THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 15, Issue of April 9, pp. 14502–14508, 2004
Printed in U.S.A.

Interfacial Enzymology of Parvovirus Phospholipases A_2*

Received for publication, November 9, 2003, and in revised form, January 7, 2004
Published, JBC Papers in Press, January 15, 2004, DOI 10.1074/jbc.M312630200

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The capsid of parvoviruses proteins were recently shown to contain secreted phospholipase A_2 ($sPLA_2$)-like activity that is required during host cell entry. Parvoviral PLA_2 domains have little sequence identity with $sPLA_2$s and lack disulfide bonds. In the present study, after bacterial expression and purification, the biochemical characterizations of these first PLA_2s identified in viruses have been investigated, and a comparison has been made with other known PLA_2s. The specific activities of three viral PLA_2s differed by 3 orders of magnitude, with porcine parvovirus PLA_2 displaying a specific activity similar to that of the most active $sPLA_2$s (*e.g.* human group IIA) and the human AAV2 and B19 parvoviral enzymes displaying approximately 10^3 lower specific activities (similar to human $sPLA_2$ groups IIE and XIIA). These differences were not caused by weaker Ca^{2+} or interfacial binding. The specific activities of the viral PLA_2s on zwitterionic or anionic phospholipid vesicles were comparable. The viral PLA_2s did not display a preference for unsaturated *versus* saturated *sn*-2 fatty acyl chains and hydrolyzed all major classes of glycerophospholipids except phosphatidylinositol. Incubation of mammalian cells with porcine parvovirus PLA_2 led to the release of arachidonic acid into the culture medium. Interestingly, among nine previously known $sPLA_2$ inhibitors, only a subset showed inhibition of the viral PLA_2s and with weak potency, indicating that the active sites of these new enzymes are structurally distinct from those of $sPLA_2$s. Based on these distinct enzymatic and structural properties, we propose to classify the parvovirus PLA_2s within the PLA_2 superfamily as group XIII enzymes.

Parvoviruses are a family of small DNA viruses comprising two subfamilies, the Parvovirinae that infect vertebrates, including man, and the Densovirinae that infect invertebrates such as insect and shrimp species. These viruses depend, because of their limited genome content, on enzymes and precursors present during the S phase of the cell for their replicative functions and thus show a proclivity for actively dividing cells. Consequently, many parvoviruses, such as the porcine parvovirus (1) (PPV)[1] and the human B19 (2) parvovirus, are fetotropic, resulting in abortions, or have a predisposition to infect other mitosis-active tissues such as transformed cells ("oncolytic property") (3).

These nonenveloped, icosahedral viruses have a diameter of approximately 25 nm and contain a linear, single-stranded DNA genome of approximately 4–6 kb. The structure of several parvovirus capsids has been solved to nearly atomic resolution, whereas the DNA is only partly ordered (4, 5). The capsid is composed of 60 structurally equivalent protein subunits, each containing a β-barrel jelly roll in which some loops between the β-strands intertwine with loops from neighboring subunits and cover the viral surface for specific functions such as recognition of cellular receptors. Approximately 10 out of the 60 capsid proteins have N-terminal extensions that are required for infectivity, in particular for cell entry and DNA delivery to the nucleus (6–8). On the other hand, the C-terminal common structure (50–70 kDa), shared by all structural proteins of the virus, suffices to form capsids and to package DNA. The N-terminal extensions are not visualized in the x-ray structures, suggesting that they are disordered.

Recently, we have identified a phospholipase A_2 (PLA_2) motif in the N-terminal extension ("unique part") of the largest capsid protein, referred to as VP1, in approximately 30 different parvoviruses (8). This domain, or its expressed form, has been coined as VP1up (8). Its sequence similarity with $sPLA_2$s is very weak and is mainly restricted to the catalytic site histidine and aspartate residues and the G*X*G calcium-binding motif. The viral PLA_2 motifs lack cysteines, unlike all other previously characterized $sPLA_2$s. In contrast, multiple disulfide bonds are a hallmark of all nonparvoviral $sPLA_2$s and are used as the basis for their classification. Moreover, viral PLA_2s lack the long loops between the α-helices that contain the active site residues of classical $sPLA_2$s.

Recombinant VP1up produced in *Escherichia coli* was shown to display enzymatic activity toward phosphatidylcholine (8). A number of point mutations in the *E. coli*-expressed VP1up led to a significant decrease in PLA_2 enzymatic activity. These mutations were also introduced into the PPV genome to investigate the role of VP1 PLA_2 activity in the context of the viral cycle. Mutations that decrease enzymatic activity of VP1 (*e.g.*

* This work was supported by National Institutes of Health Grant HL36235 (to M. H. G.) and a grant from the Natural Sciences and Engineering Research Council of Canada (to P. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PPV, porcine parvovirus; AAV2, adeno-associated virus type 2; B19, human parvovirus B19; FABP, rat liver fatty acid-binding protein; PLA_2, phospholipase A_2; $sPLA_2$, secreted PLA_2; POPA, POPC, POPG, POPS, POPI, and POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (monosodium salt), -phosphocholine, -phosphoglycerol, -phosphoserine, -phosphoinositol, and -phosphoethanolamine; PPyrPM, 1-palmitoyl-2-pyrenedecanoyl phosphatidylmethanol; Ni-NTA, nickel-nitrilotriacetic acid; PBS, phosphate-buffered saline; MES, 4-morpholinoethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid.

TABLE I
PCR primers used to make viral PLA_2 expression constructs

	Forward	Reverse
M33-VP1up (2–128)[a]	5′-GC**GGATCC**GCGCCTCCTGCAAAAAGAGC-3′ (restriction site BamHI)	5′-C**TCTAGA**CCCTGGGTGTTTTCTCGGCG-3′ (restriction site XbaI)
PPV-VP1up (2–187)[a]	5′-GC**GGATCC**GCGCCTCCTGCAAAAAGAGC-3′ (restriction site BamHI)	5′-CG**CTCGAG**GAGTTCATTTCCTGTTGCAGAC-3′ (restriction site XhoI)
B19-VP1up (2–237)[a]	5′-CG**GGATCC**AGTAAAGAAAGTGGCAAATGG-3′ (restriction site BamHI)	5′-GC**TCTAGA**GCTTGGGTATTTTTCTGAGGCG-3′ (restriction site XbaI)
AAV-VP1up (1–148)[a]	5′-GC**GGATC**CATGGCTGCCGATGGTTATC-3′ (restriction site BamHI)	5′-GC**TCTAGA**CTTAACAGGTTCCTCAACCAG-3′ (restriction site XbaI)

[a] Amplified region of VP1up contained between the restriction sites of the primers in the amplicons.

10^4-fold) displayed a more than proportional decrease in infectivity. Confocal microscopy and *in situ* hybridization studies showed that PLA_2 knockout mutants of the virus accumulated in the late endosome and lysosome and failed to deliver the viral DNA to the nucleus (8). Moreover, complementation studies indicated that PLA_2 activity must be present in *cis* with the replication-competent DNA (*e.g.* in the same particle) for viral DNA to accumulate and replicate in the nucleus (8, 9).

Surprisingly, the PLA_2 enzymatic activity toward phosphatidylcholine detergent mixed micelles measured using either expressed viral PLA_2 or virus particles varies up to 3 orders of magnitude among different parvoviruses (8). It is not known whether this is due to intrinsic differences in the enzymatic efficiency of the VP1up domain or to other factors. To address this issue and to carry out a more detailed study of the interfacial kinetic and binding properties of parvoviral PLA_2s, we cloned and expressed in *E. coli* the VP1up proteins from the parvoviruses B19, PPV, and AAV2, each of which belong to a different genus of the *Parvovirinae* subfamily. We also studied PLA_2 enzymatic activity of a variant of the unique VP1up from PPV, lacking 58 amino acids from its C terminus (denoted M33) (8) and the sensitivity of the VP1up proteins to compounds that are potent inhibitors of previously characterized $sPLA_2$s. Finally, the ability of the VP1 proteins to liberate fatty acids from mammalian cells was investigated. This investigation could explain whether the protein is able to hydrolyze the phospholipid membrane facilitating the penetration of the virus into the cells.

EXPERIMENTAL PROCEDURES

Materials—The plasmid pBAD/Thio-TOPO vector (Invitrogen), which produces N-terminal thioredoxin fusion proteins with a C-terminal His tag in *E. coli*, was used. Nonradiolabeled and radiolabeled phospholipids were obtained from Avanti Polar Lipids Inc. and Perkin-Elmer Life Sciences, respectively. Recombinant FABP, human group IIA $sPLA_2$, and human group X $sPLA_2$ were prepared as described (10).

Cloning, Expression, and Purification of VP1up Proteins—The pBAD2TEV expression vector was constructed by inserting 5′-aga tcc gaa aac ctg tat ttt cag ggc aga tct gaa ttc gaa ctc gag ctc tct aga gaa aac ctg tat ttt cag ggc cct aga, which contains a TEV protease site ENLYFQG and restriction sites BglII, EcoRI, SfuI, XhoI, SstI, and XbaI, into the pBAD/Thio-TOPO vector using the TOPO (vaccinia virus topoisomerase-based) cloning technology (Invitrogen). The correct orientation of the insert was checked by sequencing. A protein expressed by this vector would contain thioredoxin-TEV-insert-TEV-6His from its N- to its C-terminal positions. The VP1ups shown in Table I were amplified by PCR using the primers with the appropriate restriction sites listed in Table I. The amplicons were cloned into the pBAD2TEV expression vector using BamHI and XbaI sites for M33-VP1up, B19-VP1up, and AAV2-VP1up and BamHI and XhoI sites for PPV-VP1up. The clones were analyzed to confirm their sequences, and the correct clones were used to transform *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene). The bacteria were grown in LB medium at 30 °C and induced with L-arabinose when the A_{600} reached ~0.5. The bacteria were pelleted 4 h after induction by centrifugation (8,000 × *g* for 5 min), and the pellet was lysed at 4 °C by sonication in lysis buffer (35 mM Tris-HCl, pH 8.5, 400 mM NaCl, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml E-64). The lysate was clarified by centrifugation (20,000 × *g* for 5 min) at 4 °C.

The expressed fusion proteins were purified under native conditions on a Ni-NTA column (Qiagen) at 4 °C according to the manufacturer's instructions and dialyzed in 50 mM Tris-HCl, pH 8.0, 200 mM NaCl at 4 °C. Purified fusion proteins were digested overnight at 4 °C with TEV protease (1 unit of TEV/3 μg of protein). PPV-VP1up and M33-VP1up proteins were loaded at 4 °C onto a 5-ml Hi-Trap heparin-Sepharose column (5 ml; Amersham Biosciences) at 1 ml/min. After washing with 3 column volumes of buffer (20 mM Tris-HCl, pH 8.0), a salt gradient of 0–1 M KCl in this buffer was applied at 1 ml/min for 80 min. Fractions of 1 ml were collected. The VP1up proteins eluted at ~35 min, and fractions with PLA_2 activity (determined using the spectrofluorimetric assay described below) were pooled and concentrated in a Centricon-10 (Millipore) to give a final protein concentration of 1.5–1.8 mg/ml. PPV-VP1up and M33-VP1up were judged to be ~90% pure by SDS-PAGE analysis. B19-VP1up was purified by gel filtration at 4 °C on a Superdex 75 HR 10/30 column (Amersham Biosciences) using 50 mM Tris-HCl, pH 8.0. Protein was loaded at 2 mg/ml with a flow rate 0.5 ml/min. B19-VP1up eluted at ~9 ml and was concentrated in a Centricon-10 to 1.5–1.8 mg/ml. To ensure complete removal of the His_6-tagged TEV protease, the purified VP1up proteins were incubated with Ni-NTA resin (5 mg of VP1up/ml of Ni-NTA resin) for 30 min at room temperature with gentle swirling. The mixture was centrifuged at 3,000 rpm, and the supernatant containing B19-VP1up was stored at −20 °C after adding glycerol (see below). The AAV2-VP1up (16711 Da) and thioredoxin (11714 Da) proteins eluted as a doublet during gel filtration. Thus, to avoid contamination, only fractions of the beginning of the first peak, which contains pure AAV2-VP1up, were pooled. For all four enzymes, glycerol was added to the protein suspensions to final concentrations of 30% by volume prior to storage at −20 °C. VP1up proteins were found to be stable for at least 1.5 months.

Protein concentrations were determined from the absorbance at 280 nm using $A^{1\%}$ of 5.56, 8.0, 11.7, and 15.5 for PPV-VP1up, M33-VP1up, B19-VP1up, and AAV2-VP1up, respectively (calculated from the amino acid sequences). All of the proteins were judged to have the correct molecular mass by electrospray mass spectrometry (Voyager-DE RP spectrometer (PerSeptive; Biosystems)) and by N-terminal sequencing.

Assay of VP1up PLA_2 Activity—All of the VP1up proteins were assayed for PLA_2 activity using the spectrofluorimetric assay with PPyrPM (11). The specificity of the VP1up proteins for the *sn*-2 fatty acyl chain of phosphatidylcholine was determined using the dual radiolabel approach with 1-stearoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine and 1-palmitoyl-2-[9,10-^{3}H]palmitoyl phosphatidylcholine as described (12).

Immunogold Detection of PLA_2-containing Domain of VP1up on PPV Virions—PPV virus was purified as described previously (1). Both the sample submitted to a heat shock by one cycle of 4 °C/65 °C/4 °C in a PCR thermocycler for 2 min at each step and the control sample were diluted with 10 mM Tris-HCl, pH 8.1, with 1 mM EDTA buffer to an optical density of 0.01 at 260 nm. Virus in 100-μl samples was deposited onto nickel grids in a Beckman Airfuge ultracentrifuge at 20 p.s.i. for 5 min. After blotting with paper, the grids were deposited for 5 min on PBS and PBS/bovine serum albumin (1%) drops, respectively, and incubated for 2 h with VP1up-specific polyclonal antibodies raised in a rabbit against *E. coli*-expressed M33-VP1up (antiserum diluted 1/100 in PBS). The grids were then incubated, after another three washings with PBS and one washing with PBS/bovine serum albumin (1%), with gold-labeled goat anti-rabbit antibodies (diluted 1/10 colloidal Gold-affiniPure Goat anti-rabbit IgG; 12 nm, Jackson ImmunoResearch) for 30 min and a final washing (three times for 1 min in PBS and twice for 40 s in water).

pH Dependence of VP1up PLA_2 Enzymatic Activity—The pH rate profiles for the VP1up proteins were measured with co-vesicles of

1-palmitoyl-2-oleoyl phosphatidylcholine containing 1-palmitoyl-2-[9,10-^{3}H]palmitoyl phosphatidylcholine (50 Ci/mol based on total phosphatidylcholine in the assay) present at 20 μM (total phosphatidylcholine) in 100 μl of various buffers (50 mM MES, pH 5.0; 50 mM MES, pH 6.0; 50 mM HEPES, pH 7.0; 50 mM Tris, pH 8.0; and 50 mM glycine, pH 9.0) containing 1 mM $CaCl_2$. The reactions were allowed to proceed for 30 min at room temperature. Liberated free [^{3}H]palmitic acid was quantified as described (13).

Kinetic Studies with Phospholipid Vesicles—Unilamellar vesicles (0.1-micron diameter) of POPC, POPG, and POPS were prepared by extrusion in water as described (14) and stored for up to 2 days at 4 °C (or frozen and re-extruded). Phospholipid concentrations in stock solutions were determined by phosphate analysis (standard ammonium molybdate method). Vesicle hydrolysis studies were carried with the indicated concentrations of vesicles (Table II) in 1.3 ml of Hanks' balanced salt solution with Mg^{2+} and Ca^{2+} (1 mM each) containing 13 μg of FABP and 1 μM 11-dansylundecanoic acid (Molecular Probes Inc./Invitrogen) at 37 °C with magnetic stirring using the fluorimetric assay procedure described previously (10). The assays were calibrated to give moles of oleic acid formed by the addition of standard oleic acid to the reaction mixture containing all components except VP1up protein. The calcium dependence of the hydrolysis of POPG vesicles by these viral VP1up proteins was studied by using the same assay as described above except that the Ca^{2+} concentration varied from 0 (buffer with 1 mM EGTA) to 2.5 mM.

Binding of VP1up Proteins to Phospholipid Vesicles—The full details for measuring the binding of VP1up proteins to sucrose-loaded 0.1-μm phospholipid vesicles have been described for sPLA_2s (10). Binding reaction samples (100 μl) contained various concentrations of vesicles in binding buffer (5 mM MOPS, pH 7.4, 0.1 M KCl, 2 mM $CaCl_2$). The ratio of moles of total phospholipid to moles of VP1up proteins was at least 200 to avoid crowding of protein on vesicles (10). The amount of VP1up remaining in the supernatant after pelleting the vesicles by centrifugation was measured using the PPyrPM fluorimetric assay as described above.

Phospholipid Head Group Specificity Studies—Studies to measure the preference of the VP1up proteins toward phospholipids with different polar head groups were carried out using co-vesicles of POPA, POPC, POPE, POPG, POPI, and POPS as described (15).

Release of Arachidonic Acid from CHO-K1 Cells—CHO-K1 cells were cultured in Ham's F12 medium with 10% fetal calf serum, 2 mM glutamine, and the antibiotics penicillin and streptomycin. The cells were cultured in 24-well plates containing 1 ml of medium/well. When cells reached ~80% confluence, 0.1 μCi of [^{3}H]arachidonic acid (~100 Ci/mmol; American Radiolabeled Chemicals, Inc.) was added per well. After 24 h at 37 °C (humidified atmosphere with 5% CO_2), the cells were washed three times with complete medium, and 1 ml of complete medium was added. VP1up protein was then added in amounts as given in the legend to Fig. 6. The medium was removed after the indicated time period at 37 °C (see legend to Fig. 6) and centrifuged briefly to pellet any dislodged cells, and then 0.5 ml of supernatant was submitted to scintillation counting. The cell layer remaining in the well was treated with trypsin/EDTA, and the cell suspension was submitted to scintillation counting. Arachidonic acid release is expressed as a percentage of cpm released to the culture medium divided by total cpm (released plus cell associated).

RESULTS

Cloning, Expression, and Purification of Parvovirus VP1up PLA_2-like Proteins—The regions of the viral DNAs coding for PLA_2 from four different parvoviruses proteins (PPV-VP1up, M33-VP1up, AAV2-VP1up, and B19-VP1up), were amplified by PCR, and the products were inserted into a bacterial expression vector. The N-terminal thioredoxin and the C-terminal His (6) affinity tag in these fusion proteins were released from the VP1up domain by cleavage with TEV protease. Typically, 5-fold higher expression levels of fusion proteins were obtained using a host strain that contains extra copies of tRNA genes that recognize codons that are rare in *E. coli* (BL21-CodonPlus (DE3)-RIL) compared with expression in the standard strain BL21(DE3). Chromatography of M33-VP1up on a Ni-NTA column followed by a heparin-Sepharose column gave a protein that was >99% pure (Fig. 1), whereas PPV-VP1up eluted as two bands, as shown by SDS-PAGE (Fig. 1), because of the incomplete cleavage of the fusion protein by TEV protease. The slower moving band could be removed by passing the TEV digestion mixture again through a Ni-NTA column (not shown). B19-VP1up and AAV2-VP1up were purified to near homogeneity (Fig. 1) by passage over an Ni-NTA column, followed by a gel filtration column, and finally a second Ni-NTA. Using this expression system, yields of 3–5 mg/liter of bacterial culture were obtained for each protein of the four viral PLA_2s.

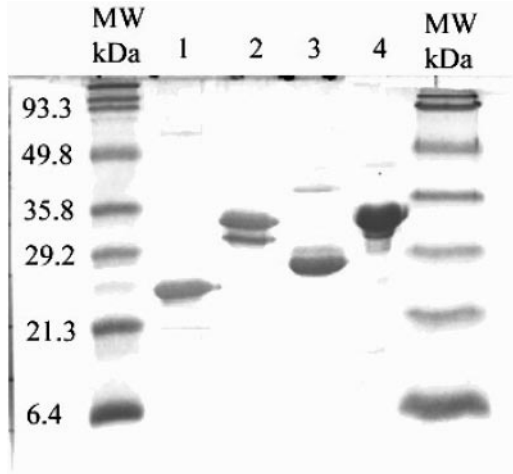

FIG. 1. **SDS-PAGE analysis of purified VP1up proteins.** *Lane 1* is 3 μg of M33-VP1up, *lane 2* is 4 μg of PPV-VP1up, *lane 3* is 2 μg of AAV2-VP1up, and *lane 4* is 2.5 μg of B19-VP1up. *MW*, molecular mass.

VP1up Proteins Are Active PLA_2s—Initially, the specific activity of the four expressed VP1up proteins were tested using the fluorimetric assay with vesicles of the anionic phospholipid PPyrPM (11). The results (Table II) showed specific activities of 9.7 and 16 μmol/(min mg), respectively, for PPV-VP1up and M33-VP1up. On the other hand, B19-VP1up and AAV2-VP1up were approximately 2 orders of magnitude less active than PPV-VP1up and M33-VP1up on PPyrPM vesicles (Table II). The kinetic data obtained with these recombinant VP1up proteins, produced in a heterologous host, established that these viral proteins are capable of functioning as PLA_2s.

PPV Externalizes Its PLA_2 Domain Following Heat Treatment—Previously, we observed that the PLA_2 activity of PPV virus was greatly enhanced when the capsid was chemically disrupted or when the virus was submitted to a thermal shock (8), suggesting that the PLA_2-bearing domain of VP1 of PPV is buried within the native virus particle and needs to be externalized for PLA_2 activity of the virus on membranes. To examine whether heat treatment of PPV exposes the PLA_2 domain on intact viral particles, we used immunogold electron microscopy to visualize the VP1up domain of PPV using a polyclonal antiserum raised in rabbits against *E. coli*-expressed M33-VP1up, and the results are summarized in Fig. 2. With purified virus, only a low level of gold particles was seen. In contrast, the detection of the VP1up domain was dramatically enhanced when viral particles were treated at 65 or 72 °C (not shown) for 2 min. The capsids dissociated when the temperature was increased to 75–85 °C (not shown). These results fit with our earlier report that the PLA_2 enzymatic activity of PPV virus particles is enhanced after heat shock and is consistent with the PLA_2 enzymatic activity of the virus particles residing in the VP1up domain.

pH Rate Profile of VP1up PLA_2s—As shown in Fig. 3, the four recombinant viral VP1up PLA_2s display similar pH dependence profiles when assayed with POPC vesicles containing a small amount of radioactive substrate 1-palmitoyl-2-[9,10-^{3}H]palmitoyl phosphatidylcholine. Maximum activity is seen in the pH range 6–7 for the four viral PLA_2s, and activity falls as the pH is lowered or raised from this range.

TABLE II
Specific activities for the hydrolysis of phospholipid vesicles by VP1up proteins

Substrate	Specific activity[a] *μmol/(min mg)*					
	M33-VP1up	PPV-VP1up	B19-VP1up	AAV2-VP1up	Human group IIA PLA_2	Human group X PLA_2
PPyrPM	16 ± 2	9.7 ± 0.6	0.071 ± 0.003	0.061 ± 0.003	70 ± 1	ND[b]
POPG	621 ± 20	290 ± 15	0.14 ± 0.02	0.50 ± 0.02	246 ± 5	ND
POPS	99 ± 7	87 ± 5	0.042 ± 0.005	0.050 ± 0.004	28 ± 1	ND
POPC	139 ± 3	88 ± 5	0.024 ± 0.003	<0.01	ND	3.6 ± 0.2

[a] Measurements with vesicles of PPyrPM were performed with 1.3 μM of substrate in 50 mM Tris, pH 8.0, 1 mM $CaCl_2$ at room temperature. For assays with vesicles of POPG, POPS, and POPC, the substrate concentration is 30 μM in 1 ml of Hanks balanced salt solution with Ca^{2+} and Mg^{2+} (1 mM each) containing 13 μg of FABP and 1 μM 11-dansylundecanoic acid at 37 °C with magnetic stirring.
[b] ND, not determined.

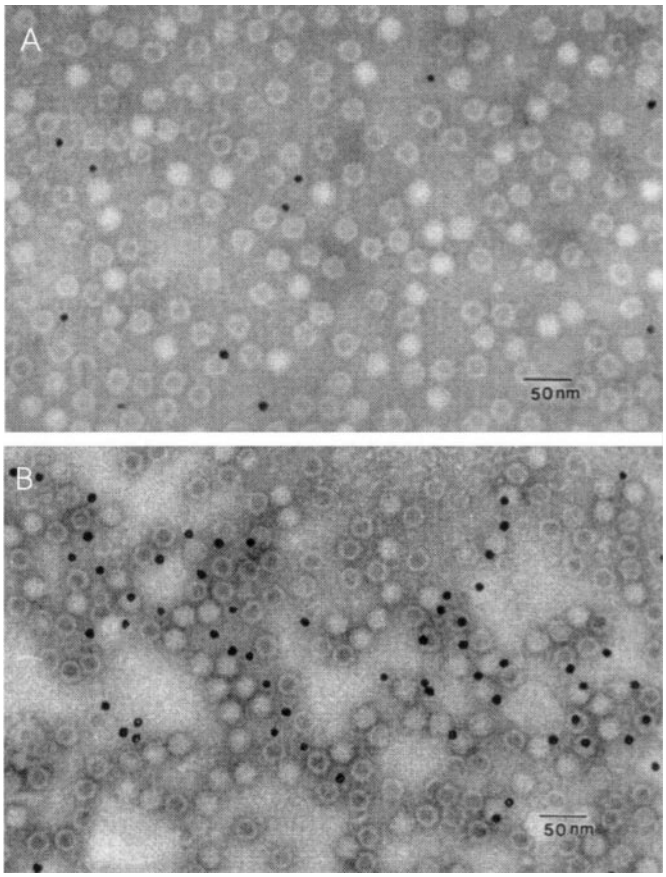

FIG. 2. **Immunogold electron microscopy detection of VP1up domain of PPV.** Virus was deposited onto nickel grids, and the VP1up domain was visualized with VP1up-specific polyclonal antiserum prepared in *E. coli* against M33-VP1up protein. *A*, purified virus before heat shock. *B*, purified virus after a 2-min heat shock at 65 °C.

Substrate Specificity of VP1up PLA$_2$—To test whether VP1up PLA_2s display specificity toward the fatty acid at the *sn*-2 position of phospholipids, we exposed co-vesicles of 1-palmitoyl-2-[9,10-^{3}H]palmitoyl phosphatidylcholine and 1-stearoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine to the various VP1up proteins and measured the ratio of [^{3}H]palmitic acid to [^{14}C]arachidonic acid after a small fraction of the total phosphatidylcholine was hydrolyzed (12). The ratio of released palmitic acid to arachidonic acid allowed the determination of the relative k_{cat}*/K_m* for the two substrates (*asterisks* denote kinetic constants for action of enzyme at the interface). This ratio was not statistically different from unity (after duplicate analysis; not shown) for each of the four VP1up PLA_2s. This result indicated that these PLA_2s do not display specificity for *sn*-2 saturated *versus* polyunsaturated fatty acyl chains, as is typical for most sPLA_2s (15).

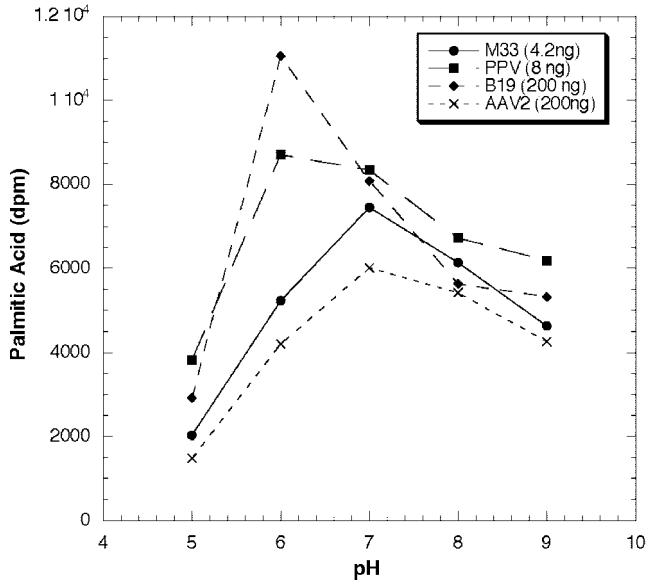

FIG. 3. **pH-rate profiles of viral PLA$_2$activities.** The pH dependence was measured with 4.2 ng of M33-VP1up, 8 ng of PPV-VP1up, 200 ng of B19-VP1up, and 400 ng of AAV-VP1up and 20 μM POPC vesicles containing a small amount of 1-palmitoyl-2-[9,10-^{3}H]palmitoyl phosphatidylcholine (final specificity radioactivity, 50 Ci/mol) (see "Experimental Procedures" for additional information).

Next, we determined the specific activities of the viral PLA_2s on vesicles of phospholipid with different polar head groups. Table II gives the values for the hydrolysis of POPC, POPS, and POPG vesicles. The specific activities on these vesicles are reflections of both the interfacial binding of these enzymes to the vesicle surface and the catalytic efficiency for the hydrolysis of different phospholipids used (15). As for sPLA_2s (15), POPG vesicles were the best substrate for the four different viral PLA_2s (Table II). PPV-VP1up and M33-VP1up PLA_2s were highly active on POPC, POPS, and POPG vesicles. Thus, the 58 amino acids that are present on the C terminus of PPV-VP1up but deleted in M33-VP1up are not required for the action on phospholipid vesicles. As with PPryPM, B19-VP1up and AAV2-VP1up were much less active than M33-VP1up and PPV-VP1up on POPC, POPS, and POPG vesicles (Table II).

We also studied the substrate specificity of viral PLA_2s on mixed vesicles of POPC, POPE, POPS, POPA, POPI, and POPG and used combined high pressure liquid chromatography electrospray ionization mass spectrometry to quantify the amount of each lysophospholipid produced before 20% of the total phospholipid in the mixed substrate vesicles had been hydrolyzed (15). In this experiment, the substrate specificity is not influenced by differential affinity of the PLA_2 to vesicles of different phospholipid composition and thus gives an indication of the intrinsic substrate specificity of the active site toward phospholipids with different polar head groups. The results shown in

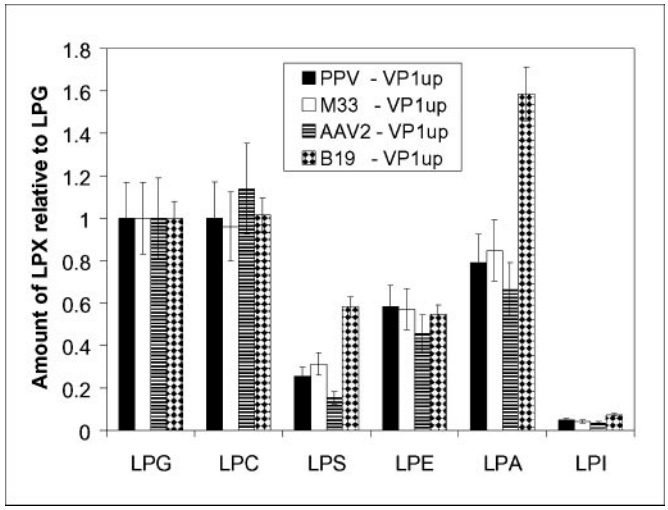

FIG. 4. **Phospholipid head group specificity of viral PLA_2s.** Mixed phospholipid vesicles containing POPA, POPC, POPE, POPG, POPI, and POPS were subjected to partial hydrolysis by four different viral VP1up proteins, and the amount of each lysophospholipid species was determined by mass spectrometry. The values are normalized to lysophosphatidylglycerol (*LPG*).

Fig. 4 indicate that all four VP1up proteins hydrolyze POPG, POPC, and POPA with high efficiency, POPE and POPS are slightly less preferred, and POPI is hydrolyzed only very poorly. Thus, it appears that parvoviral PLA_2s do not contain a well defined pocket for binding phospholipid polar head groups with high specificity.

Calcium Dependence of Phospholipid Hydrolysis by Viral PLA_2s—No detectable hydrolysis of PPyrPM was observed when calcium was omitted from the assay (in the presence of 1 mM EGTA; not shown). The results on the calcium dependence of hydrolysis of POPG vesicles by viral PLA_2s are shown in Fig. 5. These data well fit a standard hyperbolic binding equation for a single Ca^{2+} site on the protein. The obtained values of the apparent dissociation constant for Ca^{2+}, ${}^{Ca}K_d$, are 1 ± 0.3 mM for PPV-VP1up, 1 ± 0.3 mM for M33-VP1up, 0.24 ± 0.06 mM for AAV2-VP1up, and 0.14 ± 0.05 mM for B19-VP1up. Identical values of ${}^{Ca}K_d$ for M33-VP1up and PPV-VP1up indicated that the 58-amino acid C-terminal extension present in PPV-VP1up had no effect on affinity of the protein for calcium.

Interfacial Binding of VP1up Proteins to Phospholipid Vesicles—We measured the binding of VP1up proteins to sucrose-loaded phospholipid vesicles, which can be pelleted by ultracentrifugation. Diether phospholipids (dioleyl) were used to avoid hydrolysis of vesicles by bound VP1up proteins as binding studies were carried out in the presence of sufficient Ca^{2+} to saturate the enzyme based on the kinetic studies described above (2 mM). The values of the equilibrium constant for the dissociation of enzyme from vesicles (K_d) were expressed in terms of millimolar phospholipid in the outer leaflet of the vesicles (half of the total phospholipid in the binding reaction). The data in Table III show that PPV-VP1up binds to dioleyl phosphatidylcholine vesicles containing 10 or 30% dioleyl phosphatidylserine with equal affinity. AAV2-VP1up binds 2–5-fold tighter to vesicles than does PPV-VP1up. Thus, a poor interfacial binding cannot be the reason that AAV2-VP1up has low catalytic activity relative to PPV-VP1up.

Inhibition Studies with VP1up PLA_2s—Nine potent and active site-directed inhibitors of classical $sPLA_2$s were studied as potential inhibitors of the VP1up PLA_2s (inhibitor structures given in Ref. 15). Enzyme assays were carried out with PPyrPM. Remarkably, all of these inhibitors displayed little or no potency on the VP1up PLA_2s (Table IV). For example,

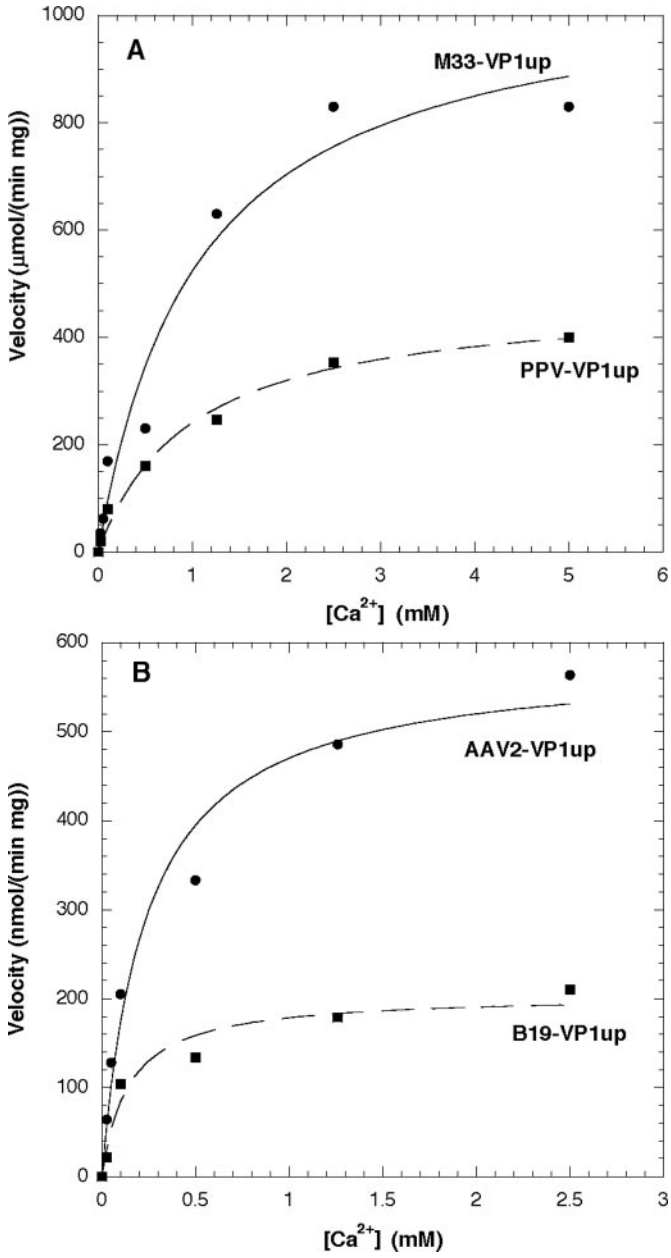

FIG. 5. **Initial velocity for the hydrolysis of POPG vesicles as a function of Ca^{2+} concentration.** *A* is for PPV-VP1up and M33-VP1up proteins, and *B* is for AAV2-VP1up and B19-VP1up proteins. The assay was carried out with the FABP assay as described under "Experimental Procedures." The *lines* are the fit of the data to the standard binding equation for the binding of Ca^{2+} to a single site on the protein.

TABLE III
Interfacial binding of PPV-VP1up and AAV2-VP1up to phospholipid vesicles

Binding studies were carried out in 5 mM MOPS, pH 7.4, 0.1 M KCl, 2 mM $CaCl_2$. Binding of M33-VP1up and B19-VP1up was not studied.

VP1up	K_d	
	10% dioleyl phosphatidylserine in dioleyl phosphatidylcholine	30% dioleyl phosphatidylserine in dioleyl phosphatidylcholine
	mM	
PPV-VP1up	0.5 ± 0.2	0.5 ± 0.1
AAV2-VP1up	0.1 ± 0.04	0.3 ± 0.1

LY311727 inhibits human group IIA PLA_2 with an IC_{50} of approximately 0.1 μM but failed to inhibit the VP1up PLA_2s, using the same assay, when tested at 25 μM. For M33- and

TABLE IV
Inhibition of VP1up proteins by known PLA_2 inhibitors

PPV-VP1up was not studied; its active site is identical to that of M33-VP1up. The amount of enzyme used was 2.2 ng for M33-VP1up, 200 ng for AAV2-VP1up, and 550 ng for B19-VP1up. The values in the table are percent inhibition and are the averages of duplicate assays.

Inhibitor	Inhibitor concentration	M33-VP1up	AAV2-VP1up	B19-VP1up
	μM	%	%	%
SB203347	20	20	NI[a]	NI
MJ50	18	50	10	NI
MJ33	20	30	22	NI
HK30	20	30	NI	NI
HK38	20	30	NI	NI
HK61	23	NI	NI	NI
Pyr-1	8	20	30	NI
DDC-1	21	NI	NI	NI
LY311727	25	NI	NI	NI

[a] NI, no inhibition detected.

AAV2-PLA_2s, measurable inhibition was seen with only a subset of the compounds, and inhibition was much weaker than that seen with classical sPLA_2s (15). None of the compounds gave detectable inhibition of B19-VP1up. Thus, it appears that although active sites of the viral PLA_2s are related to those of classical sPLA_2s, they also contain distinct features. Further studies will be required to discover highly potent inhibitors of these viral PLA_2s.

Liberation of Arachidonic Acid from Mammalian Cells by Viral PLA_2s—We studied the ability of viral PLA_2s to release arachidonic acid from membrane phospholipids of CHO-K1 cells. The cells were labeled with [^{3}H]arachidonic acid and washed to remove unesterified fatty acid. We then measured the percentage of total cellular tritium released into the culture medium after incubating the cultured cells for 2 or 4 h with exogenously added viral proteins. The results are shown in Fig. 6. Incubation of cells with as little as 100 ng/ml of PPV-VP1up and M33-VP1up led to measurable release of [^{3}H]arachidonic acid into the culture medium. No [^{3}H]arachidonic acid release above background was detected when up to 10,000 ng/ml B19-VP1up and AAV2-VP1up were added to CHO-K1 cells for 4 h (not shown). This was expected given the low specific activity of these PLA_2s on phospholipid vesicles (Table II).

DISCUSSION

In this study, we have carried out a detailed analysis of the interfacial kinetic and binding properties of four parvoviral PLA_2 domains. The specific activity of M33-PLA_2 of 621 μmol/(min mg) on POPG vesicles exceeded that for human group IIA PLA_2 on these vesicles and is among the most active sPLA_2s known (15). Human group IIA PLA_2 displays very poor activity on POPC vesicles compared with POPG vesicles because this enzyme binds poorly to zwitterionic phosphatidylcholine vesicles (10). In this case, the reaction progress curve exhibits an acceleration because enzyme binding to zwitterionic vesicles is enhanced by the build-up of anionic reaction products in these vesicles (10). In contrast, human group X PLA_2 binds well to both zwitterionic and anionic POPG vesicles, displays comparable specificity activity on both, and hydrolyzes phosphatidylcholine vesicles without an acceleration phase (10). All four viral PLA_2s behaved more like human group X PLA_2 on POPC vesicles in that no acceleration phase was observed and that the specific activities on zwitterionic and anionic phospholipid vesicles were comparable (Table II).

The VP1up proteins from AAV2 and B19 parvoviruses display specific activities toward a variety of phospholipid vesicles that are 2–3 orders of magnitude lower. It may be noted that mouse and human groups IID, IIE, and XIIA sPLA_2s also display specific activities that are 100–1,000-fold lower than

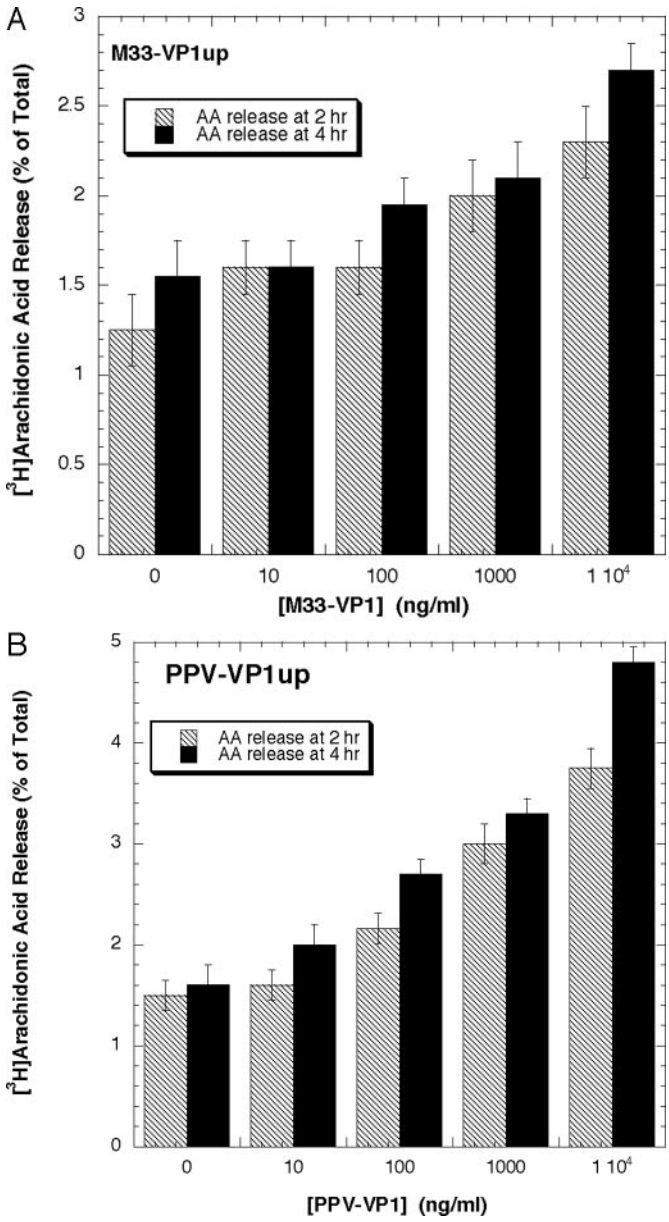

FIG. 6. **Release of [^{3}H]arachidonic acid from CHO-K1 cells treated with exogenous VP1up proteins.** The *top panel* is for M33-VP1up, and the *bottom panel* is for PPV-VP1up. Adherent CHO-K1 cells that were prelabeled with [^{3}H]arachidonic acid were treated for 2 or 4 h with the indicated amounts of VP1up PLA_2s.

the most active sPLA_2s toward phospholipid vesicles (15). The reasons for the relatively low activity of a subset of these PLA_2s is not obvious from a comparison of their amino acid sequences of these enzymes as well as the x-ray structures of a subset of them. It is possible that the physiological substrates for these low activity enzymes have structures distinct from those of standard phospholipids.

The rise in activity of the viral PLA_2s as the pH is raised from 5 up to 6 or 7 may be due to the deprotonation of the active site histidine that is predicted to be present in VP1up PLA_2s based on amino acid sequence alignment (8). Classical sPLA_2s also contain an active site histidine and display a similar rise in enzymatic activity with increasing pH with an apparent pK_a value of approximately 6–7 (16–18).

The dependence of the viral PLA_2 enzymatic activity on the calcium concentration is typical of the behavior of classical sPLA_2s (15). It should be noted that the $^{Ca}K_d$ values determined in this study are apparent values. For sPLA_2s, binding

of a single phospholipid in the active site of the enzyme requires Ca^{2+}, and active site phospholipid binding also requires that the enzyme be bound to the interface of phospholipid vesicles because long chain phospholipids have virtually no solubility in the aqueous phase (19).

It was previously found that human group X $sPLA_2$ binds to 10% dioleyl phosphatidylserine/dioleyl phosphatidylcholine vesicles with a K_d of 0.13 mM (10). In marked contrast, human group IIA $sPLA_2$ shows a dramatic dependence on the presence of >10–20% anionic phosphatidylserine for binding to vesicles (10, 20, 21), which is consistent with its well established function as a Gram-positive bactericidal agent (22–24). Gram-positive membranes are highly rich in acidic phospholipids, especially phosphatidylglycerol, and the high density of basic residues (lysines and arginines) on the surface of the group IIA enzyme is important for allowing this enzyme to penetrate the highly anionic cell wall of Gram-positive bacteria (25, 26).

With respect to interfacial binding, the VP1up proteins behave more like the human group X enzyme. This is consistent with the data in Table II showing that the viral PLA_2s display similar specific activities on POPS and POPC vesicles. This suggests that VP1up PLA_2s act on host cell membranes that are typically not rich in acidic phospholipids. VP1up domain PLA_2 activity requires submillimolar to millimolar calcium concentrations, which suggests that they act either in the extracellular fluid or inside an intracellular organelle whose aqueous lumen is derived from the extracellular fluid. On the other hand, VP1up proteins lack disulfide bonds in contrast to the classical $sPLA_2$s, which contain several disulfides and are not expected to survive the reducing environment of the cytosol of mammalian cells.

The ability of these viral PLA_2s to liberate arachidonic acid from CHO-K1 cells is reminiscent of the results obtained with human group X PLA_2 (10). Both the viral and mammalian PLA_2s are able to readily hydrolyze phosphatidylcholine vesicles (Table II), and thus it was expected that the viral PLA_2s could act on the outer monolayer of mammalian cells, which is rich in zwitterionic phospholipids. In contrast, human group IIA $sPLA_2$ binds weakly to phosphatidylcholine-rich membranes and fails to release arachidonic acid when added exogenously to mammalian cells at concentrations up to 1 μg/ml (16).

The intact PPV virus particle undergoes a structural transition after heat shock that greatly enhances the PLA_2 enzymatic activity and externalizes the VP1up PLA_2 domain (Fig. 2). It is tempting to suggest that the PPV viral capsid may be induced, at physiological temperature, to undergo a similar structural transition to expose its PLA_2 domain during virus entry into host cells because of specific environment (*e.g.* in endosome) or particular receptors at the time and location the enzyme activity is required. In contrast, VP1up of B19 virus has been reported to be always exposed (27), and its low PLA_2 activity may be required to avoid deleterious side effects of higher activities. It is also tempting to speculate that the lack of disulfides in the viral PLA_2 chains could contribute to the inability to visualize these domains in the parvovirus x-ray structures (as noted above). Parvoviruses containing point mutations in the PLA_2 catalytic residues of the VP1up proteins are no longer infectious (8, 9). Thus, PLA_2 inhibitors are expected to display antiviral activity. Unfortunately, previously described potent inhibitors of classical sPLA2s display almost no activity on VP1up enzymes. In this context, it will be interesting to determine the high resolution structure of VP1up proteins. This would also reveal the fold of these enzymes, which is difficult to predict at present because of the limited amino acid sequence homology between VP1up proteins and classical $sPLA_2$s. Given the distinct structural features of parvovirus VP1up proteins including the lack of disulfides, it is proposed that these enzymes be classified as group XIII PLA_2s to follow the recent identification of group XIIB $sPLA_2$s (28). This is consistent with the nomenclature suggested recently (29).

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Enzyme Catalysis and Regulation:

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J. Biol. Chem. 2004, 279:14502-14508.
doi: 10.1074/jbc.M312630200 originally published online January 15, 2004