

Characterization of the nonstructural protein NS80 of grass carp reovirus

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Abstract Nonstructural proteins of members of the family *Reoviridae* are believed to play significant roles in the virus replication cycle. Phylogenetic analyses indicate that the nonstructural protein NS80 of grass carp reovirus, encoded by a gene of Segment 4 (S4), is a primary determinant that is related to the formation of viroplasmic inclusion bodies (VIB), where viral replication and assembly are thought to occur. To understand the role of the NS80 protein in viral replication, an initial investigation of NS80 gene expression in both infected and transfected cells was conducted. Transmission electron microscopy results indicate that replication and assembly of GCRV occur within VIB-like structures in the perinuclear region of the cell cytoplasm. Furthermore, expression of the S4 gene in infected cells was detected with an NS80-specific antibody by western blot and immunofluorescence. Moreover, globular VIB-like structures were observed when expressing GFP-derived full-length NS80 (pEGFP-C1/NS80) and recombinants containing the C-terminal conserved region (pEGFP-C1/NS80_{335–724}) in transfected Vero. No such structures were detected in cells transfected with an N-terminal recombinant (pEGFP-C1/NS80_{1–334}), suggesting that the NS80 C-terminal conserved region may be involved in the formation of inclusion structures. These data provide a foundation for further functional studies of NS80 related to viral inclusion formation in viral replication.

Introduction

All members of the family *Reoviridae* possess a segmented double-stranded RNA (dsRNA) genome encased in multiple concentric protein capsids. They are non-enveloped, icosahedral virions with a double capsid structure, consisting of a genome comprised of 9–12 segments of dsRNA in the core. During replication and assembly of these viruses, viroplasmic inclusion bodies (VIB), or viral factories (VF), are formed throughout the cytoplasm of infected cells [7, 8, 11, 12, 32], suggesting that VIB-like structures are essential for the viral replication cycle. These VIB-like structures have a peculiarly dense consistency that distinguishes them from the adjacent cytoplasm and causes them to appear highly refractile when viewed by phase-contrast microscopy, and they contain fully or partially assembled viral particles, viral proteins, dsRNA and microtubules [4–7, 20, 23, 26]. Recent studies on mammalian orthoreoviruses (MRV) and avian orthoreoviruses (ARV) as well as other genera in the family *Reoviridae*, such as rotaviruses and orbiviruses [8, 12], have shown that the orthoreovirus nonstructural protein μ NS plays a major role in the formation of cytoplasmic inclusion structures.

The family *Reoviridae* currently includes 15 recognized genera of viruses [3, 22], including the genus *Aquareovirus*. Six aquareovirus species have been classified by the International Committee on Taxonomy of Viruses (ICTV), from *Aquareovirus A* (AQRV-A) to *Aquareovirus F* (AQRV-F) [3, 21], and an additional, seventh species, named *Aquareovirus G*, has also been recognized recently [24]. Like turreted MRV and other members of the family *Reoviridae*, GCRV, which forms a multilayered spherically structured particle, has a genome of 11 dsRNA segments, which encode 7 structural proteins (VP1–VP7) and 5 nonstructural proteins. The mature viral particle consists of

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five core proteins and two outer capsid proteins, while the five nonstructural proteins (NS80, NS38, NS31, NS26, NS16) may play roles in the viral replication cycle [2]. Unlike the nonfusogenic MRV, GCRV can produce large syncytia as a typical cytopathic effect (CPE) in *Ctenopharyngodon idellus* kidney (CIK) cell lines during infection, and mature virions are efficiently released from infected cells by lysis [14]. Studies on GCRV and other aquareovirus genomes have revealed that there is a close evolutionary relationship between members of the genera *Orthoreovirus* and *Aquareovirus* [2, 15]. Recent progress on structural reconstruction of single virus particles has further confirmed this high level of identity at the protein level [9, 10, 17], especially for the core proteins, including the functional and enzymatic domains, which are involved in maintaining the stability of the inner core shell and endogenous transcription activity among dsRNA viruses in general. Despite the significant divergence that exists between GCRV and MRV in the outer capsid protein components, the functional domains related to cell entry remain conserved. In addition to the structural proteins, non-structural proteins are believed to be indispensable for viral replication and assembly of the nascent particle. For example, NS80, which has been shown to play a key role in the formation of factory-like inclusion structures, is critical for the viral replication cycle.

Substantial progress has been made in clarifying non-structural protein function in viral replication for members of several genera in the family *Reoviridae*, including *Orthoreovirus*, *Rotavirus*, *Orbivirus* and *Phytoreovirus* [1, 4–8, 11, 18, 20, 22, 23, 29]. In MRV and ARV, the nonstructural protein μ NS expressed alone in transfected cells forms VIB-like structures that, when viewed by phase-contrast microscopy, appear to be similar to VF formed in infected cells [25], suggesting that μ NS is able to form the matrix of the factories. As a step toward understanding the molecular basis of viral factory formation in viral replication and assembly, an investigation of NS80 gene expression in both infected cells and transfected cells was carried out in this study. Ultrathin-section images obtained by transmission electron microscopy (TEM) showed that replication and assembly of GCRV took place within VIB-like structures in the perinuclear region of the cytoplasm. Additionally, expression of the NS80 gene in both infected and transfected cells was detected with specific polyclonal NS80 antibodies by both western blot (WB) and immunofluorescence (IF). Furthermore, inclusion-like structures were observed in Vero cells transfected with recombinant plasmids encoding GFP-fused, full-length NS80 (pEGFP-C1/NS80) and the conserved carboxyl-terminal region (pEGFP-C1/NS80_{335–724}). The results provide a foundation for further functional analysis of the NS80 protein.

Materials and methods

Cells and virus

A CIK cell line was prepared for the GCRV infection assays in this study. The cells were grown at 28°C in monolayers in Eagle's minimum essential medium (MEM, Invitrogen) containing 2 mM L-glutamine, supplemented with 10% of fetal bovine serum (MEM-10). Vero cells used for the transfection assay were grown at 37°C in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum, and 2 mM L-glutamine. Both penicillin (100 U/ml) and streptomycin (100 μ g/ml) antibiotics were added to the MEM and DMEM medium during cell cultivation. GCRV-873, the original isolate of GCRV, grown in CIK monolayer cells as described previously [14, 19], was used in this study.

Construction of recombinant plasmids

To construct a recombinant plasmid containing the NS80 gene, RT-PCR was performed by using the purified whole GCRV genome as a template to amplify the NS80 gene encoded on GCRV Segment 4 (S4). The primers targeting GCRV S4 were designed based on GenBank sequence AF403390 and contained specific restriction enzyme digestion sites. The recombinant plasmid expressing the C-terminal region of NS80 in *E. coli* was named pR/NS80_{335–742} and has been described previously [13]. To express the whole NS80 protein, the full-length S4 gene was cloned into the pRSET-A vector (Invitrogen, Carlsbad, USA) in this study, and this clone was named pR/NS80. The primer pair used for amplification of the full-length S4 gene was as follows: sense primer 5'CATGGATCCAA GAGCTTTGGAAGTAAC3' (*Bam*HI site underlined), and anti-sense primer 5'GCTGAATTCGACACAGAAACAC AGAGC3' (*Eco*RI site underlined). The remaining cloning steps are the same as reported previously [13]. Recombinant plasmids that contained the gene of interest were sequenced by Invitrogen Biotechnology Inc. (Shanghai, China).

To generate constructs for expression of green fluorescence protein (GFP) fused to the N terminus, three primer pairs (GCRV-S4-SN1/AS2, GCRV-S4-SN1/AS1, GCRV-S4-SN2/AS2), including forward primer 1 (GCRV-S4-SN1) 5'ATGAATTCGAAGAGCTTTGGAAGTAAC3' (*Eco*RI site underlined), forward primer 2 (GCRV-S4-SN2) 5'GAGAATTCCTCCCTCCTTACCCTTC3' (*Eco*RI site underlined), reverse primer1 (GCRV-S4-AS1):5'CAC GGATCCGATGTCTTCGGTGGGAGC3' (*Bam*HI site underlined), and reverse primer 2 (GCRV-S4-AS2) 5'AGGATCCGACACAGAAACACAGAGC3' (*Bam*HI site

underlined), were designed for RT-PCR amplification to generate full-length S4, an S4 N-terminal fragment, and an S4 C-terminal fragment, respectively. The amplified fragments were cloned into a pEGFP-C1 vector (Clontech) that had been cut with *EcoRI* and *BamHI* enzymes to generate pEGFP-C1/NS80, pEGFP-C1/NS80_{1–334} and pEGFP-C1/NS80_{335–742} recombinants. *E. coli* DH5 α cells were then transformed with the ligated products for further identification. The correctness of each construct was confirmed by sequencing (Invitrogen Biotechnology Inc., Shanghai, China).

Expression of recombinant NS80 in *E. coli* and preparation of antibody

To express recombinant NS80 in *E. coli*, BL21(DE3) cells were transformed with the pR/NS80 construct, and in vitro expression and purification of NS80 were conducted as described previously [13]. Antibodies against the recombinant NS80 protein were prepared by injecting New Zealand white rabbits subcutaneously with a mixed emulsion of 200 μ l of purified recombinant NS80 solution (about 0.5 μ g/ μ l) and an equal volume of Freund's complete adjuvant (FCA), followed by two intramuscular booster injections with an equal volume every 2 weeks. Three days later, after a final booster injection, the rabbits were bled, and sera were separated, aliquoted and stored at -80°C for further use. The antibody titer was determined by ELISA [33].

Virus replication and transmission electron microscopy examination

Virus infection of CIK cells was conducted as described elsewhere [14, 19]. When a CPE was observed in virus-infected cells, the culture supernatant of the lysed cells was harvested. The collected GCRV culture suspension was then stored at -30°C or at 4°C prior to use. For sequential analysis of GCRV replication in CIK cells, infected monolayer cells were collected at different intervals post-infection (p.i.), collected by centrifugation at 12,000 rpm for 10 min, and then stored at -30°C for further immunoblotting analysis. For ultrathin section sample preparation, harvested cells were fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 24 h at 4°C . Mock-infected CIK cell monolayers were used as a control.

GCRV particles harvested from infected culture supernatant were purified by either sucrose gradient centrifugation or CsCl density gradient centrifugation as described elsewhere [16, 17]. For TEM examination of purified virus, virion samples were deposited onto formvar-coated, carbon-stabilized copper grids (200-mesh) before they were

negatively stained with 3% (w/v) phosphotungstic acid (PTA, pH 6.8). For ultrathin section samples, GCRV-infected CIK cells pre-fixed with 2.5% paraformaldehyde were sliced, mounted on 200-mesh 0.25% formvar-coated copper grids, and double stained with 2% uranyl acetate and lead citrate. All sample grids were examined using a transmission electron microscope (Hitachi 7000-FA).

Infection and IF microscopy

To detect in vivo-expressed NS80 protein by IF, CIK cells were seeded on the day before infection at a density of 1.0×10^4 cells/cm² in six-well plates (9.6 cm² per well) containing glass coverslips. GCRV873, a third-passage infected CIK cell lysate stock of plaque-purified suspension, was used as the virus strain [14]. A virus stock at an MOI of 10–20 PFU/cell was inoculated onto confluent monolayers of CIK. After about 30–60 min of viral adsorption, cells were then washed with phosphate-buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.5) and further incubated at 28°C for maintenance in Eagle's MEM with 2% of fetal bovine serum (MEM-2) before being processed for subsequent experiments. The infected monolayer cells were collected at 6-h intervals beginning from 0 to 24 h p.i. and fixed for 10 min at -20°C in 100% methanol and then permeabilized, blocked, incubated with antibodies, and mounted as described previously [26]. Primary NS80 specific antibody was diluted in PBST and incubated with cells for 30–60 min at room temperature. After washing three times with PBS, secondary fluorescein isothiocyanate (FITC)-tagged antibodies diluted in PBST were added and incubated with cells for 30 min at room temperature. Samples were examined using an Olympus-IX51 inverted microscope equipped with phase and fluorescence optics, and images were collected digitally. All of the images were processed and prepared for presentation by using Image Pro-Express 6.3 (Olympus) and Photoshop (Adobe Systems).

Transient expression of recombinant NS80 proteins

After confirming the correct orientation of the NS80 gene in the pEGFP vector by both restriction analysis and nucleotide sequencing, the pEGFP-C1/NS80, pEGFP-C1/NS80_{1–334} and pEGFP-C1/NS80_{335–742} recombinants were purified using a QIAfilter Plasmid Maxi Kit (QIAGEN) and used to transfect Vero cells according to the transfection kit user manual (Lipofectamine 2000, Invitrogen). Briefly, 1 day before transfection, 0.5×10^5 to 2×10^5 cells were cultured in six-well plates growing in DMEM medium without antibiotics so that cells were 80–85% confluent at the time of transfection. About 4.0 μ g of plasmid DNA per

well was diluted in 250 μ l DMEM and then mixed with 10 μ l of Lipofectamine per well, diluted with the same medium. After a 20-min incubation, the mixtures were added to each well containing cells and medium. Cells were incubated at 37°C, and the medium was changed at 6 h post-transfection (pt). Transfected cells were observed around 18–24 h pt. GFP fusion proteins were detected by their intrinsic GFP fluorescence. Cell images were visualized using an Olympus-IX51 inverted microscope. In addition, the transfected cells were collected and lysed for further immunoblotting as described elsewhere [31].

SDS-PAGE and immunoblotting analysis

To analyze viral proteins expressed in infected cells, the collected cell lysates were pelleted. The pelleted cells were resuspended in 60 μ l of 1 \times PBS, lysed in sample buffer, boiled for 2 min, and subjected to SDS-PAGE. All protein samples were detected by staining with Coomassie brilliant blue R-250 (Sigma) or were transferred to a polyvinylidene fluoride (PVDF) transfer membrane using a semi-dry transfer cell (Bio-Rad) and visualized by western blotting. NS80-specific antisera (1:500) or mouse monoclonal GFP antibody (1:1,000) were employed as primary antibodies. An alkaline-phosphatase-coupled goat anti-rabbit IgG (Sigma) or rabbit anti-mouse was used as the secondary antibody at a dilution of 1:2,000. The result was observed by developing with NBT/BCIP AP substrate solution (Promega) according to the manufacturer's instructions.

Sequence comparisons

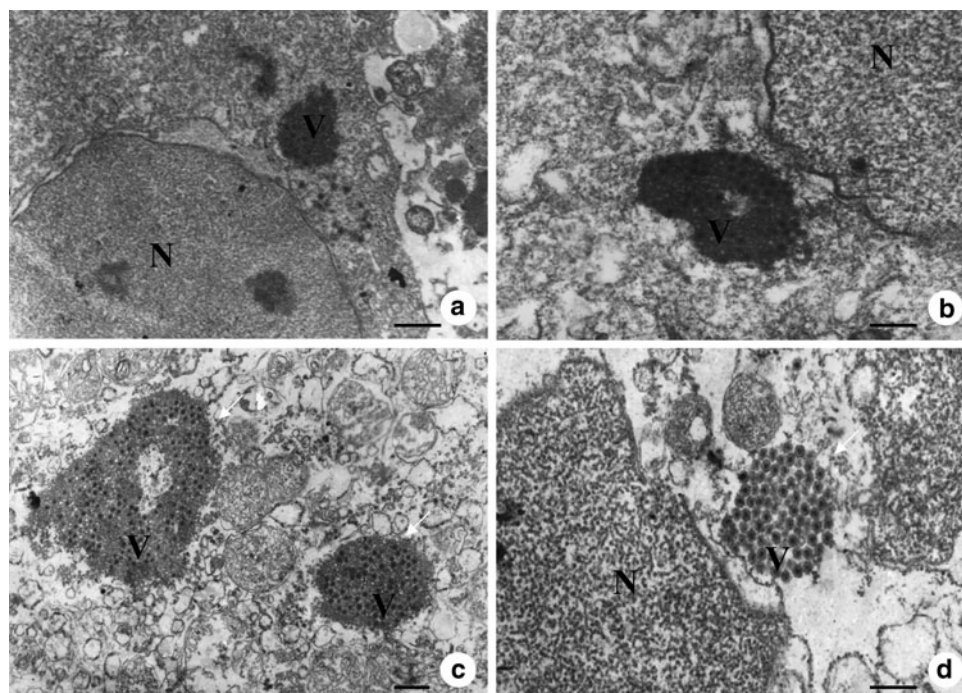
To determine conserved domains, GCRV NS80 (accession no. AF403390) was compared with the following homologous sequences: NS1 of chum salmon reovirus (aquareovirus C, CSRV, accession no. NC_007585), and μ NS of mammalian orthoreovirus T1L (accession no. AF174382), μ NS of avian orthoreovirus S1133 (accession no. AY608700). Data were analyzed by Protein Blast in NCBI (<http://blast.ncbi.nlm.nih.gov>).

Results

Viroplasmic inclusions observed during virus replication

To examine GCRV morphogenesis during replication, ultrathin-section images of virus-infected cells were viewed by TEM. As shown in Fig. 1, during GCRV replication, virus particles were found in interior regions called viroplasmic inclusions, which are not bound by a cellular membrane but are distinct from the surrounding cytoplasm. Early virus particles (about 4–8 h p.i.) of different sizes (about 35–55 nm in diameter) were observed within the dense viroplasms, as shown in Fig. 1a and b. As the infection progressed, the size and number of viroplasmic inclusions increased (Fig. 1c). Generally, inclusions were typically concentrated in the perinuclear region of the cytoplasm. At the time of late infection (18 h), mature

Fig. 1 Morphogenesis of GCRV in an infected cell. **a, b** Viroplasm (V) is formed near the nucleus (N) in the cytoplasm at 4 and 8 h p.i. **c** Extended viroplasm observed at 12 h p.i. **d** Mature virions formed within the viroplasm at 18 h in late infection. Scale bars 500 nm



virions were formed with an overall size of about 70–80 nm (Fig. 1d). Eventually, progeny virions can lead to the breakdown of infected cells so that mature particles are released efficiently into the cell culture supernatant to initiate the next cycle of replication. It appeared that once assembly had been achieved, the dense viroplasmic inclusions become thin and the mature virion was formed, indicating that virus replication and assembly occurred within the viroplasm-like inclusions (Fig. 1d).

Expression of full-length NS80 in *E. coli*

Protein NS80, which is encoded by the GCRV S4 gene, consists of 742 amino acids (about 80 kDa). When BLAST analysis was used to compare the NS80 analogue with that of CSRV in the genus *Aquareovirus* and MRV and ARV in the genus *Orthoreovirus*, identities could be found mostly within the C-terminal region of the protein (Fig. 2a). Specially, the proteins share two coiled-coil regions (513–548 and 615–700AA) in their carboxyl-proximal region, which have been shown to be important in inclusion formation by MRV [7]. Based on this analysis, to generate specific antiserum of NS80, the C-terminal region of NS80 recombinant (pR/NS80_{335–742}) was first constructed as described previously [13].

In this study, we constructed a full-length NS80 recombinant, named pR/NS80. As shown in Fig. 2b, the full-length NS80 recombinant was successfully expressed in *E. coli*, which produced a major protein of approximately 83 kDa (including an N-terminal tag of about 3 kDa) that was not present in the empty vector cell lysate, but the expression level was somewhat lower than that of NS80_{335–742}. The identity of this protein was further confirmed by immunoassays using a his-tag antibody and previously prepared NS80_{335–742} polyclonal antiserum [13]. Immunoblotting showed that the expressed NS80 cell lysate and purified NS80 protein (SDS-PAGE data not shown) were able to bind to an NS80_{335–742}-specific rabbit antibody (Fig. 2c), indicating correct expression of recombinant NS80 protein. In addition, the product of the S4 gene expressed in GCRV infected cells cross-reacted with the NS80_{335–742} antibody, suggesting that expression of the NS80 gene could be detected in GCRV-infected cells by using the prepared specific NS80_{335–742} antibody. At the same time, a small protein (about 70 kDa) was also detected with the specific antibody. To evaluate the titers of the prepared antibodies, both NS80 and NS80_{335–742} antisera were examined by ELISA (data not shown). Both of the antibodies were used in subsequent immunoassays.

NS80 expression detected in GCRV-infected cells

As reported previously, GCRV can produce large syncytia as a typical CPE when a sensitive cell line is infected [14],

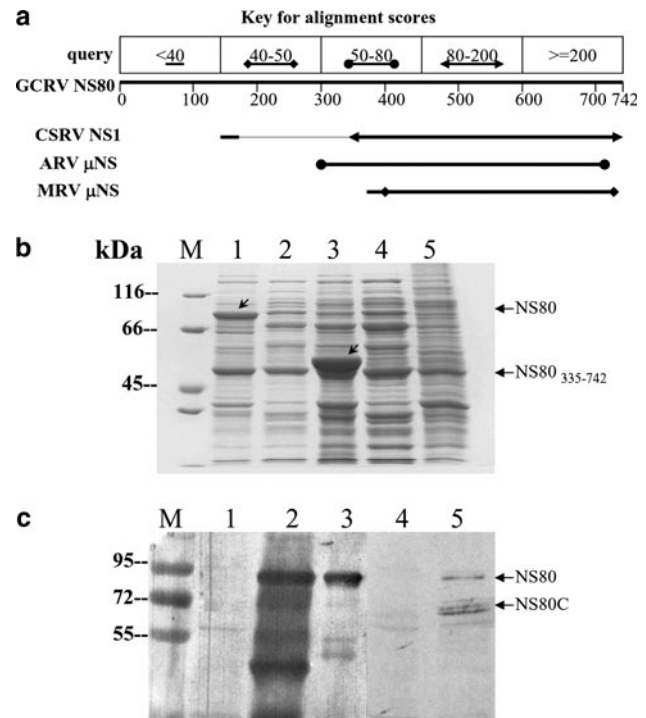


Fig. 2 Alignment of GCRV NS80 with analogous proteins and expression of NS80 protein. **a** Alignment of GCRV NS80 with analogous proteins of CSRV, ARV and MRV (see “Materials and methods” for accession numbers). The alignment shows that the C-terminal region of NS80 has a high alignment score with CSRV, ARV and MRV, and this region is conserved among the different viruses. **b** SDS-PAGE of GCRV NS80 expressed in *E. coli*. *M* standard protein marker; Lanes 1 and 2 expressed NS80 protein cell lysate pellet and supernatant induced 3 h by IPTG, respectively. Lanes 3 and 4 expressed NS80_{335–742} protein cell lysate pellet and supernatant induced 3 h by IPTG, respectively. Lane 5 cell lysate pellet of expressed empty vector (pRSET-A) induced 3 h by IPTG. **c** Western blot assay using NS80_{335–742} polyclonal antibody. *M* pre-stained standard protein marker. 1 cell lysate of expressed empty vector. 2 and 3 expressed NS80 cell lysate and purified NS80. 4 mock-infected CIK cell, 5 GCRV-infected CIK cell lysate at 24 h p.i.

in contrast to nonfosogenic MRV. To investigate whether GCRV replication is related to NS80 gene expression in infected cells, both immunoblotting and indirect IF were conducted (Figs. 3, 4). SDS-PAGE and western blot analysis (Fig. 3a, b) showed that expression of the S4 gene can be detected, resulting in a protein with a molecular weight around 80 kDa at 6, 12, 18 h p.i., suggesting that it is the full-length NS80. However, a shorter polypeptide (about 70 kDa) was also detected in all of the infected cell lysate samples (Fig. 2c, lane 5, Fig. 3b, lanes 2–5), which might be truncated NS80 or a degraded form of full-length NS80 (see “Discussion”). Furthermore, high-level expression of NS80 with inclusion-like structures was observed at 12 and 18 h p.i. by phase-contrast and IF microscopy (Fig. 4). NS80 could also be detected at 24 h p.i. in infected cell lysates by western blot (Fig. 2c, lane 5), but it was not

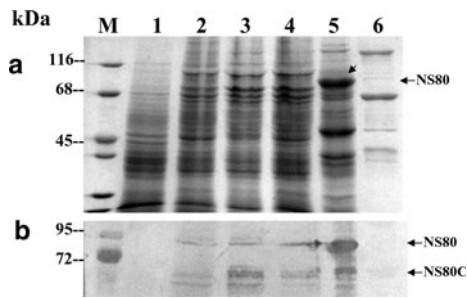


Fig. 3 SDS-PAGE and immunoblotting analysis of NS80 expression in infected cells. **a** SDS-PAGE analysis of NS80 expression in infected cells at 0, 6, 12, and 18 h p.i. *M* standard protein marker; 1–4 GCRV-infected CIK cells at 0, 6, 12, and 18 h p.i. 5 Expressed NS80 in *E. coli* cell lysate (*arrowhead*). 6 Purified GCRV virion. **b** NS80 protein was detected in infected cells by immunoblotting analysis with protein samples corresponding to those in **a** with NS80_{335–742} antibody, Lanes 1–6 in **b** correspond to lanes 1–6 in **a**

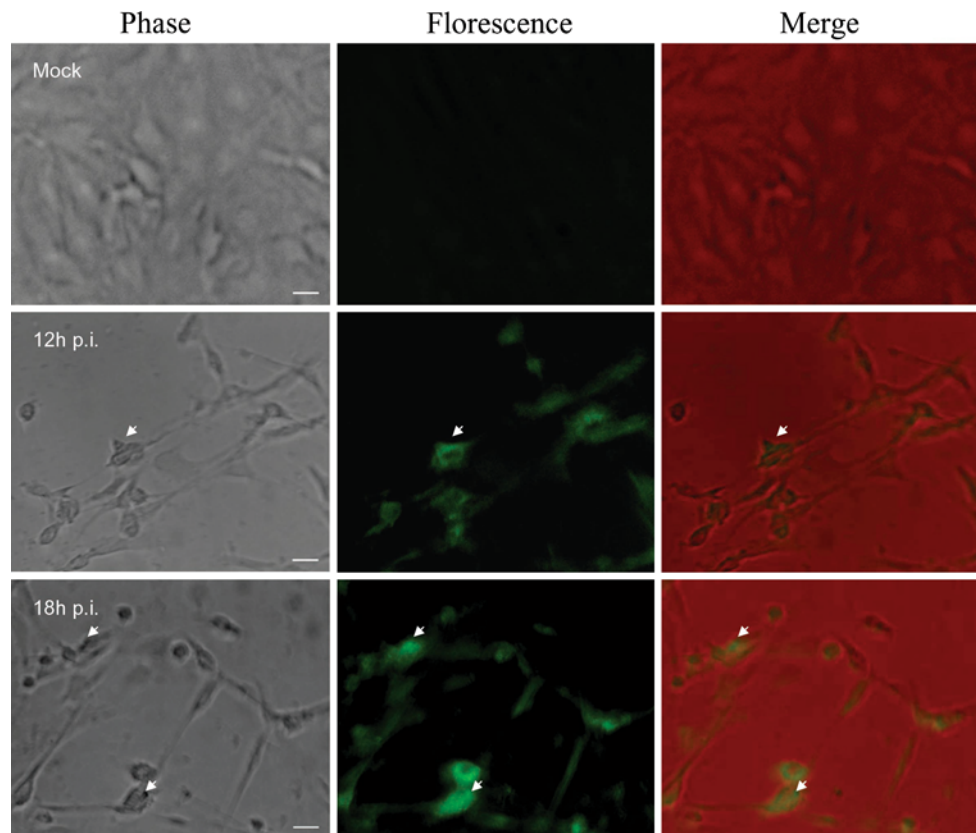
clearly detected by IF at 24 h p.i. because most of the nascent virions had been released from cells at this point. No NS80 protein could be detected in mock-infected cells or purified virus samples (Fig. 3, lanes 1, and 6).

Inclusions formed in NS80-transfected cells

By analogy to μ NS of MRV, GCRV NS80 is thought to be a key protein in the formation of viral inclusions. To test

whether the NS80 protein alone (i.e., in the absence of any other viral components in transfected cells) is able to form inclusions, Vero cells were lipo-transfected with three GFP-tagged recombinants, pEGFP-C1/NS80, pEGFP-C1/NS80_{1–334} and pEGFP-C1/NS80_{335–742}. As predicted, the recombinant full-length GFP/NS80 and GFP/NS80_{335–742} C-terminal proteins were able to form factory-like inclusions in transfected cells. Globular inclusion-like structures were observed around 18–24 h p.t due to their intrinsic green fluorescence (Fig. 5a), and these appeared similar to those formed by μ NS in MRV-infected cells [7, 22], whereas no inclusion structures were detected in cells transfected with the GFP-tagged N-terminal conserved region recombinant (pEGFP-C1/NS80_{1–334}). Likewise, green fluorescence from transfected empty EGFP-C1 was distributed throughout the cytoplasm and nucleus. To determine the correctness of the expressed recombinant protein, all of the cell lysates were subjected to SDS-PAGE, followed by immunoblotting with both GFP-specific mouse MAb and rabbit anti-NS80 serum. The results verified that the expressed GFP-derived fusion proteins from each construct were not only the right size but also produced a serological reaction with NS80 polyclonal antibody (Fig. 5b, c), indicating that the protein was expressed efficiently. This result suggests that NS80 or the C-terminal region alone is able to form factories or inclusion-like structures in transfected cells.

Fig. 4 Immunofluorescence analysis of NS80 expression in infected cells. CIK cells were infected with GCRV at a multiplicity of infection of 10 PFU/cell. Infected cells were fixed at 12 and 18 h p.i. and immunostained with rabbit anti-NS80 serum followed by goat anti-rabbit IgG conjugated to FITC. Mock-infected cells are shown in the *upper panels*, and cells infected with virus are shown in the *middle and bottom panels*. *Left, middle columns* are phase and fluorescence images, respectively. Merged images are shown in the *right column*. *Arrows* indicate inclusion-like structures. *Scale bars* 10 μ m



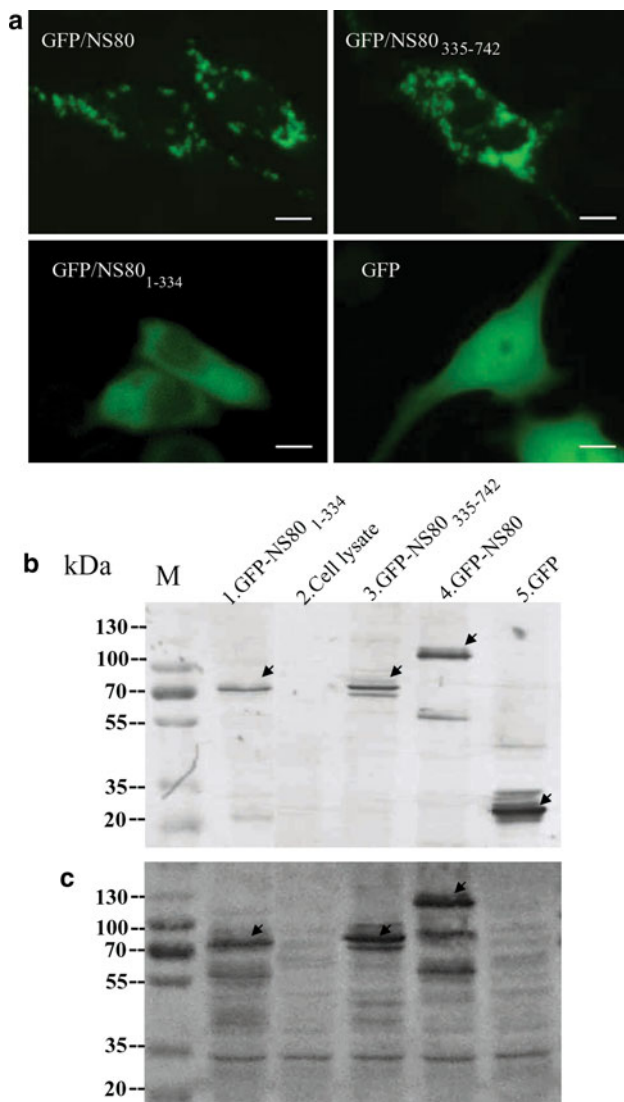


Fig. 5 Fluorescence microscopy and Immunoblotting of GFP-tagged NS80 derivatives. Vero cells were transfected with plasmids to express GFP-tagged NS80, NS80₁₋₃₃₄ and NS80₃₃₅₋₇₄₂ fusion protein and then analyzed at 20 h p.t. by using fluorescence microscopy and western blotting. **a** Observation of expressed recombinant protein by fluorescence microscopy. A viral inclusion-like structure can be detected in GFP-NS80- and GFP-NS80₃₃₅₋₇₄₂-transfected cells. As shown in the *upper left* and *upper right* panels, inclusions were visualized using the inherent fluorescence of EGFP. Vero cells transfected with GFP-NS80₁₋₃₃₄ and empty GFP vector are shown in the *lower left* and *lower right* panels, in which green fluorescence is dispersed throughout the cytoplasm and nucleus. **b, c** Immunoblotting assay using anti-GFP MAb and anti-NS80 polyclonal serum as the primary antibody and alkaline-phosphatase-coupled goat anti-rabbit IgG or rabbit anti-mouse as the secondary antibody. *M* pre-stained standard protein marker; *1* GFP-NS80₁₋₃₃₄, *2* mock-infected Vero cells, *3* GFP-NS80₃₃₅₋₇₄₂, *4* GFP-NS80, *5* GFP. The *arrows* show expressed protein of interest. *Bars* 10 μ m

Discussion

Usually, virus replication and assembly occur in specific locations within infected cells. For example, in herpes

simplex virus (dsDNA genome; family *Herpesviridae*), this occurs in nuclear inclusions, and in flock house virus (ssRNA genome; family *Nodaviridae*), it occurs on the outer mitochondrial membranes [23, 28]. For viruses in the family *Reoviridae*, previous investigations have indicated that the cytoplasmic phase-dense inclusions in reovirus-infected cells are formed and located at perinuclear sites during viral replication and assembly [7, 23, 25]. In MRV and ARV, the nonstructural protein μ NS has been identified to be the most important key factor in forming the matrix of these viral inclusions and recruiting other components to viroplasma-like structures [4–7, 20, 22, 30, 31]. However, the role of nonstructural proteins in aquareovirus virus replication has been poorly characterized to date.

GCRV, which is regarded as the most pathogenic of all of the aquareoviruses that have been isolated [27], is a tentative member of the family *Reoviridae* [2, 3, 21, 24]. Sequence analysis indicates that GCRV and MRV have a common evolutionary origin although they have different host species. Among the 12 proteins of GCRV that have been identified, 5 nonstructural proteins appear at different stages in the viral life cycle. Of these nonstructural proteins, NS80 has been identified as a homolog of the μ NS protein in MRV. To identify specific residues in NS80 that may be required for inclusion formation, we compared the deduced protein sequences of NS80 homologs from mammalian and ARV. Although BLAST analysis indicated significant divergence in the N-terminal region of NS80, there were several conserved regions that were mainly concentrated in the C-terminal region. Other aquareoviruses, such as golden shiner reovirus (GSRV), belonging to *Aquareovirus* group C and CSRV, belonging to *Aquareovirus* group A, also share conserved C-terminal domains. A C-terminal Leu residue is also conserved among the μ NS proteins of MRV and ARV [34]. These conserved regions in MRV have been identified to be critical for inclusion formation [7, 20, 22]. Based on these findings, in addition to the full-length NS80 protein, we designed a truncated NS80 recombinant from aa 335–742 to determine whether the C-terminal conserved region is essential for inclusion formation. Our results indicate that NS80₃₃₅₋₇₄₂ is sufficient for the formation of inclusion bodies in transfected cells.

It is interesting to note that we detected an additional band by immunoblotting, with a size around 70 kDa in the GCRV-infected cell lysate, and based on its molecular weight, this might be a C-terminal form of the NS80 translation product that begins at an internal in-frame initiation codon in the S4 mRNA, or it might just be a degraded form of full-length NS80. A truncated form of μ NS, μ NSC, which exists in MRV and lacks about 5 kDa from its N terminus, could also be detected in infected cells [5]. The origin of μ NSC is not certain, but it has been

proposed that it is a consequence of either secondary initiation or cleavage near Met41 in μ NS [5, 6]. A similar situation has also been found in ARV, with two μ NS isoforms recognized by μ NS-specific antibodies. In addition to the 70-kDa full-length μ NS in ARV, a 10-kDa smaller protein (about 60 kDa) was also detected in ARV infected cells that was not found to associate with the ARV σ NS protein, suggesting that the activities of μ NS and μ NSC may function at different stages of regulation of viral replication and assembly [31]. Similar to μ NSC in MRV or ARV, the roles of truncated NS80 and the possible NS80C polypeptide detected in this study may warrant further investigation.

The experiments described in this report were aimed at providing insight into the function of the GCRV nonstructural protein NS80 in viral replication. It appears that the GCRV particles were formed in the interior region of viroplasm-like inclusions. NS80, which has been deduced to be a key protein in virus replication, was detected by immunoblotting and indirect IF in both infected and transfected cells using polyclonal NS80 antiserum. Moreover, as expected, globular-like inclusions dispersed throughout the cytoplasm were observed in cells transfected with GFP-tagged full-length NS80 and a C-terminal region recombinant, suggesting that the C-terminal region from aa 335 to 742 is sufficient to form inclusions. The data provided in this study will allow us to further test the function of NS80 in the formation of the viral factory matrix in viral replication and assembly.

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