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Akt Inhibitor Akt-IV Blocks Virus Replication through an Akt-Independent Mechanism▽

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Many viruses activate the phosphatidylinositol 3′-kinase (PI3k)/Akt intracellular signaling pathway to promote viral replication. We have analyzed whether a rapidly replicating rhabdovirus, vesicular stomatitis virus (VSV), requires the PI3k/Akt signaling pathway for its replication. Through the use of chemical inhibitors of PI3k and Akt, we show that VSV replication and cytopathic effects do not require activation of these kinases. Inhibitors that block the activating phosphorylations of Akt at threonine 308 (Thr308) and serine 473 (Ser473) did not inhibit VSV protein expression or the induction of the cytopathic effects of VSV. One compound, Akt inhibitor Akt-IV, inhibited the replication of VSV, respiratory syncytial virus, and vaccinia virus but increased the phosphorylation of Akt at positions Thr308 and Ser473 and did not inhibit Akt kinase activity in vitro. Together, our data suggest that the PI3k/Akt pathway is of limited relevance to the replication of VSV but that Akt inhibitor Akt-IV is a novel broad-spectrum antiviral compound with a mechanism differing from that of its previously reported effect on the PI3k/Akt pathway. Identification of other targets for this compound may define a new approach for blocking virus replication.

One consequence of the successful replication of viruses is the alteration of cellular signaling following virus infection. Effects on the host cell can range from inhibition of cell death pathways and promotion of cell survival pathways to blocking of antiviral signaling proteins or phosphorylation cascades. Recently, significant interest has arisen in studying the abilities of different viruses to hijack the activity of a central cellular signaling pathway controlled by the activities of the phosphatidylinositol 3′-kinase (PI3k) and the protein kinase Akt (8, 11).

The PI3k/Akt pathway regulates a variety of cellular processes, including cell growth, proliferation, survival, and metabolism (14). Signaling through this pathway is initiated by receptor-mediated recruitment of catalytically active PI3k to the membrane. Active PI3k converts phosphatidylinositol 4,5-biphosphate to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 serves as a nucleation site for the colocalization of Akt with its activating kinase, PDK1, which phosphorylates Akt on threonine 308 (Thr308). This activating phosphorylation leads to a second phosphorylation event on Akt at serine 473 (Ser473) that potentiates kinase activity. Activated Akt can inhibit proapoptotic factors through phosphorylation and can activate transcription factors such as FoxO1 (29). It can also act to stimulate cellular translation through activation of mTORC1 activity, which inactivates the translation suppressor eukaryotic initiation factor 4E-BP1 (41). In addition to performing these functions, Akt can stimulate the immune response by amplifying the expression of interferon-stimulated genes (20).

The PI3k/Akt pathway has long been recognized as a pathway of significance in virus infection. Akt was originally described as an oncogene product of the Akt8 transforming retrovirus (1, 39) and has subsequently been shown to play a role in the replication of many different viruses. The polyoma virus simian virus 40 encodes a protein (small t) that inactivates PP2A, the phosphatase normally responsible for dephosphorylation and regulation of Akt. Inactivation of PP2A by small t results in Akt being maintained in an activated state. Activated Akt in turn allows for virus-mediated transformation of the cell (2). Poxviruses such as myxoma virus appear to encode a protein that can directly bind to and activate Akt (39), and in cells infected with either picornaviruses or paramyxoviruses, PI3k/Akt signaling is activated and is proposed to delay apoptosis (3, 37, 44). Similarly, influenza virus NS1 is capable of directly binding and activating the p85 subunit of PI3k, a process that is thought to delay apoptosis while virus replication is ongoing (13, 46).

It has recently been suggested that the activation of Akt (but not PI3k) is crucial for core replication functions of some viruses. Specifically, it has been suggested that the RNA-dependent RNA polymerase replication complex of all nonsegmented negative-strand (NNS) RNA viruses requires Akt-mediated phosphorylation of the viral phosphoprotein to drive RNA-dependent RNA polymerase activity (35). This hypothesis runs counter to statements in other publications which contend that PI3k and Akt activities are unimportant for replication or may even negatively impact the replication of NNS RNA viruses (4, 16, 21, 26, 28, 30, 31).

Because of the apparent contradiction of the published results, we investigated the importance of Akt for the replication of the prototype negative-strand RNA virus, vesicular stomatitis virus (VSV). To carry out this investigation, we determined the impact of small-molecule inhibitors of the PI3k/Akt pathway on VSV replication. Our results demonstrate that PI3k and Akt activities are not universally required for the replication of NNS viruses. Furthermore, our studies have identified a novel compound that has broad-spectrum antiviral effects

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that are not attributable to the alteration of known kinases within the PI3k/Akt signaling pathway.

MATERIALS AND METHODS

Virus infections. BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 7% fetal bovine serum and 2 mM glutamine. Cells were grown to 80 to 90% confluence and then infected with VSV (Indiana serotype, Orsay strain) in Dulbecco's modified Eagle's medium at a multiplicity of infection (MOI) of 10 or 0.01 PFU/cell. Cells treated with small-molecule inhibitors were first incubated with the specific inhibitor for 30 min at 37°C before virus infection in the presence of the inhibitor. VSV was grown and titers were determined in BHK-21 cells. Vaccinia virus (VACV; vTF7-3, WR strain) was grown in HeLa S3 cells, and titers were determined on CV-1 cells. Respiratory syncytial virus (RSV; human RSV-A2 strain) was grown and titers were determined in HepG2 cells.

Plaque assays. Virus titers were determined in duplicate by plaque assays of 10-fold serial dilutions ($1:10^4$ to $1:10^8$) of virus in culture medium as described previously (25).

Microscopy. Cell images were taken with a Zeiss Axiovert 200 M microscope operated with AxioVision 4 software.

Kinase assay. The in vitro kinase-profiling assay with Akt inhibitor Akt-IV was performed as described by Bain et al. (6).

Immunoblotting and detection. Infected or mock-infected cells were lysed in 35-mm six-well dishes for 5 min at 4°C by using 250 μl of NP-40 lysis buffer (Boston BioProducts Inc.) supplemented with a phosphatase inhibitor cocktail (PhosSTOP) and a protease inhibitor cocktail (Complete) as directed by the manufacturer (Roche Applied Science). Lysates were collected and spun at 10,000 × *g* for 5 min at 4°C, and then 100 μl of the supernatant was added to 20 μl of 6× sample buffer (Boston BioProducts Inc.) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Equal volumes of lysate were electrophoresed on sodium dodecyl sulfate–12 or 15% polyacrylamide gel electrophoresis gels. After electrophoresis, samples were electroblotted onto polyvinylidene difluoride membranes (0.2-μm pore size; Bio-Rad) and blocked with 5% (wt/vol) nonfat dry milk in TBS-T (Tris-buffered saline [pH 7.6], 0.1% Tween 20). Primary antibodies were diluted in 5% (wt/vol) bovine serum albumin (fraction V)–TBS-T as recommended by the antibody manufacturer (Cell Signaling Technologies, Danvers, MA). Anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G horseradish peroxidase-linked antibodies (Cell Signaling Technologies, Danvers, MA) and anti-goat horseradish peroxidase (Santa Cruz) were diluted 1:2,000 in 5% (wt/vol) nonfat dry milk in TBS-T.

Unless otherwise stated, all chemicals were purchased from Calbiochem. Antibodies to detect Akt, phospho-Akt Thr308 and phospho-Akt Ser473, 4E-BP1, and phospho-4E-BP1 Ser65 were purchased from Cell Signaling Technologies (Danvers, MA). The antibody against β-actin (1:5,000) was purchased from Santa Cruz Inc. Anti-VSV M and anti-VSV G were kind gifts from Doug Lyles (Wake Forest University) (22). Anti-RSV antibodies (Abcam) were used at a dilution of 1:10,000, and anti-A27L (Abcam) was used at a dilution of 1:250.

RESULTS

VSV replication is not inhibited by compounds that block PI3k activity. To investigate the potential antiviral effects of drugs that target the PI3k/Akt signaling pathway, we analyzed the impacts of different PI3k/Akt inhibitors on the replication of the prototype member of the order *Mononegavirales*, the rhabdovirus VSV. We initially tested the effects of wortmannin and LY294002. Both compounds are well-characterized inhibitors of PI3k, the upstream activator of Akt (38).

To determine the effects of these different compounds on virus replication, BHK-21 cells were treated with either wortmannin (5 or 10 μM) or LY294002 (10 or 20 μM). Following a 30-min drug pretreatment, the cells were infected with VSV at an MOI of 10. At 4 h postinfection (hpi), cell lysates were probed for expression of viral genes by Western blot analysis using antibodies against the VSV G and M proteins. As shown in Fig. 1A, cells that were infected with VSV showed robust expression of both VSV G and M proteins. In cells that were treated with either LY294002 or wortmannin, there was little alteration in the expression of viral proteins compared to that in untreated cells, though at high concentrations of wortmannin, G protein (only) showed somewhat lower expression. This result is likely due to an effect on the processing of glycosylated proteins by high concentrations of this drug (32).

To demonstrate that the PI3k inhibitors LY294002 and wortmannin were effectively inactivating Akt kinase activity, we sought to confirm that each drug blocked the kinase-activating phosphorylations of Akt. We evaluated Thr308 phosphorylation (the activating phosphorylation) and Ser473 phosphorylation (a potentiating phosphorylation event) by using phosphospecific antibodies. In mock-infected BHK-21 cells (Fig. 1B), we found readily detectable levels of Akt phospho-Ser473 and of Akt phospho-Thr308 (Fig. 1B, first two rows, lanes 1 and 6). Treatment with LY294002 and wortmannin had the expected effect of decreasing the phosphorylation of Akt on both of these sites and inhibiting the phosphorylation of targets downstream of Akt such as the mTOR substrate 4E-BP1 (Fig. 1B, fourth row). In a separate set of experiments, we found that virus infection did not block inhibitor-mediated dephosphorylation of Akt (data not shown).

The effects of these compounds on virus growth were tested by plaque assays, and their effects on cell rounding were observed using phase-contrast imaging. Results from growth curve experiments performed with a low MOI (0.01) showed that there was little or no effect of wortmannin or LY294002 on the replication of VSV (Fig. 1C), and analysis of cell rounding following VSV infection showed that LY294002 had little or no effect on VSV-induced cell rounding seen at 4 and 6 hpi (Fig. 1D).

Akt inhibitors show different effects on virus replication. Next, we investigated the effects of three structurally distinct Akt inhibitors, Akt-IV, Akt-V, and Akt-VIII, on VSV gene expression. Akt-V (triciribine) and Akt-VIII have been well characterized as direct inhibitors of the kinase activity of Akt (9, 18, 43). The compound Akt-IV was isolated in a high-throughput screen for inhibitors of FoxO1 translocation. Both Akt-IV and Akt-VIII have previously been suggested to have antiviral activities (35). In experiments similar to those described in the legend to Fig. 1, cells were treated with increasing concentrations of the Akt inhibitors Akt-IV, Akt-V, and Akt-VIII (0.2, 1, and 2 μM). Following inhibitor addition, cells were infected with VSV at an MOI of 10. When viral protein expression in these cells was monitored by Western blotting (Fig. 2A), we observed that inhibitor Akt-IV decreased the level of viral protein synthesis. There was a negligible decrease in VSV G and M protein expression in cells treated with 0.2 μM inhibitor, but at 1 and 2 μM, viral protein expression was dramatically inhibited.

In contrast, there was little to no effect of Akt-V or Akt-VIII on viral protein expression, regardless of the concentration of the inhibitor tested. These results were consistent with those of our plaque assays analyzing the effects of the three Akt inhibitors on VSV growth, as shown in Fig. 2B. The treatment of cells with Akt-IV decreased virus replication by more than 2 log orders at 8 and 12 hpi, but neither Akt-V nor Akt-VIII had a significant effect on virus replication. We also determined whether the treatment of cells with Akt inhibitors could inhibit virus-induced cell rounding. BHK-21 cells were treated with Akt inhibitors and either

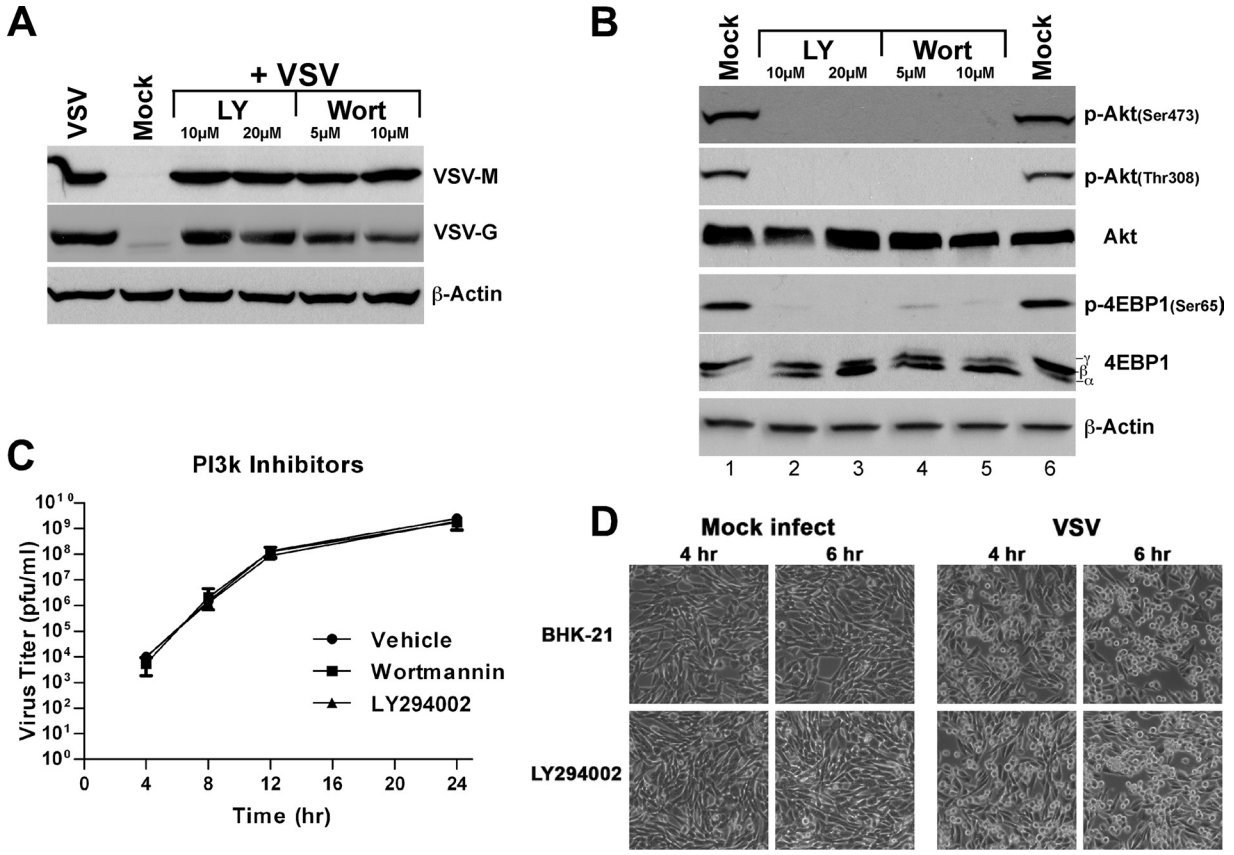

FIG. 1. Effects of PI3k inhibitors on VSV replication and cytopathic effects. (A) BHK-21 cells were pretreated with PI3k inhibitor LY294002 (LY) or wortmannin (Wort) or with vehicle (2 μl dimethyl sulfoxide [DMSO]; mock) for 30 min as indicated. Cells were then mock infected or infected with VSV (MOI of 10). At 4 hpi, cell lysates were collected and assayed by immunoblotting to determine the expression levels of VSV M and VSV G proteins. Total β-actin levels were determined to confirm loading of equal sample amounts. (B) BHK-21 cells were treated with PI3k inhibitors LY294002 and wortmannin. Cell lysates were collected at 4 h posttreatment and assayed by immunoblotting with antibodies specific to Akt, phospho-Akt Thr308 [p-Akt(Thr308)], p-Akt(Ser473), total 4E-BP1 and p-4E-BP1(Ser65), and β-actin. (C) BHK-21 cells pretreated with PI3k inhibitor LY294002 (5 μM) or wortmannin (10 μM) or with vehicle (2 μl DMSO) were infected with VSV (MOI of 0.01). Released-virus titers at the time points indicated were determined by virus plaque assays. The graph represents averages (± standard errors) of results from three experiments. (D) Cells were pretreated with a PI3k inhibitor (LY294002; 10 μM) or vehicle for 30 min and then mock infected or infected with VSV (MOI of 10). Phase-contrast images (magnification, ×10) of the BHK-21 cells in culture were taken at 4 and 6 hpi.

mock infected or infected with VSV (MOI of 10). As shown in Fig. 2C, cell rounding was not observed solely as a result of treatment with any of the Akt inhibitors. Pretreatment with Akt inhibitor Akt-V or Akt-VIII failed to inhibit or delay the VSV-induced cell rounding seen at 4 and 6 hpi. In contrast, treatment with Akt inhibitor Akt-IV before VSV infection significantly diminished cell rounding at 4 and 6 hpi.

The Akt-IV inhibitor has a novel mechanism of interacting with the Akt pathway. To further investigate why three drugs that are reported to block the enzymatic activity of the same kinase have different effects on virus replication, we sought to confirm that each drug blocked the kinase-activating phosphorylations of Akt. We measured the levels of Akt phosphorylation on residues Thr308 and Ser473 by using phosphospecific antibodies. In untreated BHK-21 cells, we found readily detectable levels of Akt phospho-Ser473 and of Akt phospho-Thr308 (Fig. 3A, top two rows, lane 10). In cells that were treated with Akt-IV, Akt-V, and Akt-VIII, 4E-BP1 phosphorylation was decreased, but to different extents, suggesting different potencies of signal blocking downstream of Akt. The most potent inhibitor of 4E-BP1 phosphorylation was Akt-IV (Fig. 3A). Importantly, we noticed a distinct difference among the effects of these drugs on Akt phosphorylation. While increasing concentrations of both Akt-V and Akt-VIII led to a decrease in detectable phosphorylation at both Thr308 and Ser473, higher concentrations of Akt-IV led to increasing phosphorylation at both residues (Fig. 3A). The quantification of band intensities demonstrates that Akt is hyperphosphorylated in cells treated with Akt-IV. Treatment of cells with 1 μM Akt-IV increased the level of Akt phosphorylation at residue Thr308 by 4.5-fold and that at residue Ser473 by 2.5-fold (Fig. 3B). This increase in Akt phosphorylation following Akt-IV addition was not cell type specific, as similar results were seen with A549 and HeLa cells (data not shown).

The increase in Akt phosphorylation following the addition of Akt-IV was unexpected based on data in previous reports (19) and led us to question whether Akt-IV's stimulation of Akt Thr308 and Akt Ser473 phosphorylation was responsible for the antiviral activity of the compound or whether Akt-IV could block Akt kinase activity but not its activating phosphory-

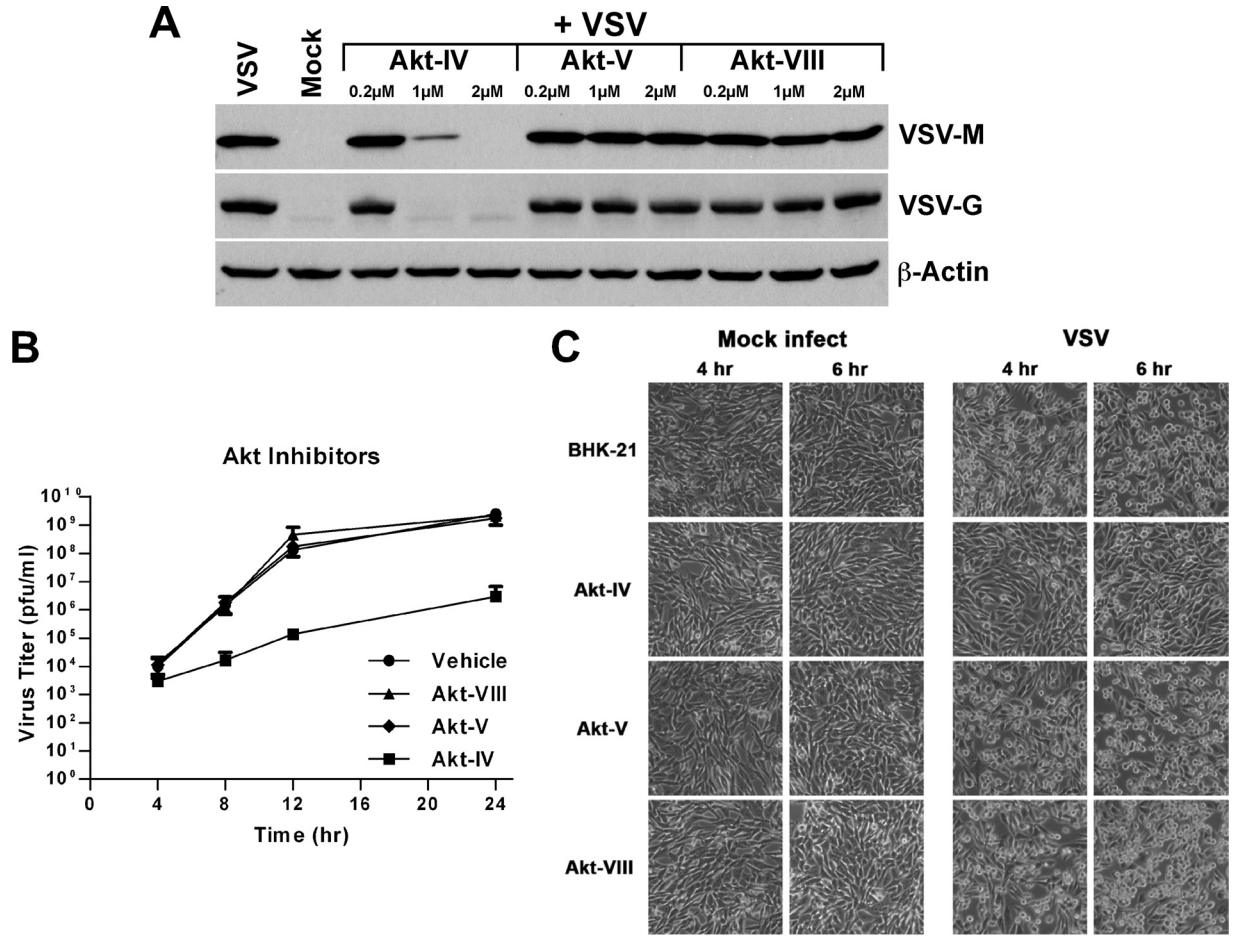

FIG. 2. Effect of Akt inhibitors on VSV replication and cytopathic effects. (A) BHK-21 cells were pretreated with Akt inhibitor Akt-IV, Akt-V, or Akt-VIII (0.2, 1, or 2 μM) or vehicle (DMSO) for 30 min and then mock infected or infected with VSV (MOI of 10) as indicated. Cell lysates were assayed by immunoblotting to determine the expression levels of VSV M and VSV G proteins. Total β-actin levels were determined as a loading control. (B) BHK-21 cells were treated with Akt inhibitor Akt-IV (1 μM), Akt-V (2 μM), or Akt-VIII (1 μM) or vehicle for 30 min and then infected with VSV (MOI of 0.01). Released-virus titers at the time points indicated were determined by virus plaque assays. The data shown are averages (± standard errors) of results from three experiments. (C) Cells were treated with an Akt inhibitor (Akt-IV at 1 μM, Akt-V at 2 μM, or Akt-VIII at 1 μM) or vehicle (1 μl DMSO) for 30 min and then mock infected or infected with VSV (MOI of 10). Phase-contrast images of the BHK-21 cells in culture were taken at 4 and 6 hpi.

lation. Because the phosphorylation of Akt Thr308 and Akt Ser473 requires PI3k activity, we sought to test the first possibility using PI3k inhibitors to block the stimulation of Akt phosphorylation by Akt-IV. Pretreatment of cells with either LY294002 or wortmannin effectively blocked the increase in Akt phosphorylation induced by Akt-IV treatment, as no detectable Akt Ser473 phosphorylation was seen following LY294002 or wortmannin pretreatment (Fig. 3C). However, despite the reduction in phosphorylation of Akt, the antiviral activity of Akt-IV was still evident (Fig. 3D).

Akt-IV does not directly block the activity of known kinases within the PI3k pathway. We wanted to determine whether the Akt-IV compound was acting directly on the kinase activity of Akt and whether the action of Akt-IV was specific to Akt. To answer these questions, we performed in vitro kinase assays in the presence and absence of Akt-IV. These assays were done with a high-throughput screening format that tested the abilities of Akt-IV to inhibit kinase phosphorylation of peptide substrates (5, 12). The screen measured the effects of the Akt-IV compound on Akt and other kinases in the Akt signaling pathway, such as PDK1 and glycogen synthase kinase 3β, as well as representative members of all of the major kinase groups (84 kinases total). At a concentration of Akt-IV of 1 μM, highly effective for virus inhibition, the compound was not inhibitory toward Akt1 or Akt2 (Table 1). Akt-IV did have a slightly inhibitory effect on the related AGC kinase group member SGK1 (~35%) and STE kinase group member MKK1 (32%). Akt-IV did not significantly affect the activities of the other kinases tested (examples of results are given in Table 1; also data not shown).

We considered that it was possible that our supply of Akt-IV compound contained impurities that were responsible for the results obtained with this compound. To examine this hypothesis, we obtained Akt-IV samples from three different companies with different compound suppliers and tested the samples in parallel. The results shown in Fig. 4 demonstrate that the three samples of Akt-IV had identical anti-VSV activities and that each stimulated the phosphorylation of Akt at Thr308 and Ser473.

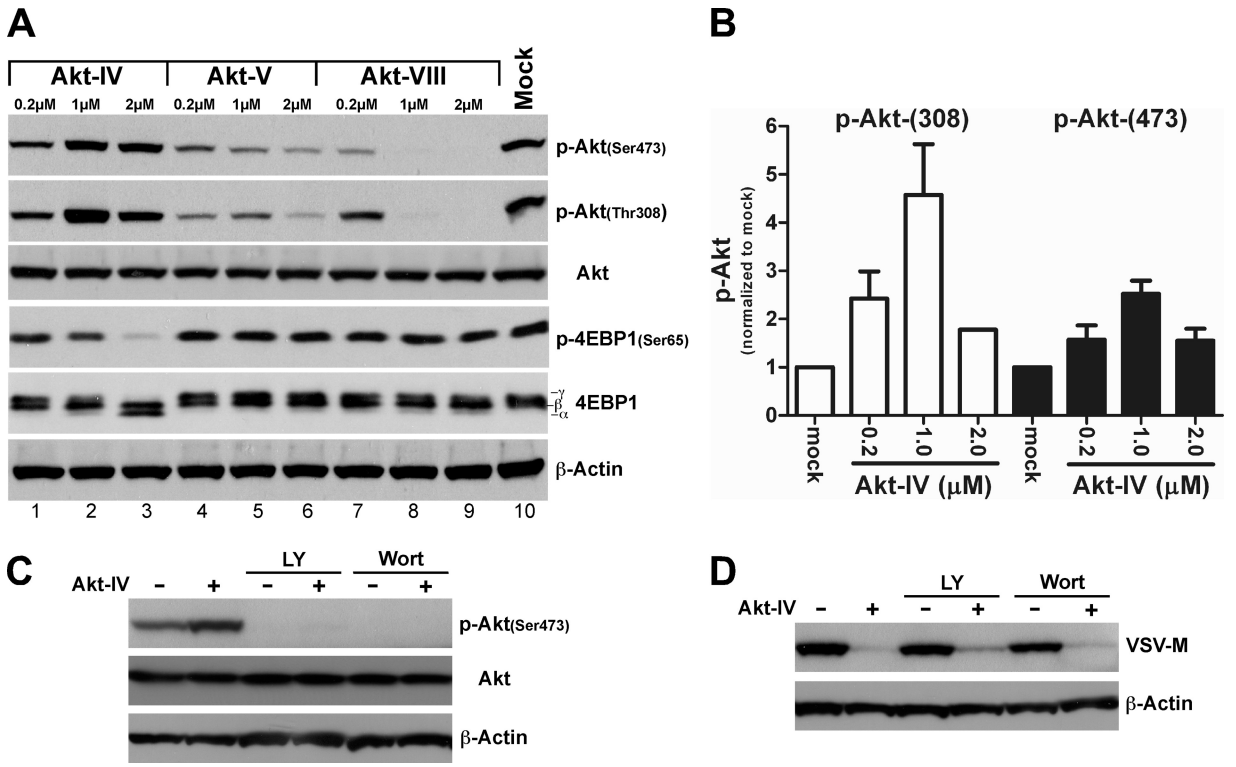

FIG. 3. Effects of PI3k and Akt inhibitors on Akt phosphorylation. (A) BHK-21 cells were treated with Akt inhibitors Akt-IV, Akt-V, and Akt-VIII. Cell lysates were collected at 4 h posttreatment and assayed by immunoblotting with antibodies specific to Akt, phospho-Akt Thr308, p-Akt(Ser473), total 4E-BP1, p-4E-BP1(Ser65), and β-actin. (B) The levels of phospho-Akt Ser473 and Thr308 in cells treated with Akt inhibitor Akt-IV were quantified by densitometry. In each case, the levels of phospho-Akt were normalized with respect to the basal levels found in cells without drug treatment (mock). Representative results from two or more independent experiments are shown. (C) BHK-21 cells were pretreated with PI3k inhibitor LY294002 (LY; 10 μM) or wortmannin (Wort; 5 μM) or with DMSO for 30 min before the addition of the Akt-IV inhibitor (1 μM). Cell lysates were collected at 4.5 h posttreatment and assayed by immunoblotting for phospho-Akt Ser473, Akt, and β-actin. +, present; −, absent. (D) Cells were pretreated with PI3k inhibitors as described in the legend to panel C except that after 30 min of Akt-IV treatment, cells were either mock infected or infected with VSV (MOI of 10). Cell lysates were collected 3.5 hpi and analyzed by immunoblotting to determine the expression levels of the VSV M protein and β-actin.

Akt-IV is inhibitory toward multiple viruses at an early stage of replication. After finding that Akt-IV inhibition of VSV replication did not appear to be dependent on the inhibition of Akt kinase activity, we chose to investigate whether the antiviral effects of Akt-IV extended to other viruses or whether they were restricted to rhabdoviruses. We tested the effects of Akt-IV addition on the replication of two other viruses, the paramyxovirus RSV and the poxvirus VACV. Obtaining results similar to those for VSV, we found that the Akt inhibitors Akt-V and Akt-VIII had little effect on the expression of either RSV (Fig. 5A) or VACV (Fig. 5B) proteins but that Akt-IV significantly inhibited gene expression by both viruses, illustrating that the compound has broad antiviral action. We did find that treatment of cells with LY294002 decreased the expression of VACV late protein A27L, consistent with other reports that this compound can inhibit VACV protein expression (33, 45).

TABLE 1. Residual activities of a diverse set of kinases following addition of 1 μM Akt inhibitor Akt-IV[a]

Kinase group	Kinase name	% Activity (mean ± SE) remaining after 1 μM Akt-IV treatment
AGC	PDK1	128 ± 1
AGC	Akt1/PKBa	99 ± 17
AGC	Akt2/PKBb	94 ± 2
AGC	SGK1	**65** ± 17
AGC	S6K1	84 ± 5
CAMK	CAMK1	94 ± 2
CK1	CK1	99 ± 2
CMGC	CK2	105 ± 12
STE	MKK1	**68** ± 1
TK	IGF-1R	96 ± 7

[a] Kinases listed are a subset of all kinases tested. Other kinases tested did not show a significant change in enzymatic activity (data not shown). Activities were determined in in vitro assays of purified kinases as described by Bain et al. (6) and are expressed relative to the level of activity pretreatment, which was set at 100%. Boldface values indicate a slightly inhibitory effect.

DISCUSSION

The results that we present in this study address the issue of whether the NSS RNA virus VSV requires PI3k/Akt activity for efficient replication. Our results demonstrate that neither the inhibition of PI3k activity nor the inhibition of Akt activity decreases VSV gene expression or virus progeny production. This observation suggests that the activity of this pathway plays a minimal role in VSV replication. This finding is consistent with a recent report showing that in invertebrates, VSV infec-

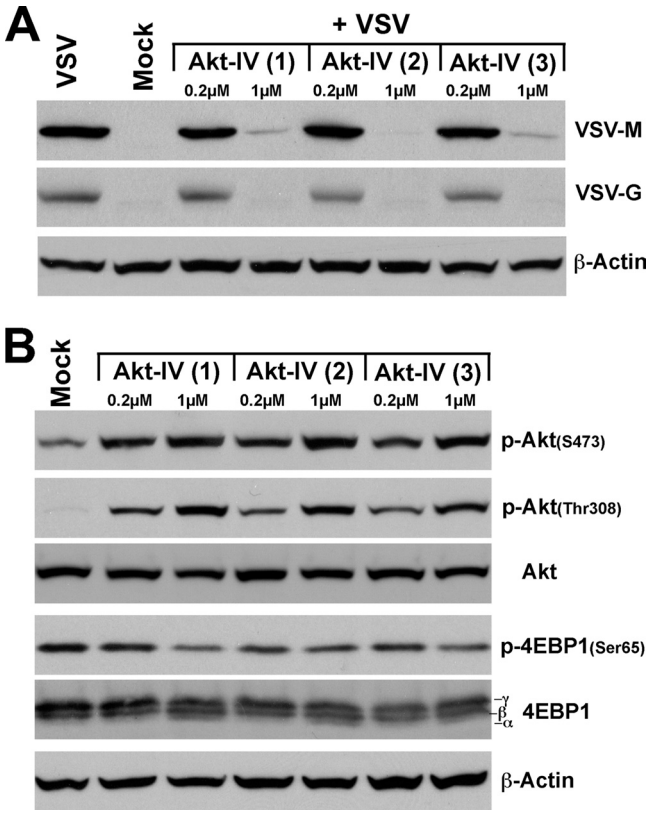

FIG. 4. Akt inhibitor Akt-IV from different sources inhibits VSV protein expression. (A) Cells were pretreated with Akt inhibitor Akt-IV purchased from Sigma-Aldrich (1), Calbiochem (2), or ChemBridge (3) for 30 min and then either mock infected or infected with VSV (MOI of 10) as indicated. Cell lysates were assayed by immunoblotting to determine the levels of VSV M protein and VSV G glycoprotein expression. β-actin levels are shown as a loading control. (B) As described in the legend to panel A, cells were treated with Akt-IV inhibitor samples from three different sources. At 4 h posttreatment, cell lysates were collected and assayed by immunoblotting with antibodies specific to phospho-Akt Thr308, p-Akt(Ser473), 4E-BP1, and p-4E-BP1(Ser65) as indicated. Total Akt and β-actin levels are shown as loading controls.

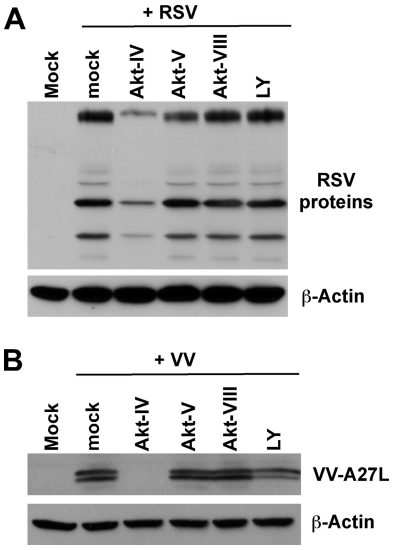

FIG. 5. Akt inhibitor Akt-IV inhibits RSV and VACV protein expression. BHK-21 cells were pretreated with Akt-IV (1 μM), Akt-V (1 μM), Akt-VIII (1 μM), LY294002 (LY; 10 μM), or vehicle for 30 min and then either mock infected or infected with RSV (MOI of 3) or VACV (VV; MOI of 2). At 18 hpi, cell lysates were collected for immunoblotting to determine the expression levels of RSV (A) and VACV (B) proteins and β-actin.

tion results in the inhibition of the PI3k/Akt signaling pathway (31).

Surprisingly, we also found contrasting actions when we examined how Akt inhibitors impacted virus replication. Treatment of cells with Akt inhibitors Akt-V and Akt-VIII did not alter VSV replication but did block the kinase-activating phophorylation events at Thr308 and Ser473 (Fig. 3A). In contrast, Akt inhibitor Akt-IV promoted Akt phosphorylation at residues Thr308 and Ser473 and showed strong inhibition of virus replication, which is in keeping with the data in an earlier report showing that this compound blocks RNA virus replication (35). These findings suggest that the action by which Akt-IV inhibits virus replication is not a result of its targeting Akt kinase activity.

Our data suggest that a revision of the proposed mechanism of action for Akt-IV is in order. Based on results of drug treatments at 10 μM, previous reports postulated that Akt-IV was acting to block phosphorylation and, thereby, activation of Akt (19). We find that at lower concentrations, Akt-IV increases the phosphorylation of Akt in multiple cell types. This increase in phosphorylation is PI3k dependent (Fig. 3C). Interestingly, our in vitro kinase assay data suggest that Akt-IV may directly activate PDK1, which phosphorylates Akt on Thr308. This potential increase in PDK1 activity may also account for the difference in the levels of Akt phosphorylation at residues Thr308 and Ser473 found in cells treated with Akt-IV (Fig. 3B).

Our observation that the Akt-IV inhibitor increases the levels of phospho-Akt suggests that the ascribed actions of this compound may be peripheral to the direct inhibition of Akt activity. The structure of the compound is consistent with the idea that Akt-IV may act as an ATP analog to block the active site of a kinase, but our screening assays did not identify Akt or any other kinase among the 80-plus kinases tested as a target. This result is consistent with findings described in other reports suggesting that Akt-IV does not alter the in vitro activity of Akt (40). The addition of Akt-IV to cells did decrease the phosphorylation of downstream Akt substrates such as 4E-BP1 (Fig. 3 and 4). The dephosphorylation of 4E-BP1 is consistent with Akt-IV's targeting signaling downstream of Akt kinase activity, perhaps at the level of mTOR.

This observation of increased phosphorylation of Akt following drug treatment is not unique to Akt-IV, as the stimulation of Akt phosphorylation has been seen previously in response to several kinase inhibitors, such as rapamycin (36) and the recently characterized Akt inhibitor Abbot compound A-443654 (24, 47). The difference in the actions of Akt-IV and A-443654 are highlighted by the results of our in vitro kinase-profiling assays; these show that Akt-IV does not directly inhibit Akt kinase activity in vitro (Table 1), while A-443654 in an identical screen does (5). Akt-IV and A-443654 both cause an increase in Akt phosphorylation and lead to the dephos-

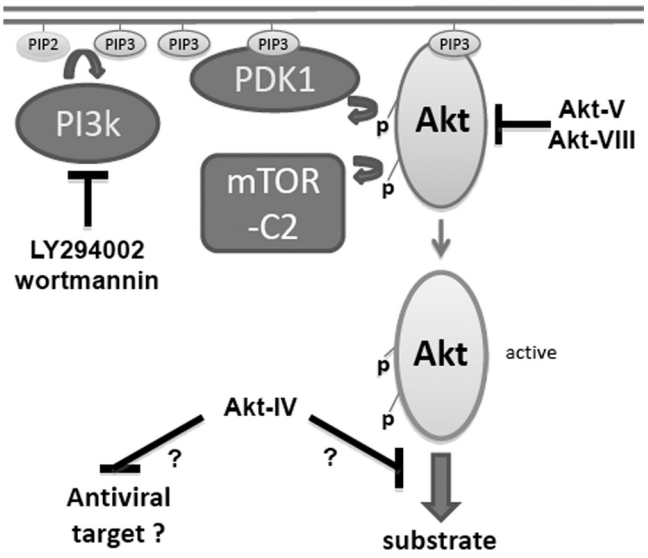

FIG. 6. Schematic of the PI3k/Akt signaling pathway depicting the possible sites of action by the PI3k inhibitors LY294002 and wortmannin and the Akt inhibitors Akt-VIII, Akt-V, and Akt-IV. PIP2, phosphatidylinositol 4,5-biphosphate; PIP3, phosphatidylinositol 3,4,5-triphosphate.

phorylation of downstream effectors (17), but their mechanisms of action must be distinct, as Akt-IV does not inhibit Akt in vitro. This pattern argues that Akt-IV has a unique mechanism of action, perhaps blocking the recruitment of a currently unidentified cofactor required for downstream signaling of Akt or inhibiting some other host cell process that is essential for viral replication (see Fig. 6).

Depicted in Fig. 6 is a simplified diagram of the PI3k/Akt signaling pathway highlighting the points at which inhibitors utilized in these experiments would exert their effects and inhibit Akt phosphorylation. The PI3k inhibitors LY294002 and wortmannin both inhibit the synthesis of PIP3, which is required for PDK1 activation of Akt. The Akt inhibitors Akt-V and Akt-VIII directly prevent phosphorylation and thus activation of Akt. Since Akt-IV does not prevent phosphorylation on Akt's activation sites or directly block kinase activity in vitro (Table 1), we propose that Akt-IV acts downstream of Akt activation and possibly at the point of substrate recognition. We also propose that the antiviral activity associated with this compound is independent of the PI3k/Akt signaling pathway and occurs by a mechanism yet to be determined.

Our results show that Akt inhibitor Akt-IV is the only Akt inhibitor we tested that blocked early replication events in VSV, RSV, and VACV infection. The simplest explanation of this activity is a non-Akt-pathway target. The compound was isolated in a high-throughput screen in vivo that was not designed to uncover compounds that specifically target Akt (19). Akt-IV, like the Akt inhibitor A-443654, may have multiple targets within the AGC kinase family (6), although data from our kinase assay screen shows no obvious candidates. Alternatively, Akt-IV may target other aspects of normal cellular function. This implication may be important for the understanding of findings from studies that have used this compound as a specific Akt inhibitor (23, 34, 42), particularly those which have found Akt-IV to be less effective than other Akt inhibitors such as Akt-V (10). Speculatively, the mechanism of antiviral action could be attributed to a block of viral entry or perhaps to inhibition either of viral RNA transcription or the translation of viral mRNAs. Further studies to determine the level of viral RNAs in the cell will help determine which stage in the viral replication cycle is affected. Notably, all three of the viruses tested here replicate in the cytoplasm. Therefore, Akt-IV may potentially block the function of a host kinase (protein) in the cytoplasm, resulting in an effect similar to one of the host antiviral responses.

Because our results and those of other researchers have established that this compound effectively inhibits the replication of multiple negative-strand RNA viruses, it would be of significant interest to determine any additional targets of this compound. It may be possible to identify the antiviral target of Akt-IV in vitro simply by increasing the number of kinase targets in the kinase-profiling assay or in vivo by using an analytical approach that combines a drug affinity pull-down assay with mass spectrometry to identify proteins associated with Akt-IV as new targets. Both approaches have been used successfully in studies to assess off-target effects of several clinical drugs that have broad-spectrum antikinase activities (7, 15, 27).

In conclusion, we demonstrate that the PI3k/Akt pathway does not appear to be necessary for VSV replication. This finding supports the conclusions of other groups that have determined that this pathway has minimal impact on negative-strand RNA virus replication (4, 16, 21, 26, 28, 30). Our studies do show that the inhibitor Akt-IV displays a mechanism of action that is different from what has been described previously and suggest that this compound deserves further study as a broad-spectrum antiviral agent. Our results show that the antiviral action of this drug is potent and sustained and blocks an early stage of viral replication. These results suggest the possibility that this compound may show a broader spectrum of antiviral activity than has been described to date. Therefore, based on our data, we propose that the Akt inhibitor Akt-IV has two distinct actions (or activities), the first being the inhibition of Akt by a unique mechanism and the second being the targeting of another, currently unknown kinase that is necessary for VSV to establish a productive replication cycle.

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