Infectious bursal disease virus activates the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway by interaction of VP5 protein with the p85α subunit of PI3K

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A R T I C L E   I N F O
Article history:
Received 23 November 2010
Returned to author for revision 2 March 2011
Accepted 4 March 2011
Available online 2 July 2011

Keywords:
IBDV
PI3K/Akt
VP5
Regulatory subunit p85α
Apoptosis

A B S T R A C T
Phosphatidylinositol 3-kinase (PI3K)/Akt signaling is commonly activated upon virus infection and has been implicated in the regulation of diverse cellular functions such as proliferation and apoptosis. The present study demonstrated for the first time that infectious bursal disease virus (IBDV), the causative agent of a highly contagious disease in chickens, can induce Akt phosphorylation in cultured cells, by a mechanism that is dependent on PI3K. Inhibition of PI3K activation greatly enhanced virus-induced cytopathic effect and apoptotic cell death as evidenced by cleavage of poly-ADP ribose polymerase and activation of caspase-3. Investigations into the mechanism of PI3K/Akt activation revealed that IBDV activates PI3K/Akt signaling through binding of the non-structural protein VP5 to regulatory subunit p85α of PI3K resulting in the suppression of premature apoptosis and improved virus growth after infection. The results presented here provide a basis for understanding molecular mechanism of IBDV infection.

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1. Introduction

Infectious bursal disease virus (IBDV) is the etiological agent of infectious bursal disease (IBD), which is a highly contagious disease in 3–15 week-old chickens. IBDV replicates in the cytoplasm of infected cells and targets the precursors of antibody-producing B lymphocytes in the bursa of Fabricius (BF). Infection by this virus leads to a severe immunosuppression in young chickens, inducing increased susceptibility to other pathogens as well as reduced efficacy of vaccination against other diseases (Lukert and Saif, 1997; Van Den Berg, 2000). Two distinct serotypes (I and II) are recognized for IBDV (McFerran et al., 1980). Serotype I virus strains differ markedly in virulence, classic and very virulent IBDV strains cause hemorrhagic inflammation of the BF whereas variant strains cause rapid bursal atrophy without evoking an inflammation response (Lukert and Saif, 1997; Snyder et al., 1992). In contrast to serotype I, serotype II viruses are naturally avirulent for chickens (Ismail et al., 1988). IBDV is one of the most important diseases affecting the poultry industry worldwide.

IBDV is a member of the genus Avibirnavirus within the family Birnaviridae, and its genome consists of two segments of double-stranded RNA, designated A (≈3.2 kb) and B (≈2.8 kb). Segment A has two partially overlapping open reading frames (ORFs). The larger ORF encodes a 110-kDa polyprotein precursor in the order NH2-pVP2-VP4-VP3-COOH. The polyprotein appears to be cotranslationally processed through the proteolytic activity of the viral protease, VP4, to generate pVP2, VP4, and VP3 (Azad et al., 1985; Hudson et al., 1986; Jagadish et al., 1988). pVP2 is further processed at its C terminus to become VP2 through the cleavage of three alanine–alanine bonds and an alanine–phenylalanine bond (Da Costa et al., 2002). VP2 and VP3 are structural proteins forming the virus capsid (Hudson et al., 1986). The smaller ORF encodes a highly conserved, basic and cysteine-rich class II membrane protein VP5 with a molecular weight of 17 kDa, which is not required for viral replication (Mundt et al., 1997). Segment B encodes VP1 which is known as a multifunctional protein with polymerase and capping enzyme activities (Spies et al., 1987; Spies and Müller, 1990).

IBDV infection has been shown to induce apoptosis both in vitro and in vivo (Lam, 1997; Ojeda et al., 1997; Tanimura and Sharma, 1998; Vasconcelos and Lam, 1994). Besides VP2, an apoptotic inducer (Fernández-Arias et al., 1997), VP5 was previously considered to be involved in the induction of apoptosis and IBDV pathogenesis (Yao et al., 1998; Yao and Vakharia, 2001). VP5 protein has also been shown to accumulate within the host plasma membrane and induce the release of viral particles (Lombardo et al., 2000; Wu et al., 2009). Furthermore, an increased data demonstrated that VP5 has an antiapoptotic function at the early stage of IBDV infection as evidenced in the VP5-deficient IBDV virus with more NF-κB activation and higher caspase-3 and -9 activities than that in the parental IBDV strain (Liu and Vakharia, 2006). However, whether there is an alternative mechanism whereby VP5 inhibits apoptosis is not clear.

Phosphatidylinositol 3-kinase (PI3K) is a heterodimeric protein/lipid kinase that consists of a regulatory subunit (usually p85α, p85β, or p55γ), and a p110 catalytic subunit (α, β, γ, or δ) (Cantrell, 2001).
Activation of PI3K causes the generation of phosphatidylinositol 3,4,5-trisphosphate from phosphatidylinositol 4,5-bisphosphate in the membrane, which functions as a second messenger to recruit pleckstrin homology domain-containing proteins, such as Akt (also known as PKB) and phosphoinositide-dependent kinase 1. Akt/PKB is a major PI3K effector and becomes further activated by phosphorylation at Thr308 and Ser473. Phosphorylated Akt plays an important role in modulating diverse downstream signaling pathways associated with cell proliferation, migration, differentiation, and the prevention of apoptosis (Datta et al., 1999; Yao and Cooper, 1995). The modulation of host cell PI3K signaling is a target for many viruses and induces a number of physiological changes within cells, from virus entry through replication and enhanced cell survival and thereby blockage of apoptosis in infected cells to oncogenic transformation as well as virion assembly (Dawson et al., 2003; Esfandiarei et al., 2004; Francois and Klotman, 2003; Fukuda and Longnecker, 2004; He et al., 2002; Nair et al., 2003; Shih et al., 2000). To date, there is still no report on the activation of PI3K/Akt induced by IBDV infection and the possible roles of the PI3K/Akt signaling pathway in the IBDV-infected cells.

Because IBDV appears to trigger apoptosis in the cultured cells at a late stage of infection and the nonstructural protein VP5 mediates antiapoptotic responses at the early stage of infection (Liu and Vakharia, 2006), the possibility that the PI3K/Akt pathway participates in the preservation of host cell survival during viral infection has prompted us to investigate the interaction between IBDV and this pathway. In the present study, we show that Akt can be phosphorylated after IBDV infection in the PI3K-dependent manner. When PI3K was blocked by a specific inhibitor LY294002, IBDV infection resulted in apoptosis at the early stage of infection; the inhibitor reduced IBDV virus production but had no effect on viral RNA transcription and protein synthesis. We also found that expression of VP5 alone was sufficient to activate the PI3K/Akt signaling but a VP5 knockout mutant IBDV did not. By using co-immunoprecipitation assay, we further found that VP5 binds to a PI3K regulatory subunit p85α, but not p85β isoform, and activates PI3K, thus causing the activation of the PI3K effector Akt. This results in more significant apoptotic responses during earlier stages of infection with the VP5 mutant. These findings suggest that IBDV VP5 helps IBDV to activate the PI3K/Akt signaling through interaction of VP5 with the regulator subunit p85α of PI3K resulting in the suppression of premature apoptosis and improved virus growth after infection.

2. Results

2.1. PI3K-dependent Akt phosphorylation was induced by IBDV infection

Because IBDV infection induced apoptosis in the infected cells late after infection (Liu and Vakharia, 2006), we speculated that a survival signal might be triggered at the early stage of viral infection. Serum starved DF-1 cells were infected with IBDV strain LM at a multiplicity of infection (MOI) of 10 TCID_{50}. Incubation with PBS served as mock-infected controls. Cell lysates prepared at the predetermined times were subjected to SDS–PAGE followed by Western blotting with the antibody specific to phosphorylated-Akt (Ser473). As shown in Fig. 1A, phosphorylated Akt was evident in virus-infected cells at 2 h and activation reached maximal levels at 10 h postinfection. Phosphorylated Akt in 1 h cells was close to that observed in the mock-infected cells (Fig. 1A). The increased phosphorylation of Akt was not concurrent with expression of viral capsid protein VP2 in the infected cells (Fig. 1A). The protein levels of total amounts of Akt remained unchanged in the IBDV-infected cells at various time points after infection when compared to that in the mock-infected cells. A loading control, β-actin in each sample, was comparable. Similar patterns of Akt activation were also observed in Vero cells following IBDV strain LM infection (data not shown), eliminating a possible cell line-specific phenomenon.

To further determine activated Akt quantitatively in the infected cells, we used a FACE assay to investigate the levels of Akt phosphorylation at the indicated time points after IBDV infection. As shown in Fig. 1B, there was a time-dependent increase in the Akt phosphorylation in the IBDV-infected cells until 10 h postinfection which decreased thereafter. At 10 h after infection, the activation of phosphorylated Akt was approximately 2.8-fold higher than that in the mock-infected cells. In addition, the levels of total Akt remain unchanged in the IBDV-infected cells at various time points after infection when compared to that in the mock-infected cells.

Akt serves as a key downstream mediator of PI3K signaling (6, 7). Thus, we continued to investigate whether the phosphorylation and activation of Akt upon IBDV infection occurs through the PI3K pathway. DF-1 cells were infected by LM strain at a MOI of 10 TCID_{50}, followed by treatment with increasing doses (5, 10, 25, and 50 μM) of LY294002, a specific PI3K inhibitor. Cell lysates were collected at 6 h postinfection and subjected to Western blotting analysis to detect the phosphorylation of Akt on Ser473. As shown in Fig. 1C, LY294002 treatment inhibited IBDV-induced Akt activation in a dose-dependent manner. By the FACE assay, reductions in Akt phosphorylation at 6 h postinfection inversely correlated with increased amounts of LY294002 (Fig. 1D). The levels of total Akt remained constant in all samples. Also, treatment of the IBDV-infected cells by wortmannin, another specific PI3K inhibitor, gave results similar to those observed for LY294002 (data not shown). These results indicated that IBDV-induced Akt phosphorylation occurs through a PI3K-dependent mechanism.

2.2. Blockage of PI3K activation enhanced cell death in IBDV-infected cells but reduced virus release

Because PI3K/Akt activation is important for cell survival (Marte and Downward, 1997), we determined whether activation of PI3K/Akt signaling pathway by IBDV infection would affect cell viability. Pretreatment of cells with LY294002 followed by IBDV infection caused CPE as early as 24 h after infection in a dose-dependent manner while the cells infected with IBDV but not pretreated with LY294002 showed minimal morphological changes (Fig. 2A). As observed for other viruses (Lee et al., 2005), we also noticed that blocking of the PI3K activity has shortened the time period of active viral production (2 days versus 3 days in the IBDV alone-infected cells) (data not shown), this is probably due to the obvious loss of cells as shown in Fig. 2A.

To further determine whether activated PI3K/Akt play any role in the replication of IBDV, we examine the effect of the PI3K/Akt on progeny virus production in the IBDV-infected DF-1 cells by treatment with the inhibitor LY294002. We infected DF-1 cells with IBDV in the presence of the inhibitor LY294002 (25 or 50 μM) and determined the virus titers in the cell culture supernatant at 8, 12, and 24 h postinfection by using a TCID_{50} assay, as shown in Fig. 2B. For virus production at 8 h postinfection, only a slight reduction of the virus titer (0.25-log-unit reduction at 50 μM of LY294002 compared to the titer of the no-treatment control) was observed. At 12 h postinfection, the inhibitor resulted in reduction of virus yield (0.5-log-unit reduction at 25 and 50 μM). At 24 h postinfection, treatment with the inhibitor LY294002 at 25 and 50 μM reduced IBDV progenies by 1.0- and 1.5-log-unit, respectively. The result suggested that blockage of PI3K activation reduced virus release.

To help delineate the mechanism of PI3K/Akt regulation of IBDV infection, we examined the effect of PI3K/Akt inhibition on viral protein expression. DF-1 cells infected with IBDV (MOI of 5) in the presence of DMSO or LY294002 (12.5–50 μM) were harvested at the predetermined time points and subjected to Western blotting using antibody against VP2 protein. As shown in Fig. 2C, synthesis of VP2 protein remains unchanged in LY294002-treated infected cells.
Infectious bursal disease virus infection induces PI3K-dependent Akt phosphorylation. (A) Whole-cell lysates from serum-starved DF-1 cells after infection with IBDV strain LM at a MOI of 10 TCID$_{50}$. IBDV-infected cells were harvested at the indicated times postinfection, and were prepared and resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted. The protein levels of Akt and its phosphorylated (Ser473) form, as well as IBDV VP2 protein, were analyzed. (B) Akt activation induced by IBDV infection was determined by using a FACE assay. DF-1 cells at the indicated time points were fixed with 4% formaldehyde and incubated with antibodies directed against Akt or its phosphorylated (Ser473) form followed by HRP-conjugated immunoglobulin G antibodies. Akt and its phosphorylated form were each assayed in triplicate. Cell numbers were normalized by using crystal violet. These results are representative of three independent experiments. Values are means±the SD from triplicate wells. *, P<0.05 for a comparison of mock-infected and IBDV-infected cells. (C) Dose-dependent inhibition of IBDV-induced Akt phosphorylation by treatment with PI3K inhibitor LY294002. DF-1 cells pretreated with increasing amounts (5, 10, 25, and 50 μM) of LY294002 for 30 min were infected with the IBDV strain LM for 60 min followed by incubation for 6 h before the cell lysates were harvested for Western blotting as described in panel A. (D) Dose-dependent inhibition of IBDV-induced Akt phosphorylation was further quantitatively determined by the FACE assay. LY294002-treated DF-1 cells at 6 h postinfection were fixed and assayed for Akt phosphorylation. These results are representative of three independent experiments. Values are means±the SD from triplicate wells. *, P<0.05 for a comparison of IBDV-infected and LY294002-treated IBDV-infected cells. p-, phosphorylated. SD, standard deviations.

Fig. 1. Infectious bursal disease virus infection induces PI3K-dependent Akt phosphorylation. (A) Whole-cell lysates from serum-starved DF-1 cells after infection with IBDV strain LM at a MOI of 10 TCID$_{50}$. IBDV-infected cells were harvested at the indicated times postinfection, and were prepared and resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted. The protein levels of Akt and its phosphorylated (Ser473) form, as well as IBDV VP2 protein, were analyzed. (B) Akt activation induced by IBDV infection was determined by using a FACE assay. DF-1 cells at the indicated time points were fixed with 4% formaldehyde and incubated with antibodies directed against Akt or its phosphorylated (Ser473) form followed by HRP-conjugated immunoglobulin G antibodies. Akt and its phosphorylated form were each assayed in triplicate. Cell numbers were normalized by using crystal violet. These results are representative of three independent experiments. Values are means±the SD from triplicate wells. *, P<0.05 for a comparison of mock-infected and IBDV-infected cells. (C) Dose-dependent inhibition of IBDV-induced Akt phosphorylation by treatment with PI3K inhibitor LY294002. DF-1 cells pretreated with increasing amounts (5, 10, 25, and 50 μM) of LY294002 for 30 min were infected with the IBDV strain LM for 60 min followed by incubation for 6 h before the cell lysates were harvested for Western blotting as described in panel A. (D) Dose-dependent inhibition of IBDV-induced Akt phosphorylation was further quantitatively determined by the FACE assay. LY294002-treated DF-1 cells at 6 h postinfection were fixed and assayed for Akt phosphorylation. These results are representative of three independent experiments. Values are means±the SD from triplicate wells. *, P<0.05 for a comparison of IBDV-infected and LY294002-treated IBDV-infected cells. p-, phosphorylated. SD, standard deviations.

In general, activation of PI3K/Akt signaling suppresses apoptotic responses via direct phosphorylation of caspase-9 and thereby inhibition of activation of this apoptotic protease (Cardone et al., 1998), as well as phosphorylation and inactivation of apoptosis-promoting activity of GSK-3β (Pap and Cooper, 1998). As demonstrated above, blocking of PI3K activation enhanced cell death in the IBDV-infected cells but reduced virus production, this might be due to increased apoptotic responses. To elucidate the role of the PI3K/Akt pathway in regulating apoptosis in the IBDV-infected cells, the phosphorylation of caspase-9 and GSK-3β as direct downstream targets of PI3K/Akt was analyzed. In the IBDV-infected cells, immunoblots of protein lysates probed with phospho-specific antibodies against caspase-9 and GSK-3β revealed increased levels of caspase-9 and GSK-3β phosphorylation at 18 h postinfection which decreased thereafter (Fig. 3A). This might be associated with increased apoptotic responses induced by VP2 (Fernández-Arias et al., 1997) at a late stage of infection. The increased levels of caspase-9 and GSK-3β phosphorylation were significantly blocked by LY294002 treatment (Fig. 3A). We also measured the cleavage of PARP, a major downstream substrate for activated caspase-9. As shown in Fig. 3A, IBDV infection also caused PARP cleavage at 18 h postinfection; however, when PI3K activation was blocked by LY294002, IBDV induced PARP cleavage at the earlier time of 12 h postinfection (Fig. 3A). A strong increase of PARP cleavage
concomitant with a reduced phosphorylation of caspase-9 is observed (Fig. 3A). This also coincides with a reduced phosphorylation of GSK-
3β. In addition, enhanced activities of caspase-3 were also detectable in the LY294002- and DMSO-treated infected cells, indicating a massive onset of an apoptotic response in the absence of PI3K activation (Fig. 3B). Thus, the results indicate that inhibition of PI3K/
Akt activation enhances the onset of premature virus-induced caspase activation and apoptosis at the early stage of IBDV infection.

2.4. Expression of VP5 results in activation of the PI3K/Akt signaling pathway

As demonstrated above, activation of the PI3K/Akt pathway was observed upon IBDV infection. IBDV VP5 has been considered to predominantly express at the early stage of infection (Mundt et al., 1995), therefore, we investigated whether VP5 protein has a potential role in activation of the PI3K/Akt pathway. For this, we generated a recombinant rLM virus and its VP5 knockout mutant, rLMVP5Δ, using reverse genetics. When DF-1 cells were infected with these two viruses, no difference in viral protein synthesis (based on VP2 expression) was observed (data not shown). However, rLMVP5Δ virus replicated slower and produced fewer progenies than its counterpart, rLM virus, at 14 h postinfection (data not shown), consistent with that of the previous report (Liu and Vakharia, 2006).

DF-1 cells were infected with wild-type LM, rLM, as well as rLMVP5Δ, and cell lysates were prepared at 8 h postinfection. The phosphorylation of Akt (Ser473) was analyzed by Western blotting. As shown in Fig. 4A, the wild-type LM as well as recombinant rLM virus infection resulted in induction of phospho-Akt compared to the VP5 mutant rLMVP5Δ, which resulted in weak phosphorylation of Akt. As expected, the PI3K inhibitor LY294002 resulted in blockage of Akt phosphorylation in the wild-type LM as well as recombinant rLM-infected cells (Fig. 4A). Similar results were obtained in Vero cells (data not shown). To rule out effects of other IBDV proteins or dsRNA-mediated effects, pCMV-VP5 plasmid was transfected in Vero cells and activated Akt was quantitatively determined. As shown in Fig. 4B, the activation of phosphorylated Akt at 24 h post-transfection showed approximately 2.9- and 1.9-fold higher than that in the mock-transfected and empty vector-transfected cells, respectively. In addition, the levels of total Akt remain unchanged in the pCMV-VP5 plasmid after transfection when compared to that in the mock-transfected as well as empty vector-transfected cells. Therefore, these results indicated that IBDV VP5 alone is sufficient to activate the PI3K/Akt signaling pathway.

2.5. VP5 protein binds to p85α regulatory subunit of PI3K

To confirm whether activation of PI3K/Akt is due to modulation of cellular signaling mechanism or to protein–protein interaction of VP5 with PI3K, VP5 and p85α or p85β full-length genes were inserted into an expression vector that allows the production of HA or myc fusion protein. Vero cells were transfected with pCMV-VP5 plasmid expressing HA-tagged VP5, analysis of proteins immunoprecipitated with the anti-HA antibody revealed that VP5 efficiently precipitated endogenous p85α rather than p85β subunit (data not shown). A reciprocal specific communoprecipitation of HA-VP5 and myc-p85α was observed with anti-myc antibody in Vero cells co-transfected with HA-VP5 and myc-p85α plasmids (Fig. 5A). As
expected, there is no specific binding of myc-p85α in the myc-p85α plasmid-alone-transfected cell lysate to anti-HA agarose (Fig. 5A). In contrast, HA-VP5 does not bind to myc-p85β in the HA-VP5 and myc-p85β plasmids-co-transfected cells (Fig. 5B). The result showed that VP5 and p85α physically and specifically interact in vivo.

2.6. VP5 protein suppresses apoptotic responses via activating PI3K/Akt signaling

VP5 has been shown to inhibit virus-induced apoptosis in the early stage of IBDV infection (Liu and Vakharia, 2006). To investigate whether VP5 protein suppresses apoptotic responses through activation of the PI3K/Akt pathway, the wild-type IBDV strain LM, recombinant rLM as well as mutant virus rLMVP5Δ at a MOI of 10 TCID50 were inoculated into DF-1 cells and PI3K/Akt activation-mediated apoptotic events were determined. At 12 h postinfection, cell lysates were prepared and subjected to Western blotting with antibodies against p-Akt (Ser473) as well as p-GSK-3β (Fig. 6A). Elevated p-Akt as well as p-GSK-3β was detected in the wild-type- and rLM-infected cells, as well as in the mock-infected cells. However, no obvious phosphorylation of caspase-9 as well as p-GSK-3β occurred in the cells infected by the rLMVP5Δ virus-infected cells as well as the wild-type- and rLM-infected cells after treatment with LY294002 (20 μM) (Fig. 6A). We then examined whether phosphorylation of caspase-9 would lead to inhibition of PARP cleavage in the mutant virus-infected cells. As shown in Fig. 6A, a cleaved PARP band was clearly visible in the rLMVP5Δ-infected cells as well as the wild-type- and rLM-infected cells after treatment with LY294002. A weak cleaved PARP band was detected in the wild-type-, rLM-, and mock-infected cells, indicating that PARP cleavage was inhibited to some extent. Equal loading of the cellular protein was monitored by determining β-actin level.

This was further confirmed by quantitating the protease activity of caspase-3 by using the fluorometric assay. There was a significant increase in caspase-3 activity (2.26- to 2.34-fold) at 12 h postinfection in the mutant virus-infected cells compared to that in the wild-type as well as rLM-infected cells. In addition, a peptide inhibitor of caspase-3 activity, Ac-DEVD-CHO, was used as an internal control to confirm assay validity. The caspase-3 activity was also measured in the cells infected with the wild-type as well as rLM virus after inhibition of PI3K/Akt activation. As expected, enhanced caspase-3 activities were observed in the wild-type as well as rLM virus-infected cells in the presence of LY294002 (Fig. 6B).

3. Discussion

PI3Ks, a family of heterodimeric enzymes are activated by binding to Src homology-2 or -3 (SH2 or SH3) domains of the proteins to the p85 or p110 subunits (Datta et al., 1999). Activated PI3K can further activate downstream protein or lipid kinases, including major target Akt. Activated Akt plays a central role in modulating diverse downstream signaling pathways associated with differentiation, proliferation, and the prevention of apoptosis (Datta et al., 1999; Yao and Cooper, 1995). In the case of viruses causing acute infections, such as dengue virus (Lee et al., 2005), influenza virus (Ehrhardt et al., 2006 & 2007), coxsackievirus B3 (Esfandiarei et al., 2004), poliovirus (Autret et al., 2008), vaccinia virus, cowpox virus (Soares et al., 2009), and rotavirus (Bagchi et al., 2010), PI3K/Akt signaling has been reported to assist viral replication by inhibiting apoptosis. In this study, we report that infectious bursal disease virus infection activates the PI3K/Akt pathway, protecting the cells from caspase-mediated apoptotic cell death during the early stage of viral infection. The PI3K/Akt signaling is involved in IBDV replication, as the PI3K inhibitor LY294002 did reduce infectious virus production. Furthermore, the PI3K/Akt pathway activation is attributed to the viral VP5 protein which can specifically interact with PI3K regulatory subunit p85α. The activation of PI3K confers an antiapoptotic signal in the VP5-expressing cells, indicating that VP5 inhibits premature apoptosis induction to ensure efficient replication via activation of the PI3K/Akt signaling.

Activation of the PI3K/Akt pathway has been demonstrated to occur during the early phase of infection by a variety of viruses and the signaling participates in virus replication via prolonging survival of infected cells and creating a favorable environment for virus growth and virion assembly (Corray, 2004; Ehrhardt and Ludwig, 2009; Ji and Liu, 2008). We examined whether the PI3K/Akt pathway plays a role in virus replication by blocking PI3K activity with the
specific inhibitor LY294002 and found that LY294002 treatment inhibited virus production in the infected cells (Fig. 2B). This indicates that activation of the PI3K/Akt pathway plays a role in supporting IBDV propagation. Similar observations were reported in other viruses, such as influenza A virus (Ehrhardt et al., 2006; Shin et al., 2007), parainfluenza virus 5 (Sun et al., 2008), vaccinia and cowpox virus (Soares et al., 2009), and so on, wherein we found that inhibition of PI3K/Akt results in reductions of virus release. To further define at which step virus replication was affected, we found that inhibition of PI3K/Akt activation did not have an effect on viral protein synthesis and the accumulation of viral-specific mRNA in the IBDV-infected cells (Fig. 3C and D). This result appears to be in disagreement with the finding that inhibition of PI3K/Akt activation reduces IBDV release from the infected cells. However, this can be explained since inhibition of IBDV-mediated PI3K/Akt activation may be resistant to the translational blockage triggered by the PI3K inhibitor as described in flavivirus (Lee et al. 2005) but blocks virus release from the infected cells at the early stage of infection, which is similar to that in the mutant IBDV virus lacking expression of VP5 protein (Liu and Vakharia, 2006). In addition, the function of VP5 protein that promotes virion release from the infected cells (Lombardo et al., 2000; Wu et al., 2009) may be blocked after inhibition of PI3K/Akt activation, whereby leading to reductions of virus production. Thus, this implicates that the nonstructural protein VP5 is involved in the IBDV-mediated PI3K/Akt activation.

By using a genetic approach, we constructed a mutant virus lacking the expression of VP5 protein and investigated the contribution of VP5 to the PI3K/Akt signaling activation. The mutant virus infection did not lead to Akt phosphorylation at Ser473, suggesting that VP5 protein is required for PI3K/Akt pathway activation. Influenza A virus infection has been shown to activate the PI3K/Akt pathway by direct interaction of the SH3 binding motif of NS1 protein with the p85α subunit of PI3K (Ehrhardt et al., 2007; Hale et al., 2008; Hale et al., 2006; Hale et al., 2010; Shi et al., 2007). Rotavirus NSP1 interacts with the p85α subunit of PI3K and modulates sustained PI3K/Akt activation in the virus-infected cells (Bagchi et al., 2010). Sequence analysis revealed that IBDV VP5 protein contains three proline motifs with the consensus sequence PXXP positioned at aa 76–79, 102–105, and 145–148. Such SH3 binding motifs are involved in the PI3K signaling and form extended helices that bind to SH3 domains found within a diverse group of signaling proteins, including the p85 subunit (Pawson, 1995). Immunoprecipitation assay demonstrated that VP5 binds specifically and efficiently to the p85α subunit of PI3K but not to the related p85β isoform (Fig. 5A and B), suggesting that activation of PI3K could be due to an interaction of VP5 with PI3K. Therefore, the mechanism of PI3K/Akt activation induced by IBDV involves a direct interaction between VP5 protein and p85α of PI3K. However, whether the interaction of one or several SH3 binding motifs of VP5 with the p85α subunit of PI3K may contribute to the PI3K/Akt signaling pathway activation in the cultured cells is to be further determined.

Activation of PI3K/Akt is a strategy adopted by many viruses to prolong cell survival and block early premature apoptosis. Activated Akt phosphorylates a large number of substrates, such as proapoptotic protease caspase-9 and GSK-3β. This can lead to phosphorylation and inactivation of the caspase-9 and GSK-3β and silences the apoptosis-promoting activities. Our results showed that the wild-type virus infection, which activates the PI3K/Akt pathway, resulting in phosphorylation of caspase-9 and GSK-3β, does not induce obvious caspase-3 apoptotic activity at the early stage of virus infection (Fig. 3A and B). The role of the PI3K/Akt pathway in delaying apoptosis in the wild-type virus was confirmed; as high levels of PARP cleavage or caspase-3 activity were earlier observed after the wild-type virus infection in the presence of PI3K inhibitor (Fig. 3A and B). Furthermore, significantly high levels of PARP cleavage or caspase-3 protease activity were observed earlier in the cells infected with the mutant virus rLMVP5Δ (Fig. 6A and B), which lacks the ability to activate PI3K/Akt pathway (Fig. 4A). These results are consistent with the reports showing a role for N5 proteins in suppressing premature apoptosis during infection with respiratory syncytial virus (Thomas et al., 2002), poliovirus (Aupert et al., 2008), influenza A virus (Ehrhardt et al., 2007; Shi et al., 2007), and rotavirus (Bagchi et al., 2010). Delayed apoptosis could encourage virus growth via retaining cellular structural integrity beneficial to optimal viral replication as well as complete formation of packaged infectious virions (Bagchi et al., 2010; Bitko et al., 2007; Kallewaard et al., 2005). Therefore, we expand the data by identification of the functional consequences of VP5-mediated PI3K activity, namely, suppressing the onset of premature virus-induced caspase activation and apoptosis in the early stage of IBDV infection.
In conclusion, these results presented here demonstrated that the signaling triggered by the PI3K/Akt pathway upon infectious bursal disease virus infection does play an important role in viral replication and regulation of apoptosis. IBDV-mediated PI3K/Akt activation is due to specific interaction of VP5 protein with PI3K regulatory subunit p85α, but not p85β isoform. The role of VP5 protein in mediating antiapoptotic responses via activation of the PI3K/Akt pathway will contribute to important information about the molecular mechanism of IBDV pathogenesis.

3. Materials and methods

3.1. Virus and cells

A DF-1 chicken embryo fibroblast (CEF) cell line (Himly et al., 1998) was maintained in Dulbecco’s Modified Eagle medium (DMEM) (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin G, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO2 incubator. Vero cells were maintained in DMEM medium supplemented with 5% FBS at 37 °C in a humidified 5% CO2 incubator and used for transfection and propagation. The IBDV strain LM (Liu et al., 2002), a CEF cell-adapted virus, was used in the study.

3.2. Reagents and antibodies

The PI3K inhibitor LY294002 and wortmannin were purchased from Calbiochem (La Jolla, CA). DF-1 cells were treated with either dimethyl sulfoxide (DMSO) which is the solvent for these inhibitors or various concentrations (5–50 μM) of these two inhibitors for 1 h prior to infection. After 1 h of virus adsorption, the virus inoculum was removed and fresh basal medium containing fresh inhibitor was added to the culture. The cytoxicity of the inhibitors on DF-1 cells was determined by trypan blue exclusion dye staining. It was noted that throughout all doses of the inhibitors used in the present study, cell viability assay showed no detectable cell death in the DF-1 cells.

Rabbit, goat, or mouse antibodies against Akt1/2/3, poly-ADP-ribose polymerase (PARP), glycogen synthase kinase-3 (GSK-3β), phosphorylated (p)-Akt (Ser473), -caspase-9 (Ser196), and β-actin were purchased from Sigma. Fluorescein isothiocyanate (FITC)- and horseradish-peroxidase (HRP)-linked secondary antibodies were purchased from Clontech. Horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Sigma. Fluorescein isothiocyanate (FITC) - and rhodamine-conjugated secondary antibodies were purchased from DAKO.

3.3. FACE

Fast activated cell-based enzyme-linked immunosorbent assay (FACE) kit to monitor the levels of Akt activation was obtained from Active Motif. Procedure was performed strictly according to the manufacturer’s instructions. Briefly, DF-1 or Vero cells seeded in 96-well plates for 1 day were infected with IBDV strain LM or transfected with plasmid pCMV-VP5 and fixed with 4% formaldehyde in phosphate-buffered saline (PBS) at the indicated time points after infection or transfection. After washing and blocking steps, the cells were reacted overnight with an anti-Akt or anti-phospho-Akt antibody. Following incubation with an HRP-conjugated secondary antibody, colorimetric analysis was performed. The A450 was determined using a plate spectrophotometer.

3.4. Real-time RT-PCR

Total cell RNAs were prepared from virus-infected DF-1 cells 12 h after being treated with 20 μM of LY294002 by using RNeasy Mini kit (Qiagen) for reverse transcription (RT)-PCR. The VP2-F5 (5′-CTGAC-TACCCGCACTCGACA-3′) and VP2-R3 (5′-CCATTGCGCACTATGA-3′) primer pair was used to amplify a 133-nucleotide region of VP2, and were normalized with β-actin primers (sense, 5′-GGAGAAATTGCGCTGACATCA-3′; antisense, 5′-CCTGAACTCTCTATGCGCA-3′) in separate reactions. Real-time RT-PCR protocol followed the instructions of an iScript one-step RT-PCR kit with SYBR Green (Bio-Rad). The RT-PCR parameters consisted of a cDNA synthesis at 95 °C for 10 min and reverse transcriptase inactivation at 95 °C for 5 min, followed by PCR cycling and detection of 95 °C for 10 s and 55 °C for 30 s (40 cycles). Each sample was run in triplicate. The relative amount of target viral mRNA was normalized to that of β-actin mRNA in the same sample.

3.5. Virus titration

The amount of IBDV produced was measured on monolayers of DF-1 cells. Cell supernatant was serially diluted and inoculated on monolayers of DF-1 cells. Following 1 h of incubation, fresh DMEM medium was added and incubated. Three days post-infection, cytopathic effect (CPE) was observed under a microscope and virus titer was determined as 50% tissue culture infective dose (TCID50) per 0.1 milliliter.

3.6. Whole cell lysates

Whole cell lysate extracts from DF-1 or Vero cells at the indicated time points after infection or transfection were prepared with the nuclear extract kit (Active Motif) according to the manufacturer’s protocol.

3.7. Generation of VP5 mutant virus

All manipulations of DNAs were performed according to standard protocols. Recombinant plasmids pCI-LMA and pCI-LMB, containing the complete genomic A and B segments, respectively, of IBDV strain LM were used as templates. To construct a mutant cDNA clone of segment A lacking the initiation codon of the VP5 gene, a set of primers (LMΔVP5-F: 5′-CGCTATCACTGGCTGAGACTAGAC-3′ and LMΔVP5-R: 5′-GGCATATGAACTGACTGCTGACTAGAC-3′) were synthesized and used for PCR amplification of the mutant plasmid pCI-LMA-VP5Δ using a QuickChange site-directed mutagenesis kit (Stratagene). The mutant clone of segment A in which a mutation of the VP5 gene initiation codon from ATG to ATC was obtained after being sequenced to confirm that no errors were introduced as a result of PCR amplification.

DF-1 cells were co-transfected with plasmids pCI-LMA or pCI-LMA-VP5Δ and pCI-LMB for generating recombinant IBDV (rLM) or mutant IBDV (rLMVP5Δ), respectively. For the infection test, the transfected cells were subjected to three successive freeze-thaw cycles. The total lysates were collected and used to infect DF-1 cells. They were then analyzed by indirect fluorescence assay (IFA) after infection. The mutant virus was passaged five times in DF-1 cells to increase virus titers.

3.8. Coimmunoprecipitation analysis

To prepare recombinant eukaryotic expression plasmids, the coding sequences of PI3K regulatory subunits p85α and p85β (GenBank accession numbers XM_424759 and XM_001233340, respectively) as well as IBDV strain LM VP5 gene were amplified by RT-PCR reaction using three pairs of primers from primary CEF cells and IBDV-infected cells. The following primers: myc-△GCGATAGTAACTAGCAGTCATCTCTAG-3′ and myc-p85α(R): 5′-TTCATTAGTTGGGAAAGG-3′; HA-VP5(F): 5′-TTTCATGACATCTGACTGGTCCTGCGAACCTAC-3′; and HA-p85β(R): 5′-TTTCATGACATCTGACTGGTCCTGCGAACCTAC-3′ were used to amplify the...
three genes, respectively. The EcoRI/Sall, EcoRI/Xhol, and EcoRI/BglII fragments p85α, p85β, and VP5 were directionally cloned between corresponding sites of eukaryotic expression vectors pCMV-myc and pCMV-HA (Clontech), downstream of the human cytomegalovirus (HCMV) promoter, to obtain myc-p85α and -p85β, as well as HA-VP5, respectively.

In vitro expression of the myc-p85α, myc-p85β, and HA-VP5 constructs was tested in transient expression experiments using Vero cells. The cells grown in 25 × 25 mm flasks were transfected or cotransfected HA-VP5 or myc-p85α or myc-p85β (2 μg of plasmid per flask), using Lipofectamine 2000 (Invitrogen), as described in the manufacturer’s protocol. After 24 h posttransfection, the expression of HA-VP5, myc-p85α or myc-p85β was demonstrated by immunoblotting analysis using rabbit anti-HA polyclonal antibody or mouse anti-myc monoclonal antibody.

Vero cell lysate extracts at 24 h after transfection with plasmid pCMV-HA-VP5 and as well as co-transfection with plasmids pCMV-HA-VP5 and pCMV-myc-p85α or pCMV-myc-p85β were prepared for immunoprecipitation analysis with profound mammalian HA tag IP/Co-IP kit (Pierce) according to the manufacturer’s protocol. Lysate extracts were incubated overnight at 4 °C with high affinity anti-HA antibody-coupled agarose. Bound proteins were eluted from agarose by boiling them with Laemmli sample buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) before Western blotting.

3.9. Western blotting

The whole cell lysate extracts prepared were diluted in 2× sample buffer and boiled for 5 min. Twenty micrograms of each extract was resolved on 10%–12% SDS–PAGE and blotted onto nitrocellulose (NC) membranes (Stratagene) with a semidry transfer cell (Bio-Rad). The membranes were blocked for 2 h at room temperature in blocking buffer. Immunoreactive bands were visualized by enhanced chemiluminescence system (Kodak Image Station 4000R).


