The PI3K/Akt pathway inhibits influenza A virus-induced Bax-mediated apoptosis by negatively regulating the JNK pathway via ASK1

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It has previously been reported that influenza A virus infection activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. In addition, it has been shown that the mutant influenza A virus PR8-SH3-mf-1, which is unable to activate the PI3K/Akt pathway, is more pro-apoptotic than the wild-type (WT) virus. However, the molecular pathways involved in regulating this process remain unknown. Here, it is reported that, although both WT and PR8-SH3-mf-1 viruses induced apoptosis, the PR8-SH3-mf-1 virus consistently showed greater potential to induce mitochondrial membrane disruption, cytochrome c release, and translocation and conformational change of Bax than the WT virus. Furthermore, the PR8-SH3-mf-1 virus was unable to phosphorylate apoptosis signal-regulating kinase 1 (ASK1) but induced higher levels of c-jun N-terminal kinase (JNK) phosphorylation than the WT virus. Blocking JNK activity could inhibit virus-induced Bax activation and apoptosis. These results reveal that, during influenza A virus infection, the PI3K/Akt pathway negatively regulates the JNK pathway via ASK1, thereby inhibiting JNK-dependent, Bax-mediated apoptosis.

INTRODUCTION

Apoptosis plays an important role in the pathogenesis of many infectious diseases, including those caused by viral infection (Razvi & Welsh, 1995). Apoptosis can be generally divided into two pathways: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. In the extrinsic pathway, stimulation of death receptors, such as Fas and tumour necrosis factor receptor 1, leads to the formation of the death-inducing signalling complex. This allows the activation of caspase-8 and/or caspase-10 and an effector caspase, caspase-3, which facilitates cell death. The intrinsic pathway is initiated in response to diverse apoptotic stimuli. Activation of this pathway leads to loss of mitochondrial membrane integrity and the consequent release of cytochrome c into the cytosol. Cytochrome c then forms a complex with Apaf-1 and procaspase-9, resulting in activation of caspase-9 and effector caspases, including caspase-3 (Danial & Korsmeyer, 2004).

Influenza A virus infection induces apoptosis in various cell types, both in vitro and in vivo (Mao et al., 2009; Mori et al., 1995; Takizawa et al., 1993). However, the mechanism by which influenza virus induces apoptosis remains unclear. Several mechanisms have been proposed, and it appears that both viral and cellular factors are required for this process. The viral neuraminidase protein was the first influenza virus protein shown to have a role in the induction of apoptosis (Schultz-Cherry & Hinshaw, 1996). The NS1 protein has also been shown to have pro-apoptotic features (Lam et al., 2008; Schultz-Cherry et al., 2001). However, these data conflict with the finding that NS1 has anti-apoptotic functions (Ehrhardt et al., 2007; Shin et al., 2007c; Zhirnov et al., 2002). PB1-F2, a recently discovered viral protein, makes cells more sensitive to apoptosis by localizing to the mitochondrial membranes and interacting with the ANT3 and VDAC1 proteins (Zamarin et al., 2005). In addition to the direct effects of viral pro- and anti-apoptotic factors, cellular pathways, including the Bcl-2 and p38 pathways, have been shown to be involved in influenza A virus-induced apoptosis (Nencioni et al., 2009; Olsen et al., 1996). Studies using equine influenza virus have shown that influenza virus infection induces apoptosis via a c-jun N-terminal kinase (JNK) or stress-activated protein kinase cascade (Lin et al., 2001).

Recently, we and others have shown that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is activated by binding of the influenza NS1 protein to the p85β regulatory subunit of PI3K (Hale et al., 2006; Li et al., 2008; Shin et al., 2007c). The mutant virus PR8-SH3-mf-1,
containing P162A/P164A/P167A mutations in NS1, was unable to activate the PI3K/Akt pathway and induced more severe apoptosis than the wild-type (WT) virus (Shin et al., 2007a), suggesting that the PI3K/Akt pathway functions to suppress virus-induced apoptosis. The current study was initiated to identify the molecular mechanisms underlying influenza virus-induced apoptosis. Specifically, we wished to determine why the mutant virus PR8-SH3-mf-1 is more pro-apoptotic than the WT virus and how the PI3K/Akt pathway inhibits apoptosis during influenza virus infection. We report here that the PR8-SH3-mf-1 virus showed greater potential to induce mitochondrial membrane disruption, cytochrome c release, and translocation and conformational change of Bax than the WT virus. Furthermore, PR8-SH3-mf-1 was unable to phosphorylate apoptosis signal-regulating kinase 1 (ASK1), but induced higher levels of JNK phosphorylation than the WT virus. Our results reveal how the PI3K/Akt pathway prevents apoptosis during influenza A virus infection.

**RESULTS**

**Kinetics of apoptosis induced by WT and PR8-SH3-mf-1 viruses**

Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of Caspase-3 is an effector caspase that facilitates cell death. Poly(ADP–ribose) polymerase (PARP) is a substrate of

In the cytosolic fraction, basal levels of cytochrome c were detected in mock-infected cells, which may be due to the stress introduced during the fractionation process. Although WT

**Influenza A virus infection results in loss of mitochondrial membrane potential and in cytochrome c release**

To examine whether the mitochondrial pathway is involved in virus-induced apoptosis, we compared the changes in mitochondrial membrane potential in WT- and mutant virus-infected cells. Madin–Darby canine kidney (MDCK) cells were infected by the viruses at m.o.i. of 1. At 16 h p.i., cells were harvested and stained with 3,3’-dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI). DiOC6 is a cell-permeant, green fluorescent lipophilic dye that is selective for the mitochondria of live cells. As shown in Fig. 2(a), 36.68 % of WT virus-infected cells displayed decreased DiOC6 uptake and were positive for PI staining, indicating they were in the late stage of apoptosis. In contrast, 51.19 % of PR8-SH3-mf-1-infected cells were in the late stage of apoptosis ($P=0.006969$). Approximately 2.89 and 36.11 % of mock-infected cells and cells treated with and staurosporine (STS; an inducer of apoptosis), respectively, were in the late stage of apoptosis.

Decreased mitochondrial membrane potential results in the release of cytochrome c from the mitochondria into the cytoplasm. We therefore assessed the levels of cytochrome c release in WT and PR8-SH3-mf-1 virus-infected cells. A549 cells were infected with the viruses at m.o.i. of 5. At 16 h p.i., cells were fractionated into cytosolic and heavy membrane fractions and subjected to Western blot analysis using anti-cytochrome c antibody. Cytochrome oxidase subunit II (Cox II) was used as a loading control for the heavy membrane fraction, and β-actin was included as a loading control for the cytosol fraction. As shown in Fig. 2(b), in the mitochondrial fraction, larger amounts of cytochrome c could be detected in mock- and WT virus-infected cells compared with PR8-SH3-mf-1-infected cells. In the cytosolic fraction, basal levels of cytochrome c were seen in mock-infected cells, which may be due to the stress introduced during the fractionation process. Although WT
virus did not induce higher levels of cytochrome c release from the mitochondria, elevated levels of cytochrome c were detected in PR8-SH3-mf-1 virus-infected cells.

**Bax is activated in influenza A virus-infected cells**

As we observed a loss of mitochondrial membrane potential in virus-infected cells, we attempted to identify the upstream proteins that trigger the mitochondrial membrane disruption. It is well known that the mitochondrial pathway is tightly regulated by the Bcl-2 family of proteins, which includes both pro-apoptotic and anti-apoptotic proteins (Kim *et al.*, 2005). Bax is one of the pro-apoptotic proteins that reside in the cytoplasm in non-apoptotic cells. In response to apoptotic stimuli, Bax is activated by a conformational change, resulting in exposure of its NH₂ terminus and translocation of the protein to the mitochondrial membrane (Lalier *et al.*, 2007). We therefore investigated Bax translocation in virus-infected A549 cells. The heavy membrane fractions prepared above were subjected to Western blotting using an anti-Bax antibody. Although Bax was detected in WT virus-infected cells (Fig. 3a, lane 2), a significantly larger amount of Bax was present in PR8-SH3-mf-1-infected cells (Fig. 3a, lane 3). Cells treated with STS were included as a positive control (Fig. 3a, lane 4). Cox II was included as a loading control.

To investigate the conformational change of Bax in virus-infected cells, total cell lysates were prepared at 16 h p.i. Active Bax was immunoprecipitated using an anti-Bax monoclonal antibody (clone 6A7) that recognizes the active form of Bax with an exposed NH₂ terminus. Immunoprecipitated proteins were subjected to Western blotting using an antibody recognizing total Bax. Bax was detected in total cell lysates from all samples (Fig. 3b,
lanes 1, 3 and 5). No active Bax was detected in the immunoprecipitates of mock-infected cells (Fig. 3b, lane 2). Although active Bax was detected in virus-infected cells (Fig. 3b, lanes 4 and 6), more active Bax was present in PR8-SH3-mf-1-infected cells. IgG levels in the immunoprecipitates served as a loading control.

![Fig. 3. Bax is activated by WT and mutant viruses.](image)

(a) Mitochondrial fraction

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(b) Immunoprecipitation

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(c) Immunofluorescence

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Bax activation was also examined using immunofluorescence microscopy. A549 cells were mock or virus infected at an m.o.i. of 5. At 16 h p.i., cells were stained with Bax (6A7) as well as anti-NP antibodies. Representative images are shown in Fig. 3(c). Active Bax was not observed in mock-infected cells, but was detected in virus-infected cells (NP-positive cells). Compared with WT virus-infected cells, there was a higher proportion of active Bax-positive fluorescent cells, and the intensity of active Bax staining was much stronger in PR8-SH3-mf-1-infected cells. STS-treated cells were used as a positive control. In all cases, Bax fluorescence displayed punctate staining, indicating that Bax was associated with the mitochondria.

The JNK pathway is involved in regulating Bax activation and apoptosis

To identify the upstream signals that regulate Bax activation upon influenza A virus infection, we focused on the JNK pathway, as its deficiency is known to cause defects in the normal function of Bax (Lei et al., 2002). We examined whether the JNK pathway was activated in virus-infected cells. A549 cells were infected with virus, and cell lysates prepared at predetermined times were subjected to Western blotting using antibodies against phospho-JNK (pJNK) and total JNK. JNK activation was seen in both WT and PR8-SH3-mf-1 virus-infected cells at 12–16 h p.i. (Fig. 4a). However, PR8-SH3-mf-1 virus induced higher levels of pJNK than the WT virus. Similar amounts of total JNK were detected in all samples, indicating that the changes in JNK phosphorylation were not due to altered levels of total JNK.

We then examined whether activated JNK was involved in regulating Bax translocation and apoptosis during influenza A virus infection. A549 cells were infected with virus at an m.o.i. of 5. After 1 h of virus adsorption, the JNK-specific inhibitor SP600125 (20 μM), or DMSO (0.4 %) as a control, was added to the medium and maintained throughout the course of infection. Cells harvested at 16 h p.i. were fractionated. Heavy membrane fractions were then subjected to Western blotting using an anti-Bax antibody. SP600125 was found to decrease the levels of Bax translocation in both WT and PR8-SH3-mf-1 virus-infected cells (Fig. 4b, lanes 4 and 6). This effect was more prominent in PR8-SH3-mf-1 virus-infected cells (Fig. 4b, lanes 5 and 6, and band density graph) than in WT virus-infected cells (Fig. 4b, lanes 3 and 4). Cox II levels were similar in all samples, indicating that the changes in Bax levels were not due to changes in mitochondrial levels in different samples.

To test whether inhibition of JNK activation inhibits apoptosis during viral infection, A549 cells were infected and treated as described above. At 16 h p.i., PARP cleavage was detected. Although cleaved PARP was not detected in mock-infected cells (Fig. 4c, lane 1), traces of cleaved PARP were detected in mock-infected, SP600125-treated cells (Fig. 4c, lane 2). As shown previously, cleaved PARP was
detected in WT and PR8-SH3-mf-1 virus-infected cells in the absence of SP600125 (Fig. 4c, lanes 3 and 5), and PARP cleavage was inhibited to a certain degree by SP600125 in virus-infected cells (Fig. 4c, lanes 4 and 6). Importantly, the inhibitory effect was more remarkable in PR8-SH3-mf-1 virus-infected cells (Fig. 4c, lanes 5 and 6). Levels of β-actin were monitored to ensure equal loading of samples.

Akt downregulates JNK through ASK1

Our ongoing experimental results showed that the PR8-SH3-mf-1 virus, which is unable to activate the PI3K/Akt pathway, induced higher levels of JNK phosphorylation than WT influenza A virus, which is able to induce Akt phosphorylation. This suggested that Akt might negatively regulate JNK activity. We then sought to identify the protein that connects the PI3K/Akt and JNK pathways. Prompted by reports that the PI3K/Akt pathway down-regulates JNK activation by negatively regulating ASK1 in non-viral systems (Aikin et al., 2004; Kim et al., 2001) as well as in poliovirus-infected cells (Autret et al., 2007), and that ASK1 regulates influenza virus infection-induced apoptosis (Maruoka et al., 2003), we examined whether ASK1 bridges the PI3K/Akt and JNK pathways. As shown in Fig. 5(a), whilst WT virus infection led to a sustained level of ASK1 phosphorylation at Ser-83 (Fig. 5(a), lanes 2, 4 and 6), no apparent phospho-ASK1 (pASK1) was seen in PR8-SH3-mf-1 virus-infected cells (Fig. 5a, lanes 3, 5 and 7). Cellular ASK1 levels were included as a loading control.

The above results were obtained by utilizing WT virus and an NS1 mutant virus that is deficient in activating the PI3K/Akt pathway. To eliminate the possibility that the observed effects were due to the functional impairment of the mutant NS1 protein rather than to the lack of PI3K/Akt activation, we performed two sets of experiments. Firstly, we infected A549 cells with WT virus and, after virus adsorption, cells were treated with 0.4 % DMSO or 20 μM LY294002, a PI3K-specific inhibitor, as described previously (Shin et al., 2007b). Cells were harvested at 16 h.p.i. and half of the cells were lysed and subjected to Western blotting using a panel of antibodies. The other half was subjected to cell fractionation. The heavy membrane fraction was then analysed by Western blotting using anti-Bax and anti-Cox II antibodies. As shown in Fig. 6(a), blocking the PI3K/Akt pathway inhibited WT virus-induced ASK1 phosphorylation and upregulated WT virus-induced JNK phosphorylation (Fig. 6a, lanes 3 and 4). In WT virus-infected, LY294002-treated cells, increased levels of Bax were translocated into the mitochondria (Fig. 6b, lanes 3 and 4). Consequently, blocking PI3K/Akt activation greatly increased WT virus-induced apoptosis, as detected by PARP cleavage (Fig. 6a, lanes 3 and 4). Levels of β-actin and Cox II in different samples served as loading controls. Secondly, we assessed whether PR8-SH3-mf-1 virus was impaired in its ability to limit interferon (IFN) production, one of the major functions of NS1 (García-Sastre et al., 1998), using an IFN bioassay. As seen in Fig. 6(c), human IFN-β at a concentration of 100 U ml⁻¹ could efficiently inhibit green fluorescent protein-linked vesicular stomatitis virus (VSV–GFP) expression. Supernatant collected from mock-infected cells caused no inhibition of VSV–GFP replication on A549 cells. Similarly, the supernatant from WT or PR8-SH3-mf-1 infection could not inhibit GFP expression from VSV–GFP. These results demonstrated that PR8-SH3-mf-1 is competent to counteract IFN production.
DISCUSSION

Recent studies have demonstrated that influenza A virus infection activates the PI3K/Akt pathway in the late phase of infection. We previously reported that mutant virus PR8-SH3-mf-1 carrying three mutations (P162A/P164A/P167A) in NS1 failed to induce PI3K/Akt activity but induced more severe apoptosis than the WT virus (Shin et al., 2007a). The current study was initiated to define how the PI3K/Akt pathway regulates influenza A virus-induced apoptosis.

We first confirmed our previous observation that both viruses are able to induce apoptosis but that PR8-SH3-mf-1 is more pro-apoptotic. Recently, Jackson et al. (2010) reported that a mutant virus in the context of influenza A/Udorn/72 carrying NS1-Y89F, which is unable to activate PI3K/Akt, is not more pro-apoptotic than WT virus. This raised the question of whether the pro-apoptotic phenotype of PR8-SH3-mf-1 is due to disabled PI3K/Akt or loss of function of NS1. Of the multiple functions of NS1, we demonstrated that PR8-SH3-mf-1 is not impaired in its ability to limit IFN production (Fig. 6c). In an attempt to examine whether the inability to activate PI3K/Akt is responsible for the pro-apoptotic phenotype by using Y89F in the background of influenza A/PR/8/34, we observed that, indeed, Y89F did not induce more apoptosis than WT virus; however, in A549 cells, the phospho-Akt (pAkt) level activated by Y89F virus was only slightly lower than WT virus but was much higher than PR8-SH3-mf-1, and, most noteworthy, in MDCK cells, Y89F induced similar levels of pAkt as the WT virus (data not shown). Thus, in our hands, Y89F was not deficient for PI3K/Akt activity, and this may explain why Y89F did not induce more apoptosis than WT virus. Although more experiments are required to elaborate the role of Y89 in activating PI3K/Akt and to demonstrate whether other functions of NS1 are affected by the 162/164/167 mutations, our data demonstrated that deficiency of PI3K/Akt activation contributes to the pro-apoptotic phenotype of PR8-SH3-mf-1 virus.

We have shown previously that influenza A virus-induced PI3K/Akt activation led to caspase-9 phosphorylation...
(Shin et al., 2007a). This prompted us to investigate whether the mitochondrial pathway was involved in influenza A virus-induced apoptosis. The results in Fig. 2 showed that WT virus infection resulted in mitochondrial membrane disruption, but that PR8-SH3-mf-1 caused more severe mitochondrial membrane damage. In agreement with the severity of the mitochondrial membrane disruption, more cytochrome c was released into the cytosol in PR8-SH3-mf-1 virus-infected cells than in WT virus-infected cells. These results suggest that the PI3K/Akt pathway is involved in regulating mitochondria-mediated apoptosis during influenza A virus infection.

The integrity of mitochondria is regulated by Bcl-2 family members, a group of proteins composed of pro-apoptotic members such as Bax, Bak, Bad and Bim and anti-apoptotic members such as Bcl-2, Bcl-xL and Bcl-w (Cory & Adams, 2002). To identify which protein(s) are involved in regulating different levels of mitochondrial dysfunction in response to WT and mutant virus infection, we compared the expression and activation profiles of several Bcl-2 family members, including Bcl-xL, Bad and Bax. Of the tested Bcl-2 family members, we could only detect differential activation of Bax in WT and PR8-SH3-mf-1 virus-infected cells (Fig. 3 and data not shown). Our experiments, which included determination of total Bax levels in the heavy membrane fractions of virus-infected cells by Western blotting and the levels of active Bax by immunoprecipitation or immunofluorescence staining with an antibody specific for active Bax, demonstrated that, whilst Bax was activated by WT virus infection, PR8-SH3-mf-1 induced significantly higher levels of active Bax (Fig. 3). While this manuscript was in preparation, McLean et al. (2009) reported that Bax is involved in influenza virus infection-induced apoptosis. Our results obtained with WT virus-infected cells are in agreement with these findings.

JNKs are a group of mitogen-activated protein kinases. The JNK pathway is activated in response to various stress stimuli including dsRNA produced during viral infection (Ludwig et al., 2002). The modulation of mitochondrial pathways of apoptosis by the JNK pathway has been documented. Studies have shown that JNK is required for the release of pro-apoptotic molecules such as cytochrome c from the mitochondria in response to UV irradiation (Tournier et al., 2000); the activated JNK pathway is sufficient to induce rapid cytochrome c release and apoptosis; and, furthermore, activated JNK fails to cause death in cells lacking Bax, suggesting that the JNK pathway regulates cell apoptosis through Bax (Lei et al., 2002). The regulation of mitochondria-mediated apoptosis by the JNK pathway has also been documented in reovirus, rotavirus and poliovirus infections (Autret et al., 2007; Clarke et al., 2004; Martin-Latil et al., 2007). Based on these observations, we investigated whether the JNK pathway plays a role in regulating influenza A virus-induced Bax activation. We first demonstrated that PR8-SH3-mf-1 induced higher levels of pJNK than the WT virus (Fig. 4a). We then examined whether the JNK pathway is required for Bax-mediated apoptosis. By blocking the JNK pathway with a specific inhibitor, Bax translocation to the mitochondria was significantly inhibited (Fig. 4b) and less apoptosis was induced, as measured by PARP cleavage (Fig. 4c). This effect was more profound in PR8-SH3-mf-1 virus-infected cells than in WT virus-infected cells. These data suggested that the JNK pathway acts upstream of Bax and is involved in the regulation of influenza A virus-induced apoptosis. The molecular mechanisms by which the JNK pathway regulates Bax activation remain unclear. Kim et al. (2006) have proposed that Bax can be phosphorylated at Thr-167 by the JNK and/or p38 pathways. Phosphorylation of Bax results in a conformational change, exposing its N terminus (activation) and its C-terminal transmembrane domain, which are required for mitochondrial translocation. Tsuruta et al. (2004) demonstrated that activated JNK promotes phosphorylation of 14-3-3, a cytoplasmic anchor of Bax. As a result of phosphorylation of 14-3-3, Bax dissociates from its cytoplasmic anchor and translocates to the mitochondrion. To illustrate how the JNK pathway regulates Bax activation in response to influenza A virus infection, we tested whether Bax is (i) phosphorylated in virus-infected cells and (ii) associated with 14-3-3 in A549 cells. We could not detect Bax phosphorylation or 14-3-3-associated Bax in the cytoplasm of virus-infected cells (data not shown). This suggests that other unknown mechanisms of Bax activation may exist.

Our next question was whether the PI3K/Akt and JNK pathways cross-talk to regulate apoptosis coordinately during influenza A virus infection. Our data showed that, whilst the WT virus activated the PI3K/Akt pathway and induced lower levels of pJNK, PR8-SH3-mf-1, which is unable to activate the PI3K/Akt pathway, induced higher levels of pJNK. This led us to speculate that the PI3K/Akt pathway may negatively regulate JNK activity during influenza A virus infection. ASK1 is a member of the mitogen-activated protein kinase kinase kinase (MKKK) family, which acts upstream of JNK kinase. ASK1 phosphorylates and activates MKK4 or MKK7 and MKK3 or MKK6, which in turn activate JNK (Ichijo et al., 1997; Wang et al., 1996). Moreover, ASK1 acts downstream of Akt and can be phosphorylated by Akt. Phosphorylated ASK1 is associated with decreased ASK1 kinase activity (Kim et al., 2001). Given these facts, we hypothesized that the PI3K/Akt pathway may phosphorylate ASK1, leading to decreased ASK1 kinase activity, and thus downregulates JNK activity. In support of our hypothesis, we detected ASK1 phosphorylation in WT virus-infected cells but not in PR8-SH3-mf-1 virus-infected cells (Fig. 5a). Inhibition of ASK1 activity led to decreased levels of pJNK (Fig. 5b).

It has been reported that activated Akt can phosphorylate caspase-9 at Ser-196, thereby inhibiting its caspase activity (Cardone et al., 1998). In a previous report, we showed that WT virus led to caspase-9 phosphorylation, whereas PR8-SH3-mf-1 virus did not (Shin et al., 2007a). Taking our data together, we propose the following model of how the PI3K/Akt pathway regulates apoptosis during influenza A
virus infection (Fig. 7). Upon influenza A virus infection, whilst the PI3K/Akt pathway is activated by binding of NS1 to the p85β subunit of PI3K, JNK is activated by dsRNA. Activated PI3K/Akt phosphorylates ASK1. Phosphorylated ASK1 has decreased kinase activity and thus downregulates JNK activity. In contrast, in PR8-SH3-mf-1 virus-infected cells, the PI3K/Akt pathway is not activated, resulting in unsuppressed JNK activity. Excess levels of JNK activity promote excessive amounts of Bax translocating to the mitochondria membrane, causing excessive amounts of cytochrome c to be released into the cytosol. Cytochrome c then forms a complex with Apaf-1 and procaspase-9, leading to the activation of effector caspases such as caspase-3. Additionally, PI3K/Akt may also inhibit apoptosis by direct phosphorylation of caspase-9.

**METHODS**

**Cells and viruses.** A549 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (FBS). MDCK cells were cultivated in minimal essential medium (Invitrogen) supplemented with 10% FBS. Influenza A/PR/8/34 (H1N1; PR8) was propagated at 37°C in 11-day-old embryonated chicken eggs. The mutant virus PR8-SH3-mf-1 was generated and propagated as described previously (Shin et al., 2007a). Virus titres were determined in MDCK cells by plaque assay.

**Antibodies and inhibitors.** Rabbit polyclonal antibodies against Bax, caspase-3, PARP, β-actin, JNK and pASK1 (Ser-83), rabbit monoclonal antibody against pJNK (Thr183/Tyr185) and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology. Anti-Bax (clone 6A7) mouse monoclonal antibody was obtained from Santa Cruz Biotechnology. Purified mouse anti-cytochrome c monoclonal antibody was obtained from BD Biosciences. Anti-cytochrome oxidase subunit II (Cox II) mouse monoclonal was obtained from Invitrogen. Anti-NP rabbit polyclonal antibody was generated in our laboratory (Shin et al., 2007b). LY294002, a specific PI3K inhibitor, and SP600125, a selective JNK inhibitor, were obtained from Sigma-Aldrich. Thioredoxin, an inhibitor of ASK1, was obtained from Calbiochem.

**Mitochondrial membrane potential measurements.** MDCK cells seeded in 35 mm dishes (5 × 10⁵ cells per dish) were infected with WT influenza A/PR/8/34 or PR8-SH3-mf-1 virus at an m.o.i. of 1. Cells were trypsinized and collected at 16 h p.i. Positive-control cells were treated with STS at a final concentration of 0.3 μM for 16 h. Cells were stained for 20 min at 37°C with DiOC6 (Invitrogen) at a final concentration of 10 nM and with 2 μg propidium iodide (PI) ml⁻¹. Data acquisition and analysis were performed with a FACSCalibur (BD Biosciences).
performed with a FACSscan (Becton Dickinson) flow cytometer and CellQuest software. Paired Student’s t-tests were applied to compare the mitochondria membrane integrity of cells infected with WT or mutant viruses.

Co-immunoprecipitation and Western blot analyses. A549 cells were mock infected or infected by the viruses at an m.o.i. of 5. At 16 h p.i., cells were harvested and lysed with lysis buffer [10 mM HEPES (pH 7.4), 150 mM NaCl and 2 % CHAPS (Sigma)] containing Complete Protease Inhibitor Cocktail (Roche). The lysates were collected, sonicated and cleared by centrifugation for 20 min at 12,000 g at 4 °C. A total of 500 μg cell lysate was incubated with 2 μg anti-Bax (6A7) antibody overnight at 4 °C. Protein G-Sepharose beads (Santa Cruz Biotechnology) were added and incubated for an additional 4 h at 4 °C. After extensive washes, the precipitated proteins were subjected to SDS-PAGE, followed by Western blotting as described previously (Shin et al., 2007b) with an anti-Bax antibody.

Preparation of subcellular fractions. A549 cells were mock infected or infected by the viruses at an m.o.i. of 5. At 16 h p.i., cells were harvested and resuspended in STE buffer [0.25 M sucrose, 5 mM Tris/HCl (pH 7.4), 1 mM EGTA, 0.002 % digitonin and Complete Protease Inhibitor Cocktail] on ice for 15 min. The suspension was centrifuged for 10 min at 10,000 g at 4 °C. The supernatant was used as the cytosolic fraction. The pellet was resuspended in buffer containing PBS and 0.5 % Triton X-100 and incubated on ice for 10 min. This suspension was centrifuged for 10 min at 10,000 g at 4 °C. The supernatant, which contained the heavy membrane fraction, was then collected.

Immunofluorescent staining. A549 cells (8 x 10^4 per well) were plated on a four-well chamber slide (Lab-Tek) and infected with WT or mutant virus at an m.o.i. of 5. For a positive control, cells were incubated with 1 μM STS for 3 h. At 16 h p.i., cells were fixed in 4 % paraformaldehyde for 30 min at room temperature and then permeabilized by incubation with 0.5 % saponin (Sigma-Aldrich) for 30 min. Cells were incubated with anti-Bax antibody (6A7) and anti-NP rabbit serum overnight at 4 °C. After being rinsed three times with 1 % BSA, 1 mM NaF in PBS, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) for 2 h at room temperature. Finally, cells were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), and images were obtained on a Leica TCS SP5 fluorescence microscope.

Bioassay to measure IFN production. The amount of IFN production in virus-infected cells was performed as described previously (Park et al., 2003), with minor modifications. A549 cells were mock infected or infected with WT or mutant virus at an m.o.i. of 5. At 16 h p.i., the supernatant was harvested and UV inactivated. A549 cells plated in 96-well plates were treated with inactivated supernatant or 100 U human IFN-β (PBL Interferon Source) ml⁻¹ for 24 h and then infected with VSV–GFP at an m.o.i. of 0.1. GFP expression was determined at 18 h p.i. under a fluorescent microscope.

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