The PI3K/Akt pathway is involved in early infection of some exogenous avian leukemia viruses

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Avian leukemia virus (ALV) is an enveloped and oncogenic retrovirus. Avian leukemia caused by the members of ALV subgroups A, B and J has become one of the major problems challenging the poultry industry in China. However, the cellular factors such as signal transduction pathways involved in ALV infection are not well defined. In this study, our data demonstrated that ALV-J strain NX0101 infection in primary chicken embryo fibroblasts or DF-1 cells was correlated with the activity and phosphorylation of Akt. Akt activation was initiated at a very early stage of infection independently of NX0101 replication. The specific phosphatidylinositol 3-kinase (PI3K) inhibitors LY294002 or wortmannin could suppress Akt phosphorylation, indicating that NX0101-induced Akt phosphorylation is PI3K-dependent. ALV-A strain GD08 or ALV-B strain CD08 infection also demonstrated a similar profile of PI3K/Akt activation. Treatment of DF-1 cells with the drug 5-((N, N-hexamethylene) amiloride that inhibits the activity of chicken Na+/H+ exchanger type 1 significantly reduced Akt activation induced by NX0101, but not by GD08 and CD08. Akt activation triggered by GD08 or CD08 was abolished by clathrin-mediated endocytosis inhibitor chlorpromazine. Receptor-mediated endocytosis inhibitor dansylcadaverine had a negligible effect on all ALV-induced Akt phosphorylation. Moreover, viral replication of ALV was suppressed by LY294002 in a dose-dependent manner, which was due to the inhibition of virus infection by LY294002. These data suggest that the activation of the PI3K/Akt signalling pathway by exogenous ALV infection plays an important role in viral entry, yet the precise mechanism remains under further investigation.

INTRODUCTION

Avian leukemia virus (ALV) is an oncogenic retrovirus that primarily infects chickens. Based on the identity of the surface region (SU) of its envelope glycoprotein, ALV is divided into different subgroups, designated A through to F and J (Coffin et al., 1992). ALV-E is endogenous virus, while all other subgroups are exogenous. Recently, the disease caused by ALV-J, ALV-A or ALV-B has become one of the major problems facing the poultry industry in China, which has been identified in the commercial meat and egg-type chickens, and also in the Chinese local breeds (Du et al., 2000; Cui et al., 2003; Cheng et al., 2010; Zhang et al., 2010). ALV can induce a rather wide variety of tumours and mortality that cause significant economic losses. The various pathotypes of tumours are mainly lymphomas, myelocytomas and haemangiomas, as well as fibrosarcomas, histiocytic sarcomas, intestinal adenocarcinoma, leiomyosarcoma and so on (Xu et al., 2004; Sun & Cui, 2007; Cheng et al., 2010). The major mechanism of ALV pathogenesis is that of typical oncogene activation by proviral insertion gene deregulation, resulting in tumour formation after chronic infection. Acute-transforming ALV variants have been discovered that have acquired an activated oncogene in their genome and these cause tumours in a short period of time (Graf & Beug, 1978; Hayward et al., 1981; Rasheed, 1995; Gong et al., 1998; Yang et al., 2007).

Exogenous ALV enters host cells primarily through the interaction of the viral envelope glycoprotein with the host cell receptor, such as the chicken Na+/H+ exchanger type 1 (cNHE1) receptor for ALV-J, TVA receptor that is a member of the low-density lipoprotein receptor family for ALV-A, and TVB5 receptor, which belongs to the tumour necrosis factor receptor superfamily for ALV-B (Weiss, 1992; Young, 1998; Chai & Bates, 2005). After binding to its receptor, exogenous ALV enters the host cells by endocytosis (Mothes et al., 2000; Diaz-Griffero et al., 2002), and then goes through the retroviral life cycle that includes reverse transcription, integration, RNA synthesis, translation, virus assembly and budding.

Viruses are intracellular obligate parasites that have evolved to exploit the host cellular machinery for their replication. There is increasing evidence that cellular signal transduc-
tion pathways are required not only for virus cell entry, but also for subsequent intracellular trafficking and viral replication, such as the enzyme phosphatidylinositol 3-kinase serine-threonine protein kinase (PI3K/Akt) pathway (Cooray, 2004). For example, human immunodeficiency virus type 1 (HIV-1) requires PI3K activity for productive infection of CD4+ T-cells. Inhibition of PI3K signalling decreases HIV infection post-viral entry and post-reverse transcription, but prior to HIV gene expression (Guntermann et al., 1999; François & Klotman, 2003).

Recent small interfering RNA screening studies have identified Akt-associated pathways are essential for HIV infection, such as the adipocytokine signalling pathway that is involved in regulation of cellular metabolism (Zhou et al., 2008). Activation of the PI3K/Akt pathway is a strategy for Zaire Ebola virus to regulate vesicular trafficking and cellular entry (Saeed et al., 2008). Adenovirus also relies on PI3K-mediated organization of the actin cytoskeleton for active internalization by αv integrins (Li et al., 1998). Non-segmented negative-sense RNA viruses utilize Akt to enhance synthesis of viral RNAs (Sun et al., 2008). Influenza A virus as well as vaccinia and cowpox virus have been found to activate the PI3K/Akt pathway to augment their efficient replication (Hale et al., 2006; Shin et al., 2007a; Soares et al., 2009).

Although the replication cycle of ALV has been characterized, the cellular factors involved in exogenous ALV infection and replication, especially signal transduction pathways, are currently unknown. Based on many examples of enrichment of the PI3K/Akt signal pathway in regulation of viral replication, we investigated the role and mechanisms of PI3K/Akt regulation during exogenous ALV propagation in vitro. Strains from ALV subgroups J, A and B were used in this study. The results would extend our current understanding of the exogenous ALV infection mechanism.

**RESULTS**

**Exogenous ALV transiently activates Akt in a PI3K-dependent manner**

Given the biological importance of the PI3K/Akt pathway, we initially investigated whether ALV-J strain NX0101, ALV-A strain GD08 and ALV-B strain CD08 infection would lead to activation of this pathway. In contrast to mock-treated cell lysate results, virus stocks of all exogenous ALV infection led to rapid increase in Akt phosphorylation within 15 min post-infection (p.i.) and decline by 30 min p.i., returning to background by 3 h p.i. (Fig. 1a–d).

In order to eliminate any secreted factors in the conditioned media associated with the viral stock which could possibly have triggered production of phosphorylated Akt (p-Akt), viral stocks were filtered and pelleted by centrifugation, and then suspended and inoculated into DF-1 cells. Fig. 1(e) shows that viral filtrate and pellet from viral stocks of NX0101 could also trigger p-Akt within 15 min p.i. of which the activation pattern was the same with that of viral stocks from the infected cell-culture supernatants. No signal detection was found in cellular extraction from DF-1 cells inoculated with supernatant after centrifugation. These results demonstrate that virus particles NX0101 could activate Akt in the early stage of cell infection.

To determine whether the phosphorylation of Akt in DF-1 cells was PI3K-dependent or -independent, the role of PI3K in Akt activation following ALV infection was then investigated. The specific PI3K inhibitors LY294002 and wortmannin were used. As expected, ALV infection increased the phosphorylation of Akt, while LY294002 or wortmannin pretreatment completely inhibited ALV-induced Akt phosphorylation at all tested concentrations (Fig. 2a–d).

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**Fig. 1.** ALV infection activates Akt phosphorylation. DF-1 cells were infected by live viral stocks of NX0101 (a; 10^4 TCID_50 0.2 ml^-1), GD08 [b; sample to positive (S/P) ratio of 1.3] or CD08 (c; S/P ratio of 1.3), or mock infected (d). Cell lysates prepared at the indicated times p.i. were subjected to SDS-PAGE and the amounts of phosphorylated Akt (p-Akt) and total Akt evaluated by Western blotting. (e) Viral stocks of NX0101 were filtered and pelleted. Viral stock, the filtrate, pellet and supernatant individually infected DF-1 cells. At 15 min p.i., cell lysates were subjected to SDS-PAGE and Western blot analysis using anti-phospho-Akt and anti-Akt antibody. The results are representative of three independent experiments.
DF-1 cell line is an immortal cell line derived from Line 0 chicken embryo fibroblasts (CEFs). While DF-1 cells have characteristics that are very similar to cultured CEFs, several studies have revealed some differences, for example the propensity of DF-1 cells to apoptosis. Here, we studied the relevance of the results with comparison to ‘normal’ cultures of CEFs. Similar to the results obtained in DF-1 cells, NX0101 also activated Akt phosphorylation as early as 15 min after infection of CEFs, and the level of Akt phosphorylation was significantly decreased by LY294002 (Fig. 2e).

**Early events in the entry process involved in PI3K/Akt pathway activation**

Taking into consideration that virus–receptor interaction and endocytic uptake are early events of the virus life cycle, we investigated whether these mechanisms might be involved in Akt activation by ALV subgroups J, A and B. Chlorpromazine is a specific inhibitor of clathrin-mediated endocytosis. Dansylcadaverine is a pharmacological inhibitor of receptor-mediated endocytosis. We tested the effect of chlorpromazine, dansylcadaverine and NHE1 inhibitor 5-(N, N-hexamethylene) amiloride (HMA) on Akt phosphorylation induced by ALV infection. Dansylcadaverine treatment had no effect on all exogenous ALV-induced Akt activation (Fig. 3a and b). However, treatment of DF-1 cells with various concentrations of chlorpromazine (above 5 μM) abolished Akt phosphorylation induced by GD08 infection (Fig. 3d), but not NX0101-induced Akt phosphorylation (Fig. 3c). Treatment of cells with HMA (above 0.5 μM) diminished Akt phosphorylation induced by NX0101 infection, but had no effect on Akt activation induced by GD08 (Fig. 3e and f). The effect of inhibitors on p-Akt activation induced by CD08 was the same with that by GD08. Akt activation triggered by ALV-J was probably

**Fig. 2.** ALV infection activates Akt phosphorylation in a PI3K-dependent manner. DF-1 cells (a, b, c and d) or primary CEF cells (e) were preincubated with LY294002 (1–20 μM), wortmannin (60 nM–1 μM) or DMSO (0.4 %, v/v) for 1 h and subsequently infected with NX0101 (10^4 TCID<sub>50</sub> 0.2 ml<sup>−1</sup>) or GD08 or CD08 (S/P ratio of 1.3) or mock infected. After 15 min, cell lysates were collected and separated by SDS-PAGE. The amounts of p-Akt and total Akt were evaluated by Western blotting. The results are representative of three independent experiments.
related to receptor engagement, and clathrin-mediated endocytosis probably mediated the Akt activation induced by ALV-A and ALV-B. These results strongly suggest that induction of the PI3K/Akt pathway is related to a very early event in the ALV entry process.

PI3K/Akt pathway regulates viral replication

Our foregoing results had indicated that ALV infection led to PI3K/Akt pathway activation. To determine whether activated Akt was necessary for exogenous ALV replication, viral production was measured by viral RNA transcription during PI3K inhibition. The real-time RT-PCR results showed that pretreatment of cells with LY294002 significantly decreased viral RNA synthesis at all concentrations tested ($P<0.05$). A 50% inhibition of NX0101 was observed with 10 μM LY294002 (Fig. 4a). While for viral RNA synthesis of GD08 and CD08 a 50% inhibition was observed with 5 μM LY294002 (Fig. 4b and c). LY294002 had an inhibitory effect on viral RNA levels of exogenous ALV in a dose-dependent manner. These data suggest that the PI3K/Akt pathway plays a role in ALV replication.

**LY294002 treatment decreases ALV transcription at an early stage of viral replication**

To determine at which point PI3K/Akt acted during the ALV replication cycle, LY294002 (10 μM) was added to DF-1 cells at −1, 0, 1, 2 and 4 h p.i., respectively. Real-time RT-PCR results showed that addition of LY294002 at −1 h p.i. or 0 h p.i. led to about 40% inhibition of viral transcription of NX0101 compared with vehicle-treated control results (Fig. 5). A 10–30% decrease of viral transcription was observed when the inhibitor was added at between 1 and 2 h p.i. No statistical differences in viral transcription were observed when the inhibitor was added at 4 h p.i. Similar to the effect of LY294002 on NX0101 replication, pretreatment of cells with LY294002 prior to virus infection or simultaneous virus infection and exposure to LY294002 could also suppress RNA replication of GD08 and CD08. These results suggest that PI3K/Akt primarily acted at an early stage of ALV infection.

**The PI3K/Akt pathway is critical for entry of ALV**

We adapted a previously described contents mixing assay that allowed rapid and quantitative measurement of

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**Fig. 3.** Pretreatment of DF-1 cells with various concentrations of dansylcadaverine (a and b), chlorpromazine (c and d) or HMA (e and f) for 30 min, respectively, followed by infection with NX0101 (about $10^4$ TCID$_{50}$, 0.2 ml$^{-1}$) or GD08 (S/P ratio of 1.3), or mock infected. After 15 min p.i., cell lysates were collected and separated by SDS-PAGE. The amounts of p-Akt and total Akt were evaluated by Western blotting.
retrovirus entry (Kolokoltsov & Davey, 2004) to investigate the role of PI3K/Akt pathway during ALV infection. This is the first time that this assay technology has been adapted to ALV. To assess their efficiency in an entry assay, viral particles NX0101 containing luciferase or not were produced, pelleted and tested on DF-1 cells. As compared with particles devoid of luciferase, a strong signal was obtained for luciferase-containing particles NX0101 (Table 1). Viral particles NX0101 containing luciferase were then further validated for specificity of entry into cells.

To investigate whether the PI3K/Akt pathway played a role in the entry step of ALV infection or was required for some other early, but post-entry step, LY294002 was tested in the entry assay. Cells treated with LY294002 exhibited significant reduction of NX0101 entry (Fig. 6). At 5 μM, LY294002 reduced entry by about 8% of that into control DMSO-treated cells, and 20 μM LY294002 reduced entry by about 55% of the control. These results suggest that the diminished viral transcription and replication of ALV by LY294002 was due to a dramatic decrease in viral entry. An early activation of the PI3K/Akt pathway during exogenous ALV infection probably plays an important role in supporting viral entry by indirect/off-target effects.

**DISCUSSION**

During virus infection, interactions between virus and host cell determine the efficiency of virus propagation. One important event from the virus’ perspective is to activate cellular signalling pathways and augment virus replication. In this study, we examined the role of the PI3K/Akt pathway during exogenous ALV infection. The results demonstrate that activation of the PI3K/Akt signalling pathway is important for optimal exogenous ALV infection in either CEF or DF-1 cells. Akt was found to be transiently phosphorylated within 15 min of ALV infection. The Akt phosphorylation levels decreased and returned to background by 3 h p.i. Akt activation induced by ALV was PI3K-dependent, which was abrogated by PI3K-specific inhibitors LY294002 and wortmannin. The fact that the level of Akt phosphorylation in ALV-infected cells analysed at later times (12 and 24 h p.i.) did not differ from that of non-infected cultures prompted us to conclude that activation of this pathway was not dependent on viral protein synthesis. Initial events in the life cycle of ALV, such as viral attachment and entry, probably trigger the PI3K/Akt pathway activation in cells. Activation of the PI3K/Akt pathway by virus attachment and entry into cells has also been described for HIV (Briand et al., 1997), enterovirus 71 (Wong et al., 2005), Ebola virus (Saeed et al., 2006), and others.
et al., 2008) and Junin virus (Linero & Scolaro, 2009). The latter authors showed that phosphorylation of Akt was induced shortly after exposure of cells to UV-inactivated Ebola virus, enterovirus 71 or Junin virus, also indicating that viral replication was not required for p-Akt induction.

Two major questions are generated by these data. One question concerns how the PI3K/Akt signalling pathway is activated by ALV. Class I PI3K are heterodimeric molecules composed of a regulatory subunit (p85 or p101) and a catalytic subunit (p110). PI3K could be activated by binding of the Src homology (SH) domain in the p85 subunit to auto-phosphorylated tyrosine kinase receptors (Skolnik et al., 1991), non-receptor tyrosine kinases (Stoyanov et al., 1995) or some viral proteins in the cytoplasm (Street et al., 2004; Shin et al., 2007b). The rapid increase in Akt phosphorylation induced by ALV suggests that activation may be mediated by receptors. Until now, the only receptors that have been reported are cNHE1 as the receptor of ALV-J, TVA receptor of ALV-A, and TVB S3 receptor of ALV-B (Young, 1998; Chai & Bates, 2006). NHE1 was found to function as a scaffold to assemble signalling complexes, to which the long cytoplasmic tail could bind, phosphorylate and activate different families of signalling molecules (Orlowski & Grinstein, 2004; Putney & Barber, 2004). For example, in renal tubular epithelial cells, NHE1-cytosolic-tail domain binding with PIP2 (phosphatidylinositol 4,5-biphosphate) and ERM (ezrin/ radixin/moesin) proteins formats a signalling complex and culminates in PI3K/Akt activation to regulate cell survival (Schelling & Abu Jawdeh, 2008). During our studies, the NHE1 inhibitor HMA was found to interfere with ALV-J infection. Integration of the proviral genome into host-cell DNA and viral RNA synthesis of NX0101 were decreased in a dose-dependent way upon incubation with HMA (0.5–20 μM). NX0101-induced Akt activation was diminished even by low concentrations of HMA, while HMA had no effect on Akt activity stimulated by CD08 or GD08 even at higher concentrations. It is interesting to speculate that binding and interaction with ALV-J NX0101 and its specific receptor on the cell surface activates the PI3K/Akt signalling pathway. Briand et al. (1997) showed that binding of HIV-1 to its receptor, the CD4 glycoprotein, induced PI3K phosphorylation in a T-cell line. The result

Table 1. Activity of luciferase-containing pseudotyped virus and in the entry assay

<table>
<thead>
<tr>
<th>Plasmid transfected</th>
<th>Luciferase activity (counts per 40 μl of virus)</th>
<th>Virus titre (S/P ratio)</th>
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<tr>
<td>Plasmid</td>
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<td>pNX0101</td>
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Fig. 5. PI3K is required early in ALV infection. DF-1 cells were treated with 10 μM LY294002 or DMSO at the indicated times during the infection of NX0101 (a), GD08 (b) or CD08 (c). At 24 h p.i., viral production was monitored by real-time RT-PCR with the primers designed for the envelope gene. Data are representative of the results of three independent experiments, both performed in triplicate. *P<0.05, **P<0.001. Error bars indicate SEM.
of immunoprecipitation indicated that CD4 complexed with the PI3K subunit p85z after HIV-1 infection.

Endocytic uptake is an early event of certain viruses’ infection. Endocytosis and low pH are essential for successful ALV-A and ALV-B entry (Mothes et al., 2000; Diaz-Griffere et al., 2002; Narayan et al., 2003). Cellular uptake of ALV-B is mediated with clathrin, as clathrin depletion significantly reduces ALV-B entry and chlorpromazine blocks uptake of the ALV-B envelope (Diaz-Griffere et al., 2005). PI3K plays an important role in regulation of early endosome fusion via activation of Rab5, a known regulator of early endosome fusion (Li et al., 1995). Activation of the PI3K/Akt pathway by ALV-A and ALV-B may be related to the endocytic process. Treatment of DF-1 cells with the inhibitor of clathrin-mediated endocytosis, chlorpromazine, abolished Akt phosphorylation induced by CD08 and GD08 infection, while treatment with dansylcadaverine (an inhibitor of receptor-mediated endocytosis) did not affect ALV-induced Akt activation. The results support the hypothesis that clathrin-mediated endocytosis is responsible for Akt activation induced by CD08 and GD08. The effect observed was in accordance with the blockade of Akt phosphorylation observed in some reports of other systems. For example, endocytosis inhibitors abolished Akt phosphorylation induced by West Nile virus (Chu et al., 2006), rhinovirus (Lau et al., 2008), Ebola virus (Saeed et al., 2008) or Junin virus (Linero & Scolaro, 2009), also suggesting that viral endocytosis may be responsible for the activation of PI3K/Akt.

The second question is about the function of the activated PI3K/Akt pathway during ALV infection. The PI3K/Akt signal pathway is known to modulate cell growth, survival and differentiation (Franke et al., 1997). There is mounting evidence that regulation of the PI3K/Akt pathway is important for virus entry (Saeed et al., 2008; Tiwari & Shukla, 2010), subsequent intracellular trafficking (Fujioke et al., 2011) and viral replication (Ehrhardt et al., 2006; Shin et al., 2007a), as PI3K or Akt inhibitors disrupt normal uptake of virus or block virus replication. Inhibition of PI3K could also result in the reduction of viral replication of ALV subgroups J, A and B in DF-1 cells, which was probably due to the blockage of viral entry since the PI3K inhibitor (LY294002) significantly reduced viral entry of NX0101 in a dose-dependent manner in DF-1 cells using the pseudotyped virus-based assay. The findings support the suggestion that the PI3K/Akt pathway is at least essential for the entry of exogenous ALV. Saeed et al. (2008) also showed that the PI3K/Akt pathway was critical for entry of Ebola virus, as both LY294002 and an Akt inhibitor significantly reduced the entry of pseudotyped virus containing luciferase at post-binding steps and a deletion assay confirmed that both p85z and p85/z were involved in viral entry. Tiwari & Shukla (2010) found that LY294002, as well as expression of a dominant-negative PI3K mutant, negatively affected HSV-1 entry.

In conclusion, we have demonstrated that infection of ALV subgroup J, A or B strains activate PI3K/Akt early in infection in a manner independent of viral replication. The PI3K/Akt pathway is essential for the entry of ALV. ALV-J induces Akt activation through virus–receptor interaction, while ALV-A and ALV-B activated Akt are related to clathrin-mediated endocytosis. The precise molecular mechanisms of ALV activation of the PI3K/Akt pathway are the focus of continuing studies by our laboratory.

**METHODS**

**Virus propagation.** The chicken embryo fibroblast (CEF) cell lines DF-1 were propagated in growth medium containing Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) with 10 % FCS (Gibco) at 37 °C and 5 % CO₂ until they reached approximately 80 % confluence. ALV-J strain NX0101 (Du et al., 2000), ALV-A strain GD08 and ALV-B strain CD08 (Zhang et al., 2009, 2010) were each proliferated in DF-1 cells, respectively. Briefly, ALV was added to DF-1 cells and incubated for 2 h at 37 °C and 5 % CO₂. After the inoculum was removed, maintenance medium containing DMEM with 2 % FCS was added and incubated at 37 °C and 5 % CO₂ for 6–7 days. Cell culture supernatants were collected at certain times. The collected supernatants were aliquoted and stored as viral stocks at −80 °C until use, or filtered through a 0.45 μm pore-size cellulose acetate filter. The filtrate was then either used directly, or was pelleted by 1 h of centrifugation at 16 000 g and the pellet was used after suspension in DMEM.

Viral spread was monitored by assaying culture supernatants for ALV capsid protein p27 by ELISA following the manufacturer’s instruction (IDEXX). The virus titres of ALV-A and ALV-B were determined by calculating the sample to positive (S/P) ratio. The virus titres of ALV-J were determined by serially diluting viral supernatants in DMEM and applying onto DF-1 cells in a 96-well plate according to the method described by Reed & Muench (1938), and expressed as TCID₅₀ per 0.2 ml. The infected cells were monitored by indirect immunofluorescence assay using mouse mAb against gp85 of ALV-J (Qin et al., 2001) as the first antibody, and DyLight 488-conjugated...
anti-mouse IgG (Jackson Immuno-Research) as the second antibody. Images were obtained on a fluorescent microscope (Leica).

**Plasmid construction.** According to the method of rapid and quantitative measurement of retrovirus entry as described previously (Kolokoltsov & Davey, 2004), the envelope-luciferase (env-luc) fusion vector was made by modifying the 3’ end of NX0101 envelope gene (GenBank accession no. DQ115805) to replace the native stop codon with an EcoRI restriction endonuclease site. This was achieved by using PCR. The primers used were 5’-CCGCGTAATCCATGGGCGAGCCCTCTTGGACGAGG-3’ and 5’-CCGGAATTCTCAGCTGCT-CCCTAACCTTGAT-3’. The firefly luciferase gene was modified to gain an EcoRI site at its 5’ end. The primers used were 5’-GATGCAATTGGAAGGCGCAAAAATATGAAAG-3’ and 5’-GATTGCGGCCGCTTACACGGCGATCTTTCCGCCCTT-3’. The latter primer also gave tandem stop codons followed by a TGCGGCCGCTTACACGGCGATCTTTCCGCCCTT-3’.

**Production of ALV-J containing env-luc fusion protein.** DF-1 cells were grown to 90% confluence on a 12-well plate in DMEM containing 10% FBS. To make virus containing the env-luc fusion protein, the cells were cotransfected with 1.6 µg plasmid pNX0101 (encoding the full genome of NX0101 in pMD18-T vector) and 1 µg pEnvLuc by using Lipofectamine 2000 (Invitrogen). After overnight incubation, the medium was replaced with fresh medium and incubated for a total of 36 h. At this time, the virus titre peaked and the supernatants were collected and filtered through a 0.45 µm-pore-size cellulose acetate filter. The filtrate was then either used directly, or was pelleted by 1 h of centrifugation at 16 000 g and the pellet was used.

**Virus infection.** Primary CEFs were prepared from 9 to 11-day-old embryos of specific-pathogen-free chicken eggs as described previously (Maas et al., 2006). CEF or DF-1 cells were mock infected or infected by incubation with diluted virus stock, respectively. Briefly, cells were seeded in triplicate wells in 10% FBS DMEM and allowed to attach in growth media at 37 °C and 5% CO2 overnight until they reached approximately 90% confluence. Virus stocks diluted in DMEM were added to CEF or DF-1 cells. Cells were incubated in virus-containing media at 37 °C and 5% CO2 for certain times.

**Time-course and concentration of inhibitors treatment.** The P13K-specific inhibitors LY294002 and wortmannin were purchased from Calbiochem. Chlorpromazine, dansylcadaverine and HMA were obtained from Sigma. Treatment of cells with LY294002 (1–50 μmol l−1), wortmannin (0.06–1 µM), chlorpromazine (5–50 µM), dansylcadaverine (10–200 µM), HMA (0.5–50 µM) or solvent DMSO (0.4 %, v/v) was performed, respectively. Chemicals were added to the DF-1 cells for 30 min or 1 h and then virus stocks infected as described above. At 24 h p.i., the cell lysates were examined for the expression level of ALV by real-time RT-PCR as described below. For time-course research, at −1, 0, 1, 2 or 4 h p.i., LY294002 was added to achieve a final 10 µM concentration. The infection was terminated at 24 h p.i. by collecting the monolayers for viral transcription analysis with real-time RT-PCR.

**Viral entry and luciferase assay.** DF-1 cells were incubated in a 24-well plate for 1 h with env-luc-containing virus. After washing cells twice with PBS, cell lysis and the measurement of reporter luciferase activity were performed by applying the luciferase assay system (Promega). Luciferase activity was measured using a Tecan Infinite M1000 luminometer (Tecan). While the signal was reduced proportionally, it remained at least 10-fold above the background of the detector.

Various concentrations of LY294002 or DMSO were incubated with DF-1 cells for 1 h before and during incubation with virus. Cells were incubated with env-luc-containing virus for 1 h, and luciferase activity was then measured as above.

**Western blot analysis.** At the indicated times, cell monolayers were washed with PBS and lysed. The lysates were collected and incubated on ice for 10 min. Lysates were cleared by centrifugation at 10 000 g for 5 min at 4 °C. The supernatants were analysed for total protein content with a BCA protein assay kit (Beyotime). Total protein (20 µg) was resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes (Roche). Membranes were blocked with 5% skimmed milk for 1 h at 37 °C, and then incubated overnight at 4 °C with specific rabbit anti-phospho-Akt (Ser473) antibody, rabbit anti-Akt antibody, rabbit anti-glyceroldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology) and mouse mAb against gp85 of ALV-J [diluted in PBS Tween 20 (PBST), 1:1000], respectively. After three rinses in PBST buffer, the membranes were incubated at 37 °C for 1 h with IRDye 700DX-conjugated anti-rabbit IgG or IRDye 800-conjugated anti-mouse IgG (1 : 8000; Rockland Immunochemicals) diluted in PBST as secondary antibody. Membranes were washed three times in PBST, then visualized and analysed with an Odyssey infrared imaging system (LI-COR Biosciences).

**RNA analysis.** At certain times p.i., after removing the supernatants, total RNA of cells was extracted with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. After treatment with DNase I (Fermentas), 1 µg total RNA was reverse transcribed using the RevertAid First strand cDNA synthesis kit (Fermentas). The cDNA generated was then used for real-time PCR amplification.

Real-time quantitative PCRs were performed on an ABI 7500 Real-time PCR System (Applied Biosystems) using Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen) according to the manufacturer’s specification. All the PCRs were carried out in 96-well plates in a final volume of 25 µl. In addition, each reaction consisted of 0.25 mM of each gene-specific primer (see Supplementary Table S1, available in JGV Online) and 2 µl 1:10 dilution of cDNA and PCR grade water. All reactions were performed in triplicate. At last, real-time quantitative PCR analysis was carried out using the 2−ΔΔCt method (Livak & Schmittgen, 2001).

**Statistical analysis.** Results were presented as means ± SEM (n = 3). Statistical comparisons were made by using Student’s t-test, and statistical significance was represented by P values of <0.05 or 0.001.

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