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The MyD116 African Swine Fever Virus Homologue Interacts with the Catalytic Subunit of Protein Phosphatase 1 and Activates Its Phosphatase Activity▽

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The DP71L protein of African swine fever virus (ASFV) shares sequence similarity with the herpes simplex virus ICP34.5 protein over a C-terminal domain. We showed that the catalytic subunit of protein phosphatase 1 (PP1) interacts specifically with the ASFV DP71L protein in a yeast two-hybrid screen. The chimeric full-length DP71L protein, from ASFV strain Badajoz 71 (BA71V), fused to glutathione *S*-transferase (DP71L-GST) was expressed in *Escherichia coli* and shown to bind specifically to the PP1-α catalytic subunit expressed as a histidine fusion protein (6×His-PP1α) in *E. coli*. The functional effects of this interaction were investigated by measuring the levels of PP1 and PP2A in ASFV-infected Vero cells. This showed that infection with wild-type ASFV strain BA71V activated PP1 between two- and threefold over that of mock-infected cells. This activation did not occur in cells infected with the BA71V isolate in which the DP71L gene had been deleted, suggesting that expression of DP71L leads to PP1 activation. In contrast, no effect was observed on the activity of PP2A following ASFV infection. We showed that infection of cells with wild-type BA71V virus resulted in decreased phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α). ICP34.5 recruits PP1 to dephosphorylate the α subunit of eukaryotic translational initiation factor 2 (also known as eIF-2α); possibly the ASFV DP71L protein has a similar function.

African swine fever virus (ASFV), the only member of the *Asfarviridae* family (10), infects cells of the mononuclear-phagocytic system, including highly differentiated fixed-tissue macrophages and reticular cells, resulting in tissue damage (the severity of damage depending on the virulence of the strain) (28). Viruses encode a plethora of proteins that interfere with the host defense mechanism (1). Analysis of the complete nucleotide sequence of the genome of the Badajoz 71 (BA71V) isolate of ASFV (29) identified proteins that are potentially capable of counteracting host defense responses. Among these is the DP71L gene. This gene was also reported (26) in the highly virulent isolate Malawi LIL 20.1 and named NL23 or l14L. The gene is present in the genomes of all pathogenic ASFV isolates analyzed and encodes either a long form (23-NL, 184 amino acids) or short form (NL-S, 70 to 72 amino acids) (26, 30). The long form of the protein is localized to the nucleolus during infection, while the short form is localized to the nucleus but not the nucleolus (15). This gene is similar to a myeloid differentiation primary response gene, MyD116 (30), a protein involved in DNA repair GADD34 and the neurovirulence-associated protein (ICP34.5) from herpes simplex virus (HSV). ICP34.5 prevents host protein shutoff mediated by double-stranded-RNA-dependent protein kinase (PKR) by recruiting protein phosphatase 1 (PP1) to dephosphorylate the α subunit of eukaryotic initiation factor 2 (eIF-2α) (5, 14, 19). GADD34 also promotes eIF-2α dephosphorylation and targets PP1α to the endoplasmic reticulum (6, 7).

The ICP34.5 protein has an additional function and binds to proliferating cell nuclear antigen (PCNA), forming a DNA-binding complex in virions with PCNA and HSV replication proteins (17). It has been proposed that ICP34.5 is required to switch PCNA to replication mode and that this is required to initiate HSV replication in nondividing cells (17). In addition, ICP34.5 has been reported to be involved in egress of HSV virions. The ICP34.5 protein contains nuclear export signal amino acid repeats consisting of phenylalanine, alanine, and threonine (PAT) which are involved in shuttling the protein to and retention in the cytoplasm (7, 22). These signals are absent from both forms of the DP71L protein. However, both forms of the DP71L protein contain a C-terminal nuclear localization signal that is present in the ICP34.5 protein (7).

Protein phosphatase 1, originally studied as phosphorylase phosphatase, is one of the major Ser/Thr protein phosphatases. It consists of a catalytic subunit of 37 kDa, which is bound to a number of different regulatory or targeting subunits. The formation of these complexes converts PP1 into many different forms, which have distinct substrate specificities and restricted subcellular locations which define its specificity, acting to regulate phosphatase activity. The existence of multiple PP1 binding proteins enables PP1 activity to be involved in a diverse range of cellular functions and reflects a strategy for its evolutionary development.

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Deletion of the short form of the DP71L gene from the virulent European isolate E70 resulted in marked attenuation of the virus in the host swine. Infection of pigs with the deletion mutant virus was characterized by a lack of clinical disease apart from a transient fever, greatly reduced viremia, and no mortality (30). In contrast, deletion of the long form of this gene from the genome of the virulent Malawi LIL 20/1 isolate had no effect on virulence, suggesting that this isolate may encode other genes which can compensate for the loss of the DP71L gene.

Identification of protein-protein interactions between viral and cellular proteins can lead to a more complete understanding of virus-mediated cellular modulation, viral immune evasion, and host range restriction, leading to novel approaches for disease control. In this report, we show that the ASFV DP71L protein interacts with the cellular PP1 protein and that the DP71L gene is required for the activation of PP1 activity that occurs following ASFV infection. This interaction could result in targeting of PP1 to dephosphorylate specific substrates, such as the α subunit of the eukaryotic initiation factor 2 (eIF-2α) and possibly nuclear proteins.

MATERIALS AND METHODS

Cell culture and viruses. Vero cells were obtained from the American Type Culture Collection (ATCC CCL-81) and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum.

The tissue culture-adapted ASFV strain BA71V was used in all experiments. Preparation of viral stocks, titrations of infectivity, and infection experiments were carried out in Vero cells. Viral DNAs were extracted from infected Vero cells.

To construct the ΔDP71L ASFV deletion mutant, the viral p72 promoter and galactosidase (GAL) gene were cloned from pINSGAL (16) into plasmid pKS (Stratagene) to create plasmid p72GAL. The two 500-bp flanking regions of the DP71L gene of the BA71V isolate were amplified by PCR and cloned into either side of the p72GAL cassette to create plasmid ΔDP71L GAL. Vero cells were infected with BA71V and then transfected with 5 μg of ΔDP71LGAL using Lipofectin according to the manufacturer's recommendations (Invitrogen). Virus was harvested after 48 h. Recombinant virus was plaque purified four times in the presence of the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate to indicate galactosidase-expressing plaques. One-step virus growth curves were performed as previously described (16).

Cloning and plasmid generation. For yeast two-hybrid analyses, DNA encoding the full-length viral DP71L protein (short form) was amplified by PCR using purified BA71V DNA as the template. The following primers harboring specific restriction sites (underlined) to facilitate the cloning process were used: 5′-GAATTCATGGGGGGGCGGC (sense) and 5′-GGATCCCTGCTGCTCCAGTAG (antisense). The 213-bp PCR product was cleaved with EcoRI-BamHI and then inserted into the multiple cloning site of the pGBT9 (Clontech) *Saccharomyces cerevisiae* shuttle vector, generating the pGBT9-DP71L plasmid.

To express the DP71L protein as a glutathione *S*-transferase (GST) fusion protein (DP71L-GST) in *Escherichia coli* BL21(DE3) cells, a DNA fragment encoding the full-length DP71L gene was subcloned, as an EcoRI-SalI fragment excised from pGBT9-DP71L, into plasmid pGEX-4T3 (Amersham Biosciences Corp.) to construct pGEX-DP71L.

The six-histidine-tagged PP1α (6×His-PP1α) expression vector, pRSETa-PP1, was constructed by cloning the amplified PCR fragment encoding the entire coding region of the porcine PP1 gene, obtained after yeast two-hybrid screening, within the pRSETa prokaryotic expression vector as a KpnI-HindIII fragment using primers 5′-**GGTACC**TTATGTCCGACAGCGAGAAGCTC (sense) and 5′-**AAGCTT**CTATTTCTTGGCTTTGGCAAAG (antisense) (restriction sites are shown in bold type).

The authenticity of the constructs and the absence of any undesired mutations were confirmed by sequence analysis.

Yeast two-hybrid screening. All media, buffers, and methods for the yeast two-hybrid assay were adopted from the Matchmaker Two-Hybrid System manual and the Yeast Protocols Handbook (both from Clontech). A full-length DP71L gene from ASFV BA71V strain fused to the Gal4 DNA binding domain (pGBT9-DP71L) was used as a bait to screen a porcine macrophage cDNA library fused to the Gal4 activation domain (pACT2 vector) (23). Transformations were conducted sequentially by the lithium acetate method into the *Saccharomyces cerevisiae* strains Y190 (*MAT***a** *ura3-52 his3-D200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4D gal80D URA3*::$GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ cyh′2 LYS2*::GAL_{UAS}-$HIS3_{TATA}$-*HIS3*) carrying *His3* and *lacZ* as reporter genes.

Moderate to fast growing transformants were evaluated for prototrophy by plating cells on selection medium lacking tryptophan, leucine, and histidine. After incubation for 7 days at 30°C, clones that allowed growth were identified and subjected to a colony lift filter assay to confirm expression of the β-galactosidase reporter gene with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate. cDNA inserts from plasmids that allowed the yeast cells containing pGBT9-DP71L to grow on selection medium were identified and further characterized by DNA sequencing. The sequences obtained were compared with the NCBI database using the BLAST program (3).

Appropriate negative controls (cotransformation with empty DNA binding domain vectors) demonstrated that none of the interactions tested were prone to self-activation (not shown).

In vitro binding assays. For preparation of GST, GST-DP71L, and 6×His-PP1α proteins, *E. coli* strain BL21(DE3) cells, transformed with bacterial expression vectors pGEX-4T3, pGEX4T3-DP71L, and pRSETa/PP1, respectively, were grown in LB medium supplemented with 50 μg/ml ampicillin to an optical density at 600 nm of 0.4 to 0.6 at 37°C. Subsequently, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cell culture at a final concentration of 1 mM, and incubation continued for an additional 4 h. Cells were harvested by centrifugation, suspended in 5 ml of lysis buffer (phosphate-buffered saline [PBS], 1% Triton X-100), and sonicated on ice. GST-DP71L or GST alone was purified by mixing with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) beads (5 ml of lysate/400 μl of 50% slurry of beads) for 1 h at 4°C. Beads were washed with PBS containing 1% Triton X-100 and with PBS alone.

For the GST-based interaction assay, equal amounts of GST fusion proteins, GST-DP71L or GST, as judged by Coomassie blue staining, attached to glutathione matrix beads were incubated for 1 h at 4°C with bacterial cell lysate, containing expressed His-tagged PP1 protein, in binding buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 0.1% NP-40 with protease inhibitor mixture [Roche Molecular Biochemical]). Beads were subsequently washed four times with binding buffer, and bound proteins were eluted with glutathione elution buffer (20 mM reduced glutathione [Amersham], 50 mM Tris, pH 8.0), fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes, and probed with mouse monoclonal anti-6×His (catalog no. 8916-1; Clontech) or mouse monoclonal anti-PP1α (catalog no. 610373; BD Biosciences) antibodies before detection by enhanced chemiluminescence (ECL kit; Amersham).

PP1 and PP2A phosphatase activity assays. Vero cells were harvested at different time points after infection (4 and 18 h) or mock infected. Infection was carried out at a multiplicity of infection (MOI) higher than 5 PFU per cell with the BA71V strain of either wild-type ASFV or ΔDP71L recombinant virus. To prepare cell extracts, the medium was removed, and cells were washed once with ice-cold PBS supplemented with 1 mM sodium orthovanadate. Subsequently, cells were suspended in cell lysis buffer (10 mM Tris-HCl, pH 7.6, 140 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, 0.5% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and a protease inhibitor cocktail from Roche) for 10 min on ice. Cells were scraped off and clarified by centrifugation, and supernatants were either used for the assay or stored at −80°C until needed.

PP1 and PP2A phosphatase activities were determined according to a previously published method (8) using purified ^{32}P-labeled phosphorylase *a* as the substrate. To prepare this substrate, phosphorylase *b* (10 mg/ml) was phosphorylated by incubation with phosphorylase kinase (0.2 mg/ml) as previously described (25). Clarified cell extracts (0.5 μg) were then preincubated for 12 min at 30°C in the absence or presence of inhibitor 2 (I2) or fostriecin (Sigma) in 50 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, and 0.1 mM EDTA in a volume of 30 μl. The phosphatase reaction was initiated by the addition of 10 μl of ^{32}P-labeled phosphorylase *a* (60,000 cpm). After 20-min incubation at 30°C, the reaction was stopped by the addition of 180 μl of 20% (wt/vol) trichloroacetic acid. The tubes were left on ice for 10 min and then centrifuged at 12,000 × *g* for 5 min at 4°C. Aliquots (180 μl) of the cleared supernatant were used to determine phosphatase activity; the amount of ^{32}P released (cpm) was determined.

Phosphorylase *a* is a substrate for both PP1 and PP2A. Therefore, to distinguish between these two protein phosphatases, assays were performed in the presence of either I2 or fostriecin, specific inhibitors of PP1 and PP2A, respectively. The doses of PP1 and PP2 inhibitors used were predetermined by a set of dose-response experiments in which the effect of each inhibitor was indepen-

dently measured. The PP1 activity inhibited by I2 (100 to 500 nM) was comparable to the activity remaining in the presence of the PP2A inhibitor fostriecin (400 to 1,000 nM). Thus, the sum of the PP1 and PP2A activities represents the total phosphorylase *a* phosphatase activity of the cell extracts. Accordingly, a concentration of 200 nM I2 was chosen to assess phosphatase activity in further experiments. PP1 activity was defined as the phosphorylase *a* phosphatase activity inhibited by I2, while PP2A activity was defined as the remaining activity.

Determination of eIF-2α phosphorylation. In order to increase the usually very low levels of phosphorylated eIF-2α (eIF-2α-P) in cells and stimulate its production, cells were treated for 30 min before harvesting the cells with 10 mM EGTA, which modifies the intracellular calcium pool by inducing PKR and eIF-2α phosphorylation (24, 25). Apart from this short modification, cell extracts, prepared in the same way as described above for the phosphatase activity assay, were analyzed using horizontal isoelectric focusing slab gels to detect eIF-2α phosphorylation status. After electrophoresis, the gels were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences), and the bands corresponding to eIF-2α and phosphorylated eIF-2α proteins were probed on immunoblots with a specific anti-eIF-2α antibody (C-20 clone) (catalog no. sc-7629; Santa Cruz) and quantified with an image analyzer with QuantityOne software (Bio-Rad).

Statistical analysis. Results are expressed as means ± standard errors (SE) for independent experiments. Statistical analysis was performed using the *t* test for comparisons between two groups or using analysis of variance and Dunnett's posttest for comparisons between three or more groups.

Nucleotide sequence accession numbers. The nucleotide sequences were deposited in databases and assigned accession numbers as follows: *Sus scrofa* mRNA for protein phosphatase 1 catalytic subunit α, NCBI accession number AB016735; *Homo sapiens* mRNA for protein phosphatase 1 catalytic subunit α, NCBI accession number BC008010; *Homo sapiens* mRNA for protein phosphatase 1 catalytic subunit γ, NCBI accession number NM_002710; and *Sus scrofa* mRNA for protein phosphatase 1 catalytic subunit α, NCBI accession number NM_001044559.

RESULTS

Identification of PP1 as a protein interacting with DP71L. Although previous published data indicated that the NL23 gene from the E70 ASFV isolate and the orthologous gene DP71L from the BA71V isolate might play a key role in ASFV pathogenesis (27, 30), the mechanism of action of the protein encoded remained unknown. Thus, the identification of new protein partners for this protein provides useful information about its function during infection. We identified proteins that interact specifically with DP71L by carrying out a yeast two-hybrid screen of a porcine macrophage cDNA library (11, 12, 23) with the full-length DP71L gene from the BA71V isolate. The Gal4BD-DP71L fusion protein did not show transactivation activity by itself when expressed in *Saccharomyces cerevisiae* (data not shown). Screening of $\sim 2 \times 10^6$ Y190 yeast transformant colonies yielded seven positive clones that grew on the selective medium (selection medium lacking tryptophan, leucine, and histidine) and showed β-galactosidase activity under a lift colony filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate. Three of these clones expressed high levels of β-galactosidase activity (data not shown) compared to the remaining clones, which were not studied further. The inserts in these three clones were different in size, but subsequent sequence analysis revealed that all three encoded different isoforms of the catalytic subunit from PP1. One of the three inserts was identified as a porcine (*Sus scrofa*) mRNA for protein phosphatase 1α (NCBI accession number AB016735). The other two sequences shared 94% identity with the α subunit (NCBI accession number BC008010) and 93% identity with the γ subunit (NCBI accession number NM_002710), respectively, of the human homologue of PP1. However, no porcine isoforms for these two latter clones have been described until now and are reported for the first time in the present work. The clone containing sequence from the catalytic α human homologue subunit was the full-length coding sequence, and this was therefore used in subsequent experiments.

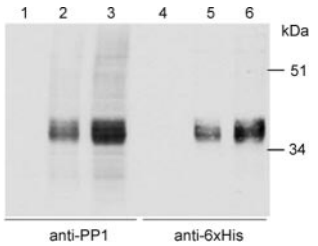

FIG. 1. In vitro association between PP1α and DP71L. GST beads (lanes 1 and 4) or GST-DP71L beads (lanes 2 and 5) were mixed with bacterial cell lysates containing His-tagged PP1 for 1 h at 4°C (see Materials and Methods). After extensive washing, proteins bound to beads were eluted and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with specific antibodies against PP1 (lanes 1 to 3) or 6×His (lanes 4 to 6). Overexpression of 6×His-PP1 as a positive control was detected with both antibodies in lanes 3 and 6, respectively. PP1 was visualized as a pulled-down band from a preloaded GST-DP71L Sepharose matrix (lanes 2 and 5), corresponding to a protein with an apparent molecular mass of ≈36 kDa, but not in the control assays using GST alone (lanes 1 and 4). The positions (in kilodaltons) of protein standards are shown to the right of the gel.

The DP71L protein product interacts with the catalytic subunit of PP1 in vitro. Yeast two-hybrid screens are excellent tools to identify interacting proteins in vivo. However, it is not possible to equate reporter gene expression unequivocally with biologically relevant interactions. To confirm the significance of the interaction between DP71L and the porcine PP1 catalytic subunit identified in the yeast two-hybrid assay, we studied the interaction in vitro. To achieve this, DP71L protein was expressed in *E. coli* as a fusion protein with glutathione *S*-transferase (GST-DP71L), and the catalytic subunit of PP1 was expressed as a fusion with polyhistidine (6×His-PP1). Recombinant GST-DP71L was immobilized on glutathione beads and incubated with a bacterial cell extract containing 6×His-PP1 protein. After extensive washing, bound proteins were eluted with glutathione elution buffer and separated on an SDS-polyacrylamide gel and visualized by Western blotting. A single protein band was specifically recovered in the GST-DP71L eluate (Fig. 1, lanes 2 and 5). This protein had an apparent M_r of ≈36,000 and reacted with the anti-PP1 monoclonal antibody on Western blots. Thus, we found that porcine PP1 was specifically eluted from the resin containing GST-DP71L. The control experiment showed that GST alone did not react with PP1 protein (Fig. 1, lanes 1 and 4). The antibody staining could not discriminate between the different isoforms of PP1. Thus, combined with the results of yeast two-hybrid analysis, these data suggest that the interaction of DP71L with PP1 is highly specific. PP1 was also specifically detected, in the same assay, when nitrocellulose membranes were probed with a monoclonal antibody recognizing the 6×His tag. Since binding took place in the absence of any other eukaryotic proteins, we postulate that the interaction between DP71L and PP1 is direct and does not require additional host proteins.

A short comparison of peptide sequences interacting with

TABLE 1. Sequences containing the VxF/W motif in some PP1c binding proteins

Gene or protein[a]	Sequence[b]	GenBank accession no.
GAC1	65-TSPEKN**VRF**AIE-76	X63941
SCD5	26-GLGPPS**VSF**DFG-37	U03492
HSV γ134.5 protein	176-PATPAR**VRF**SPH-187	M33701
Hamster GADD34	500-PLRARK**VHF**SEN-511	L28147
DP71L[c]	10-TNDTKH**VRF**AA-20	NP042844

[a] The genes or proteins with sequences containing the VxF motif were from different sources. *S. cerevisiae* (GAC1 and SCD5) and mammalian or viral genes are shown.

[b] Sequences in PP1c binding proteins containing the VxF motif are shown in bold type. Basic residues preceding and acid residues following the conserved motif VxF are underlined. The residue numbers are shown for the first and last residues in the sequences.

[c] DP71L is the BA71 ASFV homologue of the mouse MyD116 gene product.

the PP1 catalytic subunit and containing a characteristic VxF motif (9), including the PP1 binding motif in the HSV γ_1 34.5 protein sequence and a 100% identical motif present in the DP71L protein, is shown in Table 1.

Phosphatase activity in BA71V ASFV-infected cells. We performed phosphatase activity assays, using extracts from Vero cells which were either mock infected or infected at high multiplicity of infection with wild-type BA71V ASFV strain or the deletion mutant ΔDP71L virus. Cells were harvested at indicated time points after infection (4 and 18 h) in lysis buffer as described in Materials and Methods. Cell extracts were then preincubated for 20 min at 30°C in the presence of 10 μl of ^{32}P-labeled phosphorylase *a* (60,000 cpm). The reaction was stopped by the addition of 20% (wt/vol) trichloroacetic acid, and the samples were set on ice for 10 min and centrifuged. Aliquots of the supernatants were examined to determine phosphatase activity, which was measured as the amount of ^{32}P released (cpm).

To examine the physiological relevance of the DP71L-PP1 interaction and test whether this interaction resulted in activation or inhibition of the phosphatase activity, we constructed a mutant BA71V strain in which the complete coding region of the DP71L gene had been deleted (ΔDP71L). Deletion of the DP71L gene did not reduce replication of the virus compared to that of the wild-type BA71V strain as judged by virus growth curves obtained in Vero cells (Fig. 2A).

The results (Fig. 2B) show that extracts from wild-type BA71V-infected Vero cells exhibited higher levels of phosphatase activity, even at 4 h postinfection (hpi), compared to the endogenous levels from uninfected cells. When the cell extracts were assayed after 18 hpi, a two- to threefold increase of activity was observed in virus-infected cells compared to the background levels from uninfected cells. This difference was significantly different ($P < 0.001$). Moreover, phosphatase activity levels measured in extracts from ΔDP71L-infected cells were similar to the mock-infected cells, either at 4 or 18 hpi, indicating that DP71L is required for induction of phosphatase activity in ASFV infection.

Phosphorylase *a* is a substrate for both PP1 and PP2A. Therefore, to distinguish which one among the two major Ser/Thr protein phosphatases (PP1 and PP2A) could be responsible and account for the increase of phosphatase activity upon ASFV infection, phosphatase activity assays, as described above, were performed in the presence of either I2 or fostriecin, specific inhibitors of PP1 and PP2A, respectively. The doses of PP1 and PP2 inhibitors used were titrated by a set of dose-response experiments in which the effect of each inhibitor was independently measured. The PP1 activity inhibited by I2 (100 to 500 nM) was comparable to the activity remaining in the presence of the PP2A inhibitor fostriecin (400 to 1,000 nM). Thus, the sum of the PP1 and PP2A activities represents the total phosphorylase *a* phosphatase activity of the cell extracts. Accordingly, a concentration of 200 nM of I2 was chosen to assess phosphatase activity in further experiments. PP1 activity was defined as the phosphorylase *a* phosphatase activity inhibited by I2. PP2A activity was defined as the remaining activity.

As shown in Fig. 3A, the phosphatase activity due to PP2A was similar in uninfected cells and in cells infected with BA71V or ΔDP71L virus, indicating that the total phosphatase activity measured in Fig. 2B is mainly accounted for by the activity of phosphatases other than PP2A. In contrast, PP1 activity levels increased approximately threefold in cells infected with the BA71V virus (at 18 hpi), although at early times postinfection, PP1 activity remained at the background levels observed in

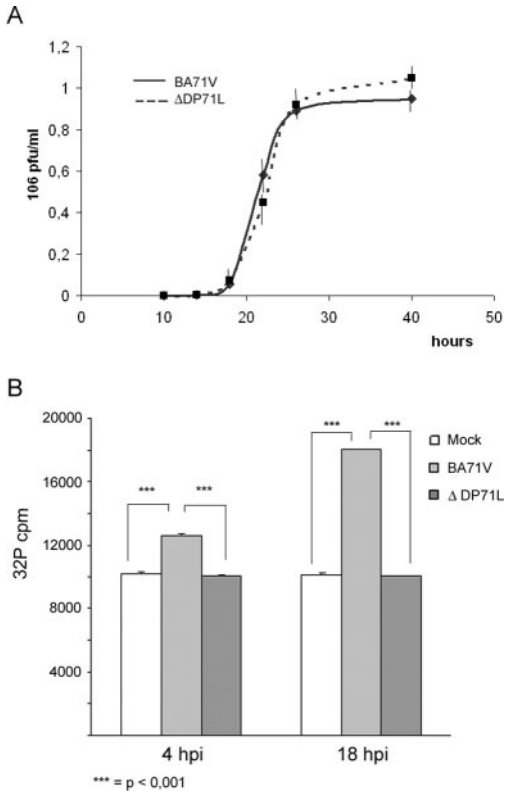

FIG. 2. (A) Growth curves for BA71V and DP71L deletion mutant virus ΔDP71L. Monolayers of Vero cells were infected with 0.1 PFU/cell, and total virus titers (10^6 PFU/ml) were analyzed by plaque assays at several times in infection. Experiments were performed in duplicate, and means ± SE (error bars) are shown. (B) BA71V infection increases total phosphatase activity. Mock-infected (control) or BA71V or ΔDP71L-infected cells for 4 h and 18 h were processed, and cell extracts were used to measure the total phosphatase activity (^{32}P cpm). Means plus SE (error bars) are shown. Values that are significantly different ($P < 0.001$) from each other are indicated (***).

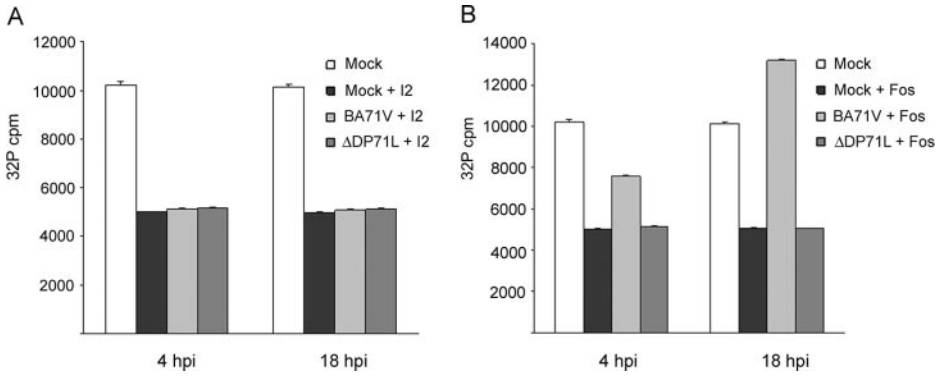

FIG. 3. BA71V infection specifically induces PP1 activation. (A) PP2α phosphatase activity was measured in cell extracts from mock-infected (control) or BA71V or ΔDP71L-infected cells in the presence of the PP2α-specific inhibitor I2. (B) Fostriecin (Fos) is a PP2α-specific inhibitor that allowed the discrimination of phosphatase activity levels specifically due to PP1 in the same cell extracts. Means plus SE (error bars) are shown.

mock-infected cells (Fig. 3B). The PP1 activity was not altered during infection with deletion mutant virus ΔDP71L, and the activity was similar to that obtained in mock-infected cells.

Since we have demonstrated the interaction between the DP71 viral gene product with PP1 as its putative partner by in vivo binding of these two proteins, using yeast two-hybrid analysis, and by in vitro binding of these two proteins, we suggest that the ASFV DP71L gene product might be responsible for the activation of protein phosphatase 1 upon infection in Vero cells.

eIF-2α-P is dephosphorylated following ASFV infection. Our results show that ASFV infection activates PP1 phosphatase activity. Possibly this may lead to dephosphorylation of specific substrates for PP1. PP1 is a ubiquitously expressed phosphatase with diverse functions. One substrate is the eukaryotic translation initiation factor (eIF-2α). Phosphorylation of eIF-2α due to PKR activation results in its inhibition, preventing the recycling of initiation factors and inhibition of protein synthesis. Many viruses have mechanisms to block PKR-mediated shutoff of host protein synthesis. The HSV ICP34.5 protein recruits PP1 to dephosphorylate eIF-2α (18). Because of the similarity of the DP71L protein with ICP34.5, we investigated whether ASFV infection may result in dephosphorylation of eIF-2α in Vero cells.

To increase phosphorylation of eIF-2α, cells were treated with 10 mM EGTA for 30 min before harvesting as described elsewhere (2, 24). Afterwards, cell extracts were prepared as described for the phosphatase activity assays.

Similar levels of total eIF-2α were found in the different cell lysates by immunoblotting using antibody that recognizes both phosphorylated and nonphosphorylated forms of eIF-2α, thus demonstrating that changes in eIF-2α phosphorylation were not due to changes in protein levels (data not shown).

As shown in Fig. 4, treating mock-infected Vero cells with EGTA results in high levels of phosphorylated eIF-2α. The background levels of eIF-2α-P in untreated Vero cells were almost undetectable. With cell extracts obtained after 4 hpi, no differences in the phosphorylation patterns were observed (data not shown). However, at 18 h postinfection, the levels of phosphorylation were reduced by more than twofold compared with background levels observed in mock-infected Vero cells. Interestingly, the decrease of eIF-2α-P dephosphorylation as a consequence of ASFV infection correlated directly and proportionally to the increase of PP1 activity, as shown in Fig. 3, and this difference is significant upon statistical analysis ($P < 0.05$). Taken together, these findings suggest that ASFV infection causes decreased phosphorylation of eIF-2α. This may result from the increased PP1 activity caused by ASFV infection.

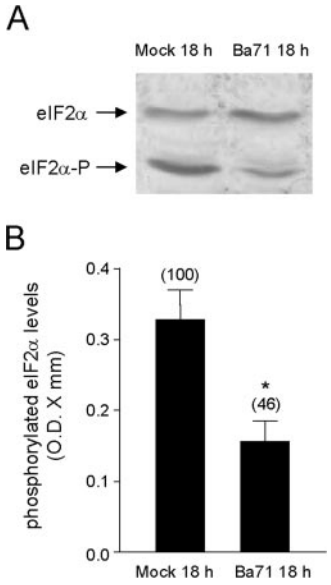

FIG. 4. eIF-2 is dephosphorylated in its α subunit upon BA71V infection. (A) Cultured mock-infected (control) or BA71V (Ba71)-infected cells for 18 h were then treated with EGTA (5 mM) for 30 min. Cells were processed, and cell extracts (65 μg of protein) were resolved in horizontal isoelectric focusing slab gels. The bands corresponding to eIF-2α and phosphorylated eIF-2α proteins were detected on immunoblots. The results of a representative experiment of three independent experiments performed in duplicate are shown. (B) Quantification of the eIF-2α-P bands in the three experiments shows eIF-2α dephosphorylation in cell extracts from BA71V-infected cells 18 h after infection. The numbers shown in parentheses are the mean values expressed as percentages. The means plus SE (error bars) are shown. The asterisk indicates that the value was significantly different ($P < 0.05$) from the mock-infected (control) value. O.D., optical density.

DISCUSSION

In this paper, we have demonstrated the interaction of an ASFV protein, DP71L, with the catalytic subunit of the phosphatase PP1 by using the yeast two-hybrid system and this interaction was confirmed by affinity chromatography. DP71L shares sequence similarity with the HSV ICP 34.5 protein over a C-terminal domain. PP1 is expressed in all eukaryotic cells and controls numerous cellular processes, including metabolism, cell division, apoptosis, and protein synthesis (8, 9). The available information suggests that most proteins interact with PP1 via multiple short sequence motifs. These PP1 binding motifs can be shared among PP1-interacting proteins, accounting for the ability of PP1 to form stable complexes with a large number of structurally unrelated proteins. More than 70 mammalian genes are known to encode proteins that interact with PP1 (8, 9). Some of these function as targeting subunits and bring PP1 in close proximity to its substrates; others modulate the activity and substrate specificity or are themselves substrates for the associated PP1.

Previous work has suggested that peptide sequences that bind to PP1 contain the motif VxF, where x is most frequently Arg (9). This motif was generally preceded by two to five basic residues immediately preceding the conserved valine. On the C-terminal side of the VxF motif, it was also evident that an acid residue was present in one of the next two residues. Thus, the consensus motif that emerges could be considered a widespread consensus sequence involved in recognition and binding of distinct regulatory subunits and proteins interacting with the catalytic subunit of PP1 (PP1c) is R/K-R/K-X_{0-2}-**V**-R/H-**F/W**-X-D/E, where V or F/W (shown in bold type) are almost invariant (9).

This docking consensus motif is present at almost 100% identity within the DP71L protein, including residues 14 to 18 (KHVRF). The only difference is the absence of an acid residue at the C-terminal side of the motif within the next two residues (shown underlined) (KHVRFA<u>AA</u>). Intriguingly, this acidic residue is also absent from the HSV γ_1 34.5 protein sequence, which also has hydrophobic residues at the second residue after the conserved motif VxF (19).

About one third of all eukaryotic proteins are controlled by phosphorylation of specific serine, threonine, and/or tyrosine residues. Most phosphorylations are reversible, implying that the phosphorylation level of a protein reflects the balance between the activities of the involved protein kinases and phosphatases. To examine the physiological relevance of the DP71L-PP1 interaction and test whether this interaction resulted in the activation or inhibition of the phosphatase activity, phosphatase activity assays were performed, using extracts from cells infected with wild-type ASFV or with a deletion mutant lacking the DP71L gene at a high multiplicity of infection. This showed that ASFV increased phosphatase activity in cells early after infection. This effect was lost in cells infected with the ASFV DP71L deletion mutant. When the two major Ser-Thr protein phosphatase activity levels were analyzed using specific inhibitors, we found that PP1 activity increased approximately threefold in cells infected with the virus (at 18 hpi), whereas at early times postinfection, PP1 activity remained at background levels. In contrast, no differences in the PP2A phosphatase activity of infected cells and mock-infected cells were found. The increased PP1 activity required expression of DP71L protein, since no increase in PP1 activity was observed in cells infected with ASFV in which the DP71L gene had been deleted. The mechanism by which PP1 activity is increased in ASFV-infected cells is unknown. PP1 is known to associate with regulatory subunits that can inhibit its phosphatase activity, for example, the I-1, NIPP1, and PNUTS proteins (21). One possibility is that the DP71L protein may displace inhibitory substrates from PP1, thus increasing PP1 phosphatase activity.

PP1 controls numerous cellular processes by the dephosphorylation of key regulatory proteins. One substrate is the eukaryotic translation initiation factor (eIF-2α). The ability of the HSV ICP34.5 and GADD34 proteins to target the catalytic subunit of PP1 to dephosphorylate eIF-2α has been previously described (4, 18). This requires the carboxyl termini of the ICP34.5 and GADD34 proteins, which share sequence similarity with DP71L (6, 20). The increase in PP1 activity we observed during ASFV infection might result in increased dephosphorylation of PP1 substrates. We demonstrated that eIF-2α phosphorylation levels were reduced by more than twofold in ASFV-infected cells compared with the background levels observed in controls, and this reduction correlated with the increase of PP1 activity. The DP71L protein may possibly act to increase dephosphorylation of eIF-2α indirectly by causing a general increase in PP1 activity or might specifically target PP1 to dephosphorylate eIF-2α. Further studies are in progress to define the role of DP71L-PP1 interaction in viral infection and associated pathogenesis.

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