AMP-activated protein kinase facilitates avian reovirus to induce mitogen-activated protein kinase (MAPK) p38 and MAPK kinase 3/6 signalling that is beneficial for virus replication

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Stimulated by energetic stress, AMP-activated protein kinase (AMPK) controls several cellular functions. It was discovered here that infection of Vero cells with avian reovirus (ARV) upregulated AMPK and mitogen-activated protein kinase (MAPK) p38 phosphorylation in a time- and dose-dependent manner. Being an energy status sensor, AMPK is potentially an upstream regulator of MAPK p38. Treatment with 5-amino-4-imidazolecarboxamide ribose (AICAR), a well-known activator of AMPK, induced phosphorylation of MAPK p38. Unlike AICAR, wortmannin or rapamycin did not induce phosphorylation of MAPK p38, suggesting that mTOR inhibition is not a determining factor in MAPK p38 phosphorylation. Inhibition of AMPK by compound C antagonized the effect of AICAR on MAPK p38 in Vero cells. Specific inhibition of AMPK by small interfering RNA or compound C also suppressed ARV-induced phosphorylation of MAPK kinase (MKK) 3/6 and MAPK p38 in Vero and DF-1 cells, thereby providing a link between AMPK signalling and the MAPK p38 pathway. The mechanism of ARV-enhanced phosphorylation of MKK 3/6 and MAPK p38 in cells was not merely due to glucose deprivation, a probable activator of AMPK. In the current study, direct inhibition of MAPK p38 by SB202190 decreased the level of ARV-induced syncytium formation in Vero and DF-1 cells, and decreased the protein levels of ARV σC, encoded by the third open reading frame of the S1 segment, is a cell attachment protein (Martinez-Costas et al., 1997) as well as an apoptosis inducer (Shih et al., 2004). The major inner capsid protein, σA, encoded by the S2 genome segment, possesses non-specific nucleotidyl phosphatase activity (Yin et al., 2002). This protein is also reported to inhibit activation of dsRNA-dependent protein kinase (PKR) by competing for dsRNA (Martinez-Costas et al., 2000; Gonzalez-Lopez et al., 2003).

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase consisting of a catalytic subunit (z) and two regulatory subunits (β and γ). A known function of the AMPK complex is to act as a sensor of energetic

INTRODUCTION
Avian reovirus (ARV), which belongs to the family Reoviridae, is an important pathogen in poultry. Sharing much similarity with mammalian reovirus (MRV), the virion particles of ARV possess two layers of capsid and ten genome segments of dsRNA. The genome segments are divided into three size classes, designated L (large), M (medium) and S (small), depending on their electrophoretic mobility, that encode at least eight structural and four non-structural proteins (Benavente & Martinez-Costas, 2007). ARVs differ from their mammalian counterparts by their ability to cause massive cell fusion through the protein p10 (Bodelon et al., 2002; Liu et al., 2008). Protein σC, encoded by the third open reading frame of the S1 segment, is a cell attachment protein (Martinez-Costas et al., 1997) as well as an apoptosis inducer (Shih et al., 2004). The major inner capsid protein, σA, encoded by the S2 genome segment, possesses non-specific nucleotidyl phosphatase activity (Yin et al., 2002). This protein is also reported to inhibit activation of dsRNA-dependent protein kinase (PKR) by competing for dsRNA (Martinez-Costas et al., 2000; Gonzalez-Lopez et al., 2003).
stress under conditions with an elevated intracellular AMP/ATP ratio. Activation of AMPK suppresses ATP consumption but increases the generation of ATP via various mechanisms such as inhibition of fatty acid synthesis and enhancement of glycolysis or fatty acid oxidation (Rutter et al., 2003; Smith et al., 2005). One known mechanism through which AMPK functions is by controlling the transcription of genes involved in nutrient metabolism (Viollet et al., 2006). Besides its roles in resource transition, AMPK also downregulates cellular protein synthesis by activating TSC2, a GTPase-activating protein that forms a complex with TSC1 and stimulates the intrinsic GTPase activity of Rheb, which results in inactivation of mTOR complex 1 (mTORC1) (Kimura et al., 2003). Being an important downstream mediator of various signalling cascades, including the phosphoinositide 3-kinase (PI3K)/Akt and AMPK pathways (Hay & Sonenberg, 2004), mTORC1 regulates translation via controlling distinct factors such as 4E-BP1, which in turn regulate the activity of translation initiation factors such as eIF-4E. When phosphorylated by mTORC1, 4E-BP1 loses its ability to sequester eIF-4E and thereby allows initiation of cap-dependent translation (Beretta et al., 1996).

It has been widely reported that AMPK potentially upregulates the function of mitogen-activated protein kinase (MAPK) p38 to modulate glucose uptake (Xi et al., 2001; Pelletier et al., 2005). Generally activated by environmental stresses and inflammatory cytokines, MAPK p38, which is a member of the mitogen-activated protein kinases, plays multiple roles in gene transcription, cytokine production and apoptosis. Infection with several viruses is known to enhance MAPK p38 signalling (Shapiro et al., 1998; Banerjee et al., 2002; Hirasawa et al., 2003). Although the function of AMPK has been well studied in metabolism, here we have demonstrated for the first time that AMPK facilitates ARV in inducing MAPK kinase (MKK) 3/6–MAPK p38 signalling that is beneficial for ARV replication. These results suggest that AMPK plays a role in the interaction between ARV and host cells.

The mTOR inhibitor rapamycin was purchased from A. G. Scientific. DMSO, the solvent used to dissolve the above chemicals except for glucose, was used as a negative control to treat cells. Two monoclonal antibodies against each of the ARV envelope proteins were produced in our laboratory (Huang et al., 2005; Hsu et al., 2006). All other antibodies including anti-AMPK, anti-phospho-AMPK (T172), anti-phospho-Akt (T308), anti-Akt, anti-MAPK p38, anti-phospho-MAPK p38 (T180/Y182), anti-4E-BP1, anti-MKK 3/6 and anti-phospho-MKK 3/6 (S189/207) were obtained from Cell Signaling Technology.

**Electrophoresis and Western blot assay.** Cells in four-well plates were washed twice with PBS and lysed with 70 µl 2.5 × Laemmli loading dye. Cells were harvested by scraping and boiled for 10 min. Equal amounts of sample were run on 10% SDS-PAGE gels and transferred to PVDF membranes. Expression of individual proteins was detected using the respective antibodies, followed by secondary antibody conjugated to horseradish peroxidase. After incubation with enhanced chemiluminescence reagents (ECL Plus; Amersham Biosciences), the membranes were exposed to X-ray films (Kodak).

**Progeny titre.** The progeny titre of ARV was determined by plaque assay. Twenty-four hours after a particular treatment, cells and the culture medium were freeze-thawed three times to release virus particles. Serially diluted supernatant was used to infect fresh cells. After incubation for 1 h, cells were washed twice with MEM to remove unabsorbed virus and further incubated in MEM for 1 h to allow virus penetration. To determine the titre of ARV, cells were maintained in fresh medium containing 20 mM NH4Cl to prevent secondary infection after washing. The number of plaques was determined using an optical microscope about 24 h later. The results are means ± SD from triplicate experiments.

**Knockdown of AMPK by RNA interference.** An RNA interference kit containing duplex small interfering RNAs (siRNAs) against AMPKs or control sequences was purchased from Upstate. Based on the manufacturer’s instructions, siRNAs were transfected into Vero cells cultured in four-well plates using Lipofectamine 2000 (Invitrogen). After 5 h incubation, 500 µl culture medium containing 10% FBS was added to each well without removing the transfection reagent. Cells were washed twice with MEM 24 h after transfection and maintained in normal culture medium before inoculation.

**METHODS**

**Cells and viruses.** Vero (African green monkey kidney) and DF-1 (spontaneously transformed chicken embryo fibroblast) cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 20 mM HEPES (pH 7.2). Cells were cultured at 37 °C in 5% CO2. Vero cells were seeded in four-well plates 1 day prior to each experiment. The experimental procedures were started after refreshing the medium when cell confluency reached about 70–80%. ARV strain S1133 was propagated in Vero cells. When the cytopathic effect (CPE) covered about 80% of the infected cells, the cells were frozen and thawed twice. The mixture was centrifuged at 3000 g for 10 min to remove cellular debris and the supernatant was stored at −70 °C for further experiments.

**Antibodies and reagents.** 5-Amino-4- imidazolcarboxamidine ribose (AICAR) and compound C were obtained from Calbiochem. DMSO and glucose were from Sigma. The PI3K inhibitors wortmannin and LY294002 were from Merck, and SB202190 was from Calbiochem.

**RESULTS AND DISCUSSION**

**ARV infection increases the levels of AMPK and MAPK p38 phosphorylation**

Upregulated by different mechanisms, AMPK is activated by multiple factors such as energetic stress, reactive oxygen species, cell confluency and some extracellular signals such as Acrp30/adiponectin (Saha et al., 2006; Jung et al., 2008). AMPK was identified as a potential upregulator of MAPK p38 in metabolism-related cells. Regardless of how AMPK is activated, maintenance of phosphorylation at Thr172 in the catalytic subunit is critical for the function of the AMPK complex (Rutter et al., 2003; Smith et al., 2005). It is unclear whether the activity of AMPK is significant in Vero cells. ARV infection raised the level of phosphorylated AMPK in a dose- and time-dependent manner (Fig. 1) in Vero cells. These results suggest a possible role for AMPK during ARV infection.
MAPK p38 regulates miscellaneous downstream factors to adjust different cellular functions. Activation of MAPK p38 during infection might lead to apoptosis or cytokine production, which may be linked to viral pathogenesis (Eliopoulos et al., 1999; Banerjee et al., 2002; Meusel & Imani, 2003). Viral infections are known to influence intracellular signalling pathways that can affect cellular function or viral replication. ARV, a known oncolytic virus, does not upregulate MAPK p38 but passively depends on Ras-activated MAPK p38 function for efficient replication in tumour cells (Norman et al., 2004). Unlike its mammalian counterpart, ARV actively raised MAPK p38 phosphorylation in a dose- and time-dependent manner (Fig. 1). Furthermore, direct inhibition of MAPK p38 by a MAPK p38 inhibitor, SB202190, suppressed ARV-induced syncytium formation in Vero cells, and decreased the levels of ARV proteins σA and σC and the progeny titre of ARV, even in the presence of wortmannin (Fig. 2a–c), indicating that MAPK p38 is a critical downstream factor involved in regulation of ARV replication. Direct inhibition of MAPK p38 by SB202190 also suppressed the formation of ARV-induced syncytia in DF-1 cells and the protein level of ARV σC (Fig. 2d, e). In addition, experiments were repeated using bovine ephemeral virus (BEFV). However, BEFV did not activate p38 signalling (data not shown). Thus, activation of MAPK p38 by an AMPK-related mechanism may be an alternative for ARV to maintain the efficiency of ARV replication in Vero and DF-1 cells.

**AICAR upregulates MAPK p38 phosphorylation in Vero cells**

AICAR is the most commonly used selective activator of AMPK, and its effects on lipid and glucose metabolism have been the major focus of many recent studies (Iglesias et al., 2004; Pencek et al., 2005). As shown in Fig. 3a, Vero cells treated with AICAR enhanced phosphorylation of AMPK and MAPK p38. Simultaneous treatment with compound C antagonized the effect of AICAR on AMPK and MAPK p38 phosphorylation, confirming that AMPK activated by AICAR upregulated phosphorylation of MAPK p38 in Vero cells. Treatment of uninfected Vero cells with AICAR also reduced 4E-BP1 phosphorylation (Fig. 3a), suggesting that AICAR mediated upregulation of AMPK activity and consequently decreased the function of mTORC1. Inhibition of AMPK by an extensively used inhibitor, compound C (Feng et al., 2005; Gaidhu et al., 2006), counteracted the effect of AICAR on 4E-BP1 (Fig. 3a). In this study, inhibition of mTORC1 directly by rapamycin or indirectly by wortmannin did not enhance phosphorylation of either AMPK or MAPK p38, although wortmannin weakly downregulated 4E-BP1 phosphorylation by inactivating PI3K (Fig. 3a). This result suggested that mTORC1 is not involved in AICAR-induced phosphorylation of MAPK p38 in Vero cells.

Unlike in liver or muscle cells, functional insight into AMPK activation in Vero cells is still lacking. These results confirmed that there is a role for AMPK, whether related to metabolism or not, in Vero cells. This effect of AICAR has also been proven in other cells (Kudchodkar et al., 2007), suggesting that the role of AMPK might not be cell-type specific.

**mTORC1 inhibition is not a determining factor in MAPK p38 phosphorylation**

mTORC1 is an important mediator by which AMPK influences cell functions such as translation. It is also probable that inhibition of mTORC1 after AMPK activation indirectly facilitates MAPK p38 phosphorylation. In Vero cells infected with a lower dose (m.o.i. of 1) of ARV to evaluate the precise role of mTORC1 inhibition on ARV propagation, treatment with rapamycin slightly increased viral protein levels, accompanied by higher levels of phosphorylated AMPK and MAPK p38 (Fig. 3b). Evaluation of ARV-induced syncytia in Vero cells and the progeny titre of ARV further confirmed that both wortmannin and rapamycin raised ARV replication levels slightly (Fig. 3c, d). In addition, inhibition of PI3K by LY294002 also resulted in reduced phosphorylation of 4E-BP1 but no negative effects on accumulation of ARV proteins and progeny titre (data not shown), suggesting that inhibition of PI3K or mTOR had no negative effects on ARV replication. Our results indicated that cap-dependent translation might be dispensable for ARV.

**Fig. 1. ARV infection enhances phosphorylation of AMPK and MAPK p38.** (a) Vero cells were mock-infected or infected with an increasing m.o.i. of ARV, as indicated. Cells were harvested for Western blot analysis of the indicated proteins at 24 h post-infection. Cells were harvested for Western blot analysis of the indicated proteins at the indicated time points. Blots are representative of three experiments.
which could explain why ARV replication is not down-regulated after AMPK activation. This observation is also supported by our recent report that propagation of ARV continued during inhibition of cap-dependent translation (Ji et al., 2009). With regard to the fact that wortmannin and rapamycin did not enhance phosphorylation of MAPK p38 in uninfected cells, the increase in the level of phosphorylated MAPK p38 by rapamycin in infected cells was undoubtedly derived from the higher levels of viral products as well as phosphorylated AMPK. In addition, ARV infection strongly enhanced phosphorylation of Akt, a potential upstream activator of mTOR (Fig. 1a), which circumvents the possibility of ARV critically relying on mTOR inhibition to induce MAPK p38 phosphorylation.

**Fig. 2.** Influence of MAPK p38 on ARV multiplication. Vero (a–c) or DF-1 (d–e) cells infected with ARV at an m.o.i. of 1 were treated with the indicated chemicals or with SB202190 at 5 or 10 μM. The cells were photographed at 24 h post-infection to assess CPE (a, d). Vero cells were harvested for Western blotting of σC and σA (b) and determination of virus titre by plaque assay at 24 h post-infection (c). DF-1 cells were harvested for Western blot analysis of the indicated proteins at 24 h post-infection (e). Arrows indicate ARV-induced syncytia in Vero or DF-1 cells. Blots are representative of three experiments.
Inhibition of AMPK impedes the ability of ARV to enhance MKK 3/6 and MAPK p38 phosphorylation

AICAR enhanced phosphorylation of MAPK p38 in Vero cells, suggesting that AMPK may be beneficial for ARV-mediated upregulation of MAPK p38. Treatment with 1 μM compound C significantly downregulated ARV-induced MAPK p38 phosphorylation (Fig. 4a). However, inhibition of AMPK with 1 μM compound C only slightly reduced ARV progeny titre (Fig. 2c), possibly because ARV is a fast-replicating virus that causes obvious CPE, such as massive syncytium formation, in a short time period. In addition to the pharmacological inhibitor, RNA interference was also utilized to understand whether knocking down AMPK would have any effect on ARV-induced MAPK p38 phosphorylation. Despite the stronger CPE of ARV in transfected cells, which caused loss of cellular proteins, phosphorylation of MAPK p38 was similarly reduced by siRNA in infected cells (Fig. 4b). In addition, treatment with different concentrations of compound C also downregulated ARV-induced phosphorylation of MAPK p38 and ARV replication in infected DF-1 cells (Fig. 4d, e). In the present study, experiments were also repeated using BEFV. However, compound C used in this study did not inhibit BEFV replication. Furthermore, although MAPK p38 is activated by various factors such as UV irradiation or heat shock, MKK 3/6 is the main upstream integrator related to MAPK p38 phosphorylation (Johnson & Lapadat, 2002). Inhibition of AMPK by compound C consistently reduced ARV-induced phosphorylation of MKK 3/6 (Fig. 4c). These results suggested that functional AMPK augments ARV in inducing MKK 3/6 and MAPK p38 phosphorylation.

In the present study, inhibition of AMPK or MAPK p38 reduced the protein levels of ARV, the amount of CPE and the progeny titre of ARV, suggesting that the AMPK–MAPK p38 signalling pathway is a major contributor towards the efficient replication of ARV. Taken together, the results of this study suggest that AMPK facilitates the MKK 3/6–MAPK p38 signalling pathway in ARV-infected Vero and DF-1 cells and hence plays a role in regulating ARV–host cell interaction. The mechanisms through which AMPK activation helps ARV to promote MKK 3/6 and MAPK p38 phosphorylation remain an interesting issue waiting to be addressed in the near future. To this end, we are carrying out studies to elucidate the mechanisms and possible factors involved in the AMPK–MKK 3/6–MAPK p38 signalling pathway.
Glucose deprivation is not the cause of ARV-induced MAPK p38 phosphorylation

A critical function of AMPK is sensing energy shortage. AMPK responds to energy shortage by enhancing resource transition. The glucose concentration in the medium has been reported as a potential determinant of AMPK activity. Glucose deprivation may upregulate AMPK function in cultured cells (Tzatsos & Tsichlis, 2007). Usually, a glucose concentration above 10 mM is considered abnormal and is used to mimic the damage caused by high glucose conditions, especially in diabetes research (Gleason et al., 2007). Under normal levels of glucose concentration, ARV was similarly capable of inducing MAPK p38 phosphorylation (Fig. 5),
indicating that ARV infection actively induced MAPK p38 phosphorylation and was not artificially influenced by a resource shortage. Although AMPK is usually considered to be involved with nutrient metabolism, here we demonstrated for the first time that ARV upregulates and benefits from AMPK in Vero and DF-1 cells.

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