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Nervous necrosis virus capsid protein exploits nucleolar phosphoprotein Nucleophosmin (B23) function for viral replication

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ABSTRACT

Nucleolar proteins facilitate the replication of certain human and animal viruses through interaction with viral proteins. In this study, an interaction between nervous necrosis virus capsid protein and nucleolar phosphoprotein B23 was identified using *in vitro* experimental approaches. The capsid protein binds to B23 early during the viral infection and accumulates in the nucleus, particularly in the nucleolus. However, over the course of the infection B23 is redistributed from the nucleoli to the nucleoplasm. siRNA-mediated knockdown of B23 reduced viral replication and cytopathic effect. Thus, B23 targets capsid protein to the nucleus and facilitates NNV replication. The results provide the first demonstration that nucleolar protein B23 has a direct role in the nodavirus replication process.

1. Introduction

Viral nervous necrosis (VNN) disease, induced by the nervous necrosis virus (NNV), has caused mass mortality in cultured marine fish at the larval stage, resulting in significant economics losses (Munday et al., 2002; Chi et al., 2003). NNV targets the nervous system, and the characteristic pathological feature of VNN disease is vacuolation of the brain and retina (Munday and Nakai, 1997). The NNV genome is composed of two positive single-stranded RNAs that lack poly-A tails. RNA1 encodes an RNA-dependent RNA polymerase (RdRp), and RNA2 encodes the capsid protein (Tan et al., 2001). A subgenome of RNA1, named RNA3, encodes the B1 and B2 proteins (Sommerset and Nerland, 2004). The B1 protein participates in anti-necrotic cell death by reducing the mitochondrial membrane potential (MMP) loss, and thus sustains cell viability (Chen et al., 2009). The B2 protein binds to newly synthesized viral double-stranded RNA, thereby preventing host RNA interference-mediated cleavage, and during the late stages of infection B2 can induce mitochondria-mediated cell death (Fenner et al., 2006; Su et al., 2014).

Nucleolar phosphoprotein Nucleophosmin (B23) is a ubiquitously expressed shuttling protein that participates in numerous cellular activities based on the phosphorylation, acetylation, ubiquitylation, and SUMOylation of its functional domains (Okuwaki, 2008). B23 has been demonstrated to bind both DNA and RNA, interact with several factors involved in the promotion of RNA processing, and prevent protein misfolding (Okuwaki et al., 2002; Lindström and Zhang, 2006). Recent studies have also highlighted that B23 is involved in additional nonclassical roles, including cell cycle regulation, cellular stress responses, and viral replication (Boisvert et al., 2007). Many viral proteins reportedly interact with B23, and are subsequently transported to the nucleus. Examples include the core protein of Hepatitis C virus (Mai et al., 2006), replication proteins of Adeno-associated virus (Bevington et al., 2007) and matrix protein of Newcastle disease virus (Duan et al., 2014). Furthermore, previous research has shown that various types of viruses can induce functional alterations in B23 that facilitate processes involved in viral infection, such as replication of viral DNA or RNA, viral assembly, and control of intracellular trafficking (Hiscox, 2002; Hiscox, 2007; Zakaryan and Stamminger, 2011).

Although B23 has an implicated role in mediating viral infection it is currently unknown if, and through which mechanism, nodavirus recruits B23. In order to investigate this we first performed a co-immunoprecipitation assay with extracts of NNV-infected cells to determine whether NNV capsid protein interacts with B23. Next, a glutathione-S-transferase (GST) pull-down assay was used to confirm a direct interaction. Additionally, we were able to demonstrate that NNV capsid protein accumulates in the nucleus by binding to B23 early in infection and that B23 is redistributed from the nucleolus to the nucleoplasm at a later point in the infection. Further characterization revealed that knockdown of B23 reduces the cytopathic effect (CPE) and replication efficiency

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Table 1
Primers and siRNA sequences used in this study.

Primer name	Sequences (5′-3′)
Capsid-HisF	CGGGGATCCGACGATAGTCATGCCCCGCG
Capsid-HisR	CGAGCGGCCGCAAGCTTCCATGGTACGCAAAG
Capsid-GSTF	CGGGGATCCACCATG GCTAGA GGTAAACAAAAT
Capsid-GSTR	CGAGCGGCCGCATTATTGCCGACGATAGCTCT
B23-HisF	GACGACAAG GGATCCAGAAGGTGCGTCCCTGCAT
B23-HisR	GCTC GCGGCCGC-CTGACAGCGCCTCCAACAC
B23-GSTF	GACGACAAG GGATCCGAAGATTCGGATGGACA
B23-GSTR	GCTC GCGGCCGCTTAAAGAGACTTCCTCCACTGC
Capsid-RT-F	GCGCGTCGACATGGTACGCAAAGGTGA
Capsid-RT-R	GCGCGCAAGCTTTTAGTTTTCCGAGTC
B23-RT-F	TAAGGATCCTTAACCACCTTTTTCTATAC
B23-RT-R	GCCTAAGGATCCTTAGCCGGCAGCCGA
GADPH-RT-F	ATCACAGCCACACAGAAGACGG
GADPH-RT-R	CTTTCCCCACAGCCTTAGCAGC
B23-RNAi1	CAGUUUCACUAGGUGGAUUUGAGAU
B23-RNAi2	GAGCCAAAGACGAAUUACAUGUUGU
B23-RNAi3	CACCACCAUUUGUCUUGAGGUUAAA
Control siRNA	AUCUCAAAUCCACCUAGUGAAACUG

of NVV. Therefore, the interaction of B23 with NNV capsid protein may represent an important mechanism in NNV replication.

2. Materials and methods

2.1. Production of plasmids

To generate his- and GST full-length NNV capsid proteins, PCR were performed to amplified ORF of NNV capsid gene from the full-length cDNA clone of NNV using specific primers (Table 1). The PCR product was then digested with restriction enzymes and inserted into pGEX6p-1 vector. The B23 open reading frame (ORF) was amplified by PCR using the cDNA of B23 of orange-spotted grouper *Epinephelus coioides* (Genbank accession number KU904400) as the template and then inserted into the pET28a and pGEX6p-1vectors using specific primers (Table 1).

2.2. Cell lines and viruses

The GS cells (Qin et al., 2006) were derived from spleen tissue of the grouper fish, and cultured at 28 °C in Leibovitz's L15 medium supplemented with 5% fetal bovine serum (FBS). RGNNV, a genotype of NNV, was selected for further study. The RGNNV-C strain, isolated from NNV-infected grouper (Epinephelus akaara), was used in this study (Liu et al., 2012). The virus was proliferated in GS cells with a multiplicity of infection (MOI) of 10, after which infected cells were incubated at 28 °C in L-15 medium supplemented with 5% FBS. Once a complete cytopathic effect (CPE) was observed the cells were centrifuged at 12,000 x g for 10 min, then the supernatant was collected for virus titration in the GS cells.

2.3. Expression and purification of recombinant proteins

The full-length recombinant His-B23 plasmid pET28a was expressed in *E. coli* BL21 and purified following Mai et al. (2014a). The full-length recombinant plasmids pGEX6p-1-capsid and pGEX6p-1-B23 were expressed in *E. coli* BL21 and purified using standard molecular procedures. All proteins used in the experiments were determined to have at least 95% purity by SDS-PAGE and Coomassie brilliant blue R-250 staining. Bovine serum albumin was used as the standard in the measurement of protein concentrations.

2.4. Preparation of polyclonal antibody

The antibody against B23 protein was prepared in-house for this study. Purified His-B23 protein was injected intradermally as an antigen to immunize a rabbit. The rabbit received injections once every 10 days over an eight-week period. For the first injection, 30 mg of antigen was mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO). During the following three injections 30 mg of antigen was mixed with an equal volume of Freund's incomplete adjuvant (Sigma-Aldrich). Four days after the final injection, the antiserum was collected from the rabbit through exsanguination. The titer count was determined to be 1:15,000 using an enzyme-linked immunosorbent assay (ELISA).

2.5. Immunoprecipitation and immunoblotting

The GS cells were infected with NNV (MOI of 10) for 24 h, and total proteins were harvested using lysis buffer. Proteins extracted from noninfected GS cells and purified NNV were used as the controls. After centrifugation, the total proteins were incubated with anti-capsid (k233) or anti-B23 antibody for 2 h. Immunoprecipitation (IP) and Western blotting (WB) assays were conducted using an ImmunoCruz IP/WB optima F system (Santa Cruz) according to the manufacturer's instructions.

2.6. GST pull-down assay

GS cells were lysed in ice-cold lysis buffer (Takara, Shiga, Japan), after which 1 mg of cell extract was incubated with 10 μg of GST-capsid, or GST together with glutathione-Sepharose, at 4 °C for 3 h. The beads were extensively washed in lysis buffer, size-fractionated by SDS-PAGE, and immunoblotted using anti-B23 antibody. Similarly, the GST pull-down assay using GST-B23 protein and exogenous his-capsid protein was performed as described above.

2.7. Immunofluorescence imaging and analysis

GS cells were infected with NNV at an MOI of 10 and prepared for Immunofluorescence analysis at 5 hpi, 8 hpi and 12 hpi, respectively. At the indicated times, cells were washed twice with PBS, fixed with 3.7% formaldehyde for 10 min, and subjected to brief permeabilization with 0.5% Triton X-100 in PBS. The cells were blocked for 30 min in PBS containing 10% FCS and then incubated with anti-B23 and anti-capsid antibodies diluted in PBS containing 10% FCS for 1 h. Next, the cover slips were washed three times with PBS and then incubated with either Alexa Fluor 488 goat anti-mouse immunoglobulin G or Alexa Fluor 594 goat anti-rabbit immunoglobulin G antibody (Invitrogen, Waltham, MA), as appropriate for the primary antibody used, for 1 h. The cells were counterstained with DAPI to detect nuclei and imaged using a confocal laser scanning microscopy, TCS SP8 (Leica, Wetzlar, Germany).

2.8. B23 RNA knockdown and virus infection

B23 short-interfering RNAs (B23-siRNAs), negative siRNA control (Cat. no. 12935-400) and transfection reagents were purchased from Invitrogen. The siRNA sequences were listed in Table 1. To study the effect of B23 knockdown on the replication of NNV, GS cells were transfected with B23 siRNA for 24 h and then infected with NNV(MOI = 10). The cultured cells were collected at five time-points (6, 12, 24, 48, 72 hpi), and virus titers were determined based on 50% tissue culture infective dose (TCID50) in GS cells. Total cellular RNA was extracted for Real-time reverse transcription (RT)-PCR analysis of the expression of NNV genes.