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# Activation of the PI3K/Akt Pathway Early during Vaccinia and Cowpox Virus Infections Is Required for both Host Survival and Viral Replication▽

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**Viral manipulation of the transduction pathways associated with key cellular functions such as actin remodeling, microtubule stabilization, and survival may favor a productive viral infection. Here we show that consistent with the vaccinia virus (VACV) and cowpox virus (CPXV) requirement for cytoskeleton alterations early during the infection cycle, PBK/Akt was phosphorylated at S473 [Akt(S473-P)], a modification associated with the mammalian target of rapamycin complex 2 (mTORC2), which was paralleled by phosphorylation at T308 [Akt(T308-P)] by PI3K/PDK1, which is required for host survival. Notably, while VACV stimulated Akt(S473-P/T308-P) at early (1 h postinfection [p.i.]) and late (24 h p.i.) times during the infective cycle, CPXV stimulated Akt at early times only. Pharmacological and genetic inhibition of PI3K (LY294002) or Akt (Akt-X and a dominant-negative form of Akt-K179M) resulted in a significant decline in virus yield (from 80% to ≥90%). This decline was secondary to the inhibition of late viral gene expression, which in turn led to an arrest of virion morphogenesis at the immature-virion stage of the viral growth cycle. Furthermore, the cleavage of both caspase-3 and poly(ADP-ribose) polymerase and terminal deoxynucleotidyl transferase-mediated deoxyuridine nick end labeling assays confirmed that permissive, spontaneously immortalized cells such as A31 cells and mouse embryonic fibroblasts (MEFs) underwent apoptosis upon orthopoxvirus infection plus LY294002 treatment. Thus, in A31 cells and MEFs, early viral receptor-mediated signals transmitted via the PI3K/Akt pathway are required and precede the expression of viral antiapoptotic genes. Additionally, the inhibition of these signals resulted in the apoptosis of the infected cells and a significant decline in viral titers.**

The family *Poxviridae* is a family of large, linear, double-stranded DNA viruses that carry out their entire life cycle within the cytoplasmic compartment of infected cells. Vaccinia virus (VACV) is a prototypical member of the genus *Orthopoxvirus*, which also includes the closely related cowpox virus (CPXV) (12, 52). The genomes of these viruses are approximately 200 kbp in length, with a coding capacity of approximately 200 genes. The genes involved in virus-host interactions are situated at both ends of the genome and are associated with the evasion of host immune defenses (1). These evasion mechanisms operate mainly extracellularly. For example, the secretion of soluble cytokine and chemokine receptor homologues blocks the receptor recognition by intercepting the cognate cytokine/chemokine in the extracellular environment. This mechanism facilitates viral attachment and entry into cells (1, 70). Therefore, decoy receptors for alpha interferon (IFN-α), IFN-β, IFN-γ, and tumor necrosis factor alpha play an important immunomodulatory role by affecting both the host antiviral and apoptotic responses.

To counteract the host proapoptotic response, poxviruses have developed a number of antiapoptotic strategies, including the inhibition of apoptotic signals triggered by the extrinsic pathway (those mediated by death receptors such as tumor necrosis factor and Fas ligand) or the intrinsic pathway (mediated by the mitochondria and triggered upon viral infection) (1, 25, 70, 74). Many studies previously identified viral inhibitors that block specific steps of the intrinsic pathway. These include the VACV-encoded E3L, F1L, and N1L genes and the myxoma virus (MYXV)-encoded M11L gene, which block cytochrome *c* release (14, 20, 34, 39, 45, 75, 90), and the CPXV-encoded cytokine response modifier gene (CrmA) as well as the VACV-encoded SPI-2 gene, which inhibits both caspase-1 and caspase-8 (25, 58, 61, 74).

An emerging body of evidence has also highlighted the pivotal role played by intracellular signaling pathways in *Orthopoxvirus* biology (18, 48, 92). We and others have shown that poxvirus manipulation of signaling pathways can be virus specific. For example, while both VACV and CPXV stimulate the

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MEK/extracellular signal-regulated kinase (ERK)/EGR-1 pathway during a substantial length of time of their infective cycle, the pathway is required only for VACV replication, whereas its role in CPXV biology has yet to be identified (71). MYXV, a rabbit-specific poxvirus, also activates the MEK/ERK pathway in a mouse model of poxvirus-host interactions. However, this stimulation led to the expression of IFN-β, which consequently blocked virus replication and possibly explains why MYXV has such a restricted host range (87).

Another signaling molecule associated with viral replication is Akt kinase (also known as protein kinase B). The MYXV host range factor M-T5 is able to reprogram the intracellular environment, thereby increasing human tumor cell permissiveness to viral replication, which is directly associated with levels of phosphorylated Akt (88). In addition, M-T5 is functionally replaced by the host phosphatidylinositol 3-kinase (PI3K) enhancer A protein (92).

The transmission of intracellular signals mediated by the serine/threonine kinase Akt to downstream molecules in response to diverse stimuli such as growth factors, insulin, and hormones is dependent upon the phosphorylation of serine 473 (S473-P) and threonine 308 (T308-P). This phosphorylation is mediated by mammalian target of rapamycin complex 2 (mTORC2) and phosphoinositide-dependent protein kinase 1 (PDK1), which act as downstream effectors of the PI3K/Akt/mTORC1 pathway (2, 66). PI3Ks are a family of enzymes (classes I to III) that generate lipid second messengers by the phosphorylation of plasma membrane phosphoinositides. Class IA PI3Ks consist of a catalytic subunit (p110, comprising the three isoforms α, β, and δ) and an adaptor/regulatory subunit (p85, comprising the two isoforms α and β) (for a detailed review, see reference 80).

The Akt family of proteins is comprised of the three isoforms α, β, and γ, which are composed of an N-terminal pleckstrin homology domain, a central catalytic domain, and a C-terminal hydrophobic domain. Akt is recruited to the plasma membrane through the binding of its pleckstrin homology domain to the phosphatidylinositol 3,4,5-triphosphate (PIP3), which is a product of PI3K that is anchored to the plasma membrane. PDK1 is also recruited to the plasma membrane through interactions with PIP3. As both PDK1 and Akt interact with PIP3, PDK1 colocalizes with Akt and activates it by phosphorylating threonine 308 (T308-P) (2, 66). Following its activation, Akt phosphorylates a number of downstream substrates such as caspase-9, BAD, glycogen synthase kinase 3β (GSK-3β), and FKHR. This leads to the suppression of apoptosis, cell growth, survival, and proliferation (11, 16, 56).

Another downstream target of PI3K/Akt is mTOR, a serine/threonine kinase that plays a central role in the regulation of cell growth, proliferation, survival, and protein synthesis (26). mTOR kinase has recently been found to be associated with two functionally distinct complexes in mammalian cells, known as mTORC1 and mTORC2 (63, 66). Although these multiprotein complexes share molecules in common, distinct adaptor proteins are recruited into each complex: regulatory-associated protein of TOR (raptor) is recruited into mTORC1, while rapamycin-insensitive companion of TOR (rictor) is recruited into mTORC2 (33, 64). While mTORC1 controls cell growth and protein translation and has proven to be rapamycin sensitive, mTORC2 regulates the actin cytoskeleton and is assumed to be rapamycin insensitive, even though under conditions of prolonged exposure to the drug, it appears to inhibit mTORC2 assembly (29, 64, 65). Additionally, it has been demonstrated that mTORC2 regulates the activity of Akt through the phosphorylation of S473 (S473-P). S473-P appears to be required for the full activation of Akt, since S473-P has been shown to enhance the subsequent phosphorylation of T308 by PDK1 (66, 67, 94). Moreover, the phosphorylation of both S473 and T308 results in a four- to fivefold increase in Akt activity compared to T308-P by PDK1 alone (66).

The PI3K/PDK1/Akt(T308)/mTORC1 pathway regulates vital cellular processes that are important for viral replication and propagation, including cell growth, proliferation, and protein translation. This pathway is particularly important for the replication of DNA viruses, as their replication is cap dependent. However, the Akt signaling pathway can also negatively affect viral replication. The stress response downstream of Akt signaling, including hypoxia and energy and amino acid depletion, inhibits mTORC1 (5, 9, 69). Therefore, DNA viruses must overcome these constraints to translate their mRNAs.

Pharmacological disruption of the PI3K/Akt pathway with the specific PI3K inhibitor LY294002 (2-morpholino-8-phenyl-4*H*-1-benzopyran-4-one) (82) has been reported to not only increase the cleavage of downstream molecules associated with proapoptotic activity [e.g., poly(ADP-ribose) polymerase (PARP) and the executioner caspase-3] (38, 41) but also promote microtubule stabilization, actin filament remodeling/cell migration, and bleb formation/viral infectivity (10, 35, 49, 54, 59).

Because the PI3K/Akt and PI3K/Akt/mTOR pathways influence diverse cellular functions and possibly a healthy antiviral response, usurping these pathways could support an increase in viral replication. In support of this, a number of reports have demonstrated that either the PI3K/Akt or the PI3K/Akt/mTOR pathway plays a role in the replication of many viruses including flavivirus (38), hepatitis C virus (27), human immunodeficiency virus type 1 (93), human papillomavirus (44, 96), respiratory syncytial virus (77), coxsackievirus B3 (19), Epstein-Barr virus (17, 50, 73), human cytomegalovirus (36, 37, 72), herpes simplex virus type 1 (7, 83), varicella-zoster virus (60), Kaposi's sarcoma-associated herpesvirus (89), adenovirus (55), and simian virus 40 (SV40) (95). With this in mind, we also investigated whether the PI3K/Akt pathway played a pivotal role in orthopoxvirus biology. In this study, we show that the VACV- and CPXV-stimulated PI3K/Akt pathway not only contributes to the prevention of host-cell death but also plays a beneficial role in the viral replication cycle.

## MATERIALS AND METHODS

**Cell culture, antibodies, and chemicals.** A spontaneously immortalized cell line (A31), which is derived from mouse BALB/c 3T3 cells, wild-type mouse embryonic fibroblasts (MEFs) (81), SV40 LT-immortalized MEFs (MEFs-LT) (kindly provided by C. Ronald Kahn, Joslin Diabetes Center), and BSC-40 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 7.5% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Cultilab; Campinas, São Paulo, Brazil) and antibiotics in 5% $CO_2$ at 37°C. After reaching 80 to 90% confluence, the medium was then changed to 1% FBS, and the cells were incubated for 12 h. Antibodies against phospho-Akt(Ser473/Thr308); ERK1/2; PARP, which recognizes full-length PARP (116 kDa) and the large (89 kDa) and small (24 kDa) fragments of PARP resulting from caspase cleavage; and caspase-3, which detects full-length caspase-3 (35 kDa) and the large fragment of caspase-3 resulting from cleavage (17 kDa), and cleaved caspase-3 (Asp175),

which detects the large fragment of cleaved caspase-3 (17 to 19 kDa), were purchased from Cell Signaling Technology (Beverly, MA). LY294002, a pharmacological inhibitor of PI3K; rapamycin, a pharmacological inhibitor of mTORC1; the pancaspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk); the viral DNA synthesis inhibitor cytosine arabinoside (Ara C), and anti-β-actin antibody were purchased from Sigma-Aldrich (São Paulo, Brazil). The inhibitor of Akt, Akt-X {10-[4′-(*N*-diethylamino)butyl]-2-chlorophenoxazine}, and Geneticin (G418) were purchased from Calbiochem (São Paulo, Brazil). The specific antibodies for the viral H3L, D8L, A14L, and F18R proteins were a generous gift from B. Moss (NIAID, Bethesda, MD). An antibody that detects CrmA (SPI-2) was obtained from D. Pickup (Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC).

**Viruses and viral infection.** Wild-type VACV strain WR and CPXV strain BR were propagated in BSC-40 cells and highly purified by sucrose gradient sedimentation as previously described (31). The infective form of intracellular mature VACV and CPXV, which represents the majority of the infectious viral progeny, was used to carry out the experiments presented in this study. For experiments involving UV-irradiated viruses, viral stocks were exposed to a UV lamp producing irradiation predominantly at 365 nm for 5 to 10 min. UV-irradiated viruses were then tested for virus infectivity. Viruses that were unable to form plaques or in which late viral gene expression could not be detected by Western blotting were considered to be UV inactivated. Viral infections were carried out when cell cultures reached 80 to 90% confluence. Cells were infected in the absence of FBS at the indicated multiplicity of infection (MOI) for the times shown. Cells were treated with the indicated drugs for 30 min prior to viral infection and then incubated in the continued presence of drug for the indicated times.

**Virus infectivity assays.** A31 cells, MEFs, and MEFs-LT cells were cultured and starved as described above at a density of $5 \times 10^5$ cells per well in a six-well culture dish and then exposed to virus. Infections of A31 cells were carried out at an MOI of 10 for 3, 6, 12, 24, 36, and 48 h either in the absence or in the presence of LY294002 (20 μM) or at the same MOI for 12 h or 24 h either in the absence or in the presence of rapamycin (50 nM) or Akt-X (15 μM), respectively. MEFs and MEFs-LT were infected for 24 h either in the absence or in the presence of LY294002 as indicated above. Cultures were then washed with cold phosphate-buffered saline (PBS), and cells were disrupted by freezing and thawing. Virus was collected from the supernatant of centrifuged cells and assayed for infectivity as described previously (15). Each experiment was run in duplicate, and the results are reported as average values. The data were confirmed by at least three independent experiments with identical results.

**AKT dominant-negative cell lines.** Cell lines stably expressing dominant-negative Akt were generated by transfecting A31 cells with 10 μg of plasmid DNA encoding an N-terminal hemagglutinin (HA)-tagged kinase-defective Akt mutant [Akt(K179M)] (DN-Akt-HA), an Akt mutant in which the lysine residue at position 179 (the ATP binding site) was changed to a methionine (22), or the empty vector (pCDNA3) using a standard calcium phosphate transfection protocol. Transfected cells were then ring cloned after selection with 800 μg/ml Geneticin for at least 21 days. The expression of the mutant Akt protein was evaluated by Western blot analysis. Cell extracts were blotted and then probed with anti-HA or anti-Akt(S473-P) antibodies as described below.

**Electron microscopy.** A31 cells were infected with VACV or CPXV at an MOI of 2 either in the presence or in the absence of LY294002 (20 μM) and incubated at 37°C for 18 or 22 h, respectively. Cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature, scraped gently, and collected by centrifugation. The cells were then washed with cacodylate buffer, fixed with 1% osmium tetroxide, dehydrated in acetone, and then processed for conventional transmission electron microscopy. Thin sections were examined using a Morgagni transmission electron microscope operating at 80 kV.

**Western blotting. (i) Lysate preparation.** Cells were grown, starved as described above, and infected with VACV or CPXV at an MOI of 10 for the times shown. Cells were left untreated or preincubated with the indicated inhibitor for 30 min and then exposed to virus in the continued presence of the drugs as shown. Cells were then washed twice with cold PBS and lysed on ice with lysis buffer (20 mM Tris acetate [pH 7.0], 1 mM EDTA, 1% Triton X-100, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 4 μg/ml leupeptin, 1 mM sodium orthovanadate). Lysates were scraped, collected into Eppendorf tubes, and centrifuged at $13,500 \times g$ for 15 min at 4°C. Protein concentrations were determined using a Bio-Rad assay.

**(ii) Electrophoresis and immunoblotting.** Forty micrograms of the cell lysate per sample was separated by electrophoresis on a 10 or 15% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto nitrocellulose membranes as previously described (16). Membranes were blocked at room temperature for 1 h with PBS containing 5% (wt/vol) nonfat milk and 0.1% Tween 20. The membranes were washed three times with PBS containing 0.1% Tween 20 and then incubated with specific rabbit or mouse polyclonal or monoclonal antibody (1:1,000 to 1:3,000) in PBS containing 5% (wt/vol) bovine serum albumin and 0.1% Tween 20. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit (1:3,000) or anti-mouse (1:5,000) antibody. Immunoreactive bands were visualized using an ECL detection system as recommended by the manufacturer (GE Healthcare, Brazil).

**TUNEL apoptosis assay.** A31 cells were grown and starved as described above and then infected with VACV or CPXV at an MOI of 10 for 4 h. Cells were left untreated or were preincubated with LY294002 (20 μM) for 30 min and then incubated with virus in the continued presence of the drug. Cells were then fixed with 4% paraformaldehyde. A terminal deoxynucleotidyl transferase-mediated deoxyuridine nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions (TdT-FragEL DNA fragmentation kit; Calbiochem). The 3′-OH ends of the fragmented nucleosomal DNA in apoptotic cells were specifically labeled using exogenous terminal deoxynucleotidyl transferase and biotin-labeled deoxynucleoside triphosphate. Labeled ends were then detected using a streptavidin-horseradish peroxidase conjugate and diaminobenzidine. Nuclei were counterstained with methyl green. At least 300 cells (in 10 randomly captured microscopic fields at a ×600 magnification) were scored to calculate the percentage of TUNEL-positive nuclei. The data are expressed as the apoptosis index, which is the average percentage of apoptotic nuclei. The experiment was performed in triplicate and repeated two times.

**Densitometric analysis.** Levels of phosphorylated Akt and cleaved caspase-3 were quantified using densitometric analysis software (LabImage), and the levels were normalized to the levels of β-actin in the same sample. The changes in protein phosphorylation and cleavage with respect to control values were estimated. The results were expressed as the Akt-P-, cleaved caspase-3-, or H3L-A14L-to-β-actin ratio measured in arbitrary units.

## RESULTS

**VACV and CPXV stimulate Akt(S473/T308) phosphorylation.** In order to generate new progeny, poxviruses manipulate essential host signaling pathways such as the MEK/ERK pathway (4) and the PBK/Akt pathway (88) and, consequently, the intracellular environment to allow increased viral replication.

Because early events in virus-host interactions involve alterations in the cytoskeleton, from which orthopoxviruses may benefit by either virus attachment/penetration, morphogenesis, or release (43, 49, 53, 68), we initially investigated whether Akt was phosphorylated upon infection with VACV or CPXV at S473, a phosphorylation that is mediated by mTORC2. Since survival signals are also important and may benefit viral replication, we also analyzed the phosphorylation of Akt at T308, a role played by PDK1 (9, 63, 66). A31 cells were infected at an MOI of 10, and cell lysates were collected from the infected cells at 1 to 24 h postinfection (p.i.) and subjected to Western blotting using an anti-phospho-Akt antibody to evaluate the phosphorylation status of Akt. As shown in Fig. 1A (top), both VACV and CPXV were able to induce Akt phosphorylation. The amount of Akt-P was verified throughout the course of VACV infection, and the highest levels of Akt-P were observed at early (1 h p.i.) and at late (24 h p.i.) time points (Fig. 1A, lanes 11 and 14), and intermediate levels were detected at 3 and 7 h p.i. (lanes 12 and 13). In contrast, infection with CPXV was found to stimulate Akt-P(S473/T308) only at early time points (1 to 3 h p.i.) of infection (Fig. 1A, lanes 5 to 8).

We then asked whether the virus-stimulated signal leading to Akt-P(308) was mediated by the upstream kinase PI3K. LY294002, a pharmacological inhibitor of PI3K, inhibits PI3K activity through the competitive inhibition of the ATP binding site located on the regulatory subunit of PI3K (24, 79, 82), and the inhibition of Akt-P(308) may result, although indirectly, in

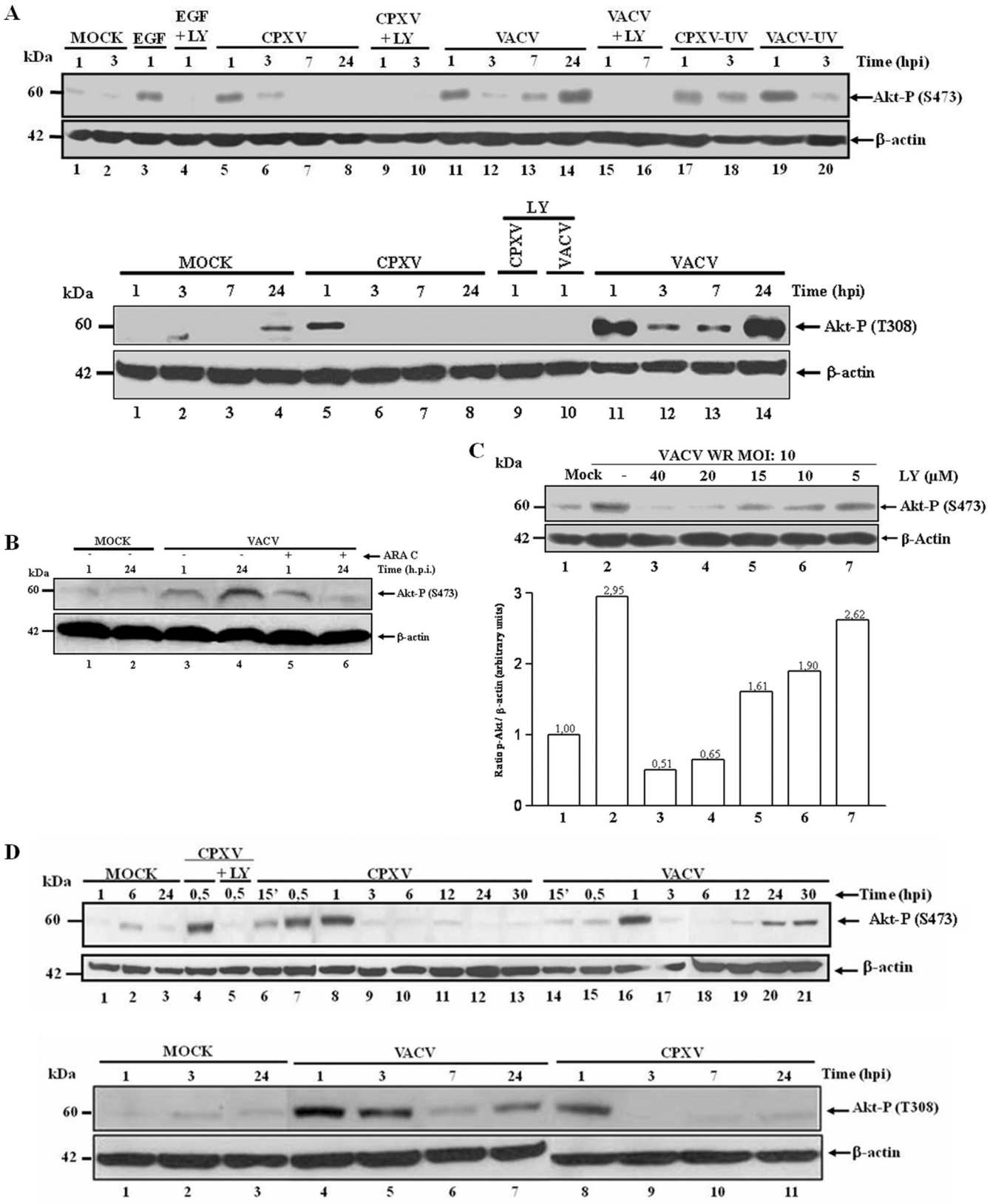


FIG. 1. VACV and CPXV induce Akt phosphorylation at S473 and T308. (A and D) A31 cells (A) and MEFs (D) were either mock infected or infected with VACV or CPXV at an MOI of 10 for the indicated times. Infected-cell lysates were subjected to Western blotting (WB) with anti-phospho-Akt(S473-P) or anti-phospho-Akt(T308-P) antibodies (top) or with anti-β actin antibody (bottom), which was used as a loading control. (A, top) Cells were infected either in the absence (lanes 5 to 8 and 11 to 14) or in the presence (lanes 9, 10, 15, and 16) of LY294002 (LY)

a reduced phosphorylation of Akt-P(473). Cells were left untreated or were preincubated with LY294002 (20 μM) for 30 min and then infected with CPXV or VACV, respectively, at an MOI of 10 for the times shown in Fig. 1A (lanes 9, 10, 15, and 16). Preincubation of the cells with LY294002 abrogated Akt-P, suggesting the requirement for PI3K in both VACV- and CPXV-induced Akt-P(T308) and, indirectly, Akt-P(S473) as well. As a control for Akt activation, we exposed the serum-starved cells to a well-known Akt stimulator, epidermal growth factor (EGF), at 100 nM. As shown in Fig. 1A, preincubation with LY294002 abrogated EGF-stimulated Akt-P(S473) (lanes 3 and 4).

Next, we raised the question as to whether the viral stimulation of Akt-P(S473) was dependent on viral replication. To inhibit replication, viruses were inactivated with UV radiation. Although viral irradiation prevents virus replication, it does not block virus penetration and the expression of some early genes (6, 51). Cells were exposed to the inactivated viruses for 1 and 3 h (Fig. 1A, top, lanes 17 to 20). Cell lysates were then collected and subjected to Western blotting with anti-phospho-Akt(S473) antibody. As shown in Fig. 1A (top), replication-competent virions, either CPXV (lanes 17 and 18) or VACV (lanes 19 and 20), were not required for the induction of Akt phosphorylation, suggesting that receptor-mediated events are sufficient to trigger the phosphorylation of Akt. Taken together, these data implied that the viral induction of Akt-P takes place before viral replication and that viral attachment and entry are sufficient for Akt-P. This is consistent with the viral requirement for actin remodeling early during the infective cycle (43, 49, 68) and viral activation of Akt-S473-P by mTORC2 as well as stimulation of early survival signals, a role played by the PI3K/PDK/Akt(T308-P) pathway (reviewed in reference 9).

Because the kinetics of Akt phosphorylation upon VACV or CPXV infection were quite unique, e.g., with Akt-P(S473/T308) being detected at late times (24 h p.i.) only upon infection with VACV, this suggested that VACV may require postreplicative gene expression in order to phosphorylate Akt. To test this hypothesis, A31 cells were incubated for 30 min with Ara C (40 μg/ml) prior to VACV infection at an MOI of 10 for the times shown. Experiments were carried out either in the absence or in the continued presence of Ara C, and cell lysates were then collected and subjected to Western blotting using anti-phospho-Akt antibody. As shown in Fig. 1B, in the presence of Ara C, Akt(S473-P) was detected only at early times but not at 24 h p.i. (compare lanes 5 and 6). As the incubation with Ara C compromises intermediate-late gene expression, this suggests that the phosphorylation of Akt late during the VACV infective cycle is dependent on post-DNA replication events. Based on this observation, we hypothesized that Akt(T308-P) at late times during the VACV infective cycle is also dependent on the same events.

In order to examine whether the pharmacological inhibition, although indirect, of Akt(S473-P) was effective in a dose-dependent manner, A31 cells were incubated with increasing concentrations (5, 10, 20, and 40 μM) of LY294002 for 30 min prior to VACV infection at an MOI of 10 for 1 h. Cell lysates were then collected and subjected to Western blotting using anti-P-Akt antibody. The pharmacological inhibition of Akt-P in response to viral infection was found to be dose dependent, as shown in Fig. 1C. Similarly, LY294002 inhibited the induction of Akt-P in a dose-dependent manner during CPXV infection (data not shown).

To further investigate whether the induction of Akt-P following infection with VACV or CPXV, and its blockade by preincubation with LY294002, occurred in cells other than A31 cells, another spontaneously immortalized cell line, MEFs (81), was grown, starved as indicated above for A31 cells, and then infected with VACV or CPXV at an MOI of 10. Cell lysates were then collected at the indicated times p.i. and analyzed by Western blotting using anti-phospho-Akt(S473/T308) antibodies to evaluate Akt-P. As demonstrated in Fig. 1D (top), the kinetics of the viral induction of Akt-P(S473/T308) and its dependence either directly or indirectly on PI3K (only the inhibition associated with CPXV infection is shown) were also confirmed with MEFs. Of note, the kinetics of Akt-P were consistent with its late stimulation occurring only upon VACV infection (Fig. 1D, top, lanes 20 and 21). The early induction of Akt-P is also consonant with the cytoskeletal alterations required during early infection. Taken together, these results were very similar to those seen with A31 cells, which strongly suggests that the findings are not cell type specific.

**The PI3K/Akt signaling pathway is required for viral growth.** In order to investigate whether the orthopoxvirus-stimulated PI3K/Akt pathway was of biological relevance to the virus, we performed a one-step viral growth curve experiment in either the presence or the absence of LY294002. A31 cells were left untreated or were treated with LY294002 (20 μM) for 30 min prior to viral infection at an MOI of 10.

(20 μM). As a control for Akt-P(S473-P), cells were stimulated with EGF (100 nM) for 30 min (lanes 3 and 4). Viral induction of Akt-P is independent of replication-competent virions. Cells were exposed to UV-inactivated virus for 1 and 3 h (lanes 17 and 18, CPXV; lanes 19 and 20, VACV). (Bottom) Cells were infected either in the absence (lanes 5 to 8 and 11 to 14) or in the presence (lanes 9 and 10) of LY294002 (20 μM) and subjected to Western blotting using an anti-Akt(T308-P) antibody as indicated. (B) VACV-induced Akt-P is dependent on post-DNA replication events. A31 cells were infected with VACV at an MOI of 10 for the times shown either in the absence (lanes 3 and 4) or in the presence (lanes 5 and 6) of Ara C (40 μg/ml). Cell lysates were then subjected to Western blotting as described above. (C) Pharmacological inhibition of virus-induced Akt-P is dose dependent. A31 cells were mock infected, infected with VACV, or incubated with 40, 20, 15, 10, or 5 μM LY294002 prior to virus infection for 1 h as indicated. The levels of phosphorylated Akt were quantified by densitometric analysis, and the phospho-Akt/β-actin ratio (arbitrary units) is shown. (D, top, upper blot) MEFs were mock infected (lanes 1 to 3), infected with CPXV (lanes 4 to 13) either in the presence (lane 5) or in the absence (lanes 4 and 6 to 13) of LY294002 (20 μM), or infected with VACV (lanes 14 to 21) and subjected to Western blotting using an anti-Akt(S473-P) antibody as indicated. (Lower blot) MEFs were mock infected (lanes 1 to 3), infected with VACV (lanes 4 to 7), or infected with CPXV (lanes 8 to 11) and subjected to Western blotting using an anti-Akt(T308-P) antibody as indicated. The molecular masses are indicated (in kDa) on the left. Similar results were obtained in three independent experiments.

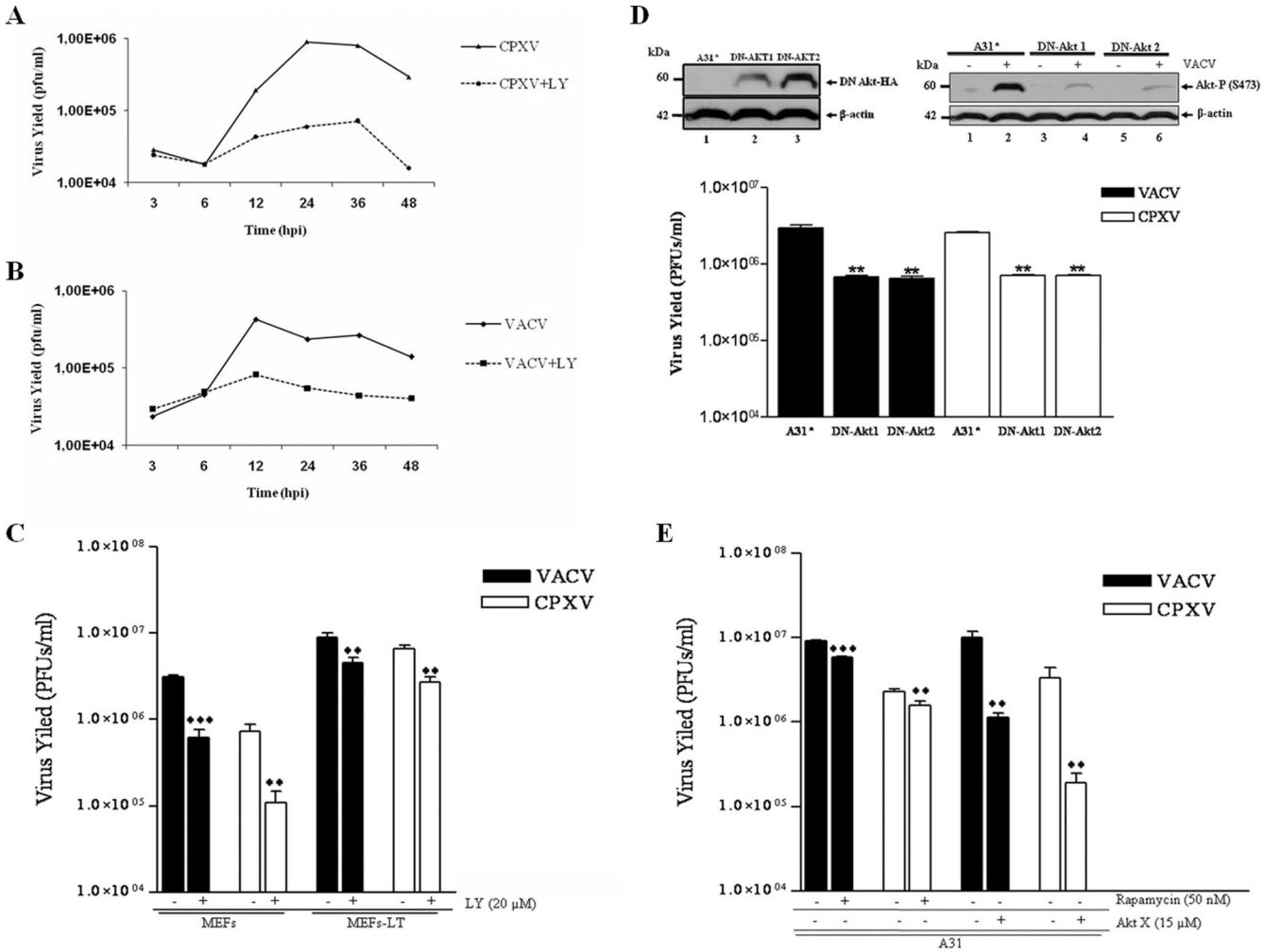


FIG. 2. The PI3K/Akt signaling pathway is required for viral replication. A31 cells were either left untreated or treated for 30 min with LY294002 (LY) (20 μM), rapamycin (50 nM), or Akt-X (15 μM) prior to virus infection and infected with CPXV or VACV at an MOI of 10 either in the absence or in the continued presence of LY294002 for 3, 6, 12, 24, 36, and 48 h; of rapamycin for 12 h; or of Akt-X for 24 h. (C) MEF cells, either spontaneously immortalized (MEFs) or MEFs-LT, were either left untreated or treated for 30 min with LY294002 (20 μM) prior to VACV or CPXV infection in the continued presence of inhibitor for 24 h. Viruses were then collected, and viral titers were determined. (D, top) Two representative cell lines transfected with a dominant-negative form of Akt [Akt(K179M)] (DN-Akt) (lanes 2 and 3) or A31 cells transfected with the empty vector (A31*) (lane 1) are shown. Cells were left uninfected or infected with VACV or CPXV at an MOI of 10 for 24 h as indicated. Lysates were collected and subjected to Western blotting with an HA-specific antibody (top left) or with anti-Akt(S473-P) antibody (top right). The bottom blots were probed with β-actin as a loading control. (D, bottom) Viral infectivity assays. Data are representative of three independent experiments with identical results (A and B) or are means of data from triplicate experiments ± SD (C, D [bottom], and E). **, $P < 0.01$; ***, $P < 0.001$. A Student's $t$ test was used in comparisons of A31* and DN-Akt cells (D) or cell lines untreated or treated with the indicated pharmacological inhibitor (C and E).

Viruses were then collected at 3, 6, 12, 24, 36, and 48 h p.i. and assayed for infectivity. The data indicate that the PI3K/Akt pathway did play a relevant role in both VACV and CPXV biologies. A significant reduction in viral titers (≥90%) was observed when either CPXV (Fig. 2A) or VACV (Fig. 2B) infection was carried out in the continued presence of the PI3K inhibitor.

In order to verify that the inhibitory effect associated with LY294002 was not restricted to A31 cells, MEFs were also infected with VACV or CPXV as described above. As shown in Fig. 2C, LY294002 caused a significant decline in viral titers (85 to 90%), thereby demonstrating that viral inhibition is not cell type specific. Next, we investigated viral replication in the context of a non-spontaneously-immortalized cell line such as MEFs-LT. Cells were infected with VACV or CPXV under the same experimental conditions used for MEFs, and the titers were then measured. Our findings revealed that virus titers were decreased by 50% (2.0-fold) and 58% (2.4-fold), respectively.

Next, to rule out the possibility of a nonspecific pharmacological inhibition of PI3K/Akt, we generated cell lines stably expressing the dominant-negative form of Akt (DN-Akt-K179M). Clones were then monitored for the expression of HA-Akt by Western blotting using an anti-HA antibody and the VACV stimulation of Akt(S473-P). Two representative clones expressing DN-Akt are shown in Fig. 2D, top left (lanes

2 and 3), as are control cells transfected with the empty vector only (A31*) (lane 1), and in Fig. 2D, top right, where the levels of Akt-P were significantly reduced upon viral infection (lanes 4 and 6). The importance of Akt in viral replication was determined by measuring the viral titers following the infection of cells expressing DN-Akt. The representative clones were infected with VACV or CPXV at an MOI of 10, and at 24 h p.i., viruses were collected, and the titers were determined. As shown in Fig. 2D (bottom), viral titers were significantly decreased (~70 to 80%) in cells expressing DN-Akt, suggesting that the PI3K/Akt pathway does play an important role in the VACV and CPXV life cycles.

To further strengthen the involvement of Akt in mediating upstream signals after viral stimulation of PI3K, we pretreated the cell with the pharmacological inhibitor of Akt, Akt-X (15 μM), and infected the cells at an MOI of 10 with VACV or CPXV in the continued presence of the inhibitor, and at 24 h p.i., virus was collected and assayed for infectivity. Our data showed that the viral titers were significantly reduced by ≥90% (Fig. 2E). Collectively, these data strongly suggest that the PI3K/Akt pathway is beneficial for VACV and CPXV replication.

Since mTORC1, the downstream target of PI3K/PDK1/Akt(T308-P), regulates key cellular events such as survival, proliferation, and translation, and because the maintenance of translation is important for viral cap-dependent mRNA translation, we investigated whether mTORC1 could be required for VACV and CPXV replication. A31 cells were left untreated or were incubated for 30 min prior to virus infection with rapamycin (50 nM), a specific pharmacological inhibitor of mTORC1. Cells were infected in the continued presence of rapamycin at an MOI of 10, and at 12 h p.i., viruses were collected and assayed for infectivity. This period of time was chosen not only because it is the earliest time when a significant decline in virus yield was verified (Fig. 2A and B) but also to rule out the possibility of a nonspecific inhibition of mTORC2 observed after a prolonged exposure of the cells to rapamycin (65). Our findings demonstrate that the replication of VACV and CPXV was only partially affected (~35% [1.5-fold decrease]) following mTORC1 inhibition (Fig. 2E). Similar levels of inhibition were also observed following incubation with different concentrations of rapamycin (30 and 70 nM), while a higher drug concentration (100 nM) appeared to increase the cytotoxic effect (data not shown).

**Disruption of the PI3K/Akt pathway is followed by altered expression levels of early and/or late viral genes.** To gain insight into the mechanism(s) underlying the decreased viral yield upon treatment with LY294002, experiments were designed to investigate whether early (CrmA/SPI-2) and/or late (F18R, H3L, A14L, and D8L) viral gene expression was affected following the preincubation of the cells with the inhibitor. Cells were cultured either in the presence or in the absence of LY294002 and infected with VACV or CPXV at an MOI of 10 for the indicated times. Cellular lysates were then collected and subjected to Western blotting using antibodies raised against the viral proteins CrmA/SPI-2 (Fig. 3A), F18R (Fig. 3B), H3L (Fig. 3C), A14L (Fig. 3D), and D8L (Fig. 3E). As shown in Fig. 3, viral gene expression was remarkably affected upon treatment with LY294002, and protein expression was either abrogated (Fig. 3B to D) or delayed (A and E). This emphasizes the critical role played by the PI3K/Akt pathway in the regulation of orthopoxvirus gene expression. It also provided genetic evidence that the activation of the PI3K/Akt pathway is required for viral late gene expression. Similarly, the infection of cells expressing DN-Akt demonstrated that both A14L and H3L expression levels were significantly reduced (Fig. 3G to H).

**Altered viral early and/or late gene expression is accompanied by an arrest in orthopoxvirus morphogenesis.** In order to investigate whether the altered expression of the viral early and/or late genes demonstrated in Fig. 3 was accompanied by an arrest in virion morphogenesis, cell cultures were left untreated (Fig. 4A and C) or were pretreated with LY294002 (20 μM) (B and D) and then infected with VACV (A and B) or CPXV (C and D) at an MOI of 2 for 18 and 22 h, respectively. As shown in Fig. 4, while cultures of infected cells alone (Fig. 4A and C) contained the full spectrum of normal intermediates and mature virions typically seen in virion morphogenesis, the preincubation of cells with LY294002 (B and D) resulted in an infectious cycle that was arrested at the immature-virion or immature-virion-with-nucleoids stage of the virion morphogenic cycle. Therefore, the inhibition of the PI3K/Akt pathway resulted in an arrest that equally affected the same stages of the morphogenic cycles of both orthopoxviruses. Furthermore, the arrest in virion morphogenesis is consistent with the altered viral gene expression induced by both the PI3K inhibitor and DN-Akt.

**Viral stimulation of the PI3K/Akt pathway induces the cleavage of PARP and caspase-3.** To further elucidate the role of the PI3K/Akt pathway in regulating the survival and/or apoptosis of orthopoxvirus-infected host cells, we blocked the pathway by preincubating cells with LY294002, infected the cells with virus, and then investigated whether the cleavage of host proteins associated with characteristic hallmark features of apoptosis, such as caspase-3 and PARP, was affected. Cells were mock infected or infected with VACV or CPXV at an MOI of 10 for 3, 6, 12, or 24 h in either the presence or the absence of LY294002 (20 μM). Cell lysates were then harvested and subjected to Western blotting with anti-caspase-3 (Fig. 5, top) and anti-PARP (Fig. 5, middle) antibodies. The pharmacological inhibition of the PI3K/Akt pathway resulted in the cleavage of caspase-3 and PARP in orthopoxvirus-infected cells (Fig. 5, top and middle, lanes 16 to 23). These findings strongly suggest that the viral stimulation of the PI3K/Akt pathway has an antiapoptotic/prosurvival effect.

**Inhibition of apoptosis is followed by an enhancement of the virus-stimulated survival pathway.** We have demonstrated that the pharmacological blockade of the PI3K/Akt pathway is followed by a decline in levels of orthopoxvirus replication (Fig. 2). Combined with the proapoptotic data presented in Fig. 5, these findings strongly suggest that, at least in part, the increased cytopathic effects observed in the infected cells (Fig. 6C and D, compare d and h with b and f) were due to the pharmacological inhibition of the antiapoptotic activity mediated by the PI3K/Akt pathway.

In order to further investigate this, A31 cells were preincubated for 30 min with increasing concentrations (10, 15, 20, and 40 μM) of the pancaspase inhibitor zVAD.fmk either in the absence or in the presence of LY294002 (20 μM). Cells were then infected with virus at an MOI of 10, and at

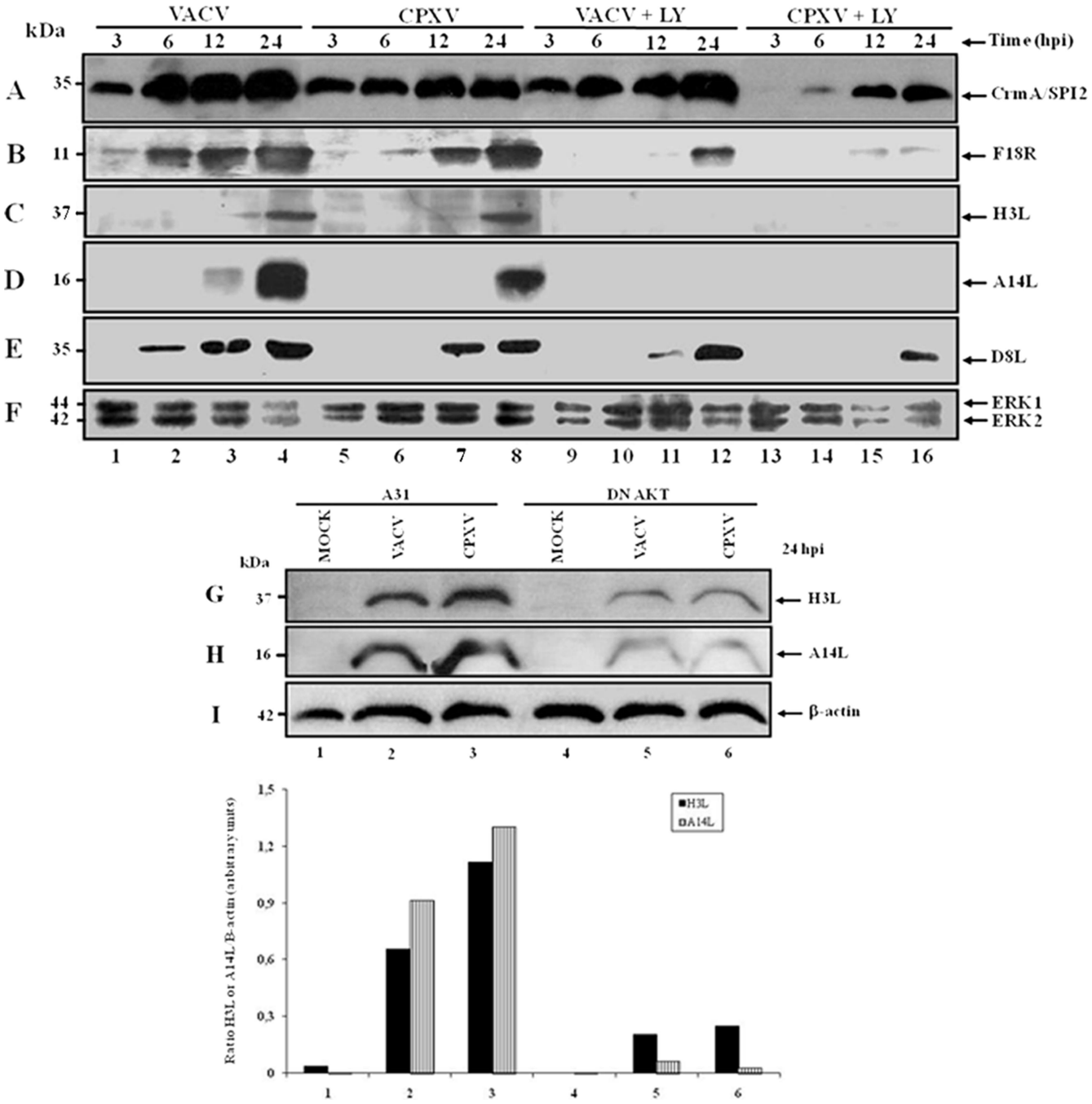


FIG. 3. Inhibition of the PI3K/Akt pathway interferes with expression of viral genes. A31 cells were left untreated or treated with LY294002 (LY) (20 μM) for 30 min prior to infection with VACV or CPXV at an MOI of 10 either in the absence or in the continued presence of LY294002. (A to E) At the various time points shown, cell lysates were harvested and analyzed by Western blotting using antibodies raised against the viral proteins CrmA (SPI-2) (A), F18R (B), H3L (C), A14L (D), and D8L (E). (F) Anti-total ERK1/2 antibody was used as an internal control for protein loading. (G and H) A31 cells transfected with the empty vector (A31*) (lanes 1 to 3) or DN-Akt2 (lanes 4 to 6) cells were infected with VACV or CPXV at an MOI of 10 for 24 h. Lysates were collected and subjected to Western blotting using anti-viral H3L (G) and A14L (H) antibodies. (I) Antibactin antibody was used as an internal control for protein loading. The levels of H3L and A14L were quantified by densitometric analysis, and the H3L/β-actin ratio and A14L/β-actin ratio (arbitrary units) are shown. Molecular masses (in kDa) are indicated on the left. Data are representative of data from at least three independent experiments with very similar results.

3 h p.i., cell lysates were collected and subjected to Western blotting with anti-Akt-S473-P antibody. As shown in Fig. 6A, incubation with zVAD.fmk (20 μM) prior to VACV infection increased the levels of Akt-P (lane 4), which were even more pronounced (1.25-fold) than the level seen with VACV infection alone (lane 1). In contrast, the pharmacological inhibition of the PI3K/Akt pathway resulted in decreased levels of Akt-P upon infection (Fig. 6A, lane 3).

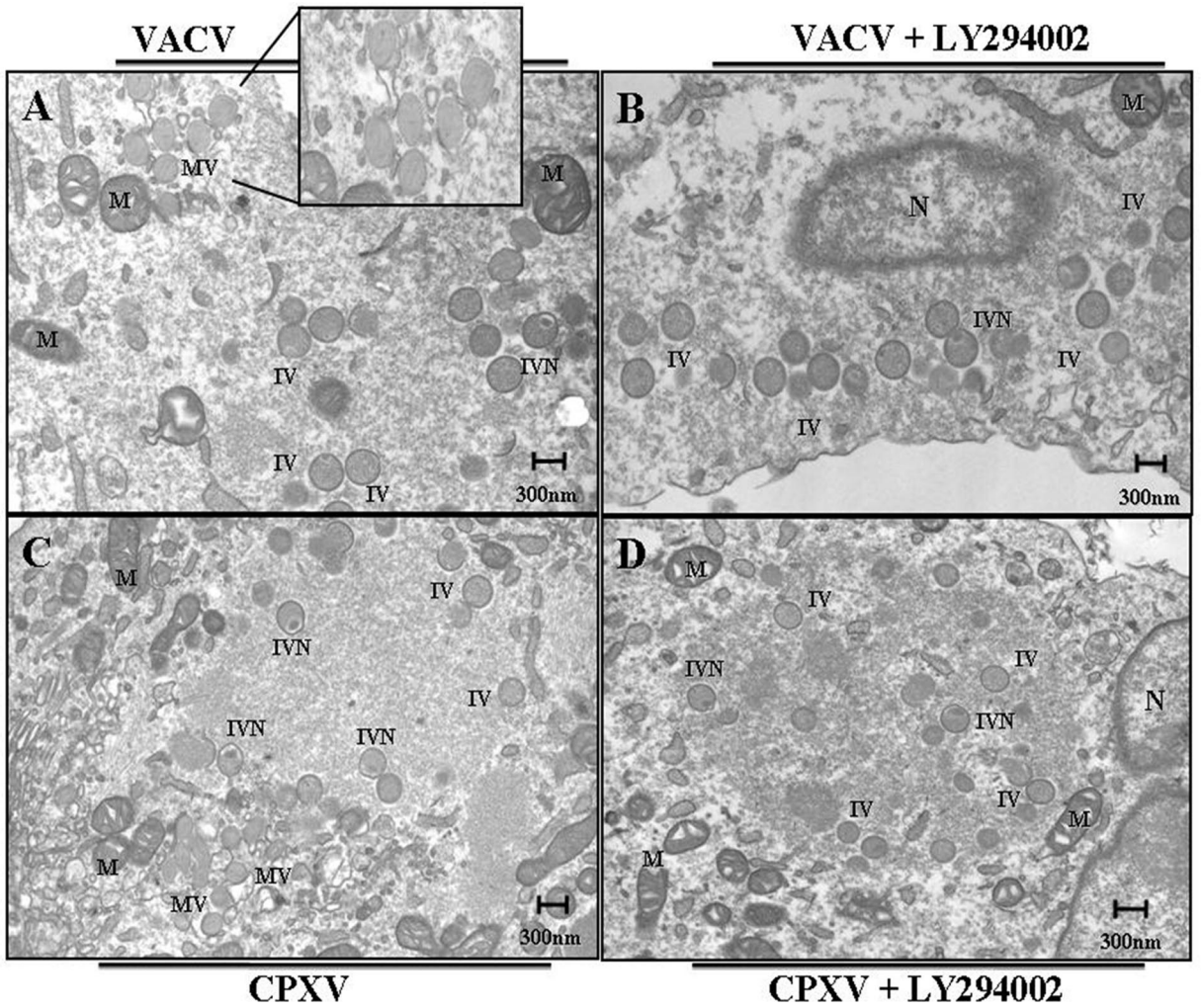


FIG. 4. Altered viral early and/or late gene expression is followed by an arrest in orthopoxvirus morphogenesis. A31 cells were infected with VACV (A and B) or CPXV (C and D) at an MOI of 2 for 18 or 22 h, respectively, either in the absence (A and C) or in the presence (B and D) of LY294002 (20 μM). Cells were then fixed and prepared for transmission electron microscopy. Electron micrographs are shown, with their scales indicated by bars. (Inset) Detail showing the mature virus. Abbreviations: IV, immature virus; IVN, immature virus with a nucleoid; MV, intracellular mature virus; N, nucleus; M, mitochondria. Data are representative of data from at least two independent experiments with similar results.

Remarkably, incubation with zVAD.fmk at 10, 15, 20, or 40 μM reversed the levels of VACV-mediated Akt phosphorylation in a dose-dependent manner even in the presence of LY294002 (Fig. 6A, lanes 5 to 8). Although not as pronounced as those verified with VACV infection, similar results were also observed when the infections were performed with CPXV (Fig. 6A, lanes 9 to 12).

Given that the inhibition of proapoptotic signals (by zVAD.fmk) is accompanied by enhanced host survival signals (e.g., increased Akt-P levels) upon VACV and CPXV infection, one would expect that under this condition, the cleavage of caspase-3 after exposure to zVAD.fmk would also be inhibited in a dose-dependent manner. To investigate this hypothesis, A31 cells were incubated with 10, 15, 20, or 40 μM of zVAD.fmk for 30 min, either in the absence or in the presence of LY294002 (20 μM), prior to viral infection at an MOI of 10 for 3 h. Cell lysates were collected and subjected to Western blotting with an anti-caspase-3 antibody that specifically detects the cleaved form of caspase-3 (17 to 19 kDa). As demonstrated in Fig. 6B, the cleavage of caspase-3, as a consequence of the inhibition of the survival pathway by LY294002 (lanes 5 to 8), was inhibited in a dose-dependent manner by the antiapoptotic compound zVAD.fmk. This would increase cell viability and, subsequently, favor viral replication. The same set of experiments was performed after infection with CPXV, and the results were similar to those found upon VACV infection (Fig. 6B, lanes 9 to 12).

With this in mind, we then investigated whether preincubation with zVAD.fmk was sufficient, at least in part, to reverse the increased cytopathic effect observed with the infections carried out in the presence of LY294002. Cells were preincubated with LY294002 (20 μM) for 30 min and then treated with zVAD.fmk (40 μM) for an additional 30 min before infection with VACV (Fig. 6C) or CPXV (Fig. 6D) at an MOI of 10 for 8 or 24 h. The infected cells were then examined by phase-contrast microscopy. Our findings

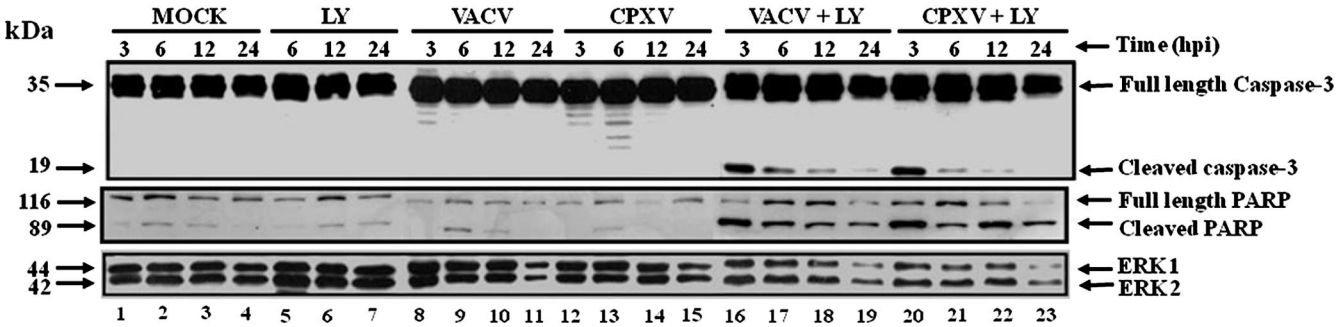


FIG. 5. Viral stimulation of the PI3K/Akt pathway has an antiapoptotic effect on host cells. A31 cells were either mock infected (lanes 1 to 4), incubated with LY294002 (LY) (20 μM) alone (lanes 5 to 7), virus infected at an MOI of 10 (lanes 8 to 15), or incubated with LY294002 prior to virus infection for 30 min and then virus infected in the continued presence of the inhibitor (lanes 16 to 23), as indicated. At various time points, cell lysates (40 μg) were harvested and immunoblotted with anti-caspase-3 (top) or with anti-PARP (middle) antibodies to detect the cleavage of precursor forms. As a control for protein loading, the membrane (middle) was stripped and reprobed with an anti-ERK1/2 antibody (bottom). Molecular masses are indicated on the left. Data are representative of data from at least two independent experiments with similar results.

suggest that the antiapoptotic signals induced upon exposure to zVAD.fmk not only decreased the cytopathic effect but also increased cell viability in both infection models (Fig. 6C and D, compare d and h with b and f). Furthermore, while the infections carried out for 24 h in the presence of LY294002 alone led to an abundant number of detached cells that were recovered from the supernatant, cells infected in the simultaneous presence of LY294002 and

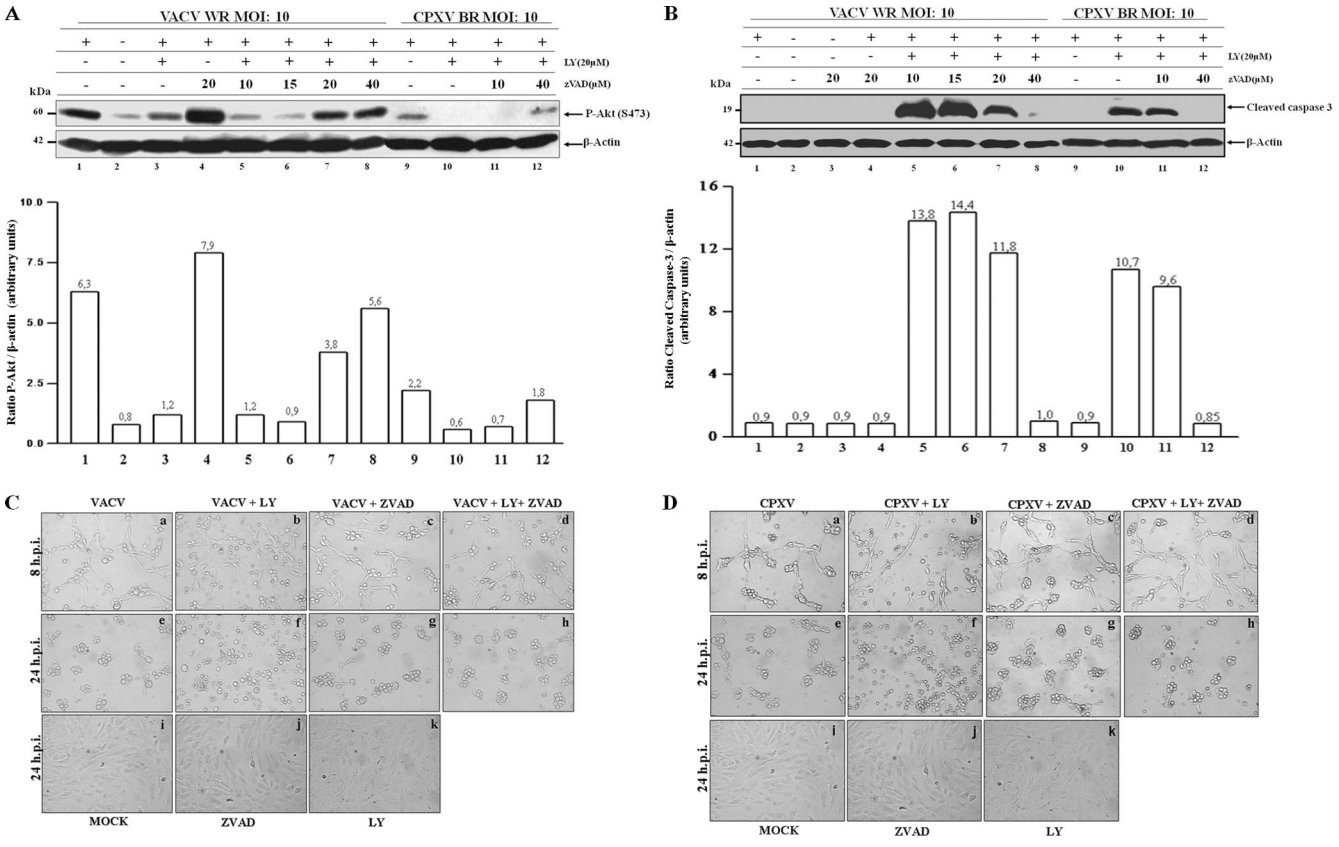


FIG. 6. Inhibition of apoptosis is followed by an enhancement of the virus-stimulated survival pathway. A31 cells were either infected with VACV or CPXV at an MOI of 10 for 3 h, incubated with LY294002 (LY) (20 μM) alone, or incubated simultaneously with zVAD.fmk (zVAD) prior to infection as indicated. Cell lysates were collected (40 μg) and were subjected to Western blotting with the indicated antibody. (A, top) The pancaspase inhibitor zVAD.fmk restores the levels of Akt-P in a dose-dependent fashion. (B, top) Inhibition of apoptosis diminishes the levels of cleaved caspase-3 in a dose-dependent manner. (A and B, bottom) An anti-β-actin antibody was used as an internal control for protein loading. Molecular masses (in kDa) are indicated on the left. The levels of phosphorylated Akt were quantified by densitometric analysis, and the phospho-Akt/β-actin ratio (arbitrary units) is shown. (C and D) Phase-contrast microscopy. A31 cells were either mock infected (i), incubated with zVAD.fmk (40 μM) alone (j), incubated with LY294002 (20 μM) alone (k), or incubated with LY294002 or LY294002 plus zVAD.fmk as indicated prior to viral infection for 30 min and then VACV or CPXV infected (a to h) in the continued presence of the inhibitor(s) for 8 and 24 h. Data were consistently reproduced in at least three independent experiments with very similar results.

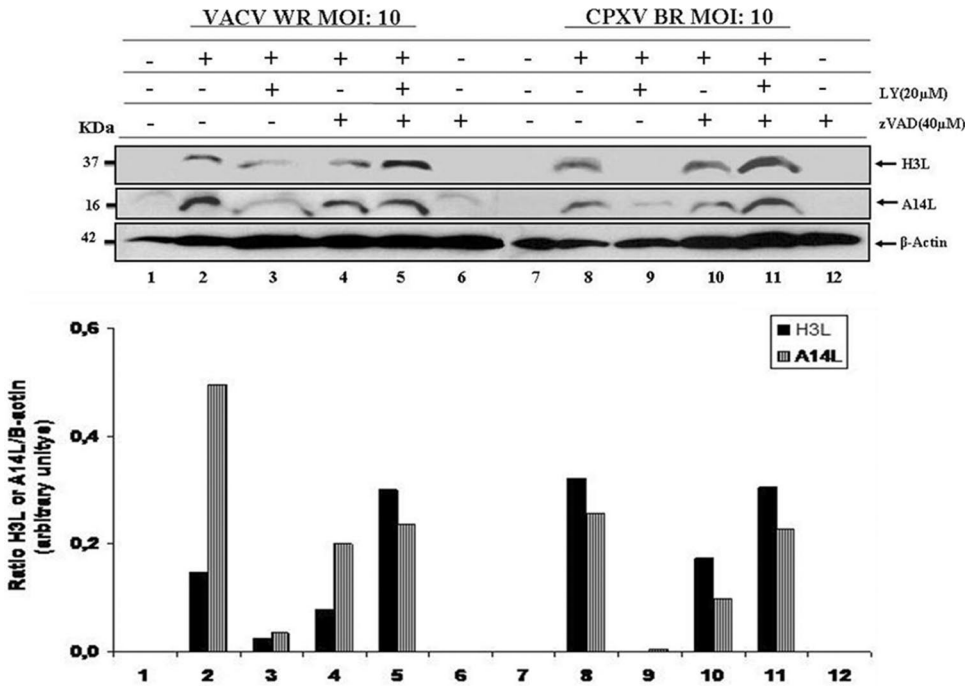


FIG. 7. Blockade of viral late protein expression by LY294002 is caspase dependent. A31 cells were preincubated with LY294002 (20 μM) (LY) for 30 min followed by treatment with zVAD.fmk (zVAD) (40 μM) for an additional 30 min prior to infection with VACV or CPXV at an MOI of 10 for 24 h, as indicated. Cell lysates were collected (40 μg) and subjected to Western blotting using anti-A14L or -H3L antibodies as indicated. (Top and middle) The pancaspase inhibitor zVAD.fmk reverses the blockade of LY294002 upon viral late protein H3L and A14L expression (lanes 5 and 11). (Bottom) Anti-β-actin antibody was used as an internal control for protein loading. The levels of H3L and A14L were quantified by densitometric analysis, and the H3L/β-actin ratio or A14L/β-actin ratio (arbitrary units) is shown. Molecular masses are indicated on the left. Data were consistently reproduced in at least two independent experiments with very similar results.

zVAD.fmk not only remained significantly more attached to the substrate but also were recovered in the supernatant to a lesser extent (data not shown). Altogether, these data indicate that the inhibition of proapoptotic signals, accompanied by enhanced host survival signals and increased cell viability upon VACV or CPXV infection, plays an important role during the viral infective cycle.

**Inhibition of viral late protein expression by LY294002 is caspase dependent.** In order to investigate whether the LY294002-mediated inhibition of viral late protein expression is due to an acceleration of apoptosis in infected cells, we compared the levels of expression of the viral proteins A14L and H3L in the absence and in the presence of zVAD.fmk. A31 cells were incubated with LY294002 (20 μM) for 30 min and then treated with zVAD.fmk (40 μM) for an additional 30 min prior to VACV or CPXV infection at an MOI of 10 for 24 h. Cell lysates were collected and subjected to Western blotting with anti-A14L or anti-H3L antibodies. Remarkably, our results indicate that the inhibition of caspase-3 cleavage (Fig. 6B) and apoptosis by the general pancaspase inhibitor zVAD.fmk reverse the inhibitory effect of LY294002 on viral A14L and H3L expression (Fig. 7). The observation that zVAD.fmk is capable of blocking caspase-3 cleavage in infected cells (Fig. 6B) in association with the reversion of A14L and H3L expression, even in the presence of LY294002 (Fig. 7, lanes 5 and 11), indicates that the regulatory effect of the PI3K/Akt pathway exerted during orthopoxvirus replication is a caspase-dependent event.

**Inhibition of the PI3K/Akt pathway by LY294002 results in apoptosis of infected cells.** In order to firmly establish that the inhibition of the PI3K/Akt pathway by LY294002 results in the apoptosis of the infected cells, a TUNEL assay was used to monitor apoptosis in individual cells. A31 cells were incubated with LY294002 (20 μM) for 30 min prior to viral infection with VACV or CPXV at an MOI of 10 for 4 and 6 h, respectively. A brown precipitate confined to the nucleus was considered to be a TUNEL-positive cell. As shown in Fig. 8A, TUNEL-positive nuclei were barely observed in the absence of LY294002 in the mock-infected cells (not shown) and in the VACV-infected (Fig.8Aa) or the CPXV-infected (Fig.8Ac) cells. However, the number of TUNEL-positive cells increased significantly only in cultures preincubated with LY294002 and infected with VACV or CPXV (Fig. 8Ab and d). The apoptosis index was calculated, and pretreatment with LY294002 resulted in a 41% (± standard deviation [SD]) and a 16% (±SD) increase in the number of apoptotic cells following infection with VACV or CPXV, respectively (Fig. 8B). To certify that the inhibition of the PI3K/Akt pathway that results in the apoptosis of A31-infected cells was not a cell-type-specific event, the same approach was used for MEFs. As demonstrated in Fig. 8Ae and f, VACV- or CPXV-infected MEFs also underwent apoptosis upon treatment with LY294002, while apoptosis was not observed in virally infected cells in the absence of LY294002 (data not shown). The apoptosis index for these cells was also determined, and it was found to be similar to that shown in Fig. 8B (data not shown). Thus, these findings not

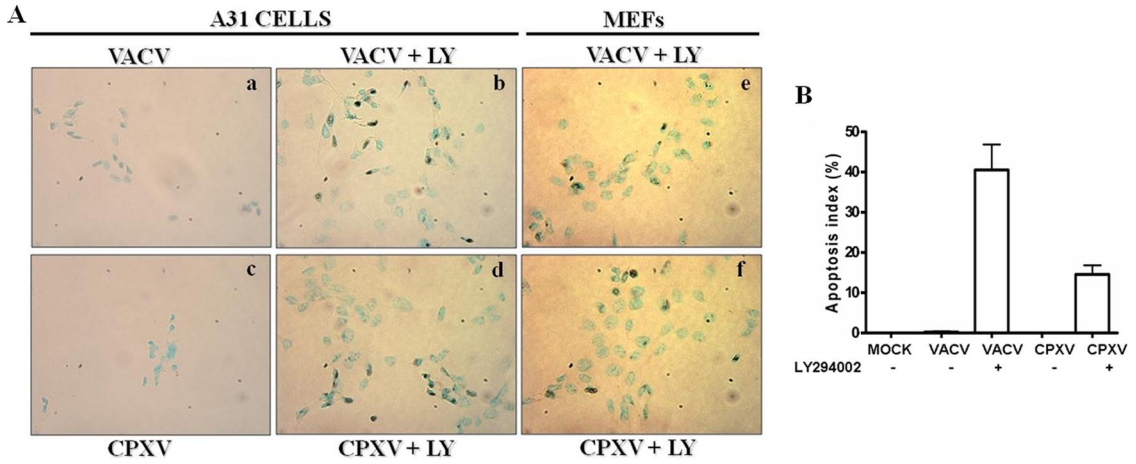


FIG. 8. (A) Pharmacological inhibition of the PI3K/Akt pathway results in apoptosis of infected cells. Shown is TUNEL staining for fragmented DNA. A31 cells were left untreated (a and c) or treated with LY294002 (LY) (20 μM) for 30 min (b and d) prior to infection with either VACV (b) or CPXV (d) at an MOI of 10 for 4 or 6 h, respectively. MEFs were treated with LY294002 for 30 min prior to VACV (e) or CPXV (f) infection as described above for A31 cells. Cells were then processed for the TUNEL assay according to the manufacturer's recommendations (Calbiochem). Brown dots are TUNEL-positive nuclei. Magnification, ×600. Data are representative of data from an experiment performed in triplicate and repeated two times with similar results. (B) Apoptosis indexes determined as the average percentages of apoptotic nuclei.

only confirm the hypothesis that the pharmacological inhibition of the PI3K/Akt pathway induced the infected spontaneously immortalized cells to undergo apoptosis but also suggest that under these conditions, CPXV appears to be more effective than VACV in inhibiting apoptosis.

## DISCUSSION

Virus replication and assembly rely on a delicate balance between host defense mechanisms and the viral manipulation of signaling pathways that govern host responses. Furthermore, the viral activation of intracellular pathways associated with the transmission of mitogenic and host survival signals can improve viral utilization of the host resources and metabolism required to efficiently produce viral progeny (13, 18).

We previously demonstrated that both VACV and CPXV stimulate the mitogen-activated protein kinase/ERK1/2 pathway throughout the viral life cycle (4, 71). However, while the stimulation of the pathway is beneficial for VACV multiplication, the biological significance of CPXV-mediated mitogen-activated protein kinase/ERK1/2 activation requires further clarification.

In the present report, we demonstrate that both VACV and CPXV stimulate a signaling pathway that results in the phosphorylation of protein kinase B/Akt on both S473 and T308, modifications that are mediated by mTORC2 and PI3K/PDK1, respectively (9, 63, 66). We also demonstrate that the incubation of cells with a pharmacological inhibitor of PI3K, LY294002, prior to viral infection impairs the signal transduction required for the induction of Akt-P in a dose-dependent manner (Fig. 1A and C). Our findings are also consistent with the hypothesis that the phosphorylation of Akt(S473/T308) is an early event that takes place soon after viral attachment/entry (e.g., 1 to 3 h p.i.) (Fig. 1D). Moreover, the induction of Akt-P does not require replication-competent virions, since infection with UV-inactivated virus also induced Akt-P (Fig. 1A). Importantly, this is consistent with the previously described viral requirement for cytoskeletal alterations early during the infection (43, 49) and the activation of mTORC2 as well as virally stimulated early survival signals. Notably, the kinetics of Akt-P induction were distinct for these orthopoxviruses. Thus, while Akt-P(S473/T308) is induced by CPXV only at early time points following infection (1 to 3 h p.i.), a time frame in which the UV-irradiated virus is also able to induce Akt-P (Fig. 1A, lanes 17 to 20), late stimulation of Akt-P upon VACV infection requires post-DNA replication events (Fig. 1B). Thus, the induction of Akt-P may be a consequence of the interaction between a viral gene product and cellular PI3K. Such an event may result in the transmission of signals to the downstream effector Akt. A similar signaling pathway has been described for the host range factor MT-5 from MYXV: the activation of Akt by MYXV is dependent on the association between MT-5 and Akt (88). However, the precise mechanisms underlying the VACV- and CPXV-mediated stimulation of the PI3K/Akt pathway remain to be elucidated.

Our data also provide evidence of the relevant role played by the PI3K/Akt pathway in the life cycle of these orthopoxviruses. Growth rates of both VACV and CPXV are significantly reduced by ≥90% (1 log unit [10-fold]) in the presence of LY294002 (Fig. 2A and B). This observation was further supported by the treatment of infected cells with the specific Akt inhibitor, Akt-X. Akt-X treatment resulted in a reduction (≥90%) in the titers of both VACV and CPXV (Fig. 2E), which is therefore of the same magnitude verified with LY294002. Furthermore, the infection of cell lines expressing DN-Akt (Fig. 2D) confirms the involvement of the PI3K/Akt pathway in viral replication (about a 70 to 80% decrease in viral titers) and rules out the possibility of a nonspecific pharmacological inhibition of LY294002. Considering that (i) PI3K is targeted by LY294002 and that PDK1 is the downstream effector of PI3K leading to Akt(T308-P) and (ii) LY294002, although indirectly, inhibits the induction of Akt(S473P), these data strongly suggest that Akt is fully activated upon infection

with these orthopoxviruses. It has also been demonstrated that viral replication declines significantly in MEFs pretreated with LY294002, confirming the biological relevance of the PI3K/Akt pathway for these orthopoxviruses (Fig. 2C). It is worth noting that while the pharmacological inhibition of PI3K/Akt (LY294002 and Akt-X) resulted in a ≥10-fold reduction in viral titers in the spontaneously immortalized cell line A31 (Fig. 2A, B, and D) and that about the same level of inhibition was also verified with MEFs (LY294002), the decline in virus yields verified after the exposure of SV40-LT MEFs to LY294002 was just partially affected (2- to 2.4-fold reduction) (Fig. 2C). Thus, these findings suggest that the preactivation state of the intracellular environment in the SV40-LT MEFs per se (9) is beneficial for virus replication and that pretreatment with LY294002 blocks only the viral increment that follows the infection, which appears to reflect the partial reduction observed.

In agreement with previously reported data (85), we also demonstrate that both VACV and CPXV require mTORC1 during the infective cycle. These findings are consistent with the viral mechanisms used to translate their cap-dependent mRNAs, a pathway that is regulated by the PI3K/PDK1/Akt(T308-P)/mTORC1 signaling pathway (reviewed in reference 9). While the overall effect of LY294002 and Akt-X on viral replication is measured by a decline of ≥90% (≥10-fold decrease) in viral titers, the blockade of mTORC1 by rapamycin (which affects mRNA translation) resulted in a reduction of ~35% (1.5-fold decrease) in virus yield, thereby suggesting that the other biological activities regulated by the PI3K/Akt pathway, beyond mTORC1 activation, seem to be coactivated in order to attend the diverse viral infective demands and, thus, maximize viral replication. However, it is known that the viral E3L and K3L genes are also activated to bypass the block of viral mRNA translation imposed by protein kinase R (12, 34, 52). Remarkably, as was described previously for herpes simplex virus (83, 84) and human cytomegalovirus (37, 86) infections, VACV also releases translation using a mechanism that operates downstream of mTOR. VACV requires mTORC1 to phosphorylate the eukaryotic translational repressor eIF4E binding protein (4E-BP). Following this phosphorylation, 4E-BP is degraded by the proteasome. Indeed, it has been demonstrated that by the inactivation of 4E-BP, VACV alters the activity of the eIF4F complex and stimulates the accumulation of eIF4F components (eIF4E and eIF4G) within the viral factories, thereby facilitating viral replication (85). While the mechanism employed by CPXV to release translation awaits further investigation, it is reasonable to assume that the strategy used by CPXV might resemble the strategy described above for VACV. Thus, we conclude that by fully activating Akt, VACV and CPXV facilitate and/or maximize their own replication.

Consistent with the viral requirement of the PI3K/Akt pathway for a productive infection, if this pathway is inhibited upstream, viral gene expression is remarkably affected (Fig. 3). While the inhibition of PI3K/Akt appears to affect viral gene expression to different extents, the delay observed with the CPXV induction of CrmA seems to be more pronounced than that observed with VACV (Fig. 3, compare lanes 9 to 12 with 13 to 16). However, the delayed expression of this viral antiapoptotic gene does not appear to affect the global CPXV antiapoptotic mechanisms, as shown in Fig. 8B. However, the mechanisms underlying this effect require further exploration. Furthermore, genetic evidence also confirms the involvement of the pathway in viral gene expression, since infection of DN-Akt cells results in a significant decrease in H3L and A14L expression levels (Fig. 3G to H). Additionally, the PI3K/Akt pathway plays an important role in virion morphogenesis, as demonstrated by the arrest that occurs at the immature-virion/immature-virion-with-nucleoid stage of the morphogenic cycle when the infections are carried out in the presence of LY294002 (Fig. 4). Similarly, the functional or genetic ablation of the A14L or H3L gene also resulted in an arrest early during virion morphogenesis (40, 62). Taken together, these data strongly demonstrate the beneficial role played by the PI3K/Akt pathway in both VACV and CPXV morphogenesis and growth.

Successful viral replication and assembly are dependent upon cell survival. Therefore, viruses have also evolved diverse mechanisms to control the pathways that govern cell survival (13, 19, 21). The PI3K/Akt pathway has been demonstrated to play a pivotal role in cell survival and proliferation (16, 78), and the inhibition of the pathway is associated with a significant decrease in host cell viability (8, 78) and virus replication (19, 30, 42, 77).

Therefore, it is reasonable to assume that VACV and CPXV stimulate the PI3K/Akt pathway to increase the viability of infected host cells in order to prolong the life span of the cell, which would allow more time for the virus to generate its progeny. As shown in Fig. 5, the inhibition of the PI3K/Akt pathway during either VACV or CPXV infection significantly increases the cleavage of proteins associated with the induction of apoptosis, such as the executioner caspase-3 and PARP (19, 38, 41) (Fig. 5, top and middle). This strongly suggests an antiapoptotic role for the pathway in the course of these orthopoxvirus infections.

Our findings also demonstrate that the pancaspase inhibitor zVAD.fmk reverses the proapoptotic signals associated with the blockade of the PI3K/Akt pathway by increasing the levels of Akt-P (Fig. 6A). This observation, in association with data showing that zVAD.fmk also reverses the cleavage of caspase-3 (Fig. 6B), reinforces the antiapoptotic role of the virus-stimulated pathway.

It has long been known that the PI3K/Akt pathway plays a critical role in cell survival and proliferation and that its disruption is associated with a significant decrease in host cell viability (8, 57). Thus, the maintenance of prosurvival and antiapoptotic signals upon viral infection is of critical importance for successful viral replication. Our data demonstrate that zVAD.fmk not only decreases the cytopathic effect associated with LY294002 during infections but also appears to increase cell adherence and viability (Fig. 6C and D), which are critical requirements for the generation of viral progeny. Furthermore, the LY294002-mediated inhibition of late viral gene expression, as demonstrated by an analysis of the A14L and H3L proteins, is a caspase-dependent process (Fig. 7). Importantly, viruses have evolved diverse means to regulate viral gene expression in either a caspase-dependent (e.g., flaviviruses [38] and orthopoxviruses [this study]) or caspase-independent (e.g., coxsackievirus) (19) manner.

Our hypothesis that the pharmacological blockade of the

PI3K/Akt pathway was, at least in part, associated with pro-survival and antiapoptotic signals (Fig. 5 to 8) was further strengthened by the observation that infected cells undergo apoptosis upon exposure to LY294002, a phenomenon that was verified not only with A31 cells but also with MEFs (Fig. 8). Therefore, receptor-mediated signals conveyed through the PI3K/Akt pathway at early times during the infection of permissive and spontaneously immortalized cells (e.g., A31 cells and MEFs) appear to be important to control cell survival and/or apoptosis. Additionally, these signals should precede the expression of viral antiapoptotic genes (CrmA, F1, E3L, and N1) because neither CPXV nor VACV seems to be capable on its own of fully preventing the cells from undergoing apoptosis in the presence of LY294002 (Fig. 8). While the activation of this pathway appears to favor VACV and CPXV replication (Fig. 2 to 5, 7, and 8), it is remarkable that the levels of Akt activation correlate not only with cancer progression (i.e., higher metabolic, survival, and proliferation activities) (76, 80) but also with the levels of permissiveness to infection with the otherwise-rabbit-specific poxvirus MYXV in a diverse set of human transformed cell lines (88, 92). Therefore, it is tempting to speculate that levels of Akt-P that have been associated with permissive transformed cells (e.g., BSC-40 and HeLa cells) may facilitate an increased level of replication of VACV and CPXV. In line with this assumption, it has consistently been verified that the viral yields following infection of HeLa or BSC-40 cells with either VACV or CPXV are at least 10-fold higher than those obtained following infection of A31 or MEF cells. Not surprising, the viral yields were only partially affected (approximately a twofold reduction) when the infection of BSC-40 cells was carried out in the continued presence of LY294002 or following the infection of SV40-LT-immortalized MEFs, which suggests that the preactivation state of Akt in these permissive lines, as well as in other transformed cell lines, is sufficient to elicit the activation of downstream signaling that is required early during the infection (Fig. 2C and our unpublished observations). However, this must be conclusively confirmed. Since most of the relevant experiments performed in vitro to elucidate the role of apoptosis during VACV or CPXV infection were carried out with transformed cell lines (e.g., HeLa, HEK 293T, BSC-40, or Jurkat cells or the monocyte/macrophage cell line J774.G8) (14, 28, 39, 74, 75, 90, 91, 97), their levels of Akt activation should be higher than those observed in spontaneously immortalized permissive cells (e.g., A31 cells or MEFs). Furthermore, in experiments in which MEFs were cultured under permissive conditions for the early VACV-mediated PI3K/Akt signals, i.e., in the absence of LY294004, it was demonstrated that the viral antiapoptotic genes were necessary and sufficient to control apoptosis (75, 90).

Therefore, it was hypothesized that under this circumstance (e.g., higher levels of Akt-P), the viruses become less dependent on the early signals elicited by the host spontaneously immortalized cell lines after viral infection, and thereby, the viral antiapoptotic repertoire could sufficiently block virus-induced apoptosis. This suggests that the early signals transmitted by the PI3K/Akt pathway upon the attachment and/or penetration of VACV or CPXV (Fig. 1A and D) would be required not only for cytoskeletal alterations but also for host survival. Because prior phosphorylation of Akt on S473 by mTORC2 was previously reported to be required for the phosphorylation of Akt on T308 by PDK1 (66, 67, 94) and the early activation of mTORC1 is necessary for host survival and viral translation (26, 85), the combination of these events, either dependently or independently, should provide the host (A31 cells and MEFs) with the necessary means to avoid apoptosis before the viral antiapoptotic repertoire could be activated. It has long been known that orthopoxviruses have evolved several strategies to regulate both the extrinsic and intrinsic apoptotic pathways (25, 74). Nonetheless, our data suggest that these viral antiapoptotic mechanisms, although necessary, are not sufficient to ensure successful viral replication (Fig. 2A to C, 3 to 5, 8, and 9). This scenario thus contrasts with that of transformed cell lines, where higher levels of Akt activation should bypass the need for virus-induced survival signals, and therefore, the virus antiapoptotic genes should be sufficient to prevent apoptosis.

Even though CPXV appears to be more effective than VACV in inhibiting apoptosis, as reflected by their apoptosis indices (16% [±SD] versus 41% [±SD], respectively), the overall effects of LY294002 on viral replication were similar, though not equal, for both viruses (Fig. 2 to 8). This suggests that the inhibition of the PI3K/Akt pathway may impact aspects of orthopoxvirus biology other than the control of survival and/or apoptosis. Indeed, the PI3K/Akt pathway regulates a variety of biological processes that are potentially important for viral replication, including actin remodeling, cell migration (3, 29, 47, 59, 64), and microtubule stabilization (10, 23, 54). Previous research demonstrated that usurping these pathways would benefit orthopoxviruses in several ways: (i) transportation of the virus through the microtubules to the cell periphery, followed by a switch from microtubule- to actin-based motility (reviewed in reference 53); (ii) transcription and translation of viral mRNAs (32, 46); (iii) alteration of cytoskeletal organization and translocation of translation factors to viral factories (32, 53, 68, 85); (iv) bleb formation and apoptotic mimicry to penetrate the cell (49); and (v) phosphorylation of Akt(S473) by mTORC2, which regulates the actin cytoskeleton (9, 37, 72). Therefore, it is not surprising that the simultaneous incubation of the infected cells with LY294002 and zVAD.fmk, which protects the cells only from apoptosis, restored the viral titers only partially (~20%) compared with the cells incubated with LY294002 alone (data not shown). This further emphasizes the global effect of the PI3K/Akt pathway on the viral life cycle beyond host survival.

In addition, it is remarkable that although VACV and CPXV belong to the same genus, they diverge in the way in which they manipulate cellular signaling pathways. While the stimulation of the PI3K/Akt pathway by both viruses facilitates viral replication, the kinetics of this activation by the viruses are quite different. While VACV stimulated the pathway during the early and late phases of the infective cycle, as determined by the need for viral late gene expression at 24 h p.i. (Fig. 1B), CPXV stimulation of the same pathway was restricted to the early phase of the viral infective cycle in both cell lines analyzed.

In conclusion, in this report, we demonstrated that the signals triggered by the PI3K/Akt pathway upon VACV and CPXV infection do play an important role in controlling cell survival and apoptosis. Nonetheless, it appears that the signals

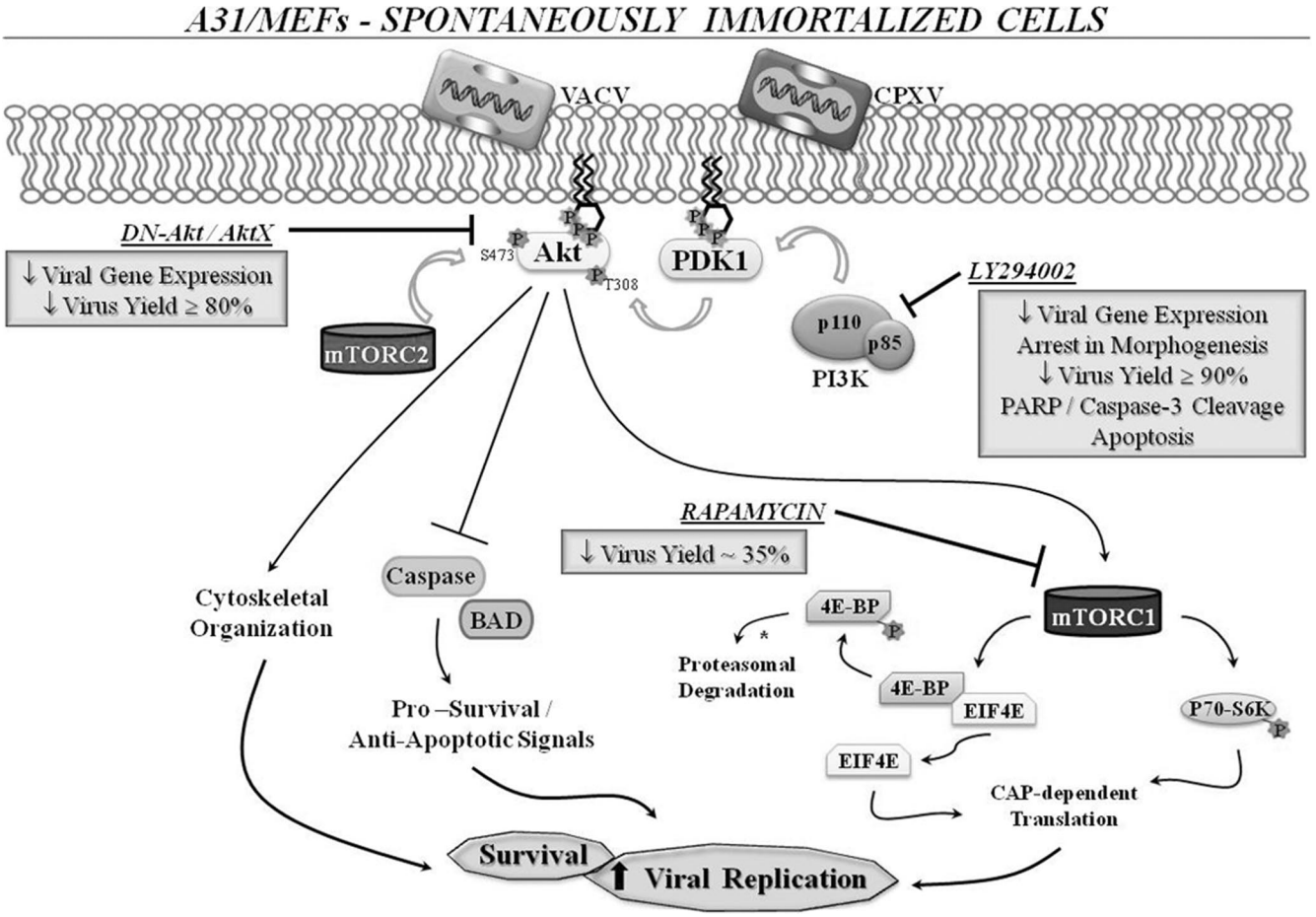


FIG. 9. Schematic representation of the VACV- and CPXV-stimulated PI3K/Akt pathway of biological relevance in spontaneously immortalized A31 and MEF cells. Upon attachment and/or penetration, VACV and CPXV activate Akt(S473-P) and Akt(T308-P) via mTORC2 and PI3K/PDK1, respectively. Thus, Akt is fully activated and transduces signals associated with diverse biological activities to attend viral demands, such as cytoskeleton alterations, survival, translation release, and microtubule stabilization, which appear to benefit virus penetration, morphogenesis, and release, thereby increasing viral replication. Boxes highlight the biological consequences after pharmacological (LY294002, rapamycin, and Akt-X) and/or dominant-negative (DN-Akt) blockade of the pathways. Viral stimulation of both the PI3K/Akt pathway early during the infective cycle and the viral antiapoptotic genes is required to impede the demise of the cells, which is followed by an increase in virus titers. In contrast, upon the blockade of the pathway, the viral antiapoptotic genes alone are not sufficient to prevent the host cells from undergoing apoptosis, which is accompanied by a decline in virus yield.

required for successful viral replication extend beyond survival and apoptosis. As the late events related to the release and/or spread of orthopoxviruses are dependent upon actin dynamics, a role associated not only with mTORC2 but also with the Src family of tyrosine kinases (reviewed in reference 53), it will be exciting to further investigate and compare the specific contributions of Akt(S473-P) and the Src family kinases to the biology of these orthopoxviruses during the late stages of their infective cycles. This could explain the more-pronounced effect of both LY294002 and Akt-X on CPXV replication (Fig. 2A to C).

A schematic representation depicting the relevant findings of this study is shown in Fig. 9. Soon after VACV and CPXV encounter a host spontaneously immortalized cell line such as A31 cells or MEFs, signals from the PI3K/Akt pathway are triggered and are required to release translation and host survival, a function associated with mTORC1, via the upstream phosphorylation of T308 by PDK1. The activation of mTORC2 [Akt(473-P)], which is required for cytoskeletal alterations and virus attachment and/or penetration, in conjunction with the phosphorylation of Akt on T308 via PI3K, appears to boost kinase activities and, thus, creates an intracellular environment that not only protects A31 cells and MEFs from undergoing apoptosis but also favors VACV and CPXV replication. Pharmacological or genetic blockade of the PI3K/Akt pathway interrupts early signal transmission, and under these circumstances, the viral antiapoptotic genes are not sufficient to prevent the cells from undergoing apoptosis.

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