VP5 autocleavage is required for efficient infection by in vitro-recoated aquareovirus particles

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Grass carp reovirus (GCRV) is a member of the genus Aquareovirus in the family Reoviridae, and contains five core proteins (VP1–VP4 and VP6) and two outer-capsid proteins (VP5 and VP7) in its particle. Previous studies have revealed that the outer-capsid proteins of reovirus are responsible for initiating infection, but the mechanism is poorly understood. Using baculovirus-expressed VP5 and VP7 to recoat purified cores, in vitro assembly of GCRV was achieved in this study. Recoated GCRV (R-GCRV) closely resembled native GCRV (N-GCRV) in particle morphology, protein composition and infectivity. Similar to N-GCRV, the infectivity of R-GCRV could be inhibited by treating cells with the weak base NH4Cl. In addition, recoated particles carrying an Asn→Ala substitution at residue 42 of VP5 (VP5N42A/VP7 R-GCRV) were no longer infectious. These results provide strong evidence that autocleavage of VP5 is critical for aquareovirus to initiate efficient infection.

The genus Aquareovirus is a tentative member of the family Reoviridae, which currently includes 15 proposed genera (King et al., 2011). Many aquareoviruses have been isolated from apparently healthy-looking fish, including the finfish and shellfish species. However, several members within the genus are identified as pathogens that are capable of causing serious disease in aquatic animals (Fang et al., 1989; Ke et al., 1990; Rangel et al., 1999). Grass carp reovirus (GCRV) has been recognized as the most pathogenic among the isolated aquareoviruses (Mohd Jaafar et al., 2008; Rangel et al., 1999). Hence, GCRV provides a useful model to understand the infection and pathogenesis of aquareoviruses in general.

The structure of GCRV is a multilayered icosahedral particle with a diameter of approximately 75–85 nm enclosing a segmented dsRNA genome. The 11 genome segments encode seven structural proteins (VP1–VP7) and five non-structural proteins. Genome sequence analysis and three-dimensional structural reconstructions by cryo-electron microscopy have indicated that there are great similarities between aquareoviruses and mammalian reoviruses (MRVs) in protein structure and particle organization (Cheng et al., 2008, 2010; Fang et al., 2005). Similar to other reoviruses in the family Reoviridae, the GCRV particle is organized mainly into two layers: an outer capsid and an inner capsid. Two hundred trimeric heterodimers of the VP5 and VP7 proteins make up the outer capsid of the virus particle, whilst the other five structural proteins (VP1–VP4 and VP6) constitute the inner capsid or core of the virion. Previous three-dimensional structure studies have revealed that the inner capsid of GCRV is constructed mainly of two proteins, VP3 and VP6, whilst the other three proteins (VP1, VP2 and VP4) are RNA polymerase-related complex proteins that possess the enzymic activities necessary for viral transcription and replication (Cheng et al., 2008; Fang et al., 2005).

Reoviruses are non-enveloped virions that lack a lipid-bilayer membrane. Based on their particle organization, they utilize an entry pathway different from that of enveloped viruses. To initiate infection in a target cell, reoviruses must disrupt the host cell membrane through a mechanism other than the membrane fusion route seen for enveloped viruses. Previous studies have revealed that MRVs need to be activated for infection by undergoing stepwise disassembly of the outer capsid to generate infectious subviral particles (ISVP) (Chandran & Nibert, 1998; Liemann et al., 2002; Sturzenbecker et al., 1987). When virions are activated to infect host cells, the outermost capsid protein σ3 of MRV is degraded by endosomal proteases, leaving μ1 exposed on the surface. The μ1 protein is found predominantly in the form of two fragments, μ1N
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(4 kDa) and μ1C (72 kDa), following putative autocleavage between residues Asn42 and Pro43 near the N terminus of the intact protein (Nibert et al., 1991, 2005). In addition, infection with intact virions, but not ISVPs, can be blocked by treating cells with a weak base, such as NH4Cl, that inhibits the proteolysis of μ1 within cells (Chandran & Nibert, 1998; Jané-Valbuena et al., 1999). Studies on the characteristics of infectious GCRV particles have demonstrated that complete digestion of VP7 and partial cleavage of VP5 can lead to enhanced infectivity of the virus, indicating that VP7 and VP5 may play important roles in GCRV entry into host cells during infection (Fang et al., 2008; Zhang et al., 2010). Recent three-dimensional structural studies of GCRV ISVP at 3.3 Å resolution revealed that autocleavage of the VP5 protein, which is myristoylated at the N terminus, occurs during conversion from the dormant to the primed state at an early stage of viral infection (Zhang et al., 2010). To gain insight into the molecular mechanism of virus infection and particle assembly, in vitro assembly of GCRV-like particles by recoating baculovirus-expressed VP5 and VP7 onto purified cores was achieved in this study. In addition, the infection characteristics of recoated WT (R-GCRV or VP5/VP7 R-GCRV) and mutant (VP5N42A/VP7 R-GCRV) particles were investigated.

Previous studies reported that the outer-capsid proteins VP5 and VP7 are required for cell entry during aquareovirus infection, and that putative autocleavage of outer-capsid protein VP5 is likely to be critical for virus entry into host cells (Fang et al., 2008; Zhang et al., 2010). To determine whether VP5 and/or VP7 were responsible for aquareovirus entry, a dual-expressed VP5 and VP7 (vAcGCRV-VP5/VP7) and singly expressed VP5 or VP7 (vAcGCRV-VP5 or vAcGCRV-VP7; data not shown) recombinant baculoviruses were constructed, respectively. In addition, to determine whether the N42 residue was responsible for autocleavage of VP5, a mutated VP5 containing an Asn→Ala substitution at residue 42 was also generated (Fig. 1a) and expressed in Spodoptera frugiperda (Sf9) cells using a recombinant baculovirus (vAcGCRV-VP5N42A/VP7). An immunofluorescence assay was carried out to confirm the subcellular location of VP5 or VP5N42A with VP7 during recombinant baculovirus infection. As shown in Fig. 1(b), compared with vAcGCRV-VP5/VP7, the co-localization of mutant VP5N42A with VP7 in infected Sf9 cells was not changed. To estimate the levels of protein expression and VP5N/VP5C cleavage, Sf9 cell lysates containing VP5 or VP5N42A co-expressed with VP7 were analysed by Western blotting (WB). The data revealed that the expression of WT VP5 resulted in two VP5-derived bands, namely intact VP5 and cleaved C-terminal VP5 (termed VP5C). By contrast, in vAcGCRV-VP5N42A/VP7-infected cells, no cleaved VP5C could be detected (Fig. 1c). Moreover, the interactions between VP5 or VP5N42A and VP7 were investigated by a co-immunoprecipitation assay. The results showed that WT VP5 or mutant VP5N42A could efficiently co-immunoprecipitate with VP7 using anti-VP7 polyclonal antibodies, and VP7 was efficiently co-immunoprecipitated with VP5 or VP5N42A by anti-VP5 polyclonal antibodies (Fig. 1d) but not by control antibody. These results indicated that the vAcGCRV-VP5/VP7 and vAcGCRV-VP5N42A/VP7 recombinant baculoviruses could express VP5 or VP5N42A and VP7 at high levels in Sf9 cells. Moreover, both VP5 and VP5N42A could interact with VP7. These results suggested that the VP5/VP7 and VP5N42A/VP7 recombinant proteins co-expressed in Sf9 cells were suitable for recoating GCRV cores in vitro.

To achieve the assembly in vitro, GCRV cores with protruding turrets were purified by CsCl gradient centrifugation and their structure confirmed using transmission electron microscopy (TEM) (Fig. 2a). Cytoplasmic extracts were also prepared from Sf9 cells infected with recombinant baculoviruses co-expressing proteins VP5 and VP7 or singly expressing VP5 or VP7. These extracts were incubated with purified GCRV cores at 28 °C for 5 h and the recoated particles purified by CsCl gradient centrifugation. Comparison by TEM of these recoated particles (R-GCRV) with native particles (N-GCRV), also purified by CsCl-based centrifugation, showed that the two types of particle had indistinguishable morphologies (Fig. 2b, c). No recoated particles were obtained when purified cores were incubated with VP5 or VP7 alone, strongly suggesting that a complex of VP5 and VP7 is required for binding to and recoating viral cores. To confirm the protein composition of R-GCRV particles, samples were analysed using SDS-PAGE (Fig. 2d). Seven structural protein components were identified whose molecular masses were consistent with those seen in similar analysis performed on N-GCRV particles. In addition, WB analysis confirmed that VP5 and VP7 had specifically bound to the cores and remained bound throughout the CsCl centrifugation (Fig. 2e). To evaluate the infectivity of recoated particles, they were tittered on Ctenopharyngodon idellus kidney (CIK) cells. As shown in Fig. 2(f), in common with N-GCRVs, R-GCRVs were 105-fold more infectious than cores on a per-particle basis. In contrast to these results, when VP5 or VP7 cell extracts (infected by vAcGCRV-VP5 or vAcGCRV-VP7) were singly incubated with cores (VP5 R-GCRV or VP7 R-GCRV), no infectivity was detected. These results indicated that infectious R-GCRV could only be produced by recoating core particles with both VP5 and VP7 proteins.

Cell entry of MRV or aquareovirus has been reported to be a multistep process involving proteolytic disassembly of virions into ISVPs (Chandran et al., 1999; Fang et al., 2008). This proteolysis of intact virions could be inhibited by treating cells with weak bases, such as NH4Cl (Chandran et al., 1999). To prove the biological similarity between R-GCRVs and N-GCRVs, cells were infected with or without 20 mM NH4Cl treatment. At 24 h post-infection (p.i.), obvious viral plaques were observed in the mock-treated cells but not in NH4Cl-treated cells (Fig. 3a). Cell supernatants were also harvested and virus titres were assayed. The results showed that more than a 105-fold
A reduction in virus titre was observed with N-GCRV or R-GCRV when the cells were treated with NH₄Cl (Fig. 3b). These results indicated that NH₄Cl could inhibit the infection with both N-GCRV and R-GCRV, confirming the similarity between them.

The infectivity of mutant VP5⁵⁴²Ａ/VP7 R-GCRV was evaluated on CIK cells. At 24 h p.i., a large number of plaques was observed in VP5/VP7 R-GCRV-infected cells but not in core- or VP5⁵⁴²Ａ/VP7 R-GCRV-infected cells (Fig. 3c). To further confirm that recoated particles with mutant VP5⁵⁴²Ａ were defective in infection, the virus titres were examined using both TCID₅₀ and real-time quantitative PCR (qPCR) assays. As shown in Fig. 3(d), the infectivity of WT R-GCRV was similar to that of N-GCRV.

In contrast, little or no infectivity was detected with either control virus cores or mutant VP5⁵⁴²Ａ/VP7 R-GCRV particles. In the qPCR assay, the mRNAs of the GCRV core proteins VP3 and VP6 were detected to assess their expression levels. High mRNA levels were detected in WT R-GCRV-infected cells but not in cells infected with mutant VP5⁵⁴²Ａ/VP7 R-GCRV, or with virus cores or cores mixed with mock Sf9 cell lysates (Fig. 3e). In addition, WB analysis showed that the expression of VP3 and VP6 could be detected in WT R-GCRV-infected cells but not in mutant VP5⁵⁴²Ａ/VP7 R-GCRV-infected cells (Fig. 3f). These results strongly indicated that the WT R-GCRVs have similar infectivity to N-GCRV but that infectivity is lost when the N42 residue is substituted to VP5⁵⁴²Ａ.
A (VP5^{N42A}), suggesting that the autocleavage of VP5 at aa 42 is indispensable for GCRV to initiate infection.

The baculovirus expression system was used for the high-level expression of recombinant VP5 and VP7 proteins, and in vitro assembly using the expressed proteins to recoat purified GCRV cores was achieved. Infectious R-GCRV could only be produced by recoating core particles with both VP5 and VP7 proteins, and not with VP5 or VP7 singly, indicating that VP7 is required for particle assembly and cell entry. These findings not only suggest that a VP5–VP7 complex can be spontaneously assembled onto purified core particles in stoichiometric amounts, but also support a proposed structure-based mechanism whereby VP7 removal triggers a VP5 conformational change and

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**Fig. 2.** Characteristics and infectivity assays of *in vitro*-recoated particles. (a) TEM image of purified GCRV cores. The boxed area in the image is enlarged to show details. The arrow indicates the turret. (b, c) TEM images of purified R-GCRV and N-GCRV. (d, e) SDS-PAGE analysis of protein components and WB analysis of VP5 and VP7 in purified R-GCRV and N-GCRV. Purified core and vAcGCRV-VP5/VP7 served as controls. (f) Infectious titre of R-GCRV determined by a p.f.u. assay. The core, N-GCRV, VP5 R-GCRV and VP7 R-GCRV served as controls. Each bar represents the mean ± SD derived from three independent experiments.
autoproteolysis, converting a virion from a dormant state to a primed ISVP (Zhang et al., 2010).

To enter host cells, a reovirus virion needs to transform into an ISVP that can penetrate the cell membrane, and this process is acid dependent (Chandran et al., 1999; Kothandaraman et al., 1998). In this study, the infectivity of N-GCRV or R-GCRV could be blocked by the weak base NH₄Cl, suggesting that N-GCRVs and R-GCRVs utilize a similar route to enter host cells. More importantly, R-GCRVs with a VP5N42A mutation were shown to be defective in infectivity and progeny protein expression in infected cells, indicating that an N42A substitution almost completely blocked the VP5 autoproteolysis. This result is consistent with a previous report in MRV where cores recoated with α3 and μ1 bearing the N42A mutation were defective for infectivity (Odegard et al., 2004), suggesting that this amino acid of μ1 plays an important role in the cleavage process.

In summary, an in vitro assembly model of aquareovirus was generated in this study. The results suggest that R-GCRV can serve as a tool for investigating virus entry. Based on the in vitro assembly model of GCRV, the functions of the outer-capsid proteins VP5 and VP7 in virus entry could be fully investigated. To our knowledge, this study has also provided the first experimental evidence for the indispensability of aquareovirus VP5 autoproteolysis in cell entry by using cores recoated with a mutated VP5 protein. These results lay a foundation for further efforts to reveal the detailed entry mechanism of aquareoviruses.

Fig. 3. VP5 autocleavage is indispensable for efficient infection of aquareovirus. (a) Plaque assays of R-GCRV and N-GCRV in the absence (−) or presence (+) of NH₄Cl. (b) Infectious titres of supernatants harvested at 24 h p.i. in (a) were determined by a p.f.u. assay. (c) Plaque assay of VP5N42A/VP7 R-GCRV. Core and VP5/VP7 R-GCRV served as controls. (d) Infectious titre of VP5N42A/VP7 R-GCRV. Core, VP5/VP7 R-GCRV and N-GCRV served as controls. (e, f) Relative expression of VP3 and VP6 in mRNA (e) or protein (f) level calculated by qPCR or WB assay, respectively. Lanes: 1, core; 2, core mixed with Sf9; 3, VP5/VP7 R-GCRV; 4, VP5N42A/VP7 R-GCRV. Actin was used as a loading control in (f). Data in (b), (d) and (e) represent the mean ± SD derived from three independent experiments.
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References


