Early phosphatidylinositol 3-kinase/Akt pathway activation limits poliovirus-induced JNK-mediated cell death.

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ABSTRACT

PV-induced apoptosis seems to play a major role in tissue injury in the central nervous system (CNS). We have previously shown that this process involves PV-induced Bax-dependent mitochondrial dysfunction mediated by early JNK activation in IMR5 neuroblastoma cells. We show here that PV simultaneously activates the phosphatidylinositol 3-kinase (PI3K)/Akt survival signaling pathway in these cells, limiting the extent of JNK activation, and thereby cell death. JNK inhibition is associated with PI3K-dependent negative regulation of the apoptosis signal-regulating kinase 1 (ASK1), which acts upstream from JNK in PV-infected IMR5 cells. In poliomyelitis, this survival pathway may limit the spread of PV-induced damage in the CNS.

Poliovirus (PV), from the *Picornaviridae* family, causes paralytic poliomyelitis — a disease in which the motor neurons are destroyed in association with PV replication. PV consists of a single-stranded positive RNA genome surrounded by a nonenveloped icosahedral protein capsid. The human PV receptor, CD155, and its simian counterparts belong to the immunoglobulin superfamily (24, 25, 31) and are related to the nectin family of adhesion molecules (28, 38).

PV is mostly transmitted via the fecal-oral route. It first infects the oropharynx and the digestive tract, and then spreads to the central nervous system (CNS) in which it mostly targets motor neurons. Studies in mouse models have shown that PV-infected motor neurons in the spinal cord die by apoptosis (10, 19). PV-induced apoptosis therefore seems to play a major role in the tissue injury occurring in the CNS.

PV triggers apoptosis *in vitro* in tissue cultures of human colon carcinoma cells (CaCo-2) (4), promonocytic cells (U937) (29), dendritic cells (41), murine L cells expressing CD155 (21, 36), HeLa cells (8, 39) and cultures of mixed mouse primary nerve cells (12) from the cerebral cortex of mice transgenic for CD155. Analyses of the apoptotic pathways induced following PV infection in several cell lines have demonstrated that mitochondria are key actors of PV-induced apoptosis. In particular, mitochondrial outer membrane permeabilization (MOMP) following PV infection leads to a loss of mitochondrial transmembrane potential and the release of proapoptotic molecules, including cytochrome *c*, from the mitochondria to the cytosol (8, 21). We recently demonstrated that MOMP in PV-infected neuronal IMR5 cells was dependent on Bax, a proapoptotic member of the Bcl-2 family. Bax activation was mediated by c-Jun NH2-terminal kinase (JNK) phosphorylation after PV infection (6). JNK activation occurred early after PV infection whereas apoptotic features were observed later in PV-infected cells. These events may involve a balance between pro- and antiapoptotic signals following PV infection. Pro- and antiapoptotic events

potentially acting in synergy or competing with each other during the reproduction cycle of PV have been described by Agol's group (1, 39). However, the mechanisms involved in maintaining this delicate balance remain unclear.

Cells become committed to undergoing apoptosis in response to a collection of multiple survival and death signals. The phosphatidylinositol 3-kinase (PI3K) signaling pathway plays a crucial role in the transmission of survival signals in various cell types (14, 26), including neurons (16). PI3K activates its downstream effector, the serine-threonine kinase Akt (also known as protein kinase B, PKB) by promoting its phosphorylation at the residues Thr308 and Ser473. Activated Akt then phosphorylates various substrates, activating antiapoptotic factors and inactivating proapoptotic factors. The role of PI3K/Akt in the regulation of cell survival and apoptosis in a number of viral infection models (11, 13, 17, 27, 30), including infection with coxsackievirus B3 (18), rhinovirus (32), foot-and-mouth disease virus (35) and enterovirus 71 (40, 43) — all members of the *Picornaviridae* family — has recently been investigated.

PV activates the PI3K/Akt survival signaling pathway in IMR5 cells

We began by determining whether PV infection of IMR5 neuroblastoma cells resulted in Akt activation. IMR5 cells were infected with PV as previously described (6). Briefly, the growth medium (DMEM supplemented with 10% FBS) was discarded. The virus was then added to monolayers at a multiplicity of infection (MOI) of ten 50% tissue culture infective dose units ($TCID_{50}$) per cell (this MOI was used for all assays performed in this study). Adsorption was allowed to proceed for 30 min at 37°C in humidified air containing 5% CO_2. Cells were then washed twice with serum-free medium to remove unbound particles and incubated with fresh DMEM supplemented with 10% FBS at 37°C. The virus was allowed to grow for the indicated times. Time zero postinfection (p.i) corresponds to the inoculation time

point. Mock-infected cells were used as a negative control. As previously described (6), both adherent and detached cells were taken into account in all experiments. Kinetics of Akt phosphorylation at serine 473 (Ser473), which is required for full Akt activation (3), was investigated in mock- and PV-infected cells. Whole-cell lysates were analyzed at the indicated times p.i. by Western blotting with a specific anti-phospho (Ser473)-Akt antibody (Fig. 1A). We checked for equal protein loading on the total Akt Western blot. The amount of phosphorylated Akt increased until 30 min p.i., and then decreased; at 4 h p.i., the amount of phosphorylated Akt present was similar to that in mock-infected cells analyzed at the same time point. To check that the virus stock used in this study did not contain host-derived components that may activate Akt signaling pathway, we depleted the virus suspension of PV using an anti-PV antibody and infected cells with either the depleted or non-depleted suspension. In contrast to cells infected with the non-depleted stock, no Akt activation (30 min p.i.) was detected in cells treated with the depleted suspension (Fig. 1A, bottom, left). We also checked that poliovirus, purified by isopycnic CsCl gradient centrifugation (9), could promote Akt activation (30 min p.i.), at an efficiency similar to that obtained with the virus preparations used in this study (Fig. 1A, bottom, right). We then investigated whether Akt activation in response to PV infection occurred through the PI3K pathway, by treating IMR5 cells with a specific PI3K inhibitor, wortmannin (5), at a concentration of 100 nM and 500 nM, 2 h before mock or virus infection. The concentration of the inhibitor was maintained during the adsorption period and PV infection. Cell lysates were collected 30 min after infection and subjected to Western blot analysis for the detection of Akt phosphorylation (Fig. 1B, top). Wortmannin inhibited Akt phosphorylation at both concentrations without altering total Akt levels. The activation of Akt in response to PV infection was illustrated by immunofluorescence staining, 30 min p.i., with the same anti-phospho (Ser473)-Akt antibody. Representative staining patterns for mock-infected and PV-infected IMR5 cells treated with

wortmannin or left untreated are presented (Fig. 1B, bottom). As expected, immunofluorescence staining was detected only in infected cells in the absence of wortmannin. Thus, the rapid PV-induced phosphorylation of Akt involves a PI3K-dependent mechanism.

We investigated whether PV adsorption onto IMR5 cells induced Akt activation in the absence of PV replication by assessing Akt phosphorylation after the addition of UV-inactivated PV (UV cross-linked at 6,000 $\mu J/cm^2$) to IMR5 cells at a dilution corresponding to an MOI of 10 $TCID_{50}$ per cell (6). The complete abolition of viral infectivity by UV light treatment was confirmed by titration assay with undiluted viral suspension. We also checked that UV inactivation did not modify virus adsorption on cells, by comparing the binding efficiency of infectious and UV light-treated PV labeled with [^{35}S]methionine (data not shown). Akt phosphorylation was induced in IMR5 cells 30 minutes after the addition of UV-inactivated PV, with an efficiency similar to that observed with infectious PV (Fig. 2). Thus, PV-cell receptor interaction alone is sufficient to induce Akt phosphorylation in the absence of viral replication.

PI3K/Akt signaling pathway limits the amplitude of Bax activation, cytochrome *c* release and apoptosis in PV-infected IMR5 cells

We assessed the role of the PI3K/Akt signaling pathway in regulating the mitochondrial pathway of apoptosis in PV-infected cells, by blocking PI3K activation with wortmannin. The mitochondrial pathway is regulated by members of the Bcl-2 family, including the proapoptotic protein Bax, which promotes the release of cytochrome *c*. Bax-mediated cell death involves several well-controlled steps, including a conformational change resulting in exposure of the NH2-terminus. Mock- and PV-infected IMR5 cells were left untreated or were treated with 100 nM wortmannin for 2 h before PV infection. The

concentration of the inhibitor was maintained throughout both PV adsorption and replication. At 8 h p.i., a time point at which Bax activation is known to occur in PV-infected cells (6), whole-cell lysates were prepared in a lysis buffer containing 1% of the zwitterionic detergent CHAPS, which has no effect on Bax conformation (22). Bax was then immunoprecipitated with an anti-Bax antibody (6A7) that specifically recognizes Bax protein with an exposed NH2 terminus. The Bax protein immunoprecipitated from mock- and PV-infected cells was visualized by Western blotting (Fig. 3A, top). No activated Bax was detected in the immunoprecipitates from mock-infected cells. Consistent with our previous report (6), Bax was immunoprecipitated with the 6A7 antibody at 8 h p.i., indicating that PV infection was responsible for inducing the change in Bax conformation. Wortmannin enhanced Bax activation in IMR5-infected cells, without affecting the total amount of Bax (Fig. 3A, bottom). The effect of wortmannin on cytochrome *c* efflux from the mitochondria of PV-infected cells was also investigated. Whole-cell extracts from mock- or PV-infected cells were fractionated at 8 h p.i., to separate the cytosolic fraction from the heavy membrane fraction, including mitochondria, as previously described (6). Cytochrome *c* release was analyzed by Western blotting the cytosolic fraction. Much more cytochrome *c* was released in response to PV infection in cells treated with wortmannin than in untreated infected cells (Fig. 3B). These results suggest that PI3K may inhibit Bax-dependent MOMP during the PV infection of IMR5 cells.

We investigated the possible involvement of PV-mediated PI3K activation in the inhibition of apoptosis, by analyzing the kinetics of apoptosis in mock infected and infected cells treated or not treated with the specific PI3K inhibitor, wortmannin (Fig. 3C). Adherent and detached cells were harvested at the indicated times p.i. and apoptosis was analyzed by assessing chromatin condensation and fragmentation by flow cytometry after acridine orange (AO) nuclear dye staining, as previously described (6). We found that levels of PV-induced

157 apoptosis were higher in infected cells treated with wortmannin than in untreated infected
158 cells. To confirm the role of PI3K/Akt signaling pathway in limiting PV-induced apoptosis,
159 we down-regulated Akt expression with a specific siRNA. Western blot analysis with a
160 specific antibody showed that Akt expression in IMR5 cells transfected with Akt siRNA was
161 significantly weaker than in cells transfected with a nontargeted control siRNA (Fig. 3D, left).
162 As expected, following PV infection (8 h p.i.), apoptosis levels were higher in Akt
163 knockdown cells than in nontargeted control siRNA-transfected cells (Fig. 3D, right). These
164 results suggest that PI3K/Akt pathway plays a role in inhibiting the mitochondrial apoptotic
165 pathway in PV-infected IMR5 cells.
166
167 **The PI3K/Akt signaling pathway does not affect PV growth, but delays PV release**
168 We evaluated the effects of PI3K/Akt signaling on the amount of total virus produced
169 in IMR5 cells, by determining the kinetics of total virus yield by $TCID_{50}$ assays in the
170 presence or absence of wortmannin. PI3K/Akt pathway inhibition had no effect on the total
171 amount of virus produced (Fig. 4). As PV-induced apoptosis levels were higher in infected
172 cells treated with wortmannin than in untreated infected cells, we assessed the possible effects
173 of the increase in apoptosis levels on externalization of the virus. Viruses were released earlier
174 in the presence of wortmannin (Fig. 4). Thus, PI3K/Akt seems to delay viral release without
175 affecting virus production.
176
177 **The PI3K/Akt signaling pathway limits JNK activation in PV-infected cells**
178 We have shown that Bax-dependent activation of the mitochondrial pathway of
179 apoptosis is mediated by early JNK activation (6). JNK activation peaks 30 min p.i and then
180 decreases in IMR5 neuroblastoma cells. It is possible that the PI3K/Akt pathway down
181 regulates the JNK pathway, as recently reported in nonviral models (2, 23).

We assessed the effects of PI3K/Akt on JNK activation in PV-infected cells, by treating cells with wortmannin. JNK activation was investigated 30 min p.i., by Western blotting whole-cell lysates with an antibody against phosphorylated forms of JNK (Fig. 5A). As expected, phosphorylated JNK was detected 30 min p.i.. Larger amounts of phosphorylated JNK were found in infected cells treated with wortmannin than in untreated cells. Thus, activation of the PI3K/Akt pathway limits JNK activation in PV-infected IMR5 cells.

JNK activation is limited by the Akt-mediated phosphorylation of ASK1 in PV-infected cells

We then examined the possibility that a kinase, upstream of JNK, was inhibited by Akt, causing the observed limited JNK phosphorylation in PV-infected cells. Apoptosis signal-regulating kinase 1 (ASK1) has been shown to be a key regulator of the JNK pathway amenable to inhibition by Akt-mediated phosphorylation at Ser83 in nonviral systems (2, 23). We assessed the possible involvement of ASK1 in JNK activation in PV-infected IMR5 cells, by down-regulating ASK1 expression using specific siRNA (37). Western blot analysis with a specific antibody showed that ASK1 levels were significantly lower in IMR5 cells transfected with ASK1 siRNA than in cells transfected with a nontargeted control siRNA (Fig. 5B, left). Moreover, following PV infection, JNK activation in ASK1 knockdown cells was weaker than in cells transfected with the nontargeted control siRNA (Fig. 5B, right). Thus, ASK1 plays an important role in JNK activation following PV infection in IMR5 cells.

We then investigated the possible limitation of ASK1 activity by PI3K/Akt-mediated phosphorylation at Ser83 in PV-infected cells. The kinetics of ASK1 phosphorylation at Ser83 in PV-infected cells was analyzed by Western blotting with a specific antibody against phosphorylated ASK1 (Fig. 5C). A transient increase in the level of ASK1 phosphorylation

was evident 30 minutes after infection, consistent with the pattern of Akt activation. Furthermore, treatment of the cells with the PI3K inhibitor wortmannin abolished the increase in ASK1 phosphorylation in PV-infected cells (Fig. 5D). Altogether, these results indicate that the PI3K/Akt pathway negatively regulates JNK activation by phosphorylating and inactivating ASK1 in PV-infected IMR5 cells.

This study provides evidence that the early PI3K/Akt survival pathway limits the magnitude of PV-induced JNK activation and cell death in IMR5 cells. We previously showed that PV-cell receptor interaction alone is sufficient to induce JNK phosphorylation, as for Akt activation. However, we also showed that JNK phosphorylation is necessary, but not sufficient, to trigger apoptosis that seems to require the active replication of PV. As previously reported by Agol's group (1, 39), several different courses of events may influence apoptosis in PV-infected cells between 30 min and 6-8 h p.i. These events may involve the interplay between cellular and viral proteins (7, 15, 20, 33, 34, 42). Thus, the early PI3K/Akt survival pathway seems to act upstream of this unidentified interplay. The PI3K/Akt pathway has been shown to play an antiapoptotic role in several viral infections (11). However, this is the first report, to our knowledge, of the limitation of JNK activation by PI3K/Akt mediating a survival pathway during a viral infection. We have also shown that the cross-talk between the PI3K/Akt and JNK pathways involved ASK1 inhibition. In poliomyelitis, this survival pathway may limit the spread of PV-induced damage in the CNS.

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FIGURE LEGENDS

Fig. 1. PV induces early Akt phosphorylation in a PI3K-dependent manner in IMR5 neuroblastoma cells

(A) Kinetics of Akt activation in PV-infected neuronal cells. (Top) Akt activation was analyzed in whole-cell lysates at the indicated times p.i., by Western blotting with a specific anti-phospho (Ser473)-Akt antibody (Cell Signaling). Whole-cell lysates from mock-infected cells were analyzed at 30 min (first lane) and 240 min (last lane) post mock-infection, respectively. Blots were then stripped and reprobed with an antibody recognizing all forms of Akt (Cell Signaling), to confirm equal protein loading. (Bottom) Western blot analyses of Akt activation 30 min p.i.. (Left) Cells were infected with viral stock (PV) or viral stock depleted of PV (PV ^{depleted}) with anti-PV antibody. (Right) Cells were infected with viral stock (PV) or CsCl-purified PV (PV ^{purified}). **(B)** Inhibition of Akt phosphorylation during PV infection in

IMR5 cells treated with the PI3K inhibitor, wortmannin (Calbiochem, 100 nM and 500 nM). (Top) Cells were incubated or not incubated with the PI3K inhibitor for 2 h before PV infection, and the concentration of the inhibitor was maintained during the adsorption period and throughout PV infection. Levels of phospho (Ser473)-Akt in whole-cell lysates were determined by Western blotting, 30 min p.i.. Blots were then stripped and reprobed with an antibody recognizing all forms of Akt, to confirm equal protein loading. (Bottom) Mock- and PV-infected IMR5 cells (30 min p.i.), treated or not treated with wortmannin (100 nM), were stained for immunofluorescence with a specific antibody against phospho (Ser473)-Akt and a secondary, fluorescein isothiocyanate-conjugated antibody (green) (middle panel). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue) (left panel). Merge, overlay of the DAPI image with the anti-phospho (Ser473)-Akt image (right panel).

Fig. 2. UV-inactivated PV induces early Akt activation in IMR5 cells. Akt activation was analyzed by Western blotting whole-cell lysates from cells infected with infectious or UV-inactivated PV (30 min p.i.) with specific anti-phospho (Ser473)-Akt antibody. Blots were then stripped and reprobed with an antibody recognizing all forms of Akt, to confirm equal protein loading.

Fig. 3. Inhibition of the PI3K/Akt signaling pathway enhances PV-induced apoptosis in IMR5 cells

(A) Enhancement of Bax activation in PV-infected cells treated with wortmannin. (Top) Cells were uninfected or infected with PV (8 h p.i.) in the presence or absence of wortmannin (100 nM). Cells were lysed in immunoprecipitation buffer. Conformationally active Bax protein was immunoprecipitated (IP) with anti-Bax 6A7 antibody (Santa-Cruz) and precipitates were immunoblotted with anti-Bax antibody. The asterisk indicates immunoglobulin light chains.

(Bottom) Whole-cell lysates not incubated with 6A7 antibody were similarly tested for total Bax by immunoblotting with a specific antibody (Upstate) to check that the amounts of Bax protein in samples before immunoprecipitation were equivalent. Actin was used as a control for protein loading. **(B)** Greater cytochrome *c* (Cyt *c*) release in PV-infected cells treated with wortmannin. Cytochrome *c* release was analyzed in cytosolic fractions of mock-infected and PV-infected IMR5 cells (8 h p.i.) treated or not treated with wortmannin (100 nM) by Western blotting with a specific antibody (BD Pharmingen). Actin was used as a protein loading control. Protein levels were determined by densitometry and plotted as ratios relative to the actin levels. **(C)** Enhancement of apoptosis in PV-infected cells treated with wortmannin. Mock-infected and PV-infected IMR5 cells treated (black) or not treated (light gray) with wortmannin (100 nM) were analyzed at the indicated times p.i. by flow cytometry after Acridine Orange (AO, Molecular Probes) staining, and the increase (*n*-fold) in apoptosis was calculated as the ratio of the percentage of PV-infected IMR5 cells that were apoptotic to the percentage of mock-infected cells that were apoptotic. Data are means from three independent experiments. Error bars represent the standard errors of the means. *, $P<0.05$ by Student's *t* test comparing untreated IMR5 cells to treated IMR5 cells. **(D)** Higher levels of apoptosis were observed after the knockdown of Akt expression in PV-infected cells. (Left) IMR5 cells were transfected with Akt siRNA (Cell Signaling) or nontargeted control siRNA (Cell Signaling) or left untreated. Akt protein was then assayed by immunoblotting with extracts from nontargeted control siRNA-transfected, Akt siRNA-transfected or untreated cells. Actin was used as a protein loading control. (Right) Cells were uninfected or were infected (8 h p.i.) with PV 72 h after transfection, and cells were analyzed by flow cytometry after AO staining and the increase (*n*-fold) in apoptosis was calculated as the ratio of the percentage of PV-infected IMR5 cells that were apoptotic to the percentage of mock-infected cells that were apoptotic. Data are means from three independent experiments. Error bars

represent the standard errors of the means. *, P<0.05 by Student's *t* test comparing untreated IMR5 cells to treated IMR5 cells.

Fig. 4. Effect of PI3K/Akt signaling inhibition on PV growth and externalization

IMR5 cells were infected with PV in the presence or absence of wortmannin (100 nM). Total virus yield (extracellular and intracellular) was determined by $TCID_{50}$ assay at the indicated times after three cycles of freezing and thawing to release intracellular viruses. Extracellular virus titer was determined from the supernatant of PV-infected cells at the indicated times after the removal of detached cells by centrifugation. Each point represents the mean virus titers for two independent experiments. Standard errors of the mean are indicated, *P<0.05 by a Student *t* test comparing untreated to treated IMR5 cells.

Fig. 5. The PI3K/Akt signaling pathway limits JNK activation by promoting ASK1 phosphorylation in PV-infected IMR5 cells

(A) JNK activation levels are higher in PV-infected cells treated with wortmannin. Cells were uninfected or infected with PV (30 min p.i.) in the presence or absence of wortmannin (100 nM). JNK activation was analyzed in whole-cell lysates, by Western blotting with a specific anti-phospho (Thr183/Tyr185)-JNK (p46 [JNK1] and p54 [JNK2/3]) antibody, as previously described (6). Blots were then stripped and reprobed with an antibody recognizing all forms of JNK, to confirm equal protein loading. **(B)** Inhibition of JNK activation after the knockdown of ASK1 expression in PV-infected IMR5 cells. (Left) IMR5 cells were transfected with ASK1 siRNA (37) or nontargeted control siRNA (Cell Signaling) or left untreated. ASK1 protein was then assayed by immunoblotting with extracts from nontargeted control siRNA-transfected, ASK1 siRNA-transfected or untreated cells. Actin was used as a protein loading control. (Right) Untreated, nontargeted control and ASK1 siRNA transfected

IMR5 cells were uninfected or infected with PV. JNK activation was analyzed (30 min p.i.) in whole-cell lysates, by Western blotting with a specific anti-phospho (Thr183/Tyr185)-JNK antibody. Blots were then stripped and reprobed with an antibody recognizing all forms of JNK, to confirm equal protein loading. Phosphorylated JNK protein levels were determined by densitometry and plotted as the ratios, relative to the levels of total JNK. Phosphorylated JNK levels following PV infection in untreated cells were taken as 100%. Data are means from three independent experiments. Error bars represent the standard errors of the means. *, $P<0.05$ by Student's *t* test comparing nontargeted control siRNA-transfected IMR5 cells to ASK1 transfected IMR5 cells. **(C)** Phosphorylation of ASK1 in PV-infected neuronal cells. ASK1 phosphorylation was analyzed in whole-cell lysates at the indicated times p.i., by Western blotting with a specific anti-phospho (Ser83)-ASK1 antibody (Cell Signaling). Blots were then stripped and reprobed with an antibody recognizing all forms of ASK1 (Cell Signaling), to confirm equal protein loading. **(D)** Inhibition of PV-induced ASK1 phosphorylation by the PI3K/Akt pathway inhibitor wortmannin. Cells were uninfected or infected with PV in the presence or absence of wortmannin (100 nM). ASK1 phosphorylation was analyzed (30 min p.i.) in whole-cell lysates by Western blotting with a specific anti-phospho (Ser83)-ASK1 antibody. Blots were then stripped and reprobed with an antibody recognizing all forms of ASK1, to confirm equal protein loading. Phosphorylated ASK1 protein levels were determined by densitometry, and plotted as the ratios relative to the levels of total ASK1.

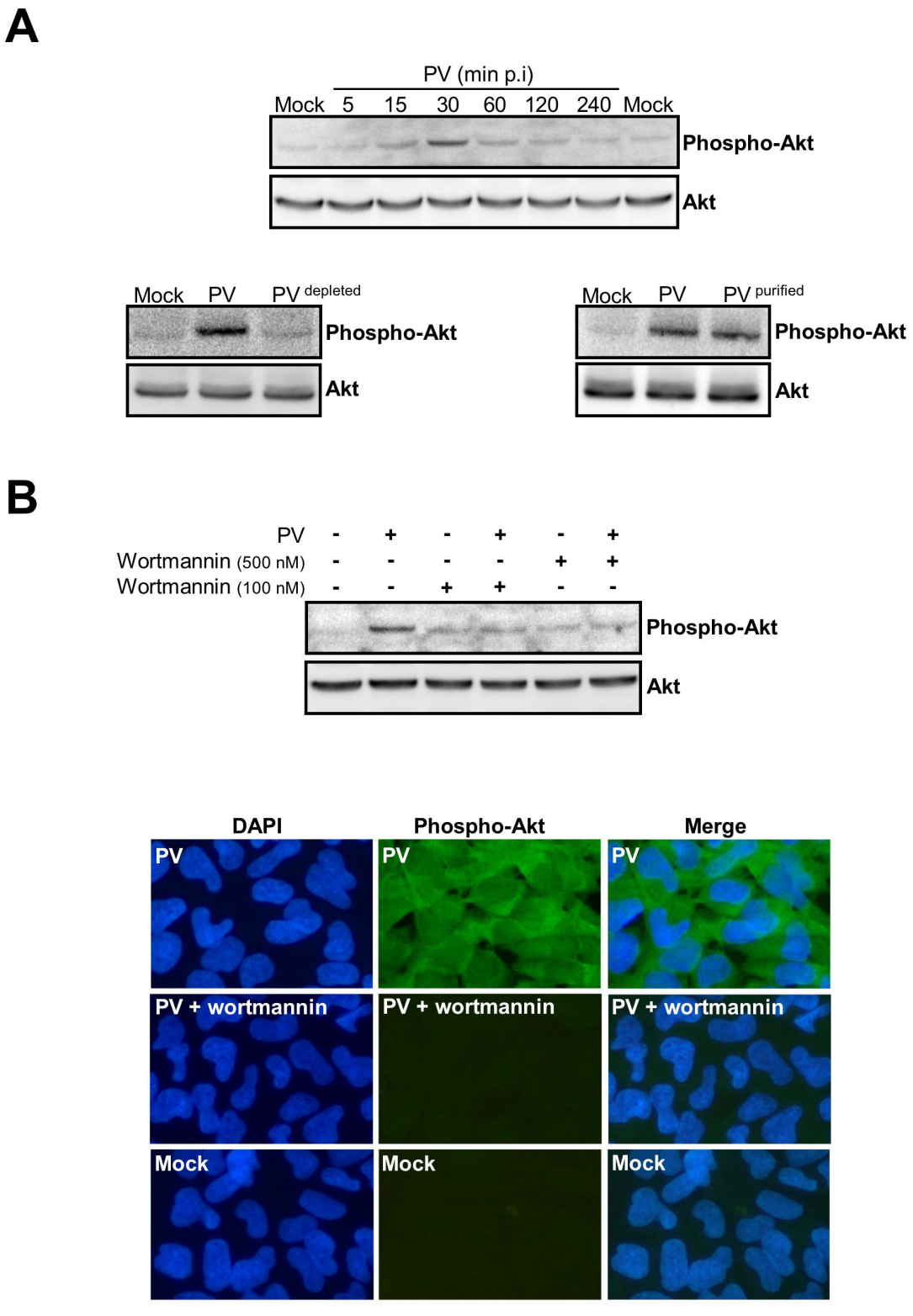
A
PV (min p.i)
Mock 5 15 30 60 120 240 Mock
Phospho-Akt
Akt
Mock PV PV depleted
Phospho-Akt
Akt
Mock PV PV purified
Phospho-Akt
Akt
B
PV - + - + - +
Wortmannin (500 nM) - - - - + +
Wortmannin (100 nM) - - + + - -
Phospho-Akt
Akt
DAPI
Phospho-Akt
Merge
PV
PV + wortmannin
Mock

Figure 1

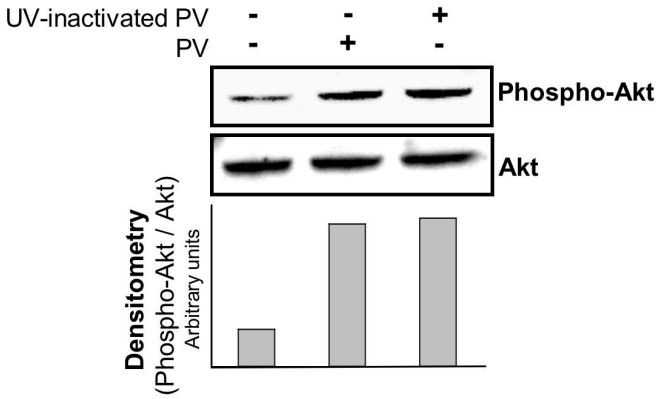
UV-inactivated PV - - +
PV - + -
Phospho-Akt
Akt
Densitometry
(Phospho-Akt / Akt)
Arbitrary units

Figure 2

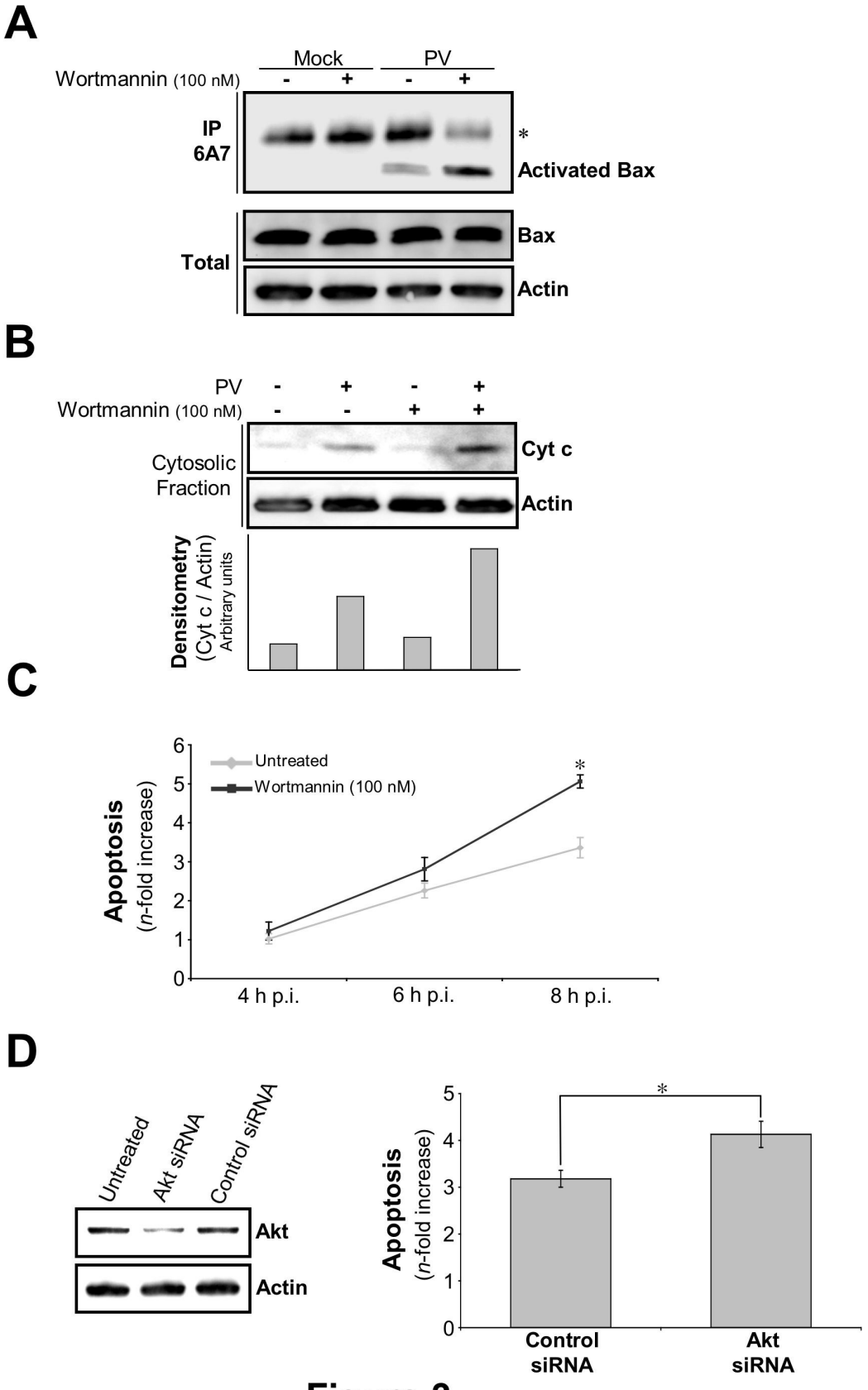
A
Mock
PV
Wortmannin (100 nM)
- + - +
IP
6A7
*
Activated Bax
Total
Bax
Actin
B
PV - + - +
Wortmannin (100 nM) - - + +
Cytosolic
Fraction
Cyt c
Actin
Densitometry
(Cyt c / Actin)
Arbitrary units
C
Apoptosis
(n-fold increase)
Untreated
Wortmannin (100 nM)
*
0
1
2
3
4
5
6
4 h p.i.
6 h p.i.
8 h p.i.
D
Untreated
Akt siRNA
Control siRNA
Akt
Actin
Apoptosis
(n-fold increase)
*
0
1
2
3
4
5
Control
siRNA
Akt
siRNA

Figure 3

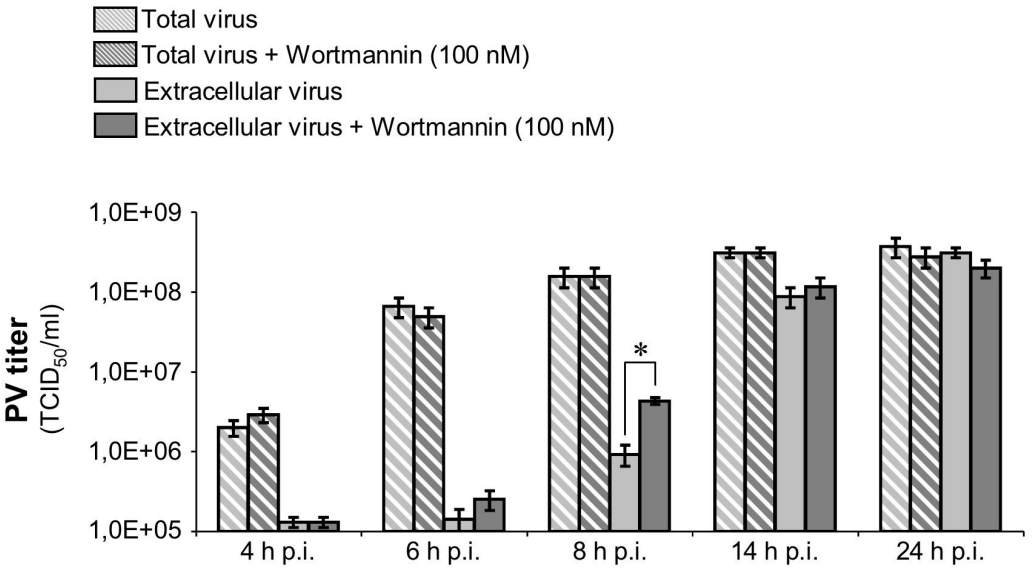
Total virus
Total virus + Wortmannin (100 nM)
Extracellular virus
Extracellular virus + Wortmannin (100 nM)
PV titer
(TCID50/ml)
1,0E+09
1,0E+08
1,0E+07
1,0E+06
1,0E+05
*
4 h p.i.
6 h p.i.
8 h p.i.
14 h p.i.
24 h p.i.

Figure 4

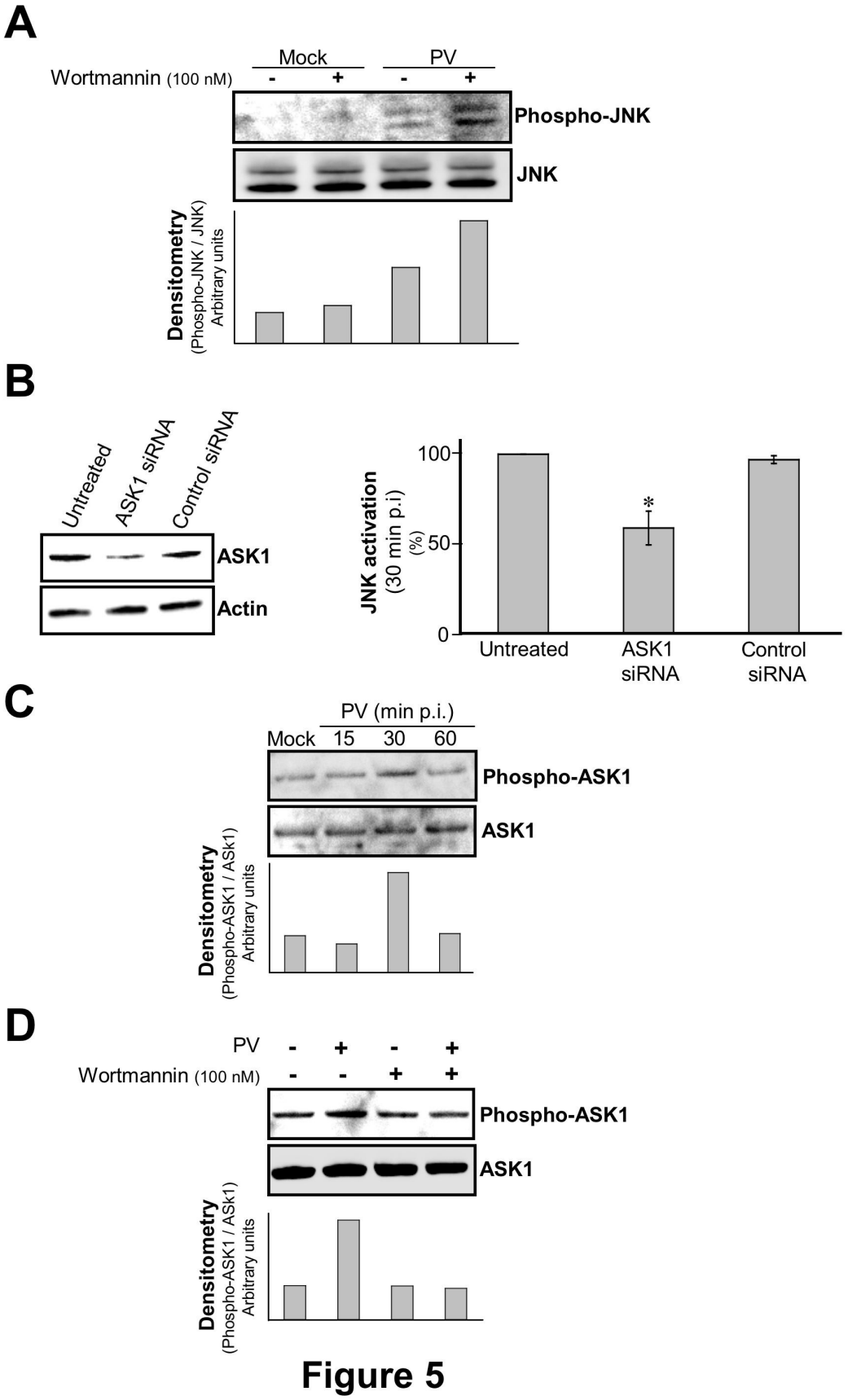
A
Mock
PV
Wortmannin (100 nM)
- + - +
Phospho-JNK
JNK
Densitometry
(Phospho-JNK / JNK)
Arbitrary units
B
Untreated
ASK1 siRNA
Control siRNA
ASK1
Actin
JNK activation
(30 min p.i)
(%)
100
50
0
*
Untreated
ASK1 siRNA
Control siRNA
C
PV (min p.i.)
Mock 15 30 60
Phospho-ASK1
ASK1
Densitometry
(Phospho-ASK1 / ASk1)
Arbitrary units
D
PV - + - +
Wortmannin (100 nM) - - + +
Phospho-ASK1
ASK1
Densitometry
(Phospho-ASK1 / ASk1)
Arbitrary units

Figure 5