Differential regulation of antiviral and proinflammatory cytokines and suppression of Fas-mediated apoptosis by NS1 of H9N2 avian influenza virus in chicken macrophages

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The NS1 protein is known to suppress immune responses in influenza virus-infected hosts. However, the role of NS1 in apoptosis in infected cells is disputed. In this study, through the use of a mutant A/​pheasant/California/2373/1998 (H9N2) avian influenza virus (AIV) with a truncated NS1, we have demonstrated that a functional NS1 protein suppresses the induction of interferons in chicken macrophages. However, NS1 appeared to be irrelevant to the regulation of cytokines interleukin (IL)-1β and IL-6, indicating that distinct mechanisms may be employed in the regulation of antiviral and proinflammatory cytokines in chicken immune cells. Our study also showed that this H9N2 AIV induced apoptosis extrinsically through the Fas/Fas ligand (FasL)-mediated pathway. We found that NS1 suppressed the apoptotic process through suppression of the induction of FasL, but not tumour necrosis factor-α or TNF-related apoptosis-inducing ligand. Furthermore, our data indicated that the disruption of a potential binding site for the p85β subunit of phosphoinositide 3-kinase in the carboxyl terminus of NS1, while having no effect on the regulation of IFN induction, may contribute to the suppression of Fas/FasL-mediated apoptosis. Therefore, suppression of Fas/FasL-mediated apoptosis by NS1 is one of the critical mechanisms necessary to increase infectivity in AIV-infected chicken macrophages.

INTRODUCTION

Avian influenza is one of the major causes of morbidity in poultry and poses a potential threat for human infection (Perdue & Swayne, 2005; Perez et al., 2005). The molecular mechanisms of the immune responses that eliminate avian influenza virus and allow it to survive following infection in birds remain poorly understood. Both innate and adaptive immunity, leading to the induction of specific antibodies and cell-mediated immune responses, are presumably essential in viral clearance. Antiviral cytokines such as interferons (IFNs) are considered critical in host defence.

Apoptosis is a strictly programmed cellular process which can be triggered by viral infection as well as many other factors. Both intrinsic and extrinsic pathways can be involved in triggering and progression events depending on the infecting virus and the host cell type. The tumour necrosis factor (TNF) members, including the Fas ligand (FasL), TNF-α and TNF-related apoptosis-inducing ligand (TRAIL), and their receptors play key roles in the extrinsic apoptotic pathway in a variety of cell types. Influenza viruses induce apoptosis in the cells of various mammalian species (Fesq et al., 1994; Hinshaw et al., 1994; Hofmann et al., 1997; Morris et al., 1999, 2005; Takizawa et al., 1993) and in chicken cells (Ito et al., 2002). Both intrinsic and
extrinsic pathways can be involved in apoptosis, which presumably promotes infection and alters viral pathogenesis in the host (Brydon et al., 2005; Brydon et al., 2003; Hofmann et al., 1997; Lowy, 2003; Uiprasertkul et al., 2007).

To date, influenza virus double-strand RNA (dsRNA) (Takizawa et al., 1996) and a number of viral proteins, such as PB1-F2 (Chanturiya et al., 2004; Chen et al., 2001), NA (Morris et al., 1999, 2002) and NS1 (Lam et al., 2008; Schultz-Cherry et al., 2001; Zhirkov et al., 2002), have been implicated in apoptotic regulation in cells. Among these, influenza virus dsRNA was shown to activate the dsRNA-activated protein kinase (PKR) which can result in the upregulation of FasL. NA can activate transforming growth factor-β. PB1-F2 targets the mitochondrial membrane resulting in the release of cytochrome c into the cytosol, thus initiating the intrinsic apoptotic pathway. NS1 is thought to be the master modulator of the host immune defence and is also capable of regulating apoptosis. The role of NS1 in apoptosis, however, appears to be complex (Lowy, 2003). While some studies have shown that NS1 can induce apoptosis to various degrees in infected cells (Lam et al., 2008; Schultz-Cherry et al., 2001), other studies have demonstrated that NS1 can suppress apoptosis (Ehrhardt et al., 2007a, b; Shin et al., 2007; Zhirkov et al., 2002). The complex roles of NS1 in the regulation of apoptosis have provoked an ongoing debate about its role in influenza-infected cells.

In this report, we focused on the infection of a low pathogenic avian influenza (LPAI) virus strain, A/peasant/California/2373/1998 (H9N2), in chicken macrophages. Studies have been carried out to characterize the regulation of both innate and adaptive immune responses in chicken macrophages and lungs by this virus (Xing et al., 2008). However, little was known about the mechanism by which apoptosis was induced and IFNs were suppressed in chicken immune cells. Using a mutant virus with a truncated NS1 generated by using the reverse genetics approach, we have tried to understand how the H9N2 NS1 protein regulates apoptosis, viral replication and antiviral cytokine expression in chicken macrophages.

METHODS

Reagents and antibodies. The HTC monocyte/macrophage cell line was obtained from Dr N.C. Rath (Agricultural Research Service, USDA, Fayetteville, USA) (Rath et al., 2003). These cells were derived from peripheral blood monocyte/macrophage cells and were grown under the conditions described previously (Rath et al., 2003). Madin–Darby canine kidney (MDCK) and human kidney 293T cell lines were purchased from ATCC and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Chicken blood was obtained from domestic pheasants during LPAI outbreaks in California (Woolcock et al., 2003); its eight genomic segments have been completely sequenced (J. Li and C. J. Cardona, unpublished). Recombinant viruses were generated with an eight-plasmid reverse genetics system (Hoffmann et al., 2001, 2002; Song et al., 2007). Briefly, viruses were rescued in 293T/MDCK co-cultures transfected with pDP2000 (Song et al., 2007; Wan & Perez, 2007) in the TnaseT-LTI reagent (Mirus), each harbouring one of the eight viral segment cDNAs. Recombinant viruses were grown in 9-day-old embryos from specific pathogen-free (SPF) hens (Charles River) and allantoic fluids (AIF) were harvested 48 h after inoculation. The viruses in AIF were titrated with a standard haemagglutination (HA) test (Hirst, 1948) and infectious viral titres were determined in MDCK cells by plaque assay (Gaush & Smith, 1968).

HTC cells (5×10^5) were plated on 10 cm tissue culture plates 16 h before infection. Cells were infected with wild-type (wt) or delNS1 viruses at an m.o.i. of 1 in serum-free RPMI 1640 containing L-[(toluene-4-sulphonamido)-2-phenyl] ethyl chloromethyl ketone (TPCK) trypsin (1 μg mL^-1) and incubated at 37°C with 5% CO2. Three culture plates were set for each time point while infection was carried out.

Real-time RT-PCR. The mRNA levels for IFN-β, IFN-β, interleukin (IL)-1β, IL-6, TNFα, FasL, TRAIL and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes in control and infected HTC cultures were analysed by a two-step real-time RT-PCR. Total RNA (1 μg), which was prepared from the cells with the RNeasy kit (Qiagen), was used for each RT reaction with the QuantiTect Reverse Transcription kit (Qiagen), following the manufacturer’s instruction. The RT reaction was carried out after the RNA preparation had been treated with DNase I at 42°C for 2 min, to eliminate genomic DNA contamination. Real-time PCR was conducted with 1 μl cDNA in a total volume of 25 μl with the iQ SYBR Green Supermix (Bio-Rad), following the manufacturer’s instructions. Relative expression values were normalized using an internal GAPDH control. The fold change of relative gene expression levels was calculated following the formula: 2^[(ΔΔCt of gene)−(ΔΔCt of GAPDH)]. Melting curves were analysed to determine the specificity of each reaction. All reactions were conducted in duplicate for each sample and the mean was calculated for each gene target. The sequences for the primers used for real-time RT-PCR were chosen using a web-based software Primer3 (http://frodo.wi.mit.edu/primer3/input.htm; v.0.4.0) and have been described previously (Xing et al., 2008).

Immunofluorescence staining and TUNEL assay. HTC cells were grown on four-chamber slides, fixed and permeabilized with 2% paraformaldehyde and 0.5% Triton X-100, followed by incubation at 4°C for 30 min with FITC-labelled anti-NP antibody (ViroStat) diluted at 1:20 in PBS. The cells were washed three times, 10 min each, with PBS, after which they were air-dried; 5% propyl gallate (Sigma) in 70% glycerol was used to wet the cells, which were then applied to a slide, covered with a coverslip and viewed with a Nikon Eclipse E400 fluorescence microscope. The TUNEL staining was performed on the pretreated cells with FITC-conjugated dUTP labelling using an in situ cell death detection kit (Roche Diagnostics).

Flow cytometric analysis of apoptosis. HTC cells infected with wt or delNS1 viruses were trypsinized and washed three times with PBS containing 0.5% BSA. An aliquot of cells (1×10^7) was stained with FITC-conjugated annexin V and propidium iodide (PI) (BD Pharmingen) in a volume of 100 μl on ice for 30 min. The cells

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were washed three times with PBS + 0.5 % BSA, followed by flow cytometric analysis on a FACScan cell sorter (Becton Dickinson). A standard 25,000 counts was performed for each assay.

**Western blot analysis.** Protein lysates were prepared by lysing uninfected and infected HTC cells in a hypotonic buffer as described previously (Sternsdorf et al., 1997; Xing et al., 2004). Cytosolic and nuclear fractions were prepared and separated by using 15 % SDS-PAGE. Proteins were transferred to an Immun-Blot PVDF membrane (Bio-Rad) and Western blot analysis was performed according to a standard protocol using goat anti-influenza A NS1 and anti-influenza A M1 antibodies (1:500), followed by an AP-conjugated rabbit anti-goat IgG antibody. BCIP/NBT reagents (Sigma) were used to develop colorimetric signals on the membrane. The membrane was also blotted with a monoclonal anti-actin antibody (Santa Cruz) as an input control.

**RESULTS**

**Susceptibility of NS1-deficient H9N2 virus in chicken macrophages**

The viruses used were generated with an eight-plasmid reverse genetics system (Hoffmann et al., 2001, 2002). Wt and mutant viruses were rescued in 293T/MDCK co-cultures transfected with pDP2000 plasmids harbouring eight viral segment cDNAs cloned from the H9N2 virus (A/Ph/California/2373/1998). The delNS1 virus was rescued in cultures co-transfected with the NS cDNA which was truncated by the introduction of a stop codon at position 180 through site-directed mutagenesis. Thus, delNS1 is a mutant virus with a truncated NS1 protein (contains only the N-terminal 60 aa). Rescued viruses were grown by inoculating 9-day-old SPF embryonated eggs and the resultant VAF was titrated on MDCK cells with a standard plaque assay. The plaques of the wt were significantly larger in size than those of delNS1 viruses on MDCK cells (data not shown).

The chicken monocyte/macrophage cell line HTC (Rath et al., 2003; Xing et al., 2008) was susceptible to both wt and delNS1 H9N2 virus infections; cytopathic effect (CPE) was observed 12 h post-infection (p.i.) at an m.o.i. of 1 (Fig. 1a). Full-length NS1 was evident from Western blot analysis of wt virus-infected cells but not of delNS1-infected HTC cells (Fig. 1b), while viral matrix was detected in both wt- and delNS1-infected cultures. A truncated NS1 fragment of 6.5 kDa (N-terminal 60 aa) was not detected, probably due to the reactivity of the antibody used in the Western blot.

**NS1 significantly affected viral replication in chicken macrophages**

While marked differences in viral replication between wt and delNS1 viruses were not observed in 9-day-old chicken embryos (Fig. 1c), the delNS1 H9N2 virus replicated at a much lower efficiency than the wt in HTC cultures (Fig. 1d). In the HTC cultures infected with the viruses at an m.o.i of 1 in serum-free RPMI 1640 (with 1 μg TPCK-trypsin ml⁻¹), the wt infectious viruses were initially detected at 8 h p.i. in the culture media and peaked at 2.5 × 10⁵ p.f.u. ml⁻¹ 24 h p.i. delNS1 appeared later and the peak titre was between 6 × 10² and 1 × 10³ p.f.u. ml⁻¹ at the same time point (Fig. 1d). The difference demonstrates the critical role for NS1 in viral infectivity in chicken macrophages.

**Fig. 1.** Replication of wt and delNS1 H9N2 viruses in chicken macrophages. (a) Morphological changes (×200 magnification) of HTC cultures infected with wt and delNS1 viruses at 18 h p.i. ctl, Control. (b) Western blot analysis of full-length NS1 expression in wt- and delNS1-infected, and uninfected (uninf.) cells. Cell lysates prepared as either cytosolic (C) or nucleic (N) fractions from both wt- and delNS1-infected cells were fractionated by 15 % SDS-PAGE and proteins were transferred to a PVDF membrane. An anti-influenza A NS1 antibody was used to probe the membrane. The membrane was also probed with anti-influenza M1 and anti-actin antibodies. (c) Replication of wt and delNS1 viruses in 9-day-old chicken embryos. Titration of the viruses in allantoic fluid from inoculated eggs was performed in MDCK cells with a standard plaque assay. (d) Replication of wt and delNS1 viruses in chicken macrophages. The viruses were harvested in culture supernatants at various time points and titrated in MDCK cells.
Antiviral cytokines were suppressed by the NS1 protein

We have previously reported that antiviral and pro-inflammatory cytokines are regulated in H9N2-infected HTC cultures (Xing et al., 2008). Interestingly, antiviral cytokines, in particular IFNs, are only slightly upregulated (Xing et al., 2008). We hypothesized that, even though the infection itself induced the production of cytokines, many cytokines, including IFNs, were ultimately suppressed in influenza-infected chicken macrophages, mediated by NS1. In this study, total RNA was prepared at various time points p.i. from HTC cultures infected with wt and delNS1 viruses, from which cDNA was synthesized and the transcription levels of various genes were determined. The results showed that IFN-β was upregulated less than twofold at 10 h and increased up to 8.2-fold at 24 h p.i. in wt-infected cells. However, IFN-β expression was upregulated to eightfold at 10 h and increased up to 21.5-fold at 24 h p.i. in delNS1-infected macrophages (Fig. 2a). Likewise, IFN-γ was almost at baseline levels at 10 h and increased to 6.8-fold at 24 h p.i. in wt-infected cells. But, the IFN-γ level was upregulated to 11-fold at 10 h and increased up to 16.3-fold at 24 h p.i. in delNS1-infected cells (Fig. 2a). These results demonstrate that NS1 produced by H9N2 suppressed the induction of IFNs in chicken macrophages.

We examined the expression of other cytokines as well and interestingly, we found that NS1 seemed not to have a role in the regulation of a few pro-inflammatory cytokines. For example, IL-1β was upregulated up to 6.2-fold at 6 h p.i in the wt infection; upregulation was also observed at similar levels in delNS1 infection (Fig. 2b). In both wt virus and delNS1 infections, the expression of IL-6 was initially downregulated by twofold and returned to the basal level in a similar pattern (Fig. 2b). We also observed that the expression of TNF-α changed only within two- to threefold between wt and delNS1 infections (Fig. 3e). Taken together, these results indicate that, while IFNs are strongly regulated by NS1, pro-inflammatory cytokines in chicken macrophages are likely regulated through a different mechanism.

Inhibition of apoptosis in H9N2-infected chicken macrophages by NS1 protein

A TUNEL assay was performed on HTC cultures that were uninfected or infected with wt or delNS1 viruses at an m.o.i. of 1 after the cells had been fixed with 2% paraformaldehyde for 18 h p.i. and permeabilized with 0.5% Triton X-100. The results indicate that a large proportion of the cells in both wt- and delNS1-infected HTC cultures were apoptotic (Fig. 3a). At 18 h p.i., there was more annexin V+ PI+ staining, which indicates dead cells, in delNS1-infected macrophages (14.3%) than in wt-infected cells (6.53%) (Fig. 3b). Moreover, there were 35.9% cells with annexin V+ staining alone (as a marker of early apoptosis) in delNS1-infected cells, while only 14.8% of wt-infected cells were annexin V+. We further quantified the data from the FACS analysis. The difference in apoptotic cells between wt- and delNS1-infected cultures at 18 h p.i. was significant (P<0.05, n=3) (Fig. 3c).

Cell lysates were prepared from wt- and delNS1-infected HTC cells and separated by using 15% SDS-PAGE. Proteins were transferred to a PVDF membrane by Western blot and probed with an anti-cleaved caspase 3 antibody. Cleaved 19 and 17 kDa peptides of active caspase 3 were evident in both wt- and delNS1-infected HTC cells, but not in uninfected cells (Fig. 3d). It appeared that more cleaved caspase 3 was generated earlier (12 and 18 h p.i.) in delNS1-infected cells than in wt-infected cells (Fig. 3d).
Taken together with the fact that virus titres in delNS1-infected cells were much lower than those in wt-infected cells (Fig. 1d), apoptosis induced in delNS1-infected cells was significantly more robust than in wt-infected cells. Thus, apoptosis in wt H9N2-infected chicken macrophages appeared to be suppressed by NS1.

**NS1 suppressed the Fas/FasL-mediated apoptotic pathway in macrophages**

To understand the mechanism of apoptosis induced in H9N2-infected chicken macrophages, we examined the expression of FasL, TRAIL and TNF-α in wt- and delNS1-infected cells with real-time RT-PCR. The results showed that there were no significant changes in TNF-α expression at 10 h p.i. in either wt- or delNS1-infected cells. A moderate upregulation of TNF-α was observed at 24 h p.i. in wt and delNS1-infected cells (1.8- and 2.6-fold, respectively) (Fig. 3e). Interestingly, a downregulation of TRAIL expression in wt- and delNS1-infected cells was observed 10 h p.i. (4.2- and 5.1-fold, respectively) and the downregulation increased up to 10.3-fold in wt-infected cells and 26.8-fold in delNS1-infected cells at 24 h (Fig. 3e). Based on these data, we reasoned that neither TNF-α nor TRAIL was involved in H9N2-induced apoptosis in HTC cultures. On the other hand, FasL was upregulated by 25.1-fold at 10 h and 62.5-fold at 24 h p.i. in wt-infected cells. This suggests that, Fas/FasL-mediated apoptosis may play a critical role in H9N2-induced apoptosis in HTC cells. Moreover, FasL was upregulated by 52.6-fold at 10 h and 173.2-fold at 24 h p.i. in delNS1-infected cells (Fig. 3e), indicating that the truncation of NS1 increased the expression of FasL and sensitized the cell to apoptosis. Based on these data we concluded that NS1 suppressed the expression of FasL and reduced H9N2 virus-induced apoptosis in chicken macrophages.

To understand further how NS1 suppresses apoptosis, we generated an NS1 mutant virus which had a point mutation (from P164 to L164) in a potential binding site for the p85β regulatory subunit of PI3K in the carboxyl terminus of NS1, which is conserved among influenza A virus NS1 (Fig. 4a). The mutant virus is designated NS(P164L). This substitution did not affect the stability of NS1, as full-length NS1 protein was detected in HTC cells infected with NS(P164L) in a Western blot using the anti-influenza A NS1 antibody (Fig. 4b). As reported previously (Shin et al., 2007), this is a putative SH3-binding motif (164PXXP) which interacted with the p85β subunit of PI3K and played an important role in providing...
cell survival signal. It was found later that the SH3-domain binding motif in NS1 is a different one located at 212PXXP (Heikkinen et al., 2008). Therefore, P164 is probably involved in the interaction of NS1 and p85β through the inter-SH2 linker region (Hale et al., 2008), leading to the activation of PI3K. We used FITC-annexin V staining in HTC cultures and found that, regardless of the exact binding site on p85β, a higher percentage of the cells infected with the NS(P164L) virus were apoptotic (15.3%) compared with the cells infected with wt virus (8.2%) at 16 h p.i. (data not shown). Also at 16 h p.i., the expression of FasL was increased to up to 115-fold in NS(P164L)-infected cells compared with wt-infected cells, in which it was increased up to 48-fold compared with baseline levels (Fig. 4c).

To examine whether this point mutation affected the infectivity of the mutant virus, we infected HTC cells with wt and NS(P164L) viruses at an m.o.i. of 1 and the infectious viruses in the medium were harvested at different times p.i. Our data showed that infectious virus titres were between 4.2 × 10^4 and 5.1 × 10^4 p.f.u. ml^-1 from 18 to 24 h p.i. in NS(P164L)-infected cells, while the titres were between 5.4 × 10^5 and 5.6 × 10^5 p.f.u. ml^-1 in wt-infected cells at the same time points (Fig. 5a). We noted that there was no obvious disregulation of IFN induction in NS(P164L)-infected cells (Fig. 5b). Taken together, it appears that disruption of this potential p85β binding site sensitized the cells to Fas/FasL-mediated apoptosis and decreased viral replication efficiency in chicken macrophages.

DISCUSSION

The H9N2 NS1 protein is a potent modulator of the host immune responses in chicken macrophages (Xing et al., 2008). We have previously demonstrated that a variety of immune-related genes, such as MHC class I and II antigens and IL-4, as well as multiple pro-inflammatory cytokines and chemokines and antiviral IFNs, either are slightly induced or remain unchanged in this cell type in response to infection. It was found that the antiviral cytokines in influenza-infected cells were suppressed and previous data have demonstrated that NS1 played a critical role in this suppression (Garcia-Sastre et al., 1998; Geiss et al., 2002; Talon et al., 2000; Wang et al., 2000). Our data indicate that this is also the case with the suppression of IFN-β and -γ in H9N2-infected chicken macrophages. However, in these studies, NS1 appeared to be irrelevant to the regulation of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, indicating that distinct mechanisms may

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**Fig. 4.** Effect on FasL transcription by disruption of NS1 binding to PI3K p85β in NS(P164L) mutant virus-infected HTC cultures. (a) Sequence alignment of a conserved p85β binding motif in the carboxyl terminus of influenza NS1 genes. The altered sequence of the NS(P164L) mutant virus, with a substitution in NS1, is shown. The viruses listed are as follows: A/Puerto Rico/8/1934 (H1N1); A/mallard/Alberta/205/1998 (H2N3); A/Florida/UR06-0597/2007 (H3N2); A/duck/Shiga/8/2004 (H4N6); A/chicken/Vietnam/20/2003 (H5N1); A/chicken/California/1973/2001 (H6N2); A/mallard/Delaware/22/2006 (H7N3); A/mallard/Alaska/708/2005 (H8N4); A/chicken/California/2373/1998 (H9N2). (b) Western blot analysis of NS1 protein expression in the HTC cells infected with wt and NS(P164L) viruses. Cell lysates were prepared from the cytosolic (C) and nucleic (N) fraction of uninfected cells (1) and cells infected with wt (2) and NS(P164L) (3) viruses. (c) Real-time RT-PCR analysis and fold changes in transcription levels of the FasL gene at 8 and 16 h p.i. Fold changes were calculated based on the difference in Ct values, which were normalized against GAPDH. Reactions were conducted in duplicate for each sample. Each reaction was repeated at least three times; bars, so.
be employed in the antiviral and proinflammatory cytokine regulation in infected chicken immune cells.

An induction of apoptosis by influenza viruses of various subtypes and strains has been observed in cultured cells (Fesq et al., 1994; Hinshaw et al., 1994; Hofmann et al., 1997; Takizawa et al., 1993) and in vivo (Mori et al., 1995). Although thought to impact viral replication and pathogenesis, the precise mechanisms and pathways of the observed phenomena are not fully understood. The process may be virus- and cell-type-specific. In this study, we have shown that the Fas/FasL-mediated apoptotic pathway may be the main extrinsic pathway in H9N2-induced apoptosis in chicken macrophages, while TNF-α and TRAIL pathways may be irrelevant. Although it does not clarify the existing contradictory data on NS1 regulation of apoptosis, our data suggest that H9N2 NS1 suppressed apoptosis through decreased induction of FasL in chicken macrophages.

Recent studies indicated that infection with either A/Puerto Rico/8/1934 (H1N1) (hereafter called H1N1) or the highly pathogenic A/Hong Kong/483/97 (hereafter H5N1/97) could induce apoptosis in human primary macrophages (Mok et al., 2007) and that in H5N1/97-infected macrophages, virus-induced apoptosis had a delayed onset compared with H1N1-infected cells. This delay in onset of apoptosis may promote a longer survival of the virus in cells and may contribute to the pathogenesis of highly pathogenic H5N1 infection (Lam et al., 2008; Mok et al., 2007). However, the role that NS1 plays in the induction of apoptosis remains controversial. Overexpression of NS1 induced apoptosis in MDCK and HeLa cells (Schultz-Cherry et al., 2001). Likewise, overexpression of NS1 of H5N1/97 virus can trigger apoptosis in airway epithelial cells (Lam et al., 2008). Evidence has accumulated, suggesting that the NS1 of the H1N1 virus downregulated apoptosis in MDCK, chicken fibroblasts (Zhirnov et al., 2002) and human macrophages (Stasakova et al., 2005) and activated the cellular PI3 kinase/Akt signalling pathway (Ehrhardt et al., 2007a, b; Hale et al., 2006, 2008; Heikkinen et al., 2008; Shin et al., 2007), which mediates anti-apoptotic responses and promotes cell survival. Additional studies will be needed to reconcile the contradictory data on NS1 regulation of apoptosis. It is noted that when apoptosis has been induced, NS1 has been overexpressed in cultured cells (Lam et al., 2008; Schultz-Cherry et al., 2001). In contrast, the suppression of apoptosis by NS1 has been observed in those studies in which NS1-deficient or truncated mutant viruses were used, including the H9N2 virus used in this study.

PI3K is a heterodimer of a p110 catalytic subunit and a p85β (or α) regulatory subunit, and the activation of PI3K by NS1 is through its binding to the regulatory p85β subunit (Hale et al., 2006; Heikkinen et al., 2008; Shin et al., 2007). There is one SH3 domain and two SH2 domains in p85β. It is thought that an interaction between the SH3 domain of p85β and a proline-rich putative SH3 domain binding motif in NS1 activates PI3K (Heikkinen et al., 2008; Shin et al., 2007). Two putative SH3 binding motifs in the carboxyl terminus of NS1, 164PXXP and 212PXXP, have been identified and the former has been found to bind to the SH3 domain (Shin et al., 2007). However, Heikkinen et al. (2008) recently determined that it was the latter that was bound to the SH3 domain. On the other hand, Hale et al. (2008) demonstrated that the interaction between p85β and NS1 is mediated only through the inter-SH2 domain of p85β and the carboxyl terminus of NS1 (Hale et al., 2008), within which both 164PXXP and 212PXXP fall. This means that the interaction between p85β and NS1 is independent of SH3 domain binding. At this stage, it is clear that whatever the trigger, the interaction between NS1 and PI3K, which is subsequently activated through the phosphorylation of the p85β regulatory subunit, provides the survival signal and delays the onset of apoptosis.

The NS1-mediated suppression of FasL mRNA expression and apoptosis in H9N2-infected chicken macrophages could mechanistically occur through the PI3K/Akt signaling pathway. We speculate that in chicken macrophages, functional NS1 activates PI3K and subsequently activates Akt, which phosphorylates the Forkhead family of transcription factors such as FKHL1, which are then retained in the cytoplasm and not translocated to the nucleus to activate Fas/FasL transcription, as has been demonstrated in mammalian cells (Brunet et al., 1999; Ciechomska et al., 2003). Active PI3K/Akt can also inhibit apoptosis by stimulating the expression of Bcl-2 members (del Peso et al., 1997; Datta et al., 1997) and inhibiting cytochrome c release and caspase 9 activation (Cardone et al., 1998). In contrast, the suppression of PI3K/Akt activation while functional NS1 is absent will deplo-

**Fig. 5.** Effect on virus replication by disruption of NS1 binding to PI3K p85β in NS(P164L) mutant virus-infected HTC cultures. (a) Replication of wt and NS(P164L) viruses in HTC cultures. The culture supernatants were sampled at 6, 12, 18 and 24 h.p.i. and virus titration was performed by plaque assay in MDCK cells, in triplicate. (b) Total RNA was prepared from HTC cells infected with virus titration was performed by plaque assay in MDCK cells, in culture supernatants were sampled at 6, 12, 18 and 24 h.p.i. and replication of wt and NS(P164L) viruses in HTC cultures. The PI3K p85β (Fesq domain) induced apoptosis in MDCK and HeLa cells (Schultz-Cherry et al., 2001).
sphorylate FKHRL1 and promote its translocation into the nucleus and subsequently activate the transcription of Fas/FasL. It is important to note that FasL mRNA was expressed at a modest level even in wt H9N2-infected chicken macrophages. This is probably due to the activation of the NFκB signalling pathway, which has been seen in influenza-infected cells (Flory et al., 2000; Pahl & Baeuerle, 1995; Ronni et al., 1997), leading to the activation of pro-apoptotic components including FasL and TRAIL (Wurzer et al., 2004).

In addition to the role of the PI3K p85β-binding sites, we believe that the whole NS1 protein inhibits the apoptotic process as well and additional mechanisms may be involved. Takizawa and colleagues used a synthetic dsRNA to demonstrate that PKR was involved in FasL upregulation in influenza-infected cells (Takizawa, 1996; Takizawa et al., 1996). It is widely accepted that influenza NS1 binds PKR (Hatada et al., 1999; Li et al., 2006; Lu et al., 1995; Min et al., 2007; Wang & Krug, 1996). The whole NS1 protein, therefore, may suppress FasL expression through binding and blocking PKR. It should be considered that the blockage of PKR is also critical for the induction of PKR-mediated IFN expression in the host cell (Bergmann et al., 2000, 2001).

In this study, we observed that cells were sensitized to apoptosis in the absence of full-length NS1, even though virus replication was significantly impeded. This observation suggests the importance of the timing of cell death during influenza replication; if activated too early in the influenza life cycle, there is not enough time for replication and proper assembly of virus progeny. It may be useful to consider this process as an avenue for the development of a novel class of anti-influenza virus therapeutics, such as the inhibitors of PI3K, through which infected cells are exposed to additional stimuli which sensitize them to apoptosis. This type of approach, while potentially harmful to patients with regard to enhanced apoptosis-related pathogenesis, may promote infected cells to undergo premature apoptosis before virus progeny can be effectively packaged and released from the cell and therefore inhibit viral replication in the host.

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Apoptosis in avian influenza virus infection


