCV (Mad1 strain) | + | 71 | NC_001699 | (Frisque et al., 1984) |
| KI (isolate Stockholm 60) | – | | NC_009238 | (Allander et al., 2007) |
| WU (strain B0) | – | | NC_009539 | (Gaynor et al., 2007) |
| MCPyV (isolate MCC350) | – | | NC_010277 | (Feng et al., 2008) |
| HPyV-6 (isolate 607a) | – | | NC_014406 | (Schowalter et al., 2010) |
| HPyV-7 (isolate 713a) | – | | NC_014407 | (Schowalter et al., 2010) |
| Trichodysplasia spinulosa-associated polyomavirus | – | | NC_014361 | (van der Meijden et al., 2010) |
| HPyV-9 | – | | NC_015150 | (Scuda et al., 2011) |
| Monkey | | | | |
| SV40 | + | 66 | NC_001669 | (Fiers et al., 1978) |
| SV40-Ri257 | + | 90 | FN812745 | (Fagrouch et al., 2011) |
| Baboon polyomavirus 1 (isolate SA12) | + | 68 | NC_007611 | (Cantalupo et al., 2005) |
| Chimpanzee polyomavirus | + | 64/65/74 | FR692334-6 | (Deuzing et al., 2010) |
| Gorilla gorilla gorilla polyomavirus | – | | HQ385752 | (Leendertz et al., 2011) |
| Squirrel monkey polyomavirus | + | 70 | NC_009951 | (Verschoor et al., 2008) |
| Lymphotropic polyomavirus[b] | – | | NC_004763 | (Pawlita et al., 1985( |
| Orangutan polyomavirus (Borneo isolate) | – | | NC_013439 | (Groenewoud et al., 2010) |
| Orangutan polyomavirus (Sumatra isolate) | – | | FN356901 | (Groenewoud et al., 2010) |
| *Carnivora* | | | | |
| California sea lion polyomavirus (strain CSL6994) | + | 50 | NC_013796 | (Colegrove et al., 2010) |
| *Artiodactyla* | | | | |
| Bovine polyomavirus | + | 118 | NC_001442 | (Schuurman et al., 1990) |
| *Rodentio* | | | | |
| Murine polyomavirus | – | | NC_001515 | (De Simone et al., 1985) |
| Murine pneumotropic virus (strain Kilham) | – | | NC_001505 | (Mayer and Dorries, 1991) |
| Hamster polyomavirus | – | | NC_001663 | (Delmas et al., 1992) |
| Mastomys polyomavirus | + | 154 | AB588640 | (Orba et al., 2011) |
| *Chiroptera* | | | | |
| Myotis polyomavirus (little brown bat; isolate 14) | + | 30 | NC_011310 | (Misra et al., 2009) |
| **Aves** | | | | |
| Canary polyomavirus | – | | GU345044 | (Halami et al., 2010) |
| Budgerigar fledging or avian polyomavirus | – | | NC_004764 | (Rott et al., 1998) |
| Finch polyomavirus | – | | NC_007923 | (Johne et al., 2006) |
| Crow polyomavirus | – | | NC_007922 | (Johne et al., 2006) |
| Goose hemorrhagic polyomavirus | – | | NC_004800 | (Johne and Müller, 2001) |

[a] Number of amino acid residues.
[b] African green monkey polyomavirus.

express additional proteins (Abend et al., 2009; Trowbridge and Frisque 1995; Zerrahn et al., 1993). One of these polypeptides was first identified in the SV40 genome and is encoded by an open reading frame upstream of the genes for the capsid proteins. Because the existence of this gene product could not be demonstrated at first, this gene was referred to as *agnogene* and its putative 66 amino acids protein as agnoprotein (derived from the Greek "agnosis" meaning without knowledge) (Jay et al., 1981). Later studies confirmed the existence of the SV40 agnoprotein and expression of agnoprotein or the presence of a putative *agnogene* has been described for several other polyomaviruses (Table 1). This review will elaborate on the structure and function of agnoprotein and will discuss the importance of this protein for polyomaviruses.

## Biochemical properties of the polyomavirus agnoprotein

### *Primary structure*

The genomes of the first identified human polyomaviruses BK and JC (BKV or BKPyV and JCV or JCPyV according to the recommendations of the International Committee on Taxonomy of Viruses; (Johne et al., 2011)) contain the *agnogene* and express this protein in infected cells (Rinaldo et al., 1998; Okada et al., 2001; Safak et al., 2001). BKPyV and JCPyV agnoproteins display high similarities with the SV40 agnoprotein, especially in the N-terminal region (Fig. 1). In contrast, none of the recently detected human polyomaviruses KI, WU, MCPyV, HPyV 6, HPyV7, TSPyV, and HPyV9 seem to encode agnoprotein (Allander et al., 2007; Feng et al., 2008; Gaynor et al., 2007; Schowalter et al., 2010; Scuda et al., 2011; van der Meijden et al., 2010). Some other mammalian polyomaviruses encode agnoprotein (Table 1). The late region of *Avipolyomavirus* genomes encode a fourth protein that was originally designated as agnoprotein (Johne and Müller, 2001). However, this structural protein is present in the virion and has no functional homology to the agnoprotein of mammalian polyomaviruses and is therefore currently referred to as VP4 (Johne and Müller, 2001).

Two striking features of agnoprotein from the different polyomaviruses are the high variability in length, and their sequence homology (Fig. 1). The length of agnoprotein ranges from 30 amino acids in bat polyomavirus to 118 residues in bovine polyomavirus, while amino acid sequence identity diverges from no to almost 90% identity (Table 2). Interestingly, the agnoproteins of BKPyV and JCPyV are $>50\%$ identical to agnoprotein of the monkey polyomaviruses SV40 and SA12. The close resemblance between the agnoproteins of

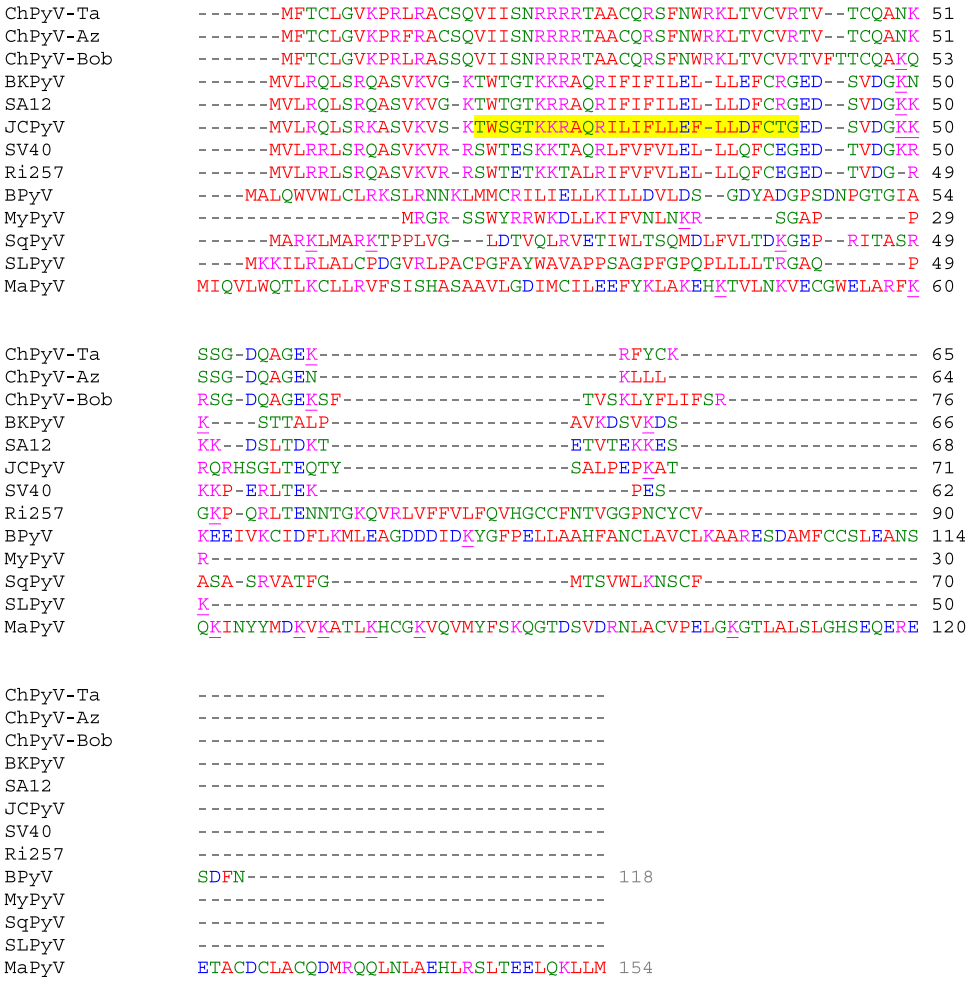


**Fig. 1.** Alignment of the amino acid sequences of the agnoprotein from different polyomaviruses. The single letter amino acid code is used. Red represents small and hydrophobic amino acids (A, F, I, L, M, P, V, W), blue symbolizes acidic amino acids (D, E), magenta corresponds to basic amino acids (K, R), and green stands for hydroxyl, amine and basic amino acids (C, G, H, N, Q, S, T, Y). An asterisk indicates an identical amino acid. The domain required for oligomerization is shaded in yellow. Putative ubiquitination sites are underlined (Tung and Ho, 2008; Lee et al., 2011). The Clustal series of programs were used for multiple alignments (Chenna et al., 2003).

**Table 2**
Amino acid identity (in %) between agnoprotein of different polyomaviruses. The EMBOSS needle pairwise sequence alignment algorithm was used.

| Agno | BKPyV | JCPyV | SV40 | Ri257 | SA12 | ChPyV1 | ChPyV2 | ChPyV3 | SqPyV | SLPyV | BPyV | MaPyV | MyPyV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BKPyV | 100 | 60 | 59.1 | 37.8 | 68.4 | 3.9 | 6.7 | 7.2 | 12.4 | 4.9 | 12.4 | 11.9 | 12.1 |
| JCPyV | | 100 | 52.1 | 38.2 | 66.2 | 9.3 | 5.5 | 6.7 | 8.7 | 4.6 | 14.7 | 8.1 | 4.7 |
| SV40 | | | 100 | 57.1 | 63.2 | 10.0 | 9.7 | 12.7 | 12.5 | 5.1 | 12.3 | 9.3 | 12.9 |
| Ri257 | | | | 100 | 44.1 | 12.4 | 6.6 | 9.3 | 18.2 | 3.9 | 16.3 | 2.8 | 13.2 |
| SA12 | | | | | 100 | 10.1 | 7.5 | 10.1 | 15.7 | 4.8 | 13.3 | 12.3 | 11.1 |
| ChPyV1 | | | | | | 100 | 75.0 | 71.1 | 10.4 | 1.7 | 4.4 | 5.3 | 5.2 |
| ChPyV2 | | | | | | | 100 | 89.2 | 12.0 | 1.9 | 4.4 | 10.3 | 13.8 |
| ChPyV3 | | | | | | | | 100 | 12.1 | 14.3 | 5.0 | 3.2 | 14.1 |
| SqPyV | | | | | | | | | 100 | 12.3 | 2.2 | 4.7 | 11.8 |
| SLPyV | | | | | | | | | | 100 | 0 | 1.0 | 13.3 |
| BPyV | | | | | | | | | | | 100 | 18.3 | 1.4 |
| MaPyV | | | | | | | | | | | | 100 | 1.8 |
| MyPyV | | | | | | | | | | | | | 100 |

these human and non-human PyV may suggest that these viruses from divergence from a common ancestor.

Aside from squirrel, bovine and mastomys PyV which have several methionines, all other agnoproteins contain just one methionine that functions as the start codon. Other sequence peculiarities comprise a statistical overrepresentation of the basic amino acids Arg and Lys, the hydrophobic residues Leu and Val and the phosphoacceptor sites Ser and Thr. On the other hand, His residues are mostly absent, as is the phosphoacceptor residue Tyr. The biological implications of these amino acid compositions are not clear (Fig. S1).

Several substitutions in agnoprotein of BKPyV and JCPyV have been identified in clinical isolates, while few or no data are available for the other PyV (Table S1). Most changes are single amino acid substitutions, but some isolates display deletions and insertions. The majority of the mutations seem to concentrate in the central and C-terminal regions (Table S1). It remains to be

examined whether these mutations affect the structural and functional properties of agnoprotein.

### *Post-translational modification sites and functional implications*

#### *Phosphorylation and stability*

The agnoproteins of BKPyV (Rinaldo et al., 1998; Johannessen et al., 2008), JCPyV (Okada et al., 2001; Sariyer et al., 2006) and SV40 (Jackson and Chalkley, 1981) are phosphoproteins. For BKV and JCV, phosphoacceptor sites have been mapped to Ser-7, Ser-11, and Thr-21 (Johannessen et al., 2008; Sariyer et al., 2006). Ser-7 and Ser-11 are also conserved in SV40, SV40 Ri257 and SA12 agnoprotein, while Thr-21 is conserved in SA12 agnoprotein, and replaced by Ser-21 in SV40 agnoprotein. BKPyV, JCPyV, SV40, SV40 Ri257 and SA12 contain a conserved Ser or Thr at position 45 (44 in SV40 Ri257), but the in vivo functionality of this phosphoacceptor site is not known. Agnoproteins encoded by other non-human polyomaviruses also contain putative phosphoacceptor sites, e.g., agnoproteins of the chimpanzee polyomavirus strains Ta, Az and Bob comprise conserved Thr-3, Ser-15, Ser-20, Thr-26, Ser-32, Thr-44, Thr-46/48, and Ser-53/55 (Table 3). It remains to be confirmed whether these represent genuine phosphoacceptor sites and the possible biological implications of phosphorylation of these sites are unknown. Tyrosine, another phoshoacceptor site, is absent (ChPyV-Az, BKPyV, SV40, SA12, SqPyV) or rare (1 residue in ChPyV-Ta, ChPyV-Bob, JCPyV, SV40 Ri257, SLPyV, MyPyV and 4 in MaPyV) in the agnoproteins of all known polyomaviruses (Fig. 1). The reason for this is unknown.

Single and double replacement of Thr-21 and Ser-7 plus Ser-11 with Ala in JCPyV agnoprotein generated mutant viruses that failed to propagate (Sariyer et al., 2006). Replacing Ser-11 with either non-phosphorylatable alanine or phospho-mimicking aspartic acid in BKPyV agnoprotein reduced the ability of these mutants to propagate compared to wild-type virus (Johannessen et al., 2008). JCPyV DNA encoding an agnoprotein in which Ser-7 was replaced with Leu or Ser-11 with Phe has been amplified from single patients in Russia and Ethiopia, respectively (GenBank accession numbers BAB93095.1 and BAE02911.1, respectively). A BKPyV isolate with deletion of residues 9–12 (removing Ser-11) has also been reported (Chen et al., 2004). These findings may indicate that virus genomes carrying mutations affecting these phosphoacceptor sites can still sustain viral replication. However, since the *agnogene* sequences were amplified by PCR, it is not known whether actual infectious virus particles were present in the human samples or whether viral DNA was released from dead cells. Alternatively, several viral strains may circulate in the same patient and strains that express wild-type agnoprotein can rescue strains expressing the phosphoagnoprotein mutants. Indeed, it has been shown that agnoprotein in *trans* can rescue propagation of SV40 and BKPyV strains that do not express agnoproteins in cell culture (Carswell et al., 1986; Myhre et al., 2010).

Studies with non-phosphorylatable agnoprotein mutants demonstrated that phosphorylation affects the stability of agnoprotein. Expression levels of single T21A, double S7A/S11A, and triple S7A/S11A/T21A mutant proteins were higher than those of wild-type JCPyV agnoprotein, whereas BKPyV S11A agnoprotein was less stable than wild-type or the phosphomimicking S11D mutant protein (Johannessen et al., 2008; Sariyer et al., 2006). Results from both studies suggest that agnoprotein degradation is modulated by residue-specific phosphorylation. Sariyer et al. did not examine the stability of JCPyV S11A agnoprotein, but phosphorylation of Ser-11 may increase the stability of agnoprotein, while phosphorylation of Ser-7 and Thr-21 may trigger degradation. Phosphorylation of a substrate can stimulate its ubiquitination and subsequent degradation by the proteasome (Hunter, 2007). It is not known whether phosphorylation of agnoprotein leads to its ubiquitination and subsequent degradation, but *in silico* prediction algorithms of ubiquitin-conjugated sites show that agnoproteins of different polyomaviruses contain putative lysine ubiquitin acceptor residues (Table 3, (Lee et al., 2011; Tung and Ho, 2008)).

Phosphorylation may also influence the subcellular location of agnoprotein (see "Subcellular localization of agnoprotein" section).

#### *Other post-translational modifications*

Apart from phosphorylation, no other post-translational modifications have been demonstrated to occur for agnoprotein. Putative sumoylation, acetylation and methylation sites are present in agnoprotein (Table 3, (Kiemer et al., 2005; Li et al., 2006; Ren et al., 2009; Shao et al., 2009)), but whether these modifications occur in vivo remains to be proven.

### *Secondary structure*

The central part of BKPyV, JCPyV, SV40 and SA12 agnoprotein contains a stretch of hydrophobic residues and is predicted to form an α-helix (Fig. S2). The crystal structure of agnoprotein has

**Table 3**
Biochemical properties of polyomavirus agnoprotein.

| | BKPyV | JCPyV | SV40 | SV40Ri257 | SA12 | ChPyV-Az | ChPyV-Ta | ChPyV-Bob | SqPyV | BPyV | SLPyV | MyPV | MaPyV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| aa | 66 | 71 | 61 | 118 | 68 | 64 | 65 | 76 | 70 | 114 | 50 | 30 | 154 |
| kD | 7.4 | 8.1 | 7.3 | 10.3 | 7.8 | 7.3 | 7.5 | 8.8 | 7.8 | 13.2 | 5.3 | 3.7 | 17.6 |
| pI | 10.0 | 10.1 | 10.1 | 9.7 | 9.8 | 11.2 | 10.9 | 11.6 | 10.4 | 4.6 | 10.1 | 12.0 | 7.6 |
| DNA binding | | | ss, ds | | | | | | | | | | |
| Phosphorylation | S7,11,52,62,66 T21 | S7,11,15,55 T21 Y62 | S7,11,17,21 T19,45,57,62 | S7,11,17, 62 T19,21,45,56 | | | | | | | | | |
| Acetylation | K60,64 | K9,13,69 | K13,59 | K13 | K52,65,66 | | K65 | K62 | K4 | | K2,3,50 | | K105 |
| Methylation | K22,R41 | K9,22 | K51 | | K50,51 | | K8,60 | K8,62 | | K30 | | | |
| ubiquitination | K13,22,64 | K9,13,69 | K13,49,59 | | | | | | | | | | |
| Glycosylation | S7,53,62,66 | S7,11,15, T61,71 | S7,17,62 T62 | | | | | | | | | | |
| Glycation | 22 | 9,22,23,49 | 22,52 | | | | | | | | | | |
| Subcellular localization | C, PN > N[a] | C, PN > N | C, PN > N | NT[b] | NT[b] | NT[b] | NT[b] | NT[b] | NT[b] | NT[b] | NT[b] | NT[b] | NT[b] |
| NES | 31–37 | 30–37 | 33–37 | 33–37 | 25–36 | | | | | 25–36 | | | |

The ExPASy bioinformatics program was used to calculate the theoretical pI and molecular mass values (Bjellqvist et al., 1994).

[a] C=cytoplasmic, PN=perinuclear, N=nuclear; > means more prominent.

[b] NT=not tested.

not been solved, but a computer-generated structural model of JCPyV agnoprotein predicts that the N-terminal and central regions (residues 17–43) of agnoproteins adopt an α-helical structure (Saribas et al., in press). The SV40 Ri257 variant has a hydrophobic region in the N-terminal part of its agnoprotein (this part is conserved in SV40), while the unique C-terminal region contains an additional hydrophobic region (Fig. S2). Agnoproteins of other PyV lack a pronounced hydrophobic region. Circular dichroism studies using full-length BKPyV agnoprotein have confirmed that the central part of agnoprotein adopts a helical structure (N.G., unpublished results).

JCPyV, BKPyV and SV40 agnoproteins form stable dimers and oligomers (Suzuki et al., 2010; Saribas et al., in press). Saribas and colleagues demonstrated that the amino acids spanning from 17 to 42 are absolutely required for JCPyV agnoprotein oligmerization (Saribas et al., in press). This region is extremely well-conserved in BKPyV, SV40, SA12 agnoprotein, suggesting that this domain may also be involved in oligomerization of these proteins. Interestingly, viral particles were efficiently released from SVG-A cells transfected with JCPyV DNA lacking the nucleotides encoding amino acids 17–42 of agnoprotein, but were mostly deficient in DNA content (Saribas et al., in press). SV40 mutants with deletions in agnoprotein removing this oligomerization domain replicated less efficiently than wild-type virus (Barkan and Mertz, 1981). These results suggest that agnoprotein dimerization/oligomerization is implicated in viral encapsidation of JCPyV DNA. The exact residues necessary for oligomerization have not been mapped, but JCPyV, BKPyV and SV40 strains with specific amino acid substitutions in the 17–42 region have been isolated (see Table S1), indicating that these residues may not be involved in agnoprotein oligomerization. It is, however, likely that several amino acids are required such that one single substitution will not abrogate agnoprotein oligomerization.

## Subcellular localization of agnoprotein

### *Amino acid motifs that may control nucleo-cytoplasmic localization of agnoprotein*

The small size of agnoproteins (varying between 30–154 residues with estimated molecular mass ranging from 3.7 to 17.6 kD) predicts that agnoprotein can shuttle between the cytoplasm and the nucleus (Mattaj and Englmeier, 1998). Experimental studies demonstrated that agnoprotein resides mainly in the cytoplasm and perinuclear region, although a minor fraction is also detectable in the nucleus (Carswell et al., 1986; Endo et al., 2003; Johannessen et al., 2008; Myhre et al., 2010; Nomura et al., 1983; Okada et al., 2001). The agnoproteins of BKPyV, JCPyV, SV40, and SA12 contain a putative Leu-rich nuclear export signal (NES) in the central region of the protein and a stretch of basic amino acids (R or K) which may represent a classic nuclear localization signal (NLS) (Fig. 1, (Fu et al., 2011; Lange et al., 2007)). Chimpanzee agnoprotein possesses a bipartite motif ($RRRRX_5RX_4RK$) with two stretches of basic amino acids, while the N-terminal regions of MyPyV, squirrel PyV and sea lion PyV agnoproteins are R/K-rich. A putative NES motif is lacking in agnoproteins of the latter. Studies with agnoprotein mutants in the NES are required to confirm the functionality of this motif.

Phosphorylation may regulate subcellular localization of a protein through conformational changes that expose a NES or NLS, or through provoking the binding to or the release from an interacting partner (Whitmarsh and Davis, 2000). One report indicates that the subcellular distribution of agnoprotein may be regulated in a phosphorylation-dependent manner. Okada and colleagues found that treatment of cells with H89, a cAMP-dependent protein kinase/protein kinase A (PKA) and mitogen-activated protein kinase MSK1 inhibitor (Davies et al., 2000), retained JCPyV agnoprotein in the nucleus (Okada et al., 2001). It is not known whether agnoprotein is a *bona fide* PKA/MSK1 substrate, and if so, which residues are phosphoacceptor sites that may be involved in regulating the subcellular residence of agnoprotein.

### *Cytoplasmic anchorage*

Cytoplasmic anchorage to the cytoskeleton may also explain the subcellular distribution of agnoprotein because JCPyV agnoprotein colocalizes with tubulin (Endo et al., 2003). A recent study showed that JCPyV agnoprotein also localizes to the plasma membrane and in the endoplasmatic reticulum (Suzuki et al., 2010). Its association with the plasma membrane probably relates to agnoprotein acting as a viroporin.

### *Lipid droplets*

The Hirsch group showed that BKPyV agnoprotein can be detected in lipid droplets and that this co-localization requires residues 20–42 (Unterstab et al., 2010). Lipid droplets are phospholipid monolayers derived from inside the lipid bilayer of the ER. On the monolayers of such droplets, proteins (e.g., caveolin-1) accumulate which lend structure and attribute a particular function to the droplet (Brown, 2001). The agnoproteins of BKPyV, SV40 and SA12 all contain a degenerated caveolin-1 consensus motif $\Phi XXXX\Phi XX\Phi$ or $\Phi X\Phi XXXX\Phi$ (where $\Phi$ represents *W*, *F* and *Y*) **F**X**F**XXXXXXX**F**, while JCPyV agnoprotein contains the motif LX**F**XXX**F**XXX**F** (Fig. 1). Binding of these agnoproteins to caveolin has not been reported, but the motif overlaps with the region required for co-localization of BKPyV agnoprotein with lipid droplets (Unterstab et al., 2010). α-soluble N-ethylmaleimide-sensitive fusion attachment protein (α-SNAP), another interaction partner of BKPyV agnoprotein, is found in lipids and may explain the presence of agnoprotein in lipid droplets (Johannessen et al., 2011).

## Agnoprotein interaction partners

The agnoproteins of BKPyV and JCPyV have been shown to interact with other viral and cellular proteins (Table 4). Interaction partners for agnoprotein from other PyV have not yet been identified and the low sequence identity between agnoproteins of different PyV suggests that similar interactions as those found for JCPyV and BKPyV agnoproteins are unlikely.

### *LT-ag, st-ag and PP2A*

JCVPyV agnoprotein can interact with LT-ag, st-ag and PP2A (Table 4). The physical interaction of JCPyV agnoprotein with LT-ag downregulates both LT-ag mediated viral gene expression and DNA replication (Safak et al., 2001). At the late stage of viral infection, expression and replication of the viral genome has to cease in order to prevent disproportionate production of genome copies and capsid proteins and to optimize efficient assembly (Safak et al., 2001). JCPyV agnoprotein was shown to interact directly with PP2A, which required agnoprotein residues 18–36 (Sariyer et al., 2008). PP2A dephosphorylated PKC-phosphorylated agnoprotein. The authors demonstrated that JCPyV agnoprotein also binds st-ag, but the agnoprotein domain necessary for interaction with st-ag was not mapped, implying that is not known whether PP2A and st-ag can bind their common substrate simultaneously or in a mutually exclusive manner. However,

**Table 4**
Identified interaction partners for agnoprotein.

| PyV | Interaction partner | Functional implication | Interacting domain partner | Interacting domain agnoprotein | Reference |
|---|---|---|---|---|---|
| BKPyV | α-SNAP | Interference secretion | Not determined | N-terminus | (Johannessen et al., 2011) |
| | PCNA | Inhibition DNA synthesis | | | our unpublished results |
| | p50[a] | Unknown | Unknown | Unknown | (Rinaldo et al., 1998) |
| | p75 | Unknown | Unknown | Unknown | (Rinaldo et al., 1998) |
| | p100 | Unknown | Unknown | Unknown | (Rinaldo et al., 1998) |
| JCPyV | FEZ1 | Facilitates viral release | Coiled-coil domain | Not determined | (Suzuki et al., 2005) |
| | HP1α | Nuclear egress virions | Not determined | N-terminus | (Okada et al., 2005) |
| | Ku70 | Aberrant DNA repair | Not determined | N-terminus | (Darbinyan et al., 2004) |
| | Tumor suppressor p53 | Dysregulation cell cycle | Not determined | N-terminus (aa 1–36) | (Darbinyan et al., 2002) |
| | p52[a] | Unknown | Unknown | Unknown | (Endo et al., 2003) |
| | p103 | Unknown | Unknown | Unknown | (Endo et al., 2003) |
| | p112 | Unknown | Unknown | Unknown | (Endo et al., 2003) |
| | p158 | Unknown | Unknown | Unknown | (Endo et al., 2003) |
| | HIV-1 Tat | Inhibition HIV-1 gene expression | Residues 1–50 | Residues 18–54 | (Kaniowska et al., 2006) |
| | Tubulin | Unknown | Unknown | Unknown | (Endo et al., 2003; Suzuki et al., 2005) |
| | YB-1 | Altered gene expression | C-terminal half | Residues 18–36 | (Safak et al., 2002) |
| | LT-ag | Repression viral transcription and DNA replication | Central domain | N-terminus | (Safak et al., 2001) |
| | st-ag | Disrupt PP2A:st-ag interaction? | C-terminus | N-terminus | (Sariyer et al., 2008) |
| | PP2A | Dephosphorylation of agnoprotein | Not determined | Residues 18–36 | (Sariyer et al., 2008) |

[a] JCPyV binds tubulin which has an estimated molecular mass of 54 kD. The p50 and p52 proteins known to interact with, respectively, BKPyV and JCPyV agnoprotein may therefore represent tubulin.

dephosphorylation of agnoprotein by PP2A was a result of st-ag-mediated inhibition PP2A, rather than st-ag preventing PP2A to bind to agnoprotein. JCPyV deficient in st-ag or encoding st-ag lacking its unique C-terminal domain (and thus the PP2A binding region) are replication incompetent (Sariyer et al., 2008; Bollag et al., 2010). JCPyV genomes encoding agnoproteins in which the PKC phosphoacceptor sites Ser-7, Ser-11, and Thr-21 were substituted by non-phosphorylatable Ala were also severely impaired with respect to replication (Sariyer et al., 2006, 2008). siRNA-mediated depletion of PP2A strongly reduced JCPyV replication in SVG-A cells and reduced agnoprotein levels (Sariyer et al., 2008). All these observations illustrate the biological importance of the PP2A:st-ag:agnoprotein link for the viral life cycle.

The agnoprotein phosphorylation state was shown to modulate the function and stability of agnoprotein, and to affect the propagation efficiency of the virus (Johannessen et al., 2008; Sariyer et al., 2006). Depending on the relative concentration of st-ag, PP2A, and agnoprotein, different complexes can be envisaged during the viral life cycle. This will affect the phosphorylation states of agnoprotein in a time-dependent manner. During the early phase of infection, there will be an excess of st-ag compared to agnoprotein and hence st-ag will predominantly bind and inactivate PP2A. This will impede PP2A-mediated dephosphorylation of agnoprotein. As the viral life cycle of PyV progresses, more agnoprotein will be synthesized, such that the likelihood to form agnoprotein:st-ag complexes is increased. This will prevent st-ag from binding and inactivating PP2A. As a result, PP2A may dephosphorylate agnoprotein. Strikingly, viral proteins of both RNA and DNA viruses have been shown to physically interact with PP2A and interfere with cell proliferation and survival. While HIV-1 Vpr, Adenovirus E4orf, and hepatitis C virus NS5A proteins induce cell cycle arrest and apoptosis by binding PP2A (Georgopoulou et al., 2006; Li et al., 2009; Godet et al., 2010), PP2A inactivation by Epstein-Barr virus EBNA-LP protects against apoptosis (Garibal et al., 2007). HTLV-I Tax and HPV E7 protein also binds and inactivates PP2A, but the biological consequences for the viral life cycle remain unknown (Pim et al., 2005; Hong et al., 2007).

*Heterochromatin protein 1α (HP1α)*

Besides viral proteins, agnoprotein may also recruit cellular proteins in its endeavor to optimize the host cell for viral production. One of these is heterochromatin protein 1α (HP1α). The nuclear envelope is composed of an inner and outer lipid layer spiked with several proteins. The inner nuclear membrane is connected to the nuclear lamina, which is composed of lamins A, B1, B2 and C. The lamin B receptor is a part of the inner nuclear membrane and binds HP1α. This interaction is thought to contribute to the reassembly of the nuclear envelope after cell division (Stewart et al., 2007). The N-terminal region of JCPyV agnoprotein can associate with HP1α, resulting in dissociation of HP1α from the lamin B receptor. This alters the nuclear envelope and facilitates nuclear release of progeny virus particles (Okada et al., 2005). Although the N-terminal region is 90% identical with BKPyV agnoprotein, no co-localization with HP1α in primary human renal tubule epithelial cells was observed (Myhre et al., 2010; Unterstab et al., 2010). This may indicate that the JCPyV agnoprotein amino acid residues critical for the interaction with HP1α are not conserved in BKPyV agnoprotein.

*Fasciculation and elongation protein zeta 1 (FEZ1)*

JCPyV agnoprotein was shown to bind to FEZ1, a multifunctional protein involved in neuronal differentiation, microtubule and centrosomal organization, and transport of cargoes (Maturana et al., 2010; Suzuki et al., 2005). This agnoprotein:FEZ1 association induced the dissociation of FEZ1 from microtubules. The authors speculated that FEZ1 prevents virion release and that agnoprotein may prevent this inhibition, thereby promoting the intracellular translocation of viral particles on microtubules (Suzuki et al., 2005). Interestingly, JCPyV agnoprotein co-localizes with the cytoskeletal protein tubulin, which oligomerizes to form microtubules. Although the functional consequences are not understood, the interaction between agnoprotein and tubulin could also support transport of viral particles along microtubules (Endo et al., 2003).

*Ku70*

JCPyV agnoprotein binds directly to the DNA repair enzyme Ku70, an event that inhibits DNA repair and disrupts DNA damage-induced cell cycle arrest. Circumventing cell cycle arrest associated with DNA repair may speed up the viral life cycle, while impairment of DNA repair may contribute to cellular transformation by JCPyV (Darbinyan et al., 2004).

*p53*

JCPyV agnoprotein was shown to bind p53 and this interaction led to higher levels of $p21^{Cip1/Waf-1}$ and accumulation of cells at the G2/M phase. This stalling of the cells in G2/M phase may allow efficient virion assembly (Darbinyan et al., 2002). These studies were, however, performed by ectopic overexpression of agnoprotein in mouse fibroblast NIH3T3 cells in the absence of other JCPyV proteins and may therefore not reflect a *bona fide* infection in the natural host.

*Y-box binding factor-1 (YB-1)*

JCPyV agnoprotein binds the transcription factor YB-1 through its N-terminal region (Safak et al., 2002). While association of JCPyV LT-ag with YB-1 results in synergistic activation of the late promoter, agnoprotein suppresses YB-1 mediated transcription of the early and late viral promoter (Safak et al., 1999, 2002). Hence, agnoprotein operates to shut off viral expression by targeting YB-1. The authors also demonstrated that YB-1, which is predominantly found in the cytoplasm in uninfected SVG-A cells, translocates to the nucleus during viral infection (Safak et al., 2002). Interestingly, the nuclear YB-1 concentration increased as viral life cycle proceeded, and decreased as the infection cycle approached its termination (Safak et al., 2002). These data suggest that agnoprotein may play a role in regulating the subcellular localization of YB-1 since agnoprotein levels also fluctuate during the viral life cycle.

## Functions of agnoprotein

*Regulation of gene expression*

Changes in agnoprotein expression affect the expression of other viral proteins. Deletions in SV40 agnogene, removal of the complete JCPyV *agnogene* or mutation of its start codon (the tetranucleotide CATG spanning the agnoprotein start codon was converted into TCGA) all caused a drop in the levels of VP1 and LT-ag (Barkan et al., 1987; Akan et al., 2006). Akan and co-workers argued that the *agnogene* contains critical *cis*-acting DNA elements to which transcription factors bind and regulate viral gene expression. Hence, deletion of the *agnogene* might remove these binding sites and affect transcription of the LT-ag and VP1 genes. However, as changing the tetranucleotide sequence CATG into TCGA will not destroy as many putative transcription binding sites as deleting the whole *agnogene*, it is possible that agnoprotein itself can affect the expression levels of LT-ag. How the lack of agnoprotein influences the expression of LT-ag is not completely understood, but it could be at the transcriptional level (e.g., agnoprotein interferes with the proteins involved in transcribing the early genes) or at the post-transcriptional level (e.g., agnoprotein affects the stability of LT-ag). Another mechanism by which agnogene DNA sequences can modulate the expression of VP1 is at the post-transcriptional level by acting as an upstream ORF (uORF). Several groups have shown that the synthesis of some proteins can be modulated in part by translation of uORF encoded within the leader region of mRNA (Geballe and Sachs, 2000; Morris and Geballe, 2000). Similarly, the coding sequence of agnoprotein may act as an uORF that controls the translation rate of VP1 and hence the amount of VP1 synthesized in infected cells. Indeed, Three groups independently observed higher intracellular protein levels of VP1 in cells infected with SV40, JCPyV or BKPyV mutants in which the translation initiation codon of agnoprotein was destroyed than in cells challenged with wild-type virus (Johannessen et al., 2008; Sedman et al., 1989; Suzuki et al., 2010). This indicates that the agnoprotein ORF reduces the translation of the downstream ORF VP1.

*Viral DNA replication*

LT-ag is indispensable for replication of the viral genome (Imperiale and Major, 2007). As previously mentioned, the interaction of JCPyV agnoprotein with LT-ag down-regulates viral DNA replication (Safak et al., 2001).

*Viral maturation and release*

Agnoprotein is expressed during later stages of viral infection and it can therefore be assumed that it is involved in completing the viral life cycle by stimulating viral maturation and/or viral release. Although the exact function of agnoprotein remains unresolved, several findings indicate that this protein is implicated in viral maturation and release. First, cells infected with SV40 agnoprotein deficient mutants displayed an abnormal nuclear localization of VP1. Agnoprotein was shown to facilitate the perinuclear-nuclear localization of VP1 (Carswell and Alwine, 1986; Resnick and Shenk, 1986). Transfection of cells with mutant SV40 or JCPyV genomes unable to express agnoprotein due to mutation of their ATG codon results in the release of progeny virions deficient in DNA content (Resnick and Shenk, 1986; Sariyer et al., 2006; Suzuki et al., 2010). Viral early and late protein expression and viral DNA replication are reduced compared to cells transfected with wild-type SV40 or JCPyV DNA. Expression of agnoprotein in *trans* restored viral expression and replication (Myhre et al., 2010; Sariyer et al., 2011). Vero cells transfected with the genome of a mutant BKPyV strain Dunlop that does not express agnoprotein can still produce infectious virus particles, although less efficiently than wild-type virus (Johannessen et al., 2008). On the other hand, Vero cells transfected with recombinant BKPyV Dunlop genome in which the non-coding region was replaced with non-coding regions of isolates from renal transplant patients were unable to produce infectious virions (Myhre et al., 2010). The NCCRs of these clinical BKPyV isolates were rearranged and had large deletions in the 5'end of the *agnogene*. The recombinant viral genomes expressed VP1, but not agnoprotein and after transfection, they produced virus-like particles in the nucleus which were not released from the cell. When released by artificial lysis of the cells these virus-like particles turned out to be non-infectious (Myhre et al., 2010). Deletion of JCPyV agnoprotein still resulted in efficient release of virus-like particles from SVG-A transfected cells (Sariyer et al., 2011), while Suzuki and colleagues, using the same cells, observed a strong decrease in the release of virus particles (Suzuki et al., 2010). The reason why some groups find that cells transfected JCPyV and BKPyV genomes not encoding agnoprotein release virus particles, while other investigators cannot, remains unclear.

Viruses such as poliovirus, rotavirus, HIV-1, HTLV-I, influenzavirus, Coronavirus, Sindbis virus, SARS and Bluetongue all encode proteins that promote release of progeny virions. These proteins, referred to as viroporins, are small (60–120 amino acids) proteins that possess at least one hydrophobic transmembrane domain able to form an amphipatic α-helix (Gonzalez and Carrasco, 2003).

The Sawa group showed that JCPyV agnoprotein acts as a viroporin that enhances viral release. Amino acids 8 (R) and 9 (K) were crucial for viroporin activity (Suzuki et al., 2010). This N-terminal RK motif is conserved in the agnoprotein of squirrel polyomavirus (residues 3 and 4 and residues 8 and 9) and of bovine polyomavirus (residues 11 and 12), but not in the closely related BKPyV, SV40 and SA12 (Fig. 1). Another group showed that SV40 VP4, a protein encoded by an internal open reading frame in the VP2/VP3 coding sequence, acts as a viroporin (Raghava et al., 2011). Both JCPyV and BKPyV have a putative VP4 ORF, but the expression of this protein has not been demonstrated. The other human polyomaviruses that do not express an agnoprotein also lack this VP4 ORF (Van Ghelue et al., in press). In addition to its viroporin function, the viroporins Vpu of HIV-1 and M2 of influenza virus impair the secretory pathway (Henkel et al., 2000; Tokarev and Guatelli, 2011). Similarly, BKPyV agnoprotein also interferes with secretion through its interaction with α-SNAP (Johannessen et al., 2011).

### *Interference with secretion*

Studies by our group have shown that agnoprotein binds to α-SNAP, a protein involved in secretion (Johannessen et al., 2011). Overexpression of agnoprotein inhibited secretion of the VSVG-EFGP reporter, suggesting an inhibitory role of agnoprotein in secretion. The biological importance is not yet understood, but agnoprotein may interfere with membrane integrity, prevent secretion of e.g., immunomodulators and viral antigens, or prevent premature release of incomplete virus particles.

## Pathogenic potential of agnoprotein

JCPyV agnoprotein possesses oncogenic properties in cell culture because it can interfere with apoptosis and cell cycle control through targeting p53 and $p21^{CIP-1/WAF-1}$, and perturb the activity of Ku70, Ku80, p53 and FEZ1 which are involved the DNA repair enzymes (Darbinyan et al., 2002, 2004, 2007; Suzuki et al., 2005). Agnoprotein has also been detected in several cancers, including colon cancer, primary CNS lymphoma, esophageal cancer and medulloblastoma. Some cases of medulloblastoma had no detectable LT-ag expression, reinforcing the assumption that agnoprotein may contribute to tumorigenesis (reviewed in Moens et al., 2007; Del Valle and Khalili, 2010). However, a causal role of JCPyV in cancer remains unclear (Maginnis and Atwood, 2009).

It was recently reported that agnoprotein plays a role in JCPyV-induced dysregulation of chemokine production by oligodendrocytes (Merabova et al., 2011). Stable expression of agnoprotein in the rat oligodendrocyte cell line CG4-OI compromised the release of chemokine CXCL5/LIX, and to a lesser extent IL-10, TNF-α, and Fractalkine. Neurons treated with conditioned medium from CG4-OI cells displayed a higher survival rate than cells challenged with medium from agnoprotein-expressing CG4-OI cells. Treatment of neurons with conditioned medium from agnoprotein-expressing CG4-OI cells induced apoptosis and was associated with activation of the p38MAPK and GSK3β pathways, while the ERK1/2 pathway was inhibited (Merabova et al., 2011). The mechanism responsible for reduced CXCL5/LIX levels in medium of agnoprotein-expressing CG4-OI cells was not examined, but may result from agnoprotein's ability to interfere with transcription (Khalili et al., 2005; Johannessen et al., 2008). JCPyV agnoprotein may also compromise secretion of CXCL5/LIX through interference with intracellular transport as was shown for BKPyV agnoprotein through interaction with α-SNAP (Johannessen et al., 2011).

## Agnoprotein: Reason for existence?

It is unclear why not all polyomaviruses encode agnoprotein, but the absence of agnoprotein in several members of the *Polyomaviridae* may indicate that there is little evolutionary selection pressure for the virus to possess this protein. This is further supported by the high diversity of the agnoprotein amino acid sequence between different polyomavirus strains (Table 2) and the many mutations found (Table S1). In fact, it is the least conserved protein in BKPyV strains. Analysis of 161 whole BKPyV genomes showed that the amino acid variation rate was 11.9% for agnoprotein, while it was 8.6% for VP2, 7.1% for VP2, and 4.2% for LT-ag (Luo et al., 2008).

### *Replication site*

The presence of an agnogene in the PyV genome may depend on the site of replication of the virus and the manner in which virus particles are released. The dermotropic MCPyV, HPyV6, HPyV7, and TSPyV do not encode agnoprotein. In accordance with human papillomaviruses, they may infect cells in the basal layer of cutaneous skin. For human papillomaviruses, hair follicles may represent an important site of infection (Doorbar, 2005). Interestingly, MCPyV, HPyV6, HPyV7, and TSPyV have been found in hair follicles (reviewed in Moens et al., 2011). Mature papillomavirus particles are not released until the infected cells reach the upper skin layer (Doorbar, 2005). This may also be the case for these human PyV and therefore these viruses do not depend on the viroporin properties of agnoprotein.

### *Fine-tuning the amount of virions released*

Another function of agnoproten could be to fine-tune the number of virus particles produced and released. Shedding a limited number of infectious virus particles may prevent the destruction of the infected host cell and enable PyV to evade the immune system. This may allow the virus to establish a persistent infection. The agnoproteins of BKPyV, JCPyV and SV40 may function as uORFs that reduce VP1 production, while virally encoded miRNA downregulates LT-ag expression. Both mechanisms allow the virus to control the number of progeny virions produced in an infected cell. The new human polyomaviruses MCPyV, HPyV6 and HPyV7 all lack agnoprotein, but they seem to be continuously shed in high numbers (e.g., $2 \times 10^6$ MCPyV genome equivalents and $5 \times 10^3$ HPyV6 genome equivalents) from the skin of healthy individuals (Schowalter et al., 2010). However, intracellular retention of viral antigens occurs until the infected cells reach the uppermost epithelial layers. Virions released in this way may avoid encountering the immune system. Therefore these viruses do not need agnoprotein to control the amount and the way progeny virus particles are released.

### *Co-infection dependency*

Another possible explanation could be that non-agnoprotein expressing HPyV depend on co-infection of agnoprotein-expressing HPyV to complete their life cycle. Co-infection of humans by several HPyV is not unlikely because seropositivity in the normal population is $>50\%$ for each of the known HPyV and e.g., BKPyV or JCPyV DNA have been detected in KIPyV and WUPyV positive samples (Kean et al., 2009; Moens et al., 2010; Schowalter et al., 2010; Trusch et al., 2012; van der Meijden et al., 2011). Supplementation of agnoprotein in *trans* requires that the same cell is simultaneously infected with a HPyV expressing agnoprotein and a HPyV which lacks *agnogene*.

## Conclusions and perspectives

The role of agnoprotein remains enigmatic in many ways, but abrogation of agnoprotein expression impairs viral propagation (Shenk et al., 1976; Myhre et al., 2010; Sariyer et al., 2011). Despite its small size, agnoprotein seems to be a multifunctional polypeptide implicated in viral transcription, replication, assembly and maturation and release. Agnoprotein accomplishes these tasks either by itself (viroporin), through interaction with other viral proteins (LT-ag) or by associating with cellular proteins. The functional implications of interactions with cellular partners are not completely understood. The expression and function of agnoprotein in PyV other than BKPyV, JCPyV and SV40 has not been addressed. Studies aimed to elaborate the role of these putative agnoproteins in the viral life cycle are necessary. Besides their crucial role in viral replication, BKPyV and JCPyV agnoproteins have been detected in tumors. Moreover, cell culture studies have shown that agnoprotein can affect cell cycle progression and DNA repair (Del Valle and Khalili, 2010; Khalili et al., 2005). Thus, agnoprotein may be an attractive target for viral therapy in malignant and non-malignant (e.g., nephropathy, PML) diseases. Antibodies raised against peptides encompassing the putative agnoprotein of non-HPyV may help to determine whether *agnogene* in these viral genomes is expressed. The low degree of similarity of these agnoproteins with BKPyV and JCPyV agnoprotein may indicate that they interact with non-related cellular proteins and fulfill other functions. Finally, the identification of novel PyV in other species and humans may reveal new members of the *Polyomaviridae* encoding agnoprotein and unveil the co-evolution with their hosts.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.05.024.

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