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The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity

Anne Girod,[1]† Christiane E. Wobus,[2]‡ Zoltán Zádori,[3] Martin Ried,[1] Kristin Leike,[1] Peter Tijssen,[3] Jürgen A. Kleinschmidt[2] and Michael Hallek[1,4,5]

[1] Laboratorium für Molekulare Biologie, Genzentrum, Ludwig-Maximilians-Universität München, Feodor-Lynen-Strasse 25, D-81377 München, Germany

[2] Deutsches Krebsforschungszentrum, Forschungsschwerpunkt Angewandte Tumorvirologie, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

[3] Centre de Microbiologie et Biotechnologie, INRS – Institut Armand-Frappier, Université du Québec, 531 boul. des Prairies, Laval, Quebec, Canada H7V 1B7

[4] Medizinische Klinik III, Klinikum Grosshadern, Ludwig-Maximilians-Universität München, Feodor-Lynen-Strasse 25, D-81377 München, Germany

[5] GSF – National Research Center for Environment and Health, Klinische Kooperationsgruppe Gentherapie, Hämatologikum, Marchioninistrasse 15, D-81377 München, Germany

The unique region of the VP1 protein of parvoviruses was proposed to contain a parvoviral phospholipase A2 (pvPLA2) motif. Here, PLA2 activity is shown in the unique region of adeno-associated virus type 2 (AAV-2) VP1 when expressed as an isolated domain in bacteria. Mutations in this region of the capsid protein strongly reduced the infectivity of mutant virions in comparison to wild-type AAV-2. This correlated with effects on the activity of PLA2. The mutations had no influence on capsid assembly, packaging of viral genomes into particles or binding to and entry into HeLa cells. However, a delayed onset and reduced amount of early gene expression, as measured by Rep immunofluorescence, was observed. These results suggest that pvPLA2 activity is required for a step following perinuclear accumulation of virions but prior to early gene expression.

Author for correspondence: Michael Hallek at Genzentrum, Ludwig-Maximilians-Universität München.
Fax +49 89 7095 6039.
e-mail mhallek@med3.med.uni-muenchen.de

† **Present address:** NascaCell GmbH, Bahnhofstrasse 9-15, D-82327 Tutzing, Germany.

‡ **Present address:** Department of Pathology, Washington University School of Medicine, Box 8118, 660 South Euclid Avenue, St Louis, MO 63110, USA.

Adeno-associated virus type 2 (AAV-2) is a defective human parvovirus that requires the presence of a helper virus for productive infection (Berns, 1990). The linear single-stranded DNA genome of 4679 nucleotides is packaged into an icosahedral particle of 20–24 nm in diameter. The capsid consists of three structural proteins, VP1, VP2 and VP3, in a ratio of 1:1:20, which are expressed from the same open reading frame by using alternative splicing and an atypical start codon (reviewed by Berns & Giraud, 1996; Rabinowitz & Samulski, 2000). Capsid assembly can occur independently of VP1 (Ruffing *et al.*, 1992). *Lip* mutants, which contain mutations in the unique part of VP1 (VP1up), synthesize normal amounts of replicating-form DNA and capsid proteins but give lower yields of infectious virus particles (Hermonat *et al.*, 1984; Tratschin *et al.*, 1984). However, the function of this region of VP1 during the AAV-2 life cycle remains unknown at the molecular level.

Recently, a conserved phospholipase A2 (PLA2) motif, resembling the catalytic motif of secreted PLA2 (sPLA2), was identified by sequence alignment in VP1up of most parvoviruses, including AAV-2 (Zádori *et al.*, 2001). Mutations of critical amino acid residues in the putative parvoviral PLA2 (pvPLA2) of porcine parvovirus (PPV) resulted in strongly reduced PLA2 activity and virus infectivity (Zádori *et al.*, 2001). Phospholipases are enzymes that hydrolyse phospholipids to generate free fatty acids and lysophospholipids (reviewed by Murakami *et al.*, 1997; Balsinde *et al.*, 1999). They are classified according to the bond cleaved in a phospholipid. Thus, PLA2 hydrolyses specifically the 2-acyl ester (*sn*-2) bond of phospholipid substrates to generate lysophospholipids and free fatty acids. PLA2s are found in prokaryotes, protists,

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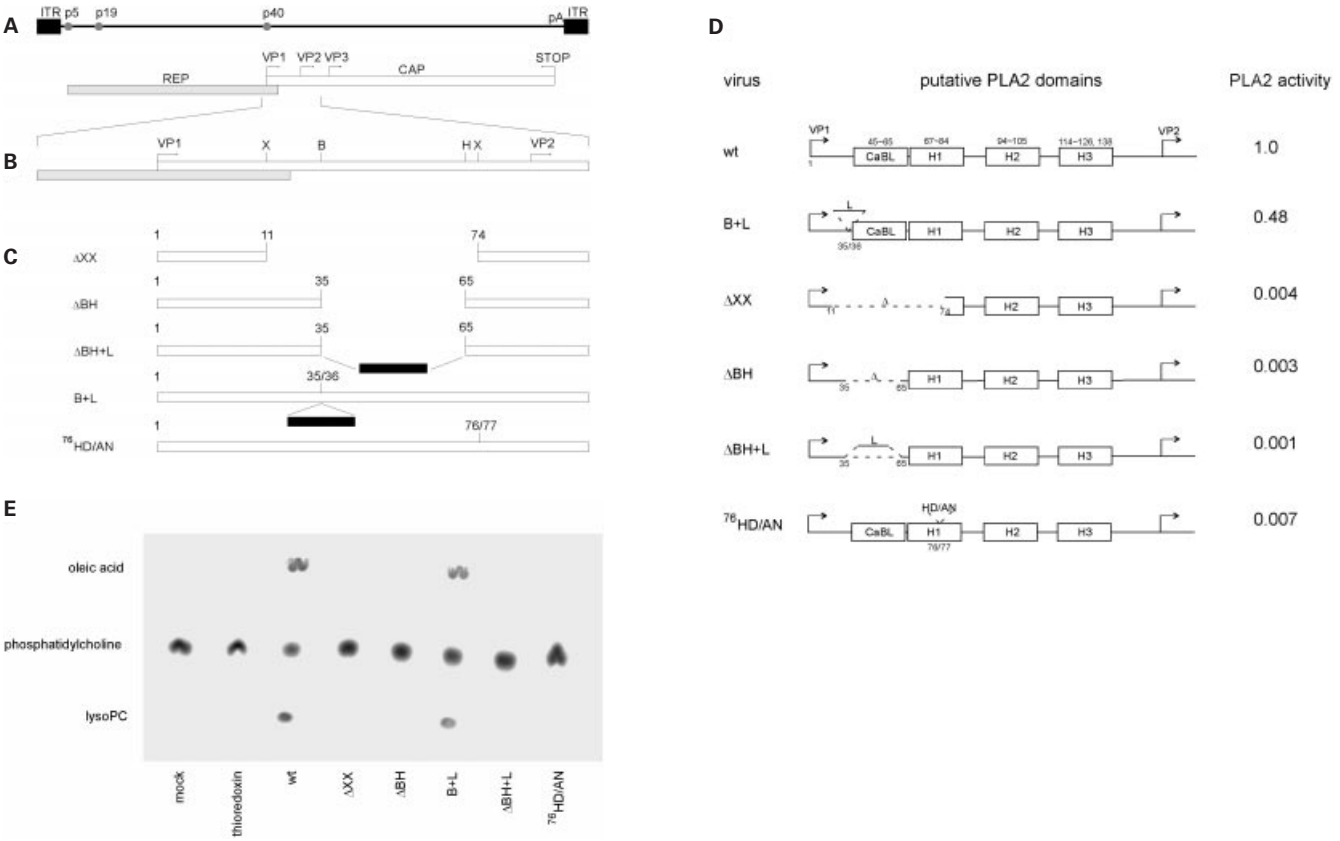


Fig. 1. Schematic representation of constructs containing mutations in the unique region of the AAV-2 VP1 capsid protein. (A) Genetic map of the wt AAV-2 DNA genome. Black boxes represent the inverted terminal repeats (ITR), other boxes indicate the *rep* and *cap* genes. The positions of the p5, p19 and p40 viral promoters, the polyadenylation signal (pA) and the initiation and stop codons of the capsid viral proteins (VP1, VP2 and VP3) are indicated. (B) Enlargement of the unique region of VP1. Restriction endonuclease sites of interest are designated as follows: X, *Xho*I; B, *Bsr*BI; H, *Hinc*II. (C) Schematic representation of the mutants used in this study. Numbers indicate the position of amino acid residues that define each deletion or insertion site, with amino acid 1 being the initiation codon of VP1. Thick black lines represent the 14-mer L14 peptide inserted at the *Bsr*BI restriction site. (D) PLA2 activity of AAV-2 wt and VP1 mutants. Schematic representation of predicted secondary structures inside the putative PLA2 domain in wt AAV-2 and different VP1 mutants, including their respective PLA2 activity. The VP1 unique portion of the AAV-2 wt genome starts at amino acid position 1. Arrows indicate start positions of VP1 and VP2. Boxes denote domains of sPLA2s present in each mutant; CaBL: calcium-binding loop; H1, H2 and H3, helixes 1, 2 and 3, respectively. Deletions (Δ), insertions of the 14-mer L14 peptide (L) and amino acid exchanges (HD to AN) are indicated by dashed lines and corresponding amino acid positions. The PLA2 activity representing the relative specific activity of each VP1up protein is based on the amount of protein required to hydrolyse 50% substrate in the mixed micelles assay. It is expressed as nmol phospholipid hydrolysed per ng enzyme. (E) A representative thin layer chromatography of VP1 mutants. Thioredoxin was used as a control. lysoPC, Lysophosphatidylcholine.

animals and plants and vary greatly in both size and structure. They are divided into three main types based on their biological properties: sPLA2, cytosolic Ca^{2+}-dependent PLA2 and intracellular Ca^{2+}-independent PLA2, resulting in many diverse functions (Dennis, 1997; Dessen, 2000).

In order to characterize the function of VP1up in the AAV-2 life cycle, we initially constructed AAV-2 deletion and insertion mutants maintaining the approximate length of VP1. Later, a double point mutation in the proposed PLA2 catalytic centre was added to our analysis (Fig. 1A–C). AAV-2 mutant constructs were generated using plasmid pUC-AV2 (Girod *et al.*, 1999) containing the full-length AAV-2 genome as the template. Mutant ΔXX, constructed for comparison (Hermonat *et al.*, 1984; Tratschin *et al.*, 1984), and mutant ΔBH were generated by removing the *Xho*I–*Xho*I (186 bp) and the *Bsr*BI–*Hinc*II (87 bp) fragments of the *cap* gene, respectively. The remaining mutants were generated using the ExSite PCR-based Site-Directed Mutagenesis kit (Stratagene), as described previously (Girod *et al.*, 1999). Mutant B + L was constructed by the insertion of the L14-encoding sequence previously used for successful AAV-2 retargeting (Girod *et al.*, 1999) at the *Bsr*BI restriction site of VP1up using primers K-ΔBH + L14 (5′CTCTGCGGGCTTTGGTGGTGGTGGGCCAGGTTT3′) and L-B + L14 (5′ CAAGCCGGCACTTTTGCCCTCCGCG-GTGATAATCCACAAGGACGGCATAAGGACGACA-GCAGGGGTCTT 3′). Mutant ΔBH + L contains a concomitant deletion of the *Bsr*BI–*Hinc*II fragment and was constructed using primers K-ΔBH + L14 and L-ΔBH + L14 (5′ CAAGC-

Table 1. Virus titres of AAV-2 wt and VP1up mutants

Virus titres are expressed as: genomic, number of AAV-2 genomes per ml; particle, number of AAV-2 particles presenting the conformational A20 epitope; infectious, number of infectious AAV-2 particles containing the viral *rep* gene.

	Virus					
Titre/ml	wt	ΔXX	B + L	ΔBH	ΔBH + L	^{76}HD/AN
Genomic	8×10^{13}	2×10^{13}	3×10^{13}	5×10^{13}	5×10^{13}	1×10^{14}
Particle	6×10^{12}	1×10^{12}	1×10^{12}	2×10^{12}	2×10^{12}	4×10^{12}
Infectious	4×10^{10}	1×10^{5}	8×10^{9}	2×10^{5}	5×10^{7}	2×10^{8}

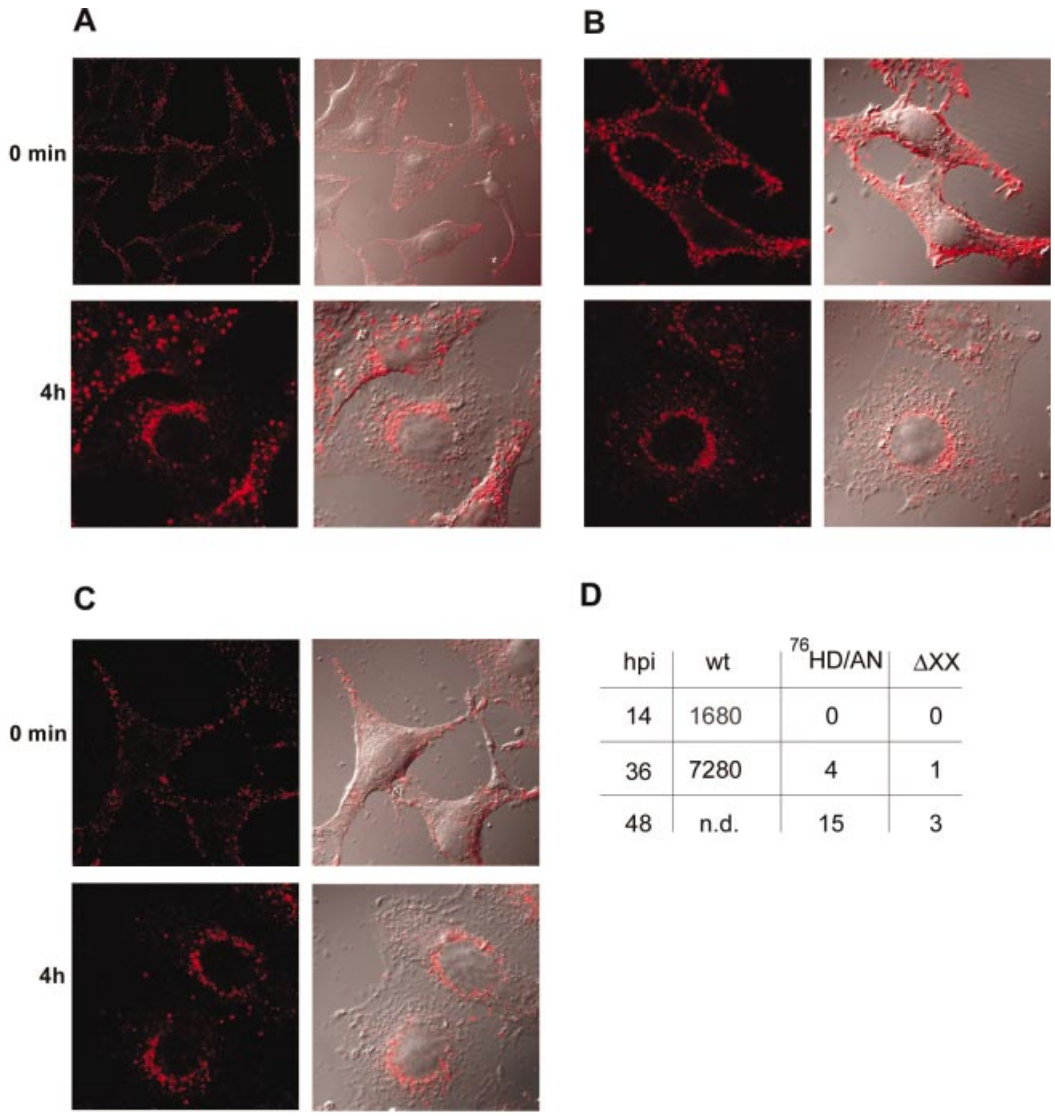
CGGCACTTTTGCCCTCCGCGGTGATAATCCACAAG-GAAACGAGGCAGACGCCGCGGCCCTC 3′). Mutant ^{76}HD/AN was generated by mutating two key residues, ^{76}HD, of the catalytic centre in the putative pvPLA2 to ^{76}AN using primers K-^{76}HD/AN (5′ GCGGCCCTCGA-GGCCAACAAAGCCTACGACCGG 3′) and L-^{76}HD/AN (5′ CCGGTCGTAGGCTTTGTTGGCCTCGAGGGCCGC 3′).

To analyse these mutants, we first tested their ability to assemble and package the viral genome. Wild-type (wt) and mutant AAV-2 virus stocks were generated and the amount of viral genome packaged was determined by dot-blot hybridizations with a random-primed Rep probe, as described previously (Girod *et al*., 1999). This analysis demonstrated that each mutant was efficiently packaged with a titre similar to that of wt AAV-2 (Table 1). In order to assess capsid assembly, VP1 mutants were first tested in an ELISA (Grimm *et al*., 1999) using the monoclonal antibody (mAb) A20 (Wistuba *et al*., 1997). All mutants were able to bind to A20 and produced similar titres of virus particles as wt AAV-2 (Table 1), indicating that the A20 epitope was conserved in all mutants. In addition, Western blot analysis showed the same relative amounts of VP1, VP2 and VP3 (1:1:20) in purified VP1 mutants and wt virions (data not shown). Taken together, these results suggested that capsid assembly and DNA packaging were unaffected in all VP1 mutants.

Second, the infectivity of VP1 mutants was analysed in HeLa cells infected with mutant viruses. Infected cells were assayed for Rep-expression 3 days post-infection (p.i.), as described previously (Girod *et al*., 1999). In contrast to mutant B + L (insertion in VP1up), the infectivity of mutants ΔXX, ΔBH and ΔBH + L, each containing deletions in VP1up, was greatly reduced compared to wt virus (Table 1). Additionally, minor amino acid changes in mutant ^{76}HD/AN lead to a reduction in infectivity of two orders of magnitude when compared to wt virus. Similar reductions in infectivity were observed for VP1up/*Lac*Z recombinant AAV-2 (rAAV-2) vectors, which contain the *Lac*Z reporter gene in a capsid carrying mutations in VP1up (data not shown). A co-infection of VP1up/*Lac*Z rAAV-2 vectors and wt AAV-2 (both at an m.o.i. of 5) did not restore the infectivity of the VP1up/*Lac*Z rAAV-2 vectors (data not shown). This suggested that this loss in infectivity could not be complemented *in trans* by co-infection with wt AAV-2, indicating a requirement for VP1up *in cis*.

To test whether the reduction in infectivity was due to defects in the putative pvPLA2, the enzymatic activity of each mutant was analysed as described previously (Zádori *et al*., 2001). Briefly, VP1up from wt and mutant AAV-2 genomes were amplified using primers AAV2-PLA2-a (5′ GCGGAT-CCATGGCTGCCGATGGTTATCTTC 3′) and AAV2-PLA2-b (5′ GCTCTAGACGTATTAGTTCCCAGACCAG 3′) and the PCR products were cloned into the pBADTBX bacterial expression vector. VP1up proteins, expressed as thioredoxin fusion proteins, were incubated with phosphatidylcholine for 10 min to measure PLA2 activity in the mixed micelles assay (Manjunath *et al*., 1994; Zádori *et al*., 2001). The relative specific PLA2 activity (Fig. 1D) was calculated after quantification of radiolabelled products separated by thin layer chromatography (Fig. 1E). The enzymatic activities of mutants ΔXX, ΔBH, ΔBH + L and ^{76}HD/AN were strongly reduced in comparison to wt but only slightly reduced in the case of mutant B + L. Sequence alignment of putative pvPLA2 and established sPLA2 domains, as presented by Zádori *et al*. (2001), was used to visualize the position of each mutation in relation to the predicted secondary structures within the putative pvPLA2 domain (Fig. 1D). This illustrated that a mutation in the catalytic centre (^{76}HD/AN) or any deletion affecting the calcium-binding loop (CaBL) (ΔXX, ΔBH and ΔBH + L) of the pvPLA2 domain strongly decreased enzyme activity. The slight reduction in enzymatic activity observed for mutant B + L suggested the region upstream of CaBL is not critical for pvPLA2 function.

In order to determine the role of the putative pvPLA2 during the AAV-2 life cycle, early steps of infection, namely binding and entry, were studied. Equal amounts (as judged by A20 ELISA) of purified wt AAV-2, ΔXX (deletion of the CaBL) and ^{76}HD/AN (mutation in the catalytic centre) mutant particles were fluorescently labelled with Cy3, as described by



hpi	wt	^{76}HD/AN	ΔXX
14	1680	0	0
36	7280	4	1
48	n.d.	15	3

Fig. 2. AAV-2 wt and VP1 mutants show similar binding and entry kinetics in HeLa cells but differ in early gene expression. (A)–(C) Binding (0 min) and entry (4 h) of Cy3-labelled wt (A), ^{76}HD/AN (B) and ΔXX (C) AAV-2 particles in HeLa cells. The focal plane was positioned in the middle of the nucleus and images were collected using a 63× lens and zoom settings 1 or 2. Fluorescent images shown on the left were merged with their respective phase contrast images using Adobe PhotoShop (version 5.0) to indicate the position of the nucleus (right panel). (D) Number of Rep-positive cells determined after a Rep-immunofluorescence staining of HeLa cells infected with wt, ^{76}HD/AN or ΔXX AAV-2. n.d., Not determined.

the manufacturer (Amersham), and incubated on HeLa cells (2000 particles per cell) for 30 min on ice. Cells were washed with PBS to remove unbound virus and fixed with 1% paraformaldehyde immediately (0 min) or after 2 or 4 h at 37 °C. Analysis of the distribution of fluorescently labelled particles by confocal microscopy showed that mutant and wt particles bound equally well to cells at 0 min (Fig. 2A–C). In addition, no significant difference in the perinuclear accumulation of the virus particles at 2 and 4 h (Fig. 2A–C; data not shown) was observed between the VP1 mutants and wt AAV-2. Similar results were obtained using A431 cells (data not shown). Taken together, these results indicated neither virus binding to cells nor virus entry and trafficking through the cytoplasm up to the point of accumulation in the nuclear periphery were affected in these mutants.

The next detectable event during a productive AAV-2 infection is early gene expression (Trempe *et al.*, 1987; Redemann *et al.*, 1989; Wistuba *et al.*, 1995). HeLa cells were co-infected with wt, ΔXX or ^{76}HD/AN mutant AAV-2 capsids (200 particles per cell) and adenovirus type 5 (m.o.i. of 2). Immunofluorescence analysis using mAb 76.3, directed against AAV-2 Rep, followed by a rhodamine-labelled sec-

ondary antibody was performed, as described previously (Wistuba *et al*., 1997), and the total amount of Rep-positive cells in each sample was determined (Fig. 2D). In the case of wt AAV-2, Rep-positive cells were detected as early as 14 h p.i. and their number increased until 36 h p.i. In contrast, no detectable signal was observed for both mutants until 36 h p.i. and it had barely increased by 48 h p.i. The time lag and low quantity of Rep expression correlated well with the reduced infectivity of both mutants. The dramatic decrease in gene expression after the removal of the CaBL motif (ΔXX) or mutation of the PLA2 active centre (^{76}HD/AN) suggested that viral PLA2 activity is needed at a step of the virus life cycle between perinuclear accumulation of AAV-2 and early gene expression.

AAV-2 is gaining increased importance as a vector for gene therapy. To continuously improve the vector design, i.e. modifying the immunoreactivity of AAV-2 capsids or targeting to specific cell types, a detailed understanding of functional domains of the AAV-2 capsid proteins is important. In this report, we focused on the unique region of VP1, which contains a PLA2 motif and a corresponding PLA2 activity that is required for the full infectivity of the virus. Analysis of individual steps in the life cycle of different VP1up mutants and wt AAV-2 leads to the following conclusions: (i) mutations in VP1up did not affect DNA replication or packaging but lead to a strong reduction in infectivity (Table 1); (ii) this reduction in virus infectivity correlated with a loss in pvPLA2 activity (Fig. 1); (iii) binding to and entry into cells was unaffected in VP1up mutants (Fig. 2A–C); (iv) however, these mutations showed drastically reduced and delayed Rep expression (Fig. 2D). Taken together, our results suggest the pvPLA2 activity is required for a step in the virus life cycle following perinuclear accumulation of virions but prior to the onset of early gene expression. Similar studies were performed with PPV where mutations in the catalytic centre of pvPLA2 had no effect on virus binding, entry or trafficking to the perinuclear late endosomal/lysosomal compartment, while mutants failed to initiate viral DNA replication in the nucleus (Zádori *et al*., 2001). Both studies suggest that the pvPLA2 activity may be required for endosome exit and viral genome transfer into the nucleus but neither the exact step nor the exact function of the enzymatic activity (i.e. signalling to the nucleus for stimulating early gene expression) during the virus life cycle are known. Furthermore, full pvPLA2 activity might require an activation step. For PPV, isolated particles exhibit pvPLA2 activity only at very high concentrations and a level of enzymatic activity in virions similar to bacterially expressed VP1up is only obtained after alkali denaturation and renaturation of particles (Zádori *et al*., 2001). This points to an activation of the pvPLA2 activity in the endosomal compartment where a partial disintegration of the AAV capsid is likely to occur.

In summary, analysis of AAV-2 capsid mutants in VP1up confirmed the presence of a viral PLA2 activity and underlined the importance of this enzymatic activity in the virus life cycle, namely the timely onset and correct amount of early gene expression. The acronym *lip* used by Hermonat *et al*. (1984) to describe mutants characterized by a 'low infectious particles' phenotype may now also be used to describe the phospho*lip*ase activity contained in VP1up. Further characterization will be needed to determine the molecular events leading to the activation of pvPLA2 as well as enzymatic targets. However, as this study showed, retaining a functional pvPLA2 domain *in cis* in any future modification of the capsid, i.e. for vector targeting, is crucial for virus infectivity. Moreover, an increase in the viral PLA activity might improve virus infectivity and thereby the efficiency of AAV-2 as a vector for gene therapy. We are currently assessing this possibility by mutating pvPLA2 and replacing it with other PLA motifs.

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References

Balsinde, J., Balboa, M. A., Insel, P. A. & Dennis, E. A. (1999). Regulation and inhibition of phospholipase A2. *Annual Review of Pharmacology and Toxicology* **39**, 175–189.

Berns, K. (1990). Parvovirus replication. *Microbiological Reviews* **54**, 316–329.

Berns, K. I. & Giraud, C. (1996). Biology of adeno-associated virus. *Current Topics in Microbiology and Immunology* **218**, 1–23.

Dennis, E. A. (1997). The growing phospholipase A2 superfamily of signal transduction enzymes. *Trends in Biochemical Sciences* **22**, 1–2.

Dessen, A. (2000). Phospholipase A_2 enzymes: structural diversity in lipid messenger metabolism. *Structure with Folding & Design* **8**, R15–R22.

Girod, A., Ried, M., Wobus, C., Lahm, H., Leike, K., Kleinschmidt, J., Deleage, G. & Hallek, M. (1999). Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nature Medicine* **5**, 1052–1056.

Grimm, D., Kern, A., Pawlita, M., Ferrari, F., Samulski, R. & Kleinschmidt, J. (1999). Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. *Gene Therapy* **6**, 1322–1330.

Hermonat, P. L., Labow, M. A., Wright, R., Berns, K. I. & Muzyczka, N. (1984). Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. *Journal of Virology* **51**, 329–339.

Manjunath, P., Soubeyrand, S., Chandonnet, L. & Roberts, K. D. (1994). Major proteins of bovine seminal plasma inhibit phospholipase A2. *Biochemical Journal* **303**, 121–128.

Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K. & Kudo, I. (1997). Regulatory functions of phospholipase A2. *Critical Reviews in Immunology* **17**, 225–283.

Rabinowitz, J. E. & Samulski, R. J. (2000). Building a better vector: the manipulation of AAV virions. *Virology* **278**, 301–308.

Redemann, B. E., Mendelson, E. & Carter, B. J. (1989). Adeno-associated virus rep protein synthesis during productive infection. *Journal of Virology* **63**, 873–882.

Ruffing, M., Zentgraf, H. & Kleinschmidt, J. A. (1992). Assembly of viruslike particles by recombinant structural proteins of adeno-associated virus type 2 in insect cells. *Journal of Virology* **66**, 6922–6930.

Tratschin, J. D., Miller, I. L. & Carter, B. J. (1984). Genetic analysis of adeno-associated virus: properties of deletion mutants constructed *in vitro* and evidence for an adeno-associated virus replication function. *Journal of Virology* **51**, 611–619.

Trempe, J. P., Mendelson, E. & Carter, B. J. (1987). Characterization of adeno-associated virus rep proteins in human cells by antibodies raised against rep expressed in *Escherichia coli*. *Virology* **161**, 18–28.

Wistuba, A., Weger, S., Kern, A. & Kleinschmidt, J. A. (1995). Intermediates of adeno-associated virus type 2 assembly: identification of soluble complexes containing Rep and Cap proteins. *Journal of Virology* **69**, 5311–5319.

Wistuba, A., Kern, A., Weger, S., Grimm, D. & Kleinschmidt, J. A. (1997). Subcellular compartmentalization of adeno-associated virus type 2 assembly. *Journal of Virology* **71**, 1341–1352.

Zádori, Z., Szelei, J., Lacoste, M.-C., Li, Y., Gariépy, S., Raymond, P., Allaire, M., Nabi, I. R. & Tijssen, P. (2001). A viral phospholipase A2 is required for parvovirus infectivity. *Developmental Cell* **1**, 291–302.

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