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The ubiquitin-proteasome system is necessary for the replication of duck Tembusu virus

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ARTICLE INFO
Keywords:
Duck tembusu virus
Ubiquitin-proteasome system
Viral replication

ABSTRACT
Duck Tembusu virus (DTMUV) is a newly emerging pathogenic flavivirus that has caused massive economic losses to the duck industry in China. The cellular factors required for DTMUV replication have been poorly studied. The ubiquitin-proteasome system (UPS), the major intracellular proteolytic pathway, mediates diverse cellular processes, including endocytosis and signal transduction, which may be involved in the entry of virus. In the present study, we explored the interplay between DTMUV replication and the UPS in BHK-21 cells and found that treatment with proteasome inhibitor (MG132 and lactacystin) significantly decreased the DTMUV progeny at the early infection stage. We further revealed that inhibition of the UPS mainly occurs on the level of viral protein expression and RNA transcription. In addition, using specific siRNAs targeting ubiquitin reduces the production of viral progeny. In the presence of MG132 the staining for the envelope protein of DTMUV was dramatically reduced in comparison with the untreated control cells. Overall, our observations reveal an important role of the UPS in multiple steps of the DTMUV infection cycle and identify the UPS as a potential drug target to modulate the impact of DTMUV infection.

1. Introduction

In 2010, a newly emerged tembusu virus (a flavivirus) related duck disease characterized by egg-drop suddenly struck south eastern China and quickly spread around the major duck-producing regions of the country, causing serious economic losses [1,2]. The infected ducks show a heavy drop in egg production accompanied by anorexia, antisocial behavior, diarrhea, and paralysis [3]. At necropsy, the infected ducks displayed severe ovarian hemorrhage, ovaritis and regression consistently. Enlarged spleen and leg muscle hemorrhage was occasionally found. The diseased egg-laying ducks had a significant reduction in egg production ranging from 20% to 60%, even up to 90% within 1–2 weeks post infection in some reported cases [4,5]. Now it has a severe impact on poultry production, causing heavy economic losses in China.

Duck tembusu virus (DTMUV) is enveloped and approximately 50 nm in diameter, and it contains a positive single-stranded RNA genome. Its genome has 10,986 nucleotides and the typical flavivirus genome organization, which consists of three structural proteins, named capsid (C), pre-membrane (prM), envelope glycoprotein(E), as well as seven nonstructural proteins (NS1, NS2A, NS2B,NS3, NS4A, NS4B, and NS5) [6,7]. The flavivirus attaches to the cell surface, mediated by the E protein, and enters into the host cells by receptor-mediated endocytosis. Low pH in the endosomal compartment triggers fusion of the viral and host cell membrane by structural reorganization of the E protein, which leads to the release of the nucleocapsid and viral RNA into the cytoplasm [8]. Viral RNA replication occurs in the rough endoplasmic reticulum (ER) and Golgi-derived membranes [9]. The newly synthesized viral RNA is either packaged within progeny virion or utilized to translate viral proteins. Flaviviruses assemble within the ER to form immature particles that exhibit prM protein. Following transport through the trans-Golgi network, furin-mediated cleavage of prM to M generates mature, infectious virions that are subsequently released by exocytosis [10].

The ubiquitin-proteasome system (UPS) is the major degradation system in the host cells. Major roles of this system consist of the degradation of misfolded or damaged proteins [11] and the regulation of the level of proteins related to the control of cell cycle progression, transcription, protein trafficking, the immune response, and signal transduction [12,13]. The UPS is formed by two major components: the
first is the proteasome, a multiprotein complex that degrades proteins, and the second component is ubiquitin, a highly conserved protein of 76 amino acids that is covalently attached to target proteins through a three-step reaction, known as ubiquitination. Protein ubiquitination is a posttranslational modification involved in several cellular functions; one of them is the delivery of the ubiquitin-tagged proteins to the proteasome for degradation. Depending on the nature of ubiquitination, this modification can modify other aspects of protein function independently of protein degradation, such as protein-protein interactions [14] and sorting of transmembrane proteins [15].

The typical virus life cycle sequentially involves cell entry, viral genome uncoating, transcription, replication, protein expression, particle assembly, and egress along with immune evasion. During viral infection, the UPS plays an important role in the regulation of cellular environmental homeostasis [16]. Generally, it is a double-edged sword in viral pathogenesis; host cells employ the UPS to degrade viral proteins to limit viral infection, meanwhile, viruses can manipulate the UPS machinery to degrade cellular restriction factors in order to facilitate viral propagation. To date, many viruses have been reported to utilize the UPS to satisfy their needs, and the UPS machinery can work in different stages of the viral life cycle, such as viral capsid uncoating [17,18], viral replication [19–21], gene transcription [22,23], viral envelopment [24], and viral progeny release [25,26]. Furthermore, in Coronavirus (CoV), the UPS was reported to play an important role during various stages of infection cycle [27], and it could potentially be developed as a drug target to modulate the impact of CoV infection in future.

In the present study, we demonstrated that the inhibition of the UPS suppresses DTMUV replication at the early infection stage through the degradation of viral copies, protein expression, and viral transcription potentially in a cell cycle-dependent manner. These results illustrate an important role of UPS in the regulation of the early stages of DTMUV replication.

2. Materials and methods

2.1. Cell culture and virus

BHK-21 (baby hamster kidney, ATCC No. CCL-10) cells were purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), then cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) in humidified 5% CO₂ environment at 37 °C, supplemented with 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin plus 10% heat-inactivated fetal calf serum (FCS) (Gibco, USA) in growth medium and 2% FCS in maintenance medium. The TMUV originally isolated in Jiangsu Province in August 2010, was cultured on BHK-21 cell lines (GenBank No. JF895923).

2.2. Reagents, antibodies and plasmids

MG132 was obtained from Selleck Chemicals. Lactacystin or MG132 and incubated for an additional 12 h. Cell viability was determined by CCK-8 assay whereby the reagent was added to the cells in a volume of 10 μl. The plate was shaken for 2 min and incubated for 1 h at 37 °C. The absorbance was measured at a wavelength of 490 nm using an ELISA plate reader.

2.4. Western blotting

BHK-21 cells seeded in 6-well plates were infected with DTMUV (MOI = 1) in the presence or absence of inhibitors. Cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM NaCl, 1% Triton X100, 0.2% sodium deoxycholate and 0.1% SDS) and electrophoresed in sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membrane (GE Healthcare). The membrane was incubated in blocking buffer (2% BSA in PBS) for 2 h at 37 °C, prior to overnight incubation with primary antibodies diluted in 2% BSA in PBS. Antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and immunoreactive protein bands were detected using the DAB Western blotting substrate (Boster, Wuhan, China). β-actin was detected simultaneously as loading control of the analysis, which immunoreacted by Western blotting substrate (Boster, Wuhan). Primary antibodies used for western blot included: anti-ubiquitin mouse monoclonal antibody (Abcam) at 1:800, anti-DTMUV E protein mouse polyclonal antibody at 1:5000, and mouse monoclonal antibody to β-actin (Sigma-Aldrich) at 1:5000, used as load control.

2.5. Real-time RT-PCR

Total RNA was extracted from BHK-21 cells treated with the indicated concentrations of the proteasome inhibitors and infected with DTMUV at a MOI of 1 using Axygen Total RNA extraction Kit (Axygen Biosciences, China) according to the manufacturer’s instructions. cDNA synthesis was performed with 1 μg of total extracted RNA using a RevertAid™ first strand cDNA synthesis kit (Fermentas, USA), according to manufacturer’s protocol.

To quantify the viral copies, absolute quantitative real-time PCR was performed using the QuantNova SYBR Green PCR Master Mix (Qiagen) in a total volume of 20 μl on an ABIprism 7500 (Applied Biosystems) according to the following steps: 5 min at 95 °C, 40 cycles of denaturation at 95 °C for 10 s, and annealing and extension at 60 °C for 30 s. For the standard curve, serial dilutions of a plasmid pcDNA3.1-NS2a constructed as described were used to quantify the virus genomic copy number.

Relative quantitative RT-PCR was performed to evaluate the RNA transcription of envelope protein. The primers, which are shown in Table 1, were designed using Lasergene sequence analysis software (DNAsStar, Inc., Madison, WI, USA). The generated cDNAs were amplified using QuantiNova SYBR Green PCR Master Mix (Qiagen) on an ABIprism 7500 (Applied Biosystems) according to the following steps: 5 min at 95 °C, 40 cycles of denaturation at 95 °C for 10 s, and annealing and extension at 60 °C for 30 s. Melting curves were obtained, and quantitative analysis of the data was performed in a relative quantification (2−ΔΔCT) study model. Parallel mock-infected samples were used.

Table 1

| Gene        | Primer | Sequence(5′→3′)          |
|-------------|--------|--------------------------|
| D-GAPDH     | F      | GCGAGCTTCAGAGGGCAGGTC    |
|             | R      | CAGCGCATACCCCATTT        |
| Tembusu E   | F      | GTGAGAATCTTAAGCTAGGAG    |
|             | R      | ACTTGGACACTGTTGTATGCC    |
| Ubb-mus-595 |        | CCUCUCUCGUAUCAAACAUUTT  |
| Ubb-mus-1273|        | GCGCAGUCUCUCGUAUATT     |
as control (relative expression = 1) and β-actin as an internal reference gene. Each sample was amplified in triplicate.

2.6. siRNA transfection

All small interfering RNAs (siRNAs) used in the present study were designed and synthesized by GenePharma (Shanghai, China). Before siRNA transfection, BHK-21 cells were seeded at 1 × 10⁵ cells/well in 24-well plates. After 24 h, the siRNAs (25 nM) were diluted in opti-MEM in a final volume of 50 μl and mixed with 50 μl of opti-MEM containing 1.25 μl of Lipofectamine reagent (Invitrogen, Carlsbad, CA) directly in the wells. The siRNA-Lipofectamine mix was incubated for 20 min at room temperature, and 400 μl MEM supplemented with 10% of FBS was then added to each well. Next, 48 h after siRNA transfection, the downstream experiments were performed. The siRNA sequences for ubiquitin were listed in Table 1.

2.7. Immunofluorescence

BHK-21 cells were seeded at a density of 1 × 10⁵ cells per well in a 24-well plates, and then infected with DTMUV (MOI = 1) in the presence or absence of MG132. At 24 h p.i., cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, followed by blocking with 1% BSA in PBS for 30 min. The cells were incubated with primary antibodies (anti-E serum 1:1000) for 2 h at room temperature. Then the cells were washed with PBS, followed by incubation with the secondary antibody, Alexa Fluor® 488 goat anti-mouse IgG (1:500, Abcam) for 30 min. Nuclei were stained with 1 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI). Samples were observed under an inverted fluorescence microscope.

2.8. Virus titration

After DTMUV infection, BHK-21 cells were incubated in the DMEM containing DMSO or inhibitors for 12 h, total viruses from cell lysates and supernatants were collected and titrated by plaque assay in triplicate samples on monolayers of BHK-21 cells. Cells were infected with 10-fold serial dilutions from samples and viral adsorption lasted 90 min in 1% FBS at 37 °C. Then the viral inoculum was removed and a 1:1 of 2% low-melting-point agarose and complete 2 × DMEM was added. Plaque visualization was developed after staining with violet crystal.

2.9. Statistical analysis

The results are presented as the means ± standard deviations (SD) and statistical analysis was performed by GraphPad's-QuickCalcs software (GraphPad).

3. Results

3.1. The alteration of free ubiquitin expression in DTMUV infection

To clarify the dynamic alterations of ubiquitin expression after DTMUV infection, at 0, 6, 12, 18 and 24 h p.i., mock-infected and DTMUV-infected cells were harvested, and the pellets were resuspended in RIPA buffer. SDS-PAGE and western blot were performed as described previously. The results indicated that the free ubiquitin expression was reduced significantly at different time points in DTMUV-infected cells compared with the mock-infected cells (Fig. 1). Meanwhile, the free ubiquitin in mock-infected cells remained unchanged at different time points. These results revealed that ubiquitin, even ubiquitin-proteasome system (UPS) may involve in DTMUV infection.

3.2. The UPS is involved in DTMUV infection in BHK-21 cells

To determine whether the UPS plays a role in DTMUV infection, we used MG132, widely used as a reversible and cell permeable proteasome inhibitor, Lactacystin, an irreversible proteasome inhibitor. Firstly, we evaluated the cytotoxic effects of these inhibitors on BHK-21 cells using a cell counting kit-8 (CCK-8, Dojindo, Japan), and selected the optimal concentration of MG132 (2 μM), lactacystin (5 μM) in the following study (Fig. 2A & 2B). We then assessed the effects of MG132 and lactacystin on the DTMUV titer. The cell supernatants were collected at 12 h post infection, and the viral copies were determined by absolute quantitative real-time PCR. Compared with DMSO-treated BHK-21 cells, MG132 inhibited the DTMUV genomic copies in the cultured cells by 40.09%, and lactacystin inhibited the DTMUV genomic copies by 47.44% (Fig. 2C). These findings indicate that UPS is required for the early stage of DTMUV replication.

3.3. Effects of proteasome inhibitors on different stages of DTMUV infection

The early step of viral infection included many events, such as binding, crossing cell membrane, cellular trafficking and uncoating, so we tried to examine which event was affected by the proteasome inhibitors. In the binding event, the number of absorbed viral particles at 4 °C was calculated by relative quantitative RT-PCR. As shown in Fig. 3A, the MG132-treated group and lactacystin-treated group showed no significant differences compared with the DMSO-treated group. It suggested that viral binding step was not affected by the proteasome inhibitors.

In the penetration of cell membrane, we measured the number of viral particles internalized into the BHK-21 cells by relative quantitative RT-PCR while the viral particles outside were digested by proteinase K to remove the virus remaining on the cell surface. The results showed proteinase K could digest viral particles outside cells effectively (Fig. 3B). After 1 h pretreatment with MG132, the cells were incubated with DTMUV at 4 °C for 1 h to allow virus binding and then incubated at 37 °C for 2 h followed by washing with PBS. Afterwards, the cells were treated with proteinase K to remove the virus remaining on the cell surface. Then the cells were collected for RNA isolation and the internalized virions were quantified by qPCR. As shown in Fig. 3C, there was no significant difference in the presence or absence of the proteasome inhibitor.

3.4. Proteasome inhibitors inhibits protein translation

We further observed the effects of MG132 or lactacystin on viral RNA transcription and protein expression. The results of transcriptional...
levels showed that viral RNA was reduced in MG132-treated cells or lactacystin-treated cells compared to the levels observed in DMSO-treated cells (Fig. 4A). To determine if MG132 and lactacystin inhibited DTMUV multiplication, BHK-21 cells were infected with DTMUV JS804 strain, and then incubated with DMEM containing DMSO or inhibitors for 12 h. Supernatants and cells were collected 12 h post-infection and stored at \(-80\) °C until analyzed by plaque assay. Cells treated with DMSO served as controls. Fig. 4B illustrated that in the presence of MG132 or lactacystin, DTMUV multiplication decreased significantly compared to the DMSO control.

In addition, a western blot analysis showed that MG132 decreased the expression of the DTMUV Envelope protein (Fig. 4C). Taken together, these results indicate that the proteasome inhibitors (MG132 or lactacystin) decrease the number of viral copies through suppression of viral RNA transcription and protein expression at the early infection phases.
To elucidate whether the ubiquitin machinery is directly associated with DTMUV infection, we used two ubiquitin-specific siRNA (siUb1273 and siUb595) to knock down the expression of the Mus Musculus ubiquitin gene (GenBank No: X51703). As shown in Fig. 5A, the viral progeny produced were reduced to 22.5% and 25.67% in the ubiquitin siRNA transfected cells compared to the negative control (NC). We further showed that viral titers were significantly reduced in the ubiquitin siRNA-transfected cells as compared to siRNA control (Fig. 5B). All of these suggest that protein ubiquitination may be a pivotal process during the DTMUV life cycle.

3.6. MG132 treatment reduced DTMUV production

In order to investigate whether MG132 treatment specifically reduced virus production at early stages of the infection cycle, we performed immunofluorescence assay to analyze the subcellular location of E protein under MG132 proteasome inhibition. After MG132 treatment, the E protein mainly localized in the cytoplasmic compartment in both DMSO-treated group and MG132-treated group, but the expression amount of E protein was decreased in the MG132-treated group (Fig. 6).
4. Discussion

The UPS plays a crucial role in the degradation of intracellular proteins and regulates the cellular signal transduction [13]. Many viruses have exploited the UPS system to their own advantage [19, 20, 27, 29]. However, the potential roles of the UPS in duck TMUV infections remain largely uncertain. In our previous study, quantitative proteomic analysis of DTMUV-infected group indicated that some genes related to the UPS were differentially expressed during DTMUV infection, including proteasome subunit beta type-7, 26s proteasome non-ATPase regulatory subunit 3 and deubiquitinating protein vcp/p135 [7]. These proteins were involved in different aspects of the UPS, including ubiquitination, deubiquitination and proteasome degradation. Also in the present study, free ubiquitin was found to be down regulated in the DTMUV-infected group (Fig. 1). All of these results suggested that the UPS was involved in DTMUV replication.

In the present study, two proteasome inhibitors, MG132 and lactacystin were used in this research, differentially, inhibition by MG132 was reversible, while inhibition by lactacystin was irreversible. When the cells treated with the two proteasome inhibitors leads to a reduction in viral activity, as determined by decreases in the number of viral copies, RNA transcription, and viral protein synthesis. Furthermore, we showed that the depletion of ubiquitin with siRNA reduces the genome copies of DTMUV, which indicates that DTMUV infection may be correlated with protein ubiquitination.

The UPS plays an important role in the different stages of the virus life cycle, including viral adsorption, viral penetration and uncoating, gene transcription, protein synthesis, assembly, and viral progeny release [16]. In our research, we demonstrated that the proteasome inhibitors decreased the number of viral copies through suppression of viral RNA transcription and protein expression at the early infection stage. These results are similar to those described for closely related viruses when the proteasome is inhibited [20, 29, 30]. Proteasome inhibitors reduce viral RNA synthesis and capsid protein expression of Coxsackie virus B3 infection [31]. Proteasome inhibitors block avian reovirus replication at an early stage in the viral life cycle, but do not affect entry and internalization [32]. For several other viruses, mouse hepatitis virus, influenza virus and herpes simplex virus, proteasome inhibitors have been shown to affect virus entry [27, 33, 34]. Vaccinia virus (VV) requires proteasome activity for the complete uncoating of the viral particle [34, 35], up to now, the mechanism by which the proteasome mediates viral uncoating occurs is unclear. To note that the particular aspects of the viral infectious cycle that influenced by proteasome inhibitors is very crucial. Nevertheless, this mechanism is different for the various viruses, so it suggested that different viruses may utilize the host UPS for various purpose, such as ubiquitin mediated degradation and/or signaling events that facilitate the establishment of a productive viral infection. Such differences in ubiquitination may also be dependent on tropism and the nature of the host cell type that is utilized in this experiment.

Ubiquitination involves the covalent conjugation of Ub to lysine residues of target proteins, regardless of viral proteins or host proteins. In this research, we found knockdown of ubiquitin markedly reduces DTMUV replication in BHK-21 cells, highlight the possibility that the DTMUV replication is modulated via protein ubiquitination. Also, Si et al. demonstrated the ubiquitination of cellular proteins is required for the effective replication of Coxsackievirus B3 [31]. In summary, ubiquitination and the UPS have been manipulated by various viruses during natural evolution to facilitate viral propagation.

In conclusion, we have shown that two proteasome inhibitors (MG132 and lactacystin) reduce DTMUV propagation, as demonstrated by decreases in the viral RNA transcription and protein expression at the early viral infection stage. Further studies are needed to focus on whether some viral proteins require ubiquitination to ensure translation and synthesis of DTMUV RNA. Such future research will aid our understanding of duck Tembusu virus biology and viral manipulation of the UPS system, which will pave the way for novel therapeutic strategies.

Disclosure of conflict of interest

None.

Acknowledgments

This work was supported by the National Key research and development program of Chian (2017YFD0500804), National Natural Science Foundation of China (No. 31502101) and Jiangsu Provincial Natural Science Foundation of China (No. BK2016006).

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