Roles of the ERK MAPK in the regulation of proinflammatory and apoptotic responses in chicken macrophages infected with H9N2 avian influenza virus

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The mitogen-activated protein kinase (MAPK) family is responsible for important signalling pathways which regulate cell activation, differentiation, apoptosis and immune responses. Studies have shown that influenza virus infection activates MAPK family members in mammals. While the extracellular signal-regulated kinase (ERK)1/2 is important for virus replication, activation of p38 controls the expression of RANTES, interleukin (IL)-8 and tumour necrosis factor (TNF)-α. In this study, we report that avian influenza virus (AIV) activates ERK, p38 and Jun-N-terminal kinases in avian species. In chicken macrophages, while ERK was required for H9N2 AIV replication, ERK regulated proinflammatory cytokines IL-1β, IL-6 and IL-8, which is distinct from what has been previously reported in mammalian cells. Moreover, ERK alone suppressed TNF-α and FasL and inhibited TNF-family-mediated extrinsic apoptosis in H9N2-infected chicken macrophages. Taken together, these findings suggest that ERK signalling may uniquely play important roles in avian host responses to AIV infection.

INTRODUCTION

Avian species are the reservoir hosts of influenza A viruses. All known subtypes of avian influenza virus (AIV) have been isolated from various species of birds with most of the subtypes causing asymptomatic infections. Low pathogenicity AIV (LPAIV), such as H9N2 viruses, have been transmitted to the poultry populations and cause infections with no or mild symptoms, while high pathogenicity AIV (HPAIV), such as the H5N1 subtype, are lethal with infections resulting in rapid death of the host. There have not been adequate studies in birds on the pathogenesis of AIV infections. Although virulence markers have been identified in influenza viruses, it is not clear why avian hosts, chickens in particular, respond so differently to HPAIV or LPAIV infections. Clinical manifestations in birds, like humans, are most likely associated with dysregulation of proinflammatory cytokines and other host responses induced by AIV (Headley et al., 1997; To et al., 2001), but magnitudes and types of the responses are largely unknown.

Although extensive studies have been carried out in mammals, research to understand how avian hosts respond to LPAIV and HPAIV immunologically and pathologically is more limited. In H9N2 AIV-infected chicken macrophages, the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8 and ah294 are upregulated, as are antiviral beta and gamma interferon (IFN-β and -γ), although IFN induction is under suppression of the non-structural protein-1 (NS1). AIV-induced apoptosis is also regulated by NS1, while Toll-like receptor 7 is downregulated in chicken macrophages. The underlying mechanisms for the regulation of cytokine and apoptotic responses in AIV-infected birds remain largely unknown, mostly because the signalling pathways governing cytokine and host responses have not yet been characterized at molecular and cellular levels in avian species. In AIV-induced apoptosis, the Fas/Fas ligand (FasL)-mediated extrinsic pathway has been identified in H9N2-infected chicken macrophages. Despite this, it is unclear why among three major tumour necrosis factor (TNF) ligands, only FasL is upregulated while TNF-α and the TNF-related apoptosis inducing ligand (TRAIL) are either unchanged or even downregulated in response to AIV infections (Xing et al., 2008b, 2009). Cytokine and apoptotic responses are presumably key elements in the pathogenesis of AIV infections.

The mitogen-activated protein kinase (MAPK) pathway is central in the control of cellular responses including host defence, inflammatory responses and cell death. The MAPK family is composed of four prototype members,
the extracellular signal-regulated kinase 1/2 (ERK1/2), p38, the Jun-N-terminal kinase or stress-activated protein kinase (JNK/SAPK) and ERK5, which are responsible for signalling pathways that regulate cell proliferation, differentiation, apoptosis and immune responses (DeSilva et al., 1996; Dong et al., 2002; Pearson et al., 2001). ERK1/2 is downstream of the growth-factor-stimulated Raf/MEK/ERK signalling cascade, while p38 and JNK/SAPK are activated by apoptosis signal-regulating kinase 1 (Garrington & Johnson, 1999). The specific p38 MAPK inhibitor SB203580 inhibits IL-1β, TNF-α and IL-6 synthesis both in vivo and in various mammalian cell lines (Badger et al., 1996; Baldassare et al., 1999; Lee & Adams, 1995), while activation of p38 can activate downstream phosphorylation of ATF-2 and HSP27, which leads to Bax expression and increased mitochondria-mediated apoptosis (Mandal et al., 2008). The JNK signalling pathway is also implicated in cell death as well as differentiation, but specific functions of the pathway depend on the cell type (Conze et al., 2002). MAPK activation has been studied extensively in influenza virus-infected mammalian cells and tissues (Ludwig et al., 2003, 2006). Influenza virus infections activate all four MAPK family members (Kujime et al., 2000; Lee et al., 2005; Ludwig et al., 2001; Pleschka et al., 2001) and the activation of ERK1/2, p38 and JNK is key in viral replication and/or proinflammatory responses in various human cells. However, no studies on MAPK pathways have been carried out in AIV infections in avian species.

We recently screened a group of commercially available MAPK antibodies and obtained and validated antibodies specific to chicken MAPK and phosphorylated MAPK. In an attempt to understand the molecular mechanisms of how proinflammatory cytokines and apoptotic responses are regulated in chickens and the role of the MAPK pathway, we examined the activation status and regulation of MAPK in chicken HTC macrophage cells.

RESULTS

MAPKs are activated in H9N2 AIV-infected chicken macrophages

HTC cells were infected with the H9N2 virus at an m.o.i of 1 and harvested at 8 h intervals post-infection (p.i.). Both soluble and insoluble fractions of cell lysates were prepared and analysed by SDS-PAGE. As shown by Western blotting with specific MAPK antibodies, ERK was found as a single protein with a molecular mass of about 42 kDa in both soluble and insoluble fractions of HTC cell lysates (Fig. 1a). A search of GenBank databases yielded only one isoform of ERK for the species Gallus gallus, suggesting that, in chickens, there is only ERK2 (referred as to ERK hereafter), as described previously (Garcia et al., 2008); this is in contrast with ERK in mammals, which has two isoforms, p44 ERK1 and p42 ERK2. We do not know if a lack of ERK1 in chickens implies any differences at the cell signalling level in response to viral infection, but apparently, chicken ERK was activated as phosphorylation of ERK (Thr202/Tyr204) was significantly increased in H9N2-infected cells at 8 and 16 h p.i. (Fig. 1a), although there was a basal level of ERK activation as detected by an anti-phospho-ERK antibody in HTC.

p38 MAPK was also activated in infected chicken macrophages. Phosphorylation of p38 was detected weakly at 8 h p.i. compared with non-infected controls. The phosphorylation of p38 increased markedly at 16 h p.i. (Fig. 1a). Two major isoforms of chicken JNK, JNK1 and JNK2 (46 and 36 kDa), were also detected in HTC cells,
consistent with mammalian JNK. There may be some minor isoform species of JNK which also exist in HTC cells (Fig. 1b), especially those that appeared at 4 h p.i. and seemed to be associated with infection. The significance of these minor isoforms will not be known until they are fully characterized. Transient phosphorylation of JNK was observed, which started at 4 h and continued through to 12 h p.i. in response to H9N2 infection, indicating that both isoforms of JNK are activated upon infection but deactivated after a few hours due to possible dephosphorylation by a MAPK phosphatase which is as yet unidentified in birds.

### Inhibition of chicken MAPK by specific inhibitors in H9N2-infected HTC cells

To explore the functions of chicken MAPK, specific MAPK inhibitors were evaluated in chicken cells. Among numerous MAPK inhibitors, which are reported to act on mammalian MAPK, we tested and selected U0126, SB203580 and InSolution JNK Inhibitor II for ERK, p38 and JNK kinases, respectively. Selection was based on effectiveness in the inhibition of AIV-induced MAPK activation and low relative toxicity, in which viable cell numbers (trypan blue exclusion) remain comparable between treated and non-treated cultures through the infection period.

As for MAPK inhibition, the HTC cells were pre-treated with the inhibitors diluted in RPMI 1640 medium 1 h prior to infection. A series of concentrations from 5 to 100 μM of U0126, SB203580 and JNK Solution Inhibitor II were tested in the cells. After the 1 h treatment, the cells were infected with H9N2 virus as described in Methods. The virus inocula were removed after 1 h adsorption and the infected cells were subsequently maintained in serum-free RPMI medium containing inhibitors at various concentrations for 8 h (and 12 h for positive and negative controls in some experiments) before the cells were lysed for Western blot analyses with the anti-phospho-ERK antibody. As shown in Fig. 2(a), the phosphorylation of ERK, resulting from H9N2 infection, was completely abolished by 10 and 20 μM U0126 at 8 h p.i. The inhibition was specific as the induced activation of ERK was not decreased by treatment with SB203580 (p38) or JNK Inhibitor II at their effective concentrations. Likewise, the phosphorylation of JNK, activated by H9N2 infection at 8 h p.i., was also significantly inhibited by InSolution JNK Inhibitor II, but was not affected by treatment with U0126 (ERK) or SB203580 (Fig. 2b).

Interestingly, even though it was rather specific, the inhibition of one type of MAPK seemed to have an impact on another one. As shown in Fig. 2(a), the phosphorylation of ERK was enhanced by treatment with either SB203580 or JNK Inhibitor II, compared with the phosphorylation of ERK at 8 h p.i. without the inhibitor treatment. This may indicate the presence of a compensatory mechanism in chicken macrophages among MAPK, in which ERK may be further activated, when p38 or JNK kinases are inhibited.

### ERK2 is essential to proinflammatory responses as well as virus replication in H9N2-infected chicken macrophages

Previous studies in influenza-infected mammalian cells indicated that among three MAPKs, only p38 MAPK was involved in regulating proinflammatory cytokine responses, including the upregulation of IL-1β, TNF-α and RANTES (Kujime et al., 2000; Lee et al., 2005). On the other hand, ERK1/2 was essential to viral replication due to its involvement in the transportation of viral NS2 protein and nucleoprotein (NP) (Ludwig et al., 2004; Pleschka et al., 2001). In chicken macrophages, our data indicate that ERK is required for viral replication as well. Unlike in previous reported mammalian cells, ERK is also critical in proinflammatory cytokine responses in chicken macrophages.

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**Fig. 2.** Inhibition of ERK and JNK phosphorylation in chicken macrophages by specific inhibitors. Cells were pre-treated with inhibitors at the indicated concentrations and infected with H9N2 virus 1 h after treatment. Cell lysates were prepared for SDS-PAGE and Western blot analyses to determine dephosphorylation and inhibition of MAPK activation. (a) ERK phosphorylation (8 h p.i.) was inhibited in the presence of U0126 (lanes 4 and 5) but not suppressed by the p38 (lanes 6 and 7) and JNK (lanes 8 and 9) inhibitors. (b) JNK phosphorylation was inhibited by InSolution JNK inhibitor II (lanes 6 and 7) but was not affected by the ERK (lanes 2 and 3) and p38 (lanes 4 and 5) inhibitors.
The HTC cells were infected with H9N2 virus at an m.o.i. of 1, following the pre-treatment with U0126 at 10 μM for 1 h. The viruses were removed after 1 h of adsorption as described in Methods and, after three washes with PBS, the cells were maintained in serum-free RPMI medium containing U0126. The viruses in the culture medium were harvested at 4, 8, 16 and 24 h p.i., and subjected to a standard plaque titration in MDCK cells. Viral M gene transcript levels in the infected cells were measured by the Taqman-based real-time PCR. As shown in Fig. 3(a), even though no significant differences (*P>0.05, t test) were observed in the copy numbers of the viral M gene at 8 and 16 h p.i., virus replication (indicated as p.f.u.) decreased significantly in U0126-treated cells at 8, 16 and 24 h p.i., indicating that ERK is required for efficient replication of H9N2 AIV in chicken macrophages.

To evaluate the expression of proinflammatory cytokines in U0126-treated cells after infection, we prepared total RNA from both uninfected and infected cells, treated with the inhibitor for various lengths of time. The total RNA was reverse transcribed for subsequent SYBR Green-based real-time PCR with specific primers (Xing et al., 2008b). We found that the expression of IL-1β and IL-8 transcripts was suppressed through the course of infection in 10 μM U0126-treated cells (Fig. 3b), indicating that the expression of IL-1β and IL-8 is positively regulated by ERK in chicken macrophages. In addition, we found that IL-6 expression remained downregulated at 8 h p.i. when HTC cells were treated with 10 μM U0126 (Fig. 3b), indicating that IL-6 is also regulated by ERK, at least partially.

Taken together, while ERK plays a key role in virus replication in chicken macrophages, it is also involved in the upregulation of proinflammatory cytokines IL-1β, IL-6 and IL-8. However, ERK seems to have no effect on the expression of antiviral IFN-β as shown in Fig. 3(c). It appears that antiviral IFN expression is not regulated by ERK in chicken macrophages.

**Effect of p38 and JNK on cytokine regulation and viral replication**

Cytokines IL-1β, IL-6 and IL-8 were all upregulated by treatment with 5 μM SB203580 at 8 and 16 h p.i., demonstrating that in chicken macrophages, p38 is important in the regulation of proinflammatory cytokines after infection (Fig. 4a). We found, however, that these cytokines were not affected by InSolution JNK inhibitor II (50 μM; data not shown), but IFN-β induction was significantly decreased in H9N2-infected cells treated with the JNK inhibitor, indicating that JNK1/2 is critical to IFN-β production in chicken macrophages (Fig. 4b). The induction of IFN-β by JNK1/2 may directly contribute to the inhibition of viral replication (Fig. 4c), as the viral titres increased (*P<0.05) in H9N2-infected HTC cells treated with InSolution JNK inhibitor II. However, no significant decrease in viral titres was observed in infected cells treated with SB203580.
ERK suppresses TNF-family-mediated extrinsic apoptosis in H9N2-infected chicken macrophages

Previous studies have shown that p38 regulates TNF-α expression in influenza virus-infected mammalian cells, although no studies have indicated that ERK1/2 affects apoptosis (Uchide et al., 2005). We have observed that the HTC cells underwent aggressive apoptosis in response to H9N2 infection (Xing et al., 2008b, 2009). However, in HTC cells treated with U0126 (10 μM), we found that apoptosis was more pronounced than in untreated controls at various time points p.i. (Fig. 5a). These findings indicate that ERK may be also implicated in the apoptosis induced in H9N2-infected chicken macrophages.

To understand the mechanism through which apoptosis increases when ERK is suppressed, real-time RT-PCR was performed to examine transcript levels of TNF family members. It was shown previously that some of the TNF family members are upregulated in H9N2-infected HTC cells, triggering the extrinsic apoptotic pathway (Xing et al., 2009). In H9N2-infected cells, 15- and 25-fold increases in FasL transcripts were detected at 8 and 16 h p.i. However, the upregulation of FasL transcripts increased to 28- and 100-fold at 8 and 16 h p.i., respectively, when the HTC cells were treated with 10 μM U0126 (Fig. 5b). As for TNF-α, the levels of its transcripts were almost unchanged in infected HTC cells at 8 and 16 h p.i. However, the upregulation of TNF-α was apparent up to 6.5-fold at 16 h p.i. in U0126-treated cells (Fig. 5b). Taken together, it appears that the induction of both FasL and TNF-α was suppressed by activated ERK in H9N2-infected chicken macrophages. Therefore, the increased expression of FasL and TNF-α may account for the observed elevation in apoptosis in U0126-treated HTC cells (Fig. 5a).

In infected HTC, the expression of TRAIL was slightly downregulated, which was not changed by treatment with U0126 (Fig. 5c). However, the downregulation of TRAIL was further increased in SB203580- and InSolution JNK Inhibitor II-treated cells, up to 10- and 17-fold, respectively, and the differences were significant (P<0.05, t test). Based on these findings, p38 and JNK appeared to upregulate the expression of TRAIL in H9N2-infected HTC cells, but ERK did not. However, TRAIL also seems to be suppressed by an unidentified signalling pathway, which may regulate TRAIL in AIV-infected chicken macrophages. In the end, even though both p38 and JNK are activated upon infection, TRAIL appears to be constantly suppressed during infection and contributes little to the extrinsic apoptosis observed in H9N2-infected HTC cells.
DISCUSSION

In summary, we have succeeded in detecting ERK, p38 and JNK kinases in chicken macrophages and found that all three MAPKs were activated in response to H9N2 AIV infection. Specific MAPK inhibitors, such as U0126, SB203580 and JNK Inhibitor II, can inhibit ERK, p38 and JNK, respectively, in HTC cells. Our results demonstrate that ERK is important to H9N2 replication in chicken cells, similarly to findings in mammalian cells. Uniquely, we have found that ERK was also essential for the expression of proinflammatory IL-1β, IL-6 and IL-8, suggesting that it may have an important role in the pathogenesis of H9N2 LPAIV in infected chickens.

MAPKs are both structurally and functionally diverse in mammals. While ERK1/2, which has two isoforms ERK1 (p44) and ERK2 (p42), is important for efficient virus replication (Ludwig et al., 2003, 2004; Marjuki et al., 2007; Pleschka et al., 2001), activation of p38 MAPK controls the expression of RANTES, IL-8 and TNF-α (Kujime et al., 2000; Lee et al., 2005; Mori et al., 2003), which appears to control proinflammatory responses after infection. The role of p38 has been further elucidated in one study demonstrating that TNF-α expression is controlled by p38 only at the post-transcriptional level in influenza-infected human choriocarion cells but p38 had no effect on virus replication (Uchide et al., 2005). The JNK pathway, on the other hand, seems to be crucial in the host innate antiviral response, by regulating the expression of IFN-β through AP-1 (Ludwig et al., 2001). Therefore, influenza virus replication is suppressed when JNK is activated, resulting in IFN-β expression (Ludwig et al., 2001). JNK is also involved in the regulation of apoptosis through the modulation of c-Jun/AP-1 and transforming growth factor-β in MDCK cells (Ludwig et al., 2003).

Previous studies have shown no evidence that ERK regulates the expression of proinflammatory cytokines in influenza-infected mammalian cells. However, our recent studies with H9N2 infection of the human monocytic cell line, THP-1, indicated that proinflammatory cytokines were also regulated by ERK (Z. Xing and others, unpublished data). ERK is evidently involved in the regulation of inflammatory responses in a few studies on other microorganisms as well. Two of the major proinflammatory cytokines, TNF-α and IL-12, produced in Plasmodium falciparum glycosylphosphatidylinositol-stimulated macrophages, can be effectively regulated by the ERK inhibitor U0126 (Zhu et al., 2009). The MAPK-activated protein kinase 2 (MK2), which is targeted by both ERK and p38, has also shown a regulatory effect on the expression of TNF-α and IL-12, and MK2 can be inhibited by U0126 (Zhu et al., 2009). Similarly, the inhibition of ERK as well as p38 pathways significantly decreased the IL-1β response induced by Vibrio cholerae and its flagellin in human
et al. (Bandyopadhaya et al., 2008). An earlier study also showed that both p38 and ERK pathways are important for the regulation of the IL-6 production in primary human glomerular mesangial cells of the nephron (Leonard et al., 1999). Functions of MAPK signalling pathways appear to be diverse in different cell types and may also vary by species. The results we observed in the regulation of proinflammatory cytokines by ERK in influenza-infected chicken macrophages contrast with findings in non-monic acid mammalian cells.

Previously, we reported that TNF family members were differentially regulated in H9N2-infected HTC cells. While FasL was upregulated aggressively after infection, the expression of TNF-α was virtually unchanged and that of TRAIL was even downregulated (Xing et al., 2009). The mechanism for this differential regulation is unknown. The results of this study, however, indicate that chicken ERK suppressed FasL and TNF-α induction and inhibited, to a certain degree, TNF-family-mediated extrinsic apoptosis induced by AIV infection. The previously observed ‘differential regulation’ may be partially explained by the observation that, while the TNF-α upregulation was totally abrogated, the suppression of FasL by activated ERK was, however, incomplete. Although still at a high level, FasL induction was suppressed to some degree in infected cells. A plausible explanation is that, as shown in mammals, FasL is strongly stimulated by the NFκB pathway. This could also be the case in chicken macrophages, which may compensate in part for suppression by an anti-apoptotic ERK pathway.

The regulation of TRAIL seems to be through a different mechanism. Unlike FasL and TNF-α, which are suppressed by ERK, TRAIL may be strongly inhibited by an unknown pathway, which can overcome moderate induction by both p38 and JNK in H9N2-infected chicken macrophages.

That TRAIL is upregulated by p38 and JNK is consistent with the same observation in human bronchial epithelial cells (NCI-H292) infected with A/Udorn/307/72 (H3N2) (Hayashi et al., 2008). In NCI-H292 treated with SB203580 and CEP-11004 (JNK inhibitor), mRNA levels of TRAIL and an adaptor protein, TNF receptor-1 associate protein, were both downregulated (Hayashi et al., 2008), indicating that p38 and JNK, in contrast with ERK, may be pro-apoptotic and promote apoptosis through the upregulation of TRAIL in chicken macrophages.

These results provide the first evidence that MAPK signalling pathways respond to AIV infection in avian species, and demonstrate the critical roles of chicken ERK MAPK in virus replication, proinflammatory and apoptotic responses in AIV infection. While ERK is essential to virus replication in mammals and birds, chicken ERK is distinctly important in eliciting proinflammatory cytokines and suppressing TNF-family-mediated intrinsic apoptosis induced by AIV infection in chicken macrophages. We are unable to interpret the differences in MAPK functions in the host responses of birds and mammals at this stage, because we have found recently that a similar regulatory effect on proinflammatory cytokines by ERK1/2 occurs in H9N2-infected human monocytic THP-1 cells (data not shown). Presumably, ERK MAPK may play a different role in monocytic cells from its role in epithelial or other cell types, a notion which needs further confirmation. Further studies which explore both the basic functions of MAPK and other signalling pathways in avian species, and the roles that they play in AIV infections, may help elucidate the specific mechanisms of AIV pathogenesis in birds.

METHODS

Cells and reagents. Chicken monocyte/macrophage HTC is a spontaneously transformed cell line, obtained from Dr N. C. Rath (Agricultural Research Service, USDA, Fayetteville, USA) (Rath et al., 2001). MDCK (Cat. CCL-34) cells were purchased from ATCC (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS). Chicken blood was from Colorado Serum. Mouse anti-ERK, rabbit anti-p38 and anti-JNK antibodies as well as rabbit anti-phospho ERK, anti-phospho p38 and anti-phospho JNK antibodies (Cell Signalling) and alkaline phosphatase (AP)-conjugated anti-rabbit and anti-mouse IgG antibody (Santa Cruz Biotechnology) were obtained. MAPK inhibitors, U0126, SB203580 and InSolution JNK Inhibitor II (Cat. 420128) were from EMD Chemicals.

Virus and virus infection. The A/peasant/California/2373/1998 (H9N2) virus, referred to as the H9N2 virus hereafter, was isolated from a domestically raised pheasant during an LPAI outbreak in California (Woolcock et al., 2001) and its eight genomic segments have been completely sequenced. The virus in virus allantoic fluid (VAF) was titrated with a standard haemagglutination test (Hirst, 1948) and infectious viral titres were determined in MDCK cells by a standard plaque assay (Gaush & Smith, 1968). For infection, the HTC cells were split and resuspended in RPMI 1640 medium (Invitrogen) containing 10% FBS and plated on 10 cm tissue culture plates (5 × 10⁵ cells per plate) 16 h before infection. The cells were infected with the H9N2 virus in VAF at an m.o.i. of 1, and after 1 h of adsorption, the virus was removed and serum-free RPMI 1640 medium was added. The cells were incubated at 37 °C with 5% CO₂ for a designated time before cell lysates were prepared.

Real-time RT-PCR. The mRNA transcript levels for IFN-β, IL-1β, IL-6, IL-8, TNF-α, FasL, TRAIL and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were analysed by a two-step real-time RT-PCR as described previously (Xing et al., 2009). Total RNA (1 μg), which was prepared from the cells using the RNaseasy kit (Qiagen), was used for each RT reaction with the QuantiTect reverse transcription kit (Qiagen) following the manufacturer’s instructions. The RT reaction was carried out with the RNA after treatment with DNase I for elimination of genomic DNA contamination at 42 °C for 2 min. Real-time PCR was conducted with 1 μl cDNA in 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 (Harper et al., 2005). For each reaction, melting curves were analysed to determine the specificity of each gene. To detect the viral RNA level, the infected cells were lysed with RLT buffer and subjected to total RNA extraction with the RNeasy kit (Qiagen). The RNA was reverse transcribed and cDNA was used for Taqman-based real-time PCR (Applied Biosystems) to measure viral
M gene transcripts in the infected cells (Spackman et al., 2003; Xing et al., 2008a).

Flow cytometric analysis of apoptosis. HTC cells infected with the virus were trypsinized and washed three times with FACS staining buffer (PBS containing 0.5% BSA). Cells (1 x 10^6) were used for staining with fluorescein-conjugated annexin V and propidium iodide (BD Pharmingen) in a volume of 100 µl, on ice for 30 min. The cells were washed three times with the FACS staining buffer, followed by flow cytometric analysis on a FACSscan cell sorter (Becton Dickinson).

Western blot analysis. Cell lysates were prepared by lysing uninfected and infected HTC cells in a hypotonic buffer with 1% NP-40 lysis buffer containing 1 mM PMSF, 1% aprotinin, 20 µg leupeptin ml^-1 and 1 mM sodium vanadate (Sigma) as described previously (Sternsdorf et al., 1997; Xing et al., 2004). Cytosolic and nuclear fractions in cell lysates were separated with low speed centrifugation (1000 g, 5 min at 4 °C) and subjected to SDS-PAGE (12%). Proteins were transferred to Immuno-Blot PVDF membrane (Bio-Rad) and Western blot analysis was performed following standard protocols using rabbit or mouse anti-MAPK antibodies (1:500), followed by an incubation with AP-conjugated anti-rabbit or anti-mouse IgG antibody. BCIP/NBT reagents (Sigma) were used for the development of colorimetric signals on the membrane. The membrane was also blotted with a monoclonal anti-actin antibody (Santa Cruz Biotechnology) for input control.

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