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The key molecular events during *Macrobrachium rosenbergii* nodavirus (MrNV) infection and replication in Sf9 insect cells

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ABSTRACT

In this study we demonstrated that *Macrobrachium rosenbergii* nodavirus (MrNV) was able to internalize and replicate in Sf9 insect cells, with levels of infection altered by substances affecting the caveolin-(CAV) mediated endocytosis pathway. The use of Sf9 cells for efficient MrNV replication and propagation was demonstrated by confocal microscopy and PCR amplification, through which early viral binding and internalization were initially detectable at 30 min post-infection; whereas at 72 h, the distinguishable sign of late-MrNV infection was observable as the gradual accumulation of a cytopathic effect (CPE) in the cells, ultimately resulting in cellular disruption. Moreover, during the early period of infection, the MrNV signals were highly co-localized with CAV1 signals of the CAV-mediated endocytosis pathway. The use of genistein as an inhibitor of the CAV-mediated endocytosis pathway significantly reduced MrNV and CAV1 co-localization, and also reduced the levels of MrNV infection in Sf9 cells as shown by PCR and ELISA. Moreover, the addition of the pathway agonist okadaic acid not only recovered but also augmented both the levels of MrNV co-localization with CAV1 and of Sf9 infection in the presence of genistein inhibition; therefore demonstrating that MrNV infection in Sf9 cells was associated with the CAV-mediated endocytosis pathway machinery.

1. Introduction

Macrobrachium rosenbergii nodavirus (MrNV) is one of the known causative agents of white tail disease (WTD) in the giant freshwater prawn *Macrobrachium rosenbergii*. Early WTD can be observed as the appearance of a whitish coloration in the muscles of post-larvae (PL) prawns. The later stages of the disease manifests as a sudden, 100% rate of mortality of PL in the rearing ponds, resulting in extensive damage to closed-hatchery giant freshwater prawn aquaculture (Azad et al., 2005; Bonami et al., 2005). Regarding the causative virus, MrNV is a 30 nm, non-enveloped icosahedral virus containing a linear bipartite positive-sense single-stranded RNA (ssRNA+) genome. Similar to other nodaviruses, MrNV has been shown to infect a broad spectrum of cells and organisms, ranging from mosquito cell cultures (C6/36) and aquatic insects to other types of prawn such as *Penaeus monodon* and *Litopenaeus vannamei* (Hayakijkosol and Owens, 2013; Sahul Hameed et al., 2004; Sudhakaran et al., 2008; Sudhakaran et al., 2007b).

The detection of MrNV infection in susceptible organisms has been performed through conventional techniques such as polymerase chain reaction (PCR) and immuno-detection (Pillai et al., 2006; Romestand and Bonami, 2003). Nevertheless, studies into the actual process of viral entry and infection at the intracellular level have been constrained by difficulties in maintaining stable primary cell cultures of aquatic organisms (Lu et al., 1995). Furthermore, these difficulties have been compounded by the lack of reliable continuous crustacean cell lines that would otherwise permit the long-term observations required for studying MrNV infection and replication pathways. Recent studies utilizing continuous mosquito cell lines have somewhat useful in studying MrNV infection and propagation; nevertheless, the maintenance of this particular cell line for long-term culture still proved problematic. To overcome the aforementioned difficulties, we have demonstrated the susceptibility of an insect cell line, Sf9, to MrNV infection; in addition, demonstrated the use of the cell line to facilitate in-depth studies of viral infection pathway.

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In general, the early phase of the infection pathway of non-enveloped viruses involves the receptor-based attachment of viral particles to the targeted cell surface, followed by the internalization of the virus into the cytoplasm. The viral entry of alpha-nodaviruses, in particular the entry of the flock house virus (FHV) into *Drosophila melanogaster* culture cells, serves as a useful starting model for further insightful investigations into the mechanism of entry of other members of the nodavirus family (Odegard et al., 2010), (Walukiewicz et al., 2006). When compared to FHV, however, limited information is available regarding the early process of MrNV infection in its natural hosts or in susceptible insect cell cultures. Given the similar icosahedral structures of both viruses, it is therefore tempting to assume that MrNV follows a similar process of endosome-dependent host-cell entry as that of its nodavirus counterparts and of non-enveloped viruses that infect mammalian cells. Small ssRNA viruses such as the picornaviruses have been extensively reported to interchangeably utilize clathrin (CLA)- and caveolin (CAV)-mediated endocytosis, whereas other similar viruses such as the poliovirus have been found to not be dependent on either pathway (Brandenburg et al., 2007; Hogle, 2002). Accordingly, further in-depth studies into the process of entry and trafficking of MrNV is required. For those purposes, the selection of host cells that reliably demonstrates susceptibility to viral infection, replication, propagation as well as permit long-term viral pathway studies would therefore be crucial.

In this study, we demonstrated the ability of MrNV to internalize and replicate in the Sf9 insect cells, the former event of which appeared to favor CAV-dependent endocytosis while the latter event was evident by a distinct cytopathic effect (CPE) and a rapid increase in virus copy number in the infected Sf9 cells. Furthermore, early MrNV infection period that is believed to be associated with CAV-mediated endocytosis is proven by inhibiting or re-activating the pathway by genistein and okadaic acid, the substances that are extensively use for studying viral infection via tyrosine-phosphate dependent caveolin-internalization pathway.

2. Materials and methods

2.1. MrNV inoculum preparation

Macrobrachium rosenbergii nodavirus (MrNV) was isolated from MrNV- infected post-larvae that were identified by the whitish discoloration of their tail muscles. The MrNV inoculum was prepared as described previously (Ravi et al., 2010). Briefly, infected tissues of *M. rosenbergii* larvae were minced and homogenized in sterile phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The crude sample was then centrifuged (400*g*, 4 °C, 10 min) to eliminate crude debris. The collected supernatant was further centrifuged (12,000*g*, 4 °C, 30 min) and subsequently passed through 0.22 μm filters. The inoculum obtained was then added immediately to Sf9 cultured cells in the virus infection experiments described below.

2.2. Sf9 cell culture and MrNV infection experiments

Sf9 cells were cultured with serum-free medium (SF-900 III SFM) containing 1× antibiotic-antimycotic (Gibco, Grand Ireland, NY) in 25 ml flasks at 27 °C, with the medium replaced every two days. After reaching >90% confluence, the cells were dislodged and sub-cultured on 6-well plates at a concentration of 2×10^6 cells/well and were further cultured for another day. The cells were pre-chilled at 4 °C, and the inoculum containing MrNV was added into the medium at a final concentration of 40 μg/ml followed by continuous, gentle shaking. After incubation (4 °C, 1 h), the excess MrNV inoculum solution was removed and replaced with new medium and incubated further at various time points: 30 min, 2hr and 72 h. To observe cell viability, cells were stained with trypan blue and observed with light microscopy. The other portion of cells was harvested to perform either protein/RNA extractions or indirect immunofluorescent staining (IIF).

Table 1
Primer pairs used in this study.

Gene	Primer sequence
MrNV (RNA2)	(F) 5′- CCATGGCTAGAGGTAAACAAAATTCTA-3′
	(R) 5′- CTCGAGCTAATGATGATGATGATGATG-3′
Sf9 actin	(F) 5′- AGAAGATCTGGCACCACACC-3′
	(R) 5′- GTCATCTTCTCTCTGTTGGCCTT-3′

2.3. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Sf9 cells were collected and homogenized in Isol-RNA lysis reagent (5-PRIME Inc., Gaithersburg, MD) following the manufacturer's protocols to obtain purified total RNA. The RNA samples were treated with RNase-free diethylpyrocarbonate (DEPC) water and subjected to RT-PCR using a Superscript III One Step RT-PCR kit (Invitrogen, Eugene, CA). The PCR amplification was run for 40 cycles as follows: preheated at 95 °C for 5 min followed by cycles of 95 °C for 60 s; 60 °C for 30 s and 72 °C for 60 s. MrNV capsid protein-specific primers (Table 1) were designed from the nucleotide sequences available in the GenBank database (accession # JQ418298). Amplification of Sf9 actin (an internal control, Table 1) was conducted in the separated PCR reaction tubes using the same PCR conditions described above. The obtaining PCR products of MrNV and actin were mixed for co-electrophoresis. The PCR bands were then visualized by ethidium bromide staining. Comparisons of RNA expression levels were performed by densitometric analyses using Image J software (Schneider et al., 2012).

2.4. Indirect immunofluorescence (IIF) and confocal microscopy

The harvested Sf9 culture cells were fixed with 4% paraformaldehyde (30 min, room temperature) and washed with PBS. Free aldehydes were quenched with 30 mM glycine in PBS and cells were then washed twice with PBS containing 0.2% Tween 20 (PBS-T). Non-specific antibody staining was blocked with 4% bovine serum albumin (BSA) and the cells were exposed to 1:500 mouse anti-MrNV antibody in blocking solution (0.5% BSA in PBS-T). This monoclonal antibody (a kind gift from Prof. Paisan Sithigorngul, Srinakarinwirot University, Thailand) has been shown to possess high specificity towards the MrNV capsid protein (Longyant et al., 2012; Wangman et al., 2012). The cells were also exposed to monoclonal antibodies against caveolin-1 (CAV1) and the clathrin (CLA) heavy chain (Santa Cruz Biotechnology Inc., Dallas, TX) at a concentration of 1–2 μg/ml. Thereafter, cells were further incubated with goat anti-mouse Alexa 594 or Alexa 488 (Invitrogen, Carlsbad, CA) at a dilution of 1:1000 and were counterstained with either DiI (1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; $DiIC_{18}(3)$) or Oregon Green 488-conjugated 1,2-dihexadecanoyl-sn-*glycero*-3 phosphoethanolamine (DHPE; 1:5000) for labeling membrane-bound organelles, and with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclear staining.

For confocal microscopy, the cells were plated on glass slides and topped with coverslips, and the images were acquired by an Olympus FV1000 confocal microscope. The samples were excited by argon and krypton laser lines and the fluorescent emissions were screened by 520 nm and 590 nm band-pass filters. A line-by-line