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PI3K/Akt/p53 pathway inhibits reovirus infection

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ABSTRACT

Viral infections activate many host signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which has recently attracted considerable interest due to its central role in modulating virus replication. This study demonstrated that the sero-type 3 reovirus strain Masked Palm Civet/China/2004 (MPC/04) could transiently activate the PI3K/Akt pathway in A549 cells at earlier time points of infection. The blockage of PI3K/Akt activation increased viral RNA synthesis and yield. The role of the downstream effectors MDM2/p53 of PI3K/Akt in regulating reovirus replication was further analyzed. We found that during reovirus infection, the level of phosphorylated MDM2 (p-MDM2) was increased and the expression of p53 was reduced. In addition, the blockage of PI3K/Akt by Ly294002 or knockdown of Akt by siRNA reduced the level of p-MDM2 and increased the level of p53. Both indicated that the downstream effectors MDM2/p53 of PI3K/Akt were activated. Pre-treatment with Nutlin, which can destroy MDM2 and p53 cross-talk and increase the expression of p53 RNA and protein, dose-dependently enhanced reovirus replication. Additionally, the overexpression of p53 alone also supported reovirus replication, and knockdown of p53 significantly inhibited viral replication. This study demonstrates that PI3K/Akt/p53 activated by mammalian reovirus can serve as a pathway for inhibiting virus replication/infection, yet the precise mechanism of this process remains under further investigation.

1. Introduction

Reoviruses are non-enveloped, double-stranded RNA viruses in the family *Reoviridae*. Members of the genus *Orthoreovirus* have been isolated from a broad range of mammalian, avian, and reptilian hosts (Fields et al., 2001; Jackson and Muldoon, 1973; Rosen et al., 1960). The mammalian orthoreoviruses (MRV) were first isolated from humans in 1951. These viruses commonly infected humans but are rarely pathogenic in adults (Tyler et al., 2004). However, recent studies have demonstrated that reoviruses that spilled over from wild animals can cause acute and severe clinical disease in humans (Cheng et al., 2009; Chua et al., 2007, 2008, 2011). These recent discoveries have raised concerns about future zoonotic reovirus infections in humans and also demonstrated the need to better understand virus-host interactions.

Mammalian reoviruses are highly tractable models for understanding virus-host interactions (Danthi et al., 2008). Studies using these viruses have improved our understanding of the pathogenesis of viral encephalitis. Viruses have evolved to exploit the host cellular machinery to ensure efficient replication. However, limited information exists about the role of many host-signaling pathways during reovirus replication.

The PI3K/Akt pathway has recently been shown to be required not only for viral cell entry but also for subsequent intracellular trafficking and viral replication (Cooray, 2004). Feng et al. reported that the induction of the PI3K/Akt signaling pathway by exogenous ALV infection regulated viral entry (Feng et al., 2011). PI3K activation was required for the human immunodeficiency virus type 1 (HIV-1) infection of CD4+ T-cells, and the suppression of PI3K signaling reduced HIV infection post-viral entry and post-reverse transcription, but prior to HIV gene expression (Francois and Klotman, 2003). The activation of the PI3K/Akt pathway is a strategy for Zaire Ebola virus to regulate vesicular trafficking and cellular entry (Saeed et al., 2008). Influenza A virus NS1 protein activated the PI3K/Akt pathway to augment its efficient replication (Ehrhardt et al., 2007; Shin et al., 2007). The direct relevance of the PI3K/Akt signaling pathway to reovirus infection is currently unknown.

Abbreviations: CHT, α-Chymotrypsin; LY, LY294002; Wort, Wortmannin; p-Akt, phosphorylated Akt; p-MDM2, phosphorylated MDM2.

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The p53 tumor suppressor protein is an important component of the PI3K/Akt signaling pathway that responds to various cellular stresses, such as DNA damage and deregulated oncogene expression (Gottlieb et al., 1996; Khanna and Jackson, 2001). Studies have demonstrated that type I interferons (IFN-α/β) enhance p53 transcription, and p53 also contributes to the immune responses that lead to the eradication of pathogens, such as viruses (Takaoka et al., 2003). p53 is involved in influenza virus-induced cell death, and inhibiting p53 could increase viral replication (Turpin et al., 2005). Another study demonstrated that p53 plays a crucial role in the cellular innate defence against the hepatitis C virus (HCV) (Dharel et al., 2008). However, the encephalomyocarditis virus (EMCV) and human parainfluenza virus type 3 (HPIV3) could down-regulate p53 in infected cells (Marques et al., 2005). Moreover, the absence of p53 inhibits the replication of both EMCV and HPIV3 (Marques et al., 2005). The role of p53 in reovirus infection is of concern.

To examine the ability of PI3K/Akt to regulate reovirus replication, we analyzed the role of this signaling pathway in sero-type 3 reovirus infection. Our study found that Akt can be phosphorylated in a PI3K-dependent manner early during infection, and the blockage of Akt activation could promote viral replication. Following the induction of PI3K/Akt, the downstream effectors MDM2/p53 of PI3K/Akt were activated during reovirus infection. The up-regulation of p53 using an inhibitor or the overexpression of p53 in cells increased reovirus production, and knockdown of p53 by siRNA reduced reovirus replication. This study demonstrates that PI3K/Akt/p53 is involved in the regulation of mammalian reovirus replication.

2. Materials and methods

2.1. Cells and viruses

The human lung-epithelial cell line A549 was cultured in F-12K medium. Mouse L929 cells were maintained in 1640 medium. Fetal bovine serum (10%) and 1% antibiotics were added to the medium, and all cells were incubated at 37 °C in 5% CO_2. Masked Palm Civet/China/Liu/2004 (MPC/04) was isolated from the masked palm civet in Yunnan, China in 2003 and propagated in L929 cells. The virus stock was purified using CsCl gradients (Hand and Tamm, 1971; Smith et al., 1969) and stored at −80 °C for the following studies.

The S1 gene from the Masked Palm Civet/China/Liu/2004 (MPC/04) shared 85.6% nucleotide identity with the S1 gene of reovirus serotype 3 Dear (T3D). A phylogenetic analysis revealed that the S1 gene of MPC/04 belonged to serotype 3. Infection studies via the intranasal inoculation of BALB/c mice indicated that the virus can cause clinical diseases and death in 80% of infected animals.

2.2. Preparation of UV-inactivated virus

The virus was diluted to 10^7 PFU/ml in DMEM without serum and irradiated in microtiter plates at a distance of 6 cm with shortwave (254 nm) UV light for 20 min (Helentjaris and Ehrenfeld, 1977). UV-irradiated virus infectivity was tested with a plaque assay. Secondly, UV-inactivated virus was inoculated into A549 cells for 2 h, and they were fixed with cold absolute methanol and kept at −20 °C for 15 min. Viral antigen was detected by indirect immunofluorescence with mouse polyclonal antibody anti-reovirus and followed by goat anti-mouse IgG-FITC secondary antibody (Sigma, St. Louis, MO, USA). The treated viruses were unable to form plaques and viral antigens could be detected by indirect immunofluorescence, which was considered to be UV-inactivated (Soares et al., 2009).

2.3. Titration

The virus titers of the samples were determined with plaque assays, as previously described (Middleton et al., 2007). Briefly, the samples were diluted and used to infect 6-well plates of L929 cells by incubation for 1 h. The monolayers were covered with 2 ml of 1% Bacto Agar (DIFCO) and serum-free medium 199 (Irvine Scientific) containing 10 μg/ml TLCK-treated α-Chymotrypsin (CHT) (Sigma–Aldrich). The plaques were counted 2 to 4 days later. CHT plaque assays were first fixed by incubation with 1 ml of 10% paraformaldehyde in PBS for 45 min at room temperature. The agar overlays were then peeled off, and the cells were stained by covering them with 0.05% crystal violet (Sigma–Aldrich) in 10% paraformaldehyde for 5 min at room temperature, followed by two washes with water.

2.4. Virus infection

Serum-starved A549 cells were washed with phosphate-buffered saline (PBS) and incubated with virus at the indicated multiplicities of infection (MOI) diluted in F-12K containing 0.2% bovine serum albumin (BSA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C for the indicated times.

2.5. Analysis of viral replication after inhibitors treatment

Serum-starved A549 cells were treated with LY294002 (LY) (10–50 μM), Wortmannin (Wort) (0.1–1 μM), Nutlin (5–20 μM) or solvent DMSO (0.4%, v/v) before virus infection for 1 h and then infected at a MOI of 5. The inoculum was aspirated, and cells were incubated with F-12K in the presence of drug. Twenty-four hours post-infection (p.i.), the virus titer in cell supernatant was determined with a plaque assay, and the virus RNA was analyzed by real-time PCR. To study the time-course of infection, either Ly294002 or Nutlin was added at the indicated time points. The infection was terminated 24 h p.i., the virus titer in cell supernatant was determined with a plaque assay and the viral RNA was analyzed by real-time PCR.

2.6. Western blot analysis

The cell monolayers were washed with PBS and lysed at the indicated times. The lysates were collected and incubated on ice for 10 min. The lysates were cleared by centrifugation at 10,000*g* for 5 min at 4 °C. The supernatants were analyzed for total protein content with a BCA protein-assay kit (Beyotime). The total protein (30 μg) was resolved by 10% SDS–PAGE and transferred onto nitrocellulose membranes (Millipore). The membranes were blocked with 5% skim milk for 1 h at 37 °C and then incubated overnight at 4 °C with specific antisera: rabbit anti-phospho-Akt antibody (Ser473) (ab81283, Abcam), rabbit anti-Akt antibody (ab32505, Abcam), rabbit anti-phospho-MDM2 antibody (Ser166) (ab170880, Abcam), rabbit anti-MDM2 antibody (ab38618, Abcam), rabbit PI3K p85 antibody (ab40755, Abcam), rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GADPH) antibody (ab22555, Abcam) and rabbit anti-p53 antibody (9282, CST). After three rinses in TBST buffer, the membranes were incubated at 37 °C for 1 h with IRDye 800DX-conjugated anti-rabbit IgG (1:8000; Rockland Immunochemicals) diluted in TBST as a secondary antibody. The membranes were washed three times in PBST, then visualized and analyzed with an Odyssey infrared imaging system (LI-COR Biosciences).