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Identification of an essential virulence gene of cyprinid herpesvirus 3

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ABSTRACT

The genus *Cyprinivirus* consists of a growing list of phylogenetically related viruses, some of which cause severe economic losses to the aquaculture industry. The archetypal member, cyprinid herpesvirus 3 (CyHV-3) causes mass mortalities worldwide in koi and common carp. A CyHV-3 mutant was described previously that is attenuated *in vivo* by a deletion affecting two genes (ORF56 and ORF57). The relative contributions of ORF56 and ORF57 to the safety and efficacy profile of this vaccine candidate have now been assessed by analysing viruses individually deleted for ORF56 or ORF57. Inoculation of these viruses into carp demonstrated that the absence of ORF56 did not affect virulence, whereas the absence of ORF57 led to an attenuation comparable to, though slightly less than, that of the doubly deleted virus. To demonstrate further the role of ORF57 as a key virulence factor, a mutant retaining the ORF57 region but unable to express the ORF57 protein was produced by inserting multiple in-frame stop codons into the coding region. Analysis of this virus *in vivo* revealed a safety and efficacy profile comparable to that of the doubly deleted virus. These findings show that ORF57 encodes an essential CyHV-3 virulence factor. They also indicate that ORF57 orthologues in other cypriniviruses may offer promising targets for the rational design of attenuated recombinant vaccines.

1. Introduction

The *Alloherpesviridae* is one of three families in the order *Herpesvirales*, and consists of herpesviruses that infect fish or amphibians (Boutier et al., 2015a). It currently contains 12 species distributed into four genera. One of these genera, *Cyprinivirus*, comprises a growing list (Doszpoly et al., 2015) of phylogenetically related viruses, some of which cause severe economic losses to the aquaculture industry. These include cyprinid herpesvirus 2 (CyHV-2; also known as goldfish hematopoietic necrosis virus), which causes a lethal disease in goldfish (*Carassius auratus*) and gibel carp (*C. gibelio*) (Xu et al., 2013, 2014; Ito and Maeno, 2014), anguillid herpesvirus 1 (AngHV-1), which is responsible for mortalities of up to 30% in cultured and wild European and Japanese eel populations (*Anguilla anguilla* and *A. japonica*) (van Beurden et al., 2012), and cyprinid herpesvirus 3 (CyHV-3; also known as koi herpesvirus), which is the etiological agent of a lethal disease in common carp (*Cyprinus carpio*) and koi carp (Adamek et al., 2014; Rakus et al., 2013).

CyHV-3 is considered to be the archetypal fish alloherpesvirus (Boutier et al., 2015a). Since its emergence in the 1990s, this virus has caused severe economic losses to the carp culture industry worldwide (Adamek et al., 2014; Rakus et al., 2013). For example, outbreaks of CyHV-3 that occurred in Indonesia in 2002 and 2003 were estimated to have resulted in economic losses amounting to US$15 million (Bondad-Reantaso et al., 2005; Sunarto et al., 2005). CyHV-3 is also provoking a societal impact in developing countries by affecting familial aquaculture and thereby limiting access to one of the cheapest sources of animal protein (Boutier et al., 2015a). In addition, it is having an ecological impact by inducing carp mortalities in natural habitats (Ito et al., 2014; Uchii et al., 2013).

Various types of vaccine candidate against CyHV-3 have been developed (Boutier et al., 2015b; O'Connor et al., 2014; Perelberg et al., 2008). Among these, attenuated vaccines seem the most promising because they are more likely to suit practical field constraints, such as the need for mass vaccination of fish weighing only a few grams each. Also, scientific advances are increasingly

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facilitating the rational design of attenuated vaccines (Rueckert and Guzman, 2012), including deleting genes from a viral genome in a precise way that precludes reversion to virulence (Meeusen et al., 2007). This approach has been taken for CyHV-3 by targeting various open reading frame (ORFs), including ORF16, ORF55, ORF123 and ORF134, which encode a G protein-coupled receptor (GPCR), thymidine kinase (TK), deoxyuridine triphosphatase (dUTPase) and interleukin-10, respectively. However, none of the recombinants lacking these genes has been shown to have a safety and efficacy profile compatible with their use as attenuated vaccines (Costes et al., 2008; Fuchs et al., 2011; Ouyang et al., 2013). This list of failures illustrates the difficulties of predicting the impact of gene deletions in alloherpesviruses, particularly as there is little genetic similarity between these viruses and the much more extensively studied members of the family *Herpesviridae* (Boutier et al., 2015a).

Recently, we reported the development of an attenuated CyHV-3 vaccine candidate compatible with mass vaccination of carp (Boutier et al., 2015b). The attenuation depends on an accidentally generated deletion affecting two divergently transcribed genes (ORF56 and ORF57), removing most of ORF56 (leaving less than 10% at the 3′ end) and part of ORF57 (leaving about 75% at the 3′ end). Using *in vivo* imaging system and quantitative PCR, we demonstrated that this doubly deleted virus and the wild-type parental virus have similar tropisms. However, compared to the parental wild-type virus, the spread of the deleted virus to the other organs was much slower, and its replication was reduced in intensity and duration (Boutier et al., 2015b). The relative contributions of ORF56 and ORF57 to the safety and efficacy profile of the doubly deleted virus are not known. The functions of these ORFs are similarly not known, although ORF57 is conserved in all cyprinivirus and ORF56 is conserved only among cyprinivirus infecting cyprinid fish (Davison et al., 2013; van Beurden et al., 2010). In the present study, we investigated the relative contributions of ORF56 and ORF57 to the safety and efficacy profile of the doubly deleted virus. The results demonstrated that ORF57 encodes an essential CyHV-3 virulence factor, and raised the possibility that ORF57 orthologues in other cyprinivirus may present promising targets for rational design of attenuated vaccines.

2. Materials and methods

2.1. Cells and viruses

CCB cells (Neukirch et al., 1999) were cultured as described previously (Boutier et al., 2015b). The CyHV-3 FL and M3 strains were isolated from the kidney tissue of koi carp that died during independent CyHV-3 outbreaks in Belgium. The FL bacterial artificial chromosome plasmid (BAC) was generated from the FL strain (Costes et al., 2008). The mutant deleted for ORF56 and ORF57 (Δ56-57) was produced from this BAC (Boutier et al., 2015b).

2.2. Production of recombinant viruses

2.2.1. Generation of recombinant BACs

Figs. 1 and 4 outline the strategies used to generate various recombinant viruses from the FL BAC (Costes et al., 2008) on the basis of *galK* positive/negative selection in bacteria (Costes et al., 2008; Warming et al., 2005). Recombination cassettes encoding *galK* were produced by PCR, using the primers listed in Table S1 and the p*galK* vector as template. The ORF57 Rev and ORF57 NS cassettes were amplified from plasmids encoding a wild-type (57Rev) and mutated (57NS) ORF57, respectively, by using the primers listed in Table S1. Details of the ORF57 NS cassette encoding in-frame stop codons in ORF57 are given in Fig. 4A.

2.2.2. Generation of recombinant viruses from BACs

Recombinant BACs were co-transfected into CCB cells with the pGEMT-TK plasmid or the pEFIN3 NLS Cre plasmid (both at a molecular ratio of 1:75) by using polyethylenimine (3 μg per 1 μg DNA) (Costes et al., 2008). Transfection with the pGEMT-TK plasmid induced recombination upstream and downstream of the BAC cassette, leading to reversion to a wild-type TK (ORF55) locus (FL BAC revertant viruses). Transfection with the pEFIN3 NLS Cre plasmid induced expression of a nuclear Cre recombinase and caused cre-loxP-mediated excision of the BAC cassette, leading to viruses expressing a truncated form of TK due to the retention of a 172 bp sequence from the BAC cassette in the TK locus (FL BAC excised viruses). Since the BAC cassette encodes the enhanced green fluorescent protein (EGFP), EGFP-negative plaques were picked and amplified in both cases. The strategies are shown in Figs. 1 and 4, which also specify in bold font the short names for the recombinant viruses that are used below.

2.2.3. Genetic characterization of recombinant viruses

CyHV-3 recombinants were characterized by restriction fragment length polymorphism (RFLP) analysis using SacI digestion, Southern blot analysis, sequencing of manipulated regions, and full-length genome sequencing as described previously (Boutier et al., 2015b; Costes et al., 2009; Ouyang et al., 2013; Rakus et al., 2017).

2.3. In vitro experiments

2.3.1. Antibodies against the ORF56 and ORF57 proteins

Mouse polyclonal antibodies (pAbs) directed against the predicted unstructured domain (analysed by using IUPred, http://iupred.enzim.hu) of the ORF56 protein (pORF56) (specified by coordinates 98049-99398, NC_009127.1) or against the full-length ORF57 protein (pORF57) (specified by coordinates 99382-100803, NC_009127.1) were produced by DNA immunization using a commercial service (DelphiGenetics). Mouse monoclonal antibody (mAb) 6B2 was selected from a bank of mouse mAbs raised against CyHV-3 structural proteins, and recognizes an epitope in the last 165 amino acid residues of pORF57 (specified by coordinates 100309-100803, NC_009127.1).

2.3.2. Indirect immunofluorescent staining

Cells were fixed in phosphate-buffered saline (PBS) containing 4% (w/v) paraformaldehyde, and permeabilized in PBS containing 0.1% (v/v) NP-40. Incubation of antibodies and washes were performed in PBS containing 10% (v/v) foetal calf serum (FCS). Mouse pAbs raised against pORF56 (diluted 1:500), mouse mAb 6B2 raised against pORF57 (diluted 1:2500), mouse pAbs raised against pORF57 (diluted 1:500), and rabbit pAbs raised against CyHV-3 structural proteins (diluted 1:1500) were used as primary antibodies (37 °C for 1 h). Alexa Fluor 488 GAM IgG(H + L) and Alexa Fluor 568 GARb IgG(H + L) (Invitrogen) were used as secondary antibodies (37 °C for 30 min). Cell nuclei were stained by using TO-PRO-3 iodide (Invitrogen, 1:1000 at room temperature for 15 min) or DAPI dilactate (Invitrogen, 1:30,000 in PBS at room temperature for 5 min). After washing, cells were mounted by using Prolong Gold antifade reagent (Invitrogen). Confocal analyses were performed by using Leica SP2 and SP5 instruments.

2.3.3. Plaque size assay

Cultures of CCB cells in six-well plates were inoculated with 100–200 plaque-forming units (PFU)/well of virus for 2 h and overlaid with Dulbecco's modified essential medium (DMEM,