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Characterization of gene deletion mutants of *Cyprinid herpesvirus 3* (koi herpesvirus) lacking the immunogenic envelope glycoproteins pORF25, pORF65, pORF148 and pORF149

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ABSTRACT

Cyprinid herpesvirus 3 (CyHV-3) or koi herpesvirus is a global pathogen causing mass mortality in koi and common carp, against which improved vaccines are urgently needed. In this study we investigated the role of four nonessential, but immunogenic envelope glycoproteins encoded by members of the ORF25 gene family (ORF25, ORF65, ORF148 and ORF149) during CyHV-3 replication. Single deletion of ORF65 did not affect *in vitro* replication, and deletion of ORF148 even slightly enhanced virus growth on common carp brain (CCB) cells. Deletions of ORF25 or ORF149 led to reduced plaque sizes and virus titers, which was due to delayed entry into host cells. An ORF148/ORF149 double deletion mutant exhibited wild-type like growth indicating opposing functions of the two proteins. Electron microscopy of CCB cells infected with either mutant did not indicate any effects on virion formation and maturation in nucleus or cytoplasm, nor on release of enveloped particles. The ORF148, ORF149 and double deletion mutants were also tested in animal experiments using juvenile carp, and proved to be insufficiently attenuated for use as live virus vaccines. However, surviving fish were protected against challenge with wild-type CyHV-3, demonstrating that these antibody inducing proteins are dispensable for an efficient immune response *in vivo*.

1. Introduction

Cultivation of common carp has a long tradition in Europe and Asia, and in several regions provides a key source of protein for human consumption. Furthermore, the breeding of colored carp varieties (koi) as ornamental fish has become a popular hobby, and a profitable business worldwide (Balon, 1995). In the 1990ies, a previously unknown virus infection led to mass mortality of common and koi carp in Israel and Europe. The causative agent was identified as a herpesvirus and designated as carp interstitial nephritis and gill necrosis virus (CNGV), or koi herpesvirus (KHV) (Bretzinger et al., 1999; Hedrick et al., 1999; Neukirch et al., 1999; Ronen et al., 2003). Due to the severity of the infection, the development of attenuated vaccines was rapidly initiated (Bretzinger et al., 1999; Hedrick et al., 1999; Neukirch et al., 1999; Ronen et al., 2003).

Analyses of genome sequences demonstrated a close relationship to carp pox virus (*Cyprinid herpesvirus 1*), and goldfish haematopoietic necrosis virus (*Cyprinid herpesvirus 2*), leading to classification as *Cyprinid herpesvirus 3* (CyHV-3) within the genus *Cyprinivirus* in the family *Alloherpesviridae* and the order *Herpesvirales* (Davison et al., 2009). The CyHV-3 genome has a size of 295 kbp including 22 kbp of direct repeat sequences at both ends, and contains approximately 155 open reading frames (ORFs) predicted to encode proteins (Aoki et al., 2007). Some of these ORFs are conserved in other herpesviruses of fish or amphibians, but homologies to mammalian and avian herpesviruses (family *Herpesviridae*) are very limited. Analysis of purified virus particles by mass spectrometry identified 40 different CyHV-3 encoded proteins, including 3 capsid components, and 13 membrane proteins (Michel et al., 2010).

The integral membrane proteins pORF25, pORF65, pORF81, pORF99, pORF136, pORF148 and pORF149 were also identified and characterized by specific antisera or monoclonal antibodies (mAb), and,

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Table 1
PCR and sequencing primers used in this study.

name	Sequence	nucleotide position
KTO25F	5'-*CAC**GAATTC***TCATGTACGAGTACCTGCG-3'	44,755 - 44,774
KTO25R	5'-*CAC**AAGCTT***CGGAGAGCATGATGAAGAGG-3'	48,347 - 48,366 (r)
KTO65F	5´-*CAC**GAATTC***TTTCAGTTCAACGACCCG-3´	121,758 - 121,777
KTO65R	5´-*CAC**AAGCTT***TACCTTCTACGGCGTGG-3´	124,935 - 124,954 (r)
KTO149R-F	5´-*CAC**GAATTC***CGAGGGAGATCAGAGTTC-3´	253,076 - 253,094
KTO149R-R	5`-*CAC**AAGCTT***GGGATGATGTCAGCGTTG-3´	259,046 - 259,064 (r)

Nucleotide positions refer to the sequence of the CyHV-3 TUMST1 genome (GenBank accession # AP008984) (Aoki et al., 2007). Additional, nonmatching nucleotides (in Italics), engineered restriction sites (bold), and reverse strand primers (r), are indicated.

except pORF81 and pORF136, proved to be modified by N-linked glycosylation (Fuchs et al., 2014; Rosenkranz et al., 2008; Vrancken et al., 2013). ORFs 25, 65, 148, and 149 constitute members of a family of distantly related genes which is conserved in cyprinid herpesviruses, and might have evolved from a common ancestor (Davison et al., 2013; Aoki et al., 2007). Indirect immunofluorescence (IF) tests of eukaryotic cells transfected with expression plasmids for ORF25, ORF65, ORF99, ORF148 or ORF149 revealed specific reactions of the respective glycoproteins with serum antibodies from experimentally and naturally KHV-infected carp (Fuchs et al., 2014). Therefore, vaccines based on KHV mutants lacking these genes might be suitable for serological differentiation between infected and vaccinated animals (DIVA) (van Oirschot, 1999). On the other hand, candidate subunit or DNA vaccines containing or encoding single CyHV-3 envelope proteins like pORF25 or pORF81 have been shown to induce protective immune responses (Cui et al., 2015; Zhou et al., 2014a, b), suggesting that their deletion might affect the protective efficacy of CyHV-3 live vaccines. Recent studies demonstrated, that the four ORF25 family genes including ORF65, ORF148, and ORF149 are not essential for CyHV-3 replication in cell culture, and that deletion of ORF25 attenuated the virus *in vivo*, but significantly reduced its protective efficacy against wild-type challenge (Vancsok et al., 2017). In contrast, single deletions of ORF65, ORF148, or ORF149 did not lead to sufficient attenuation of a virulent CyHV-3 strain in carp (Vancsok et al., 2017). Thus, other CyHV-3 recombinants possessing *e.g.* double deletions of the viral thymidine kinase (TK, ORF55) and deoxyuridine triphosphatase (dUTPase, ORF123) genes or of the adjacent ORFs 56 and 57 appeared to be more promising (Boutier et al., 2017; Schröder et al., 2018).

Whereas the previous functional analyses of CyHV-3 envelope proteins were performed with a member of the European virus lineage isolated in Belgium (Vancsok et al., 2017), we reanalyzed the potential of ORF25, ORF65, ORF148, and ORF149 deletions for vaccine development by using a less virulent, but highly cell-culture adapted East Asian type CyHV-3 strain from Taiwan (KHV-T) (Mletzko et al., 2017; Gao et al., 2018). To investigate possible additive effects on virulence, or functional redundancy we also generated an ORF148 and ORF149 double deletion mutant. Furthermore, the cell culture adaptation of KHV-T facilitated *in vitro* investigation of individual viral gene functions. The crucial roles and interactions of envelope (glyco)proteins during virus entry, virion formation, spread and release have been extensively investigated for mammalian herpesviruses (reviewed by Mettenleiter, 2002; Sathiyamoorthy et al., 2017), but little is known about their functional homologues in alloherpesviruses. We therefore studied the roles of pORF25, pORF65, pORF148, and pORF149 in entry and virus morphogenesis by penetration kinetics and electron microscopy of infected common carp brain (CCB) cells (Neukirch et al., 1999).

2. Materials and methods

2.1. Viruses and cells

KHV-T (Mletzko et al., 2017), and its derivatives were propagated in CCB cells (Neukirch et al., 1999), which were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) at 25 °C. Infected cells were incubated in medium containing only 5% FBS, and, after appearance of cytopathogenic effects, lysed by freeze-thawing and stored in aliquots at −80 °C. For plaque assays, CCB cell monolayers were incubated with serial virus dilutions for 2 h. Subsequently, the inoculum was replaced by MEM containing 5% FBS and 6 g/l of methylcellulose, and incubation at 25 °C was continued for 3–7 days.

2.2. Construction of deletion plasmids and virus recombinants

Genomic KHV-T DNA was prepared from infected cell lysates as described (Fuchs and Mettenleiter, 1996). For the generation of deletion mutants, transfer plasmids were prepared after amplification of the targeted glycoprotein gene regions from KHV-T DNA by PCR using Pfx DNA polymerase (Thermo Fisher Scientific), with the respective primer pairs (Table 1). The obtained 3629 bp (ORF25), 3210 bp (ORF65), and 6005 bp (ORF148/149) PCR products were digested at engineered *Eco*RI and *Hin*dIII sites, and inserted into the correspondingly digested vector pUC19 (New England Biolabs), resulting in plasmids pUC-KT25R, pUC-KT65R, and pUC-KT149R (Fig. 1B–D) From the latter plasmid, major parts of ORF148 and ORF149 were deleted either singly, or in combination (Fig. 1D). For the double deletion a 3048 bp *Hpa*I/*Pst*I fragment of pUC-KT149R was either removed or replaced by an expression cassette for enhanced green fluorescent protein (EGFP), which had been isolated as a 1607 bp *BamHI/EcoRI* fragment from plasmid pBl-GFP (Fuchs and Mettenleiter, 1999). Single deletions of ORF148 or ORF149 were introduced by removal or EGFP-substitutions of 635 bp *Hpa*I/*Not*I, or 1252 bp *Xcm*I/*Pst*I fragments, respectively, resulting in transfer plasmids pUC-KTΔ148(G), pUC-KTΔ149(G), pUC-KTΔ148/149(G). In a similar manner, major parts of ORF25 (Fig. 1B) and ORF65 (Fig. 1C) were removed from the original plasmids by digestion with *Bam*HI or *Bsi*WI, resulting in deletions of 1220 bp or 1169 bp, respectively. Optionally, the deleted fragments were replaced by the EGFP expression cassette, yielding plasmids pUC-KTΔ25(G) and pUC-KTΔ65(G). In all cloning experiments non-compatible fragment ends were blunted by treatment with Klenow polymerase prior to ligation.

In a first series of experiments CCB cells were cotransfected (K2 Transfection System, Biontex) with genomic KHV-T DNA and the GFP-gene containing deletion plasmids. Homologous recombination led to the corresponding GFP-expressing virus mutants KHV-TΔ148 G, KHV-TΔ149G, KHV-TΔ148/149G, KHV-TΔ25G and KHV-TΔ65G (Fig. 1) which could be purified to homogeneity by plaque assays on CCB cells, and subsequent aspiration of green fluorescent cell foci. Subsequently, genomic DNA of these KHV-T recombinants was prepared and used for cotransfections with deletion or rescue plasmids lacking a reporter gene. From the resulting non-fluorescent virus plaques deletion mutants KHV-TΔ148, KHV-TΔ149, KHV-TΔ148/149, KHV-TΔ25, and KHV-TΔ65, as well the revertants KHV-T148R, KHV-T149R, KHV-T148/149R, KHV-T25R and KHV-T65R (Fig. 1) were isolated. Genomic DNA of all generated CyHV-3 recombinants was analyzed by restriction and Southern blot analyses (not shown), and the modified genome regions

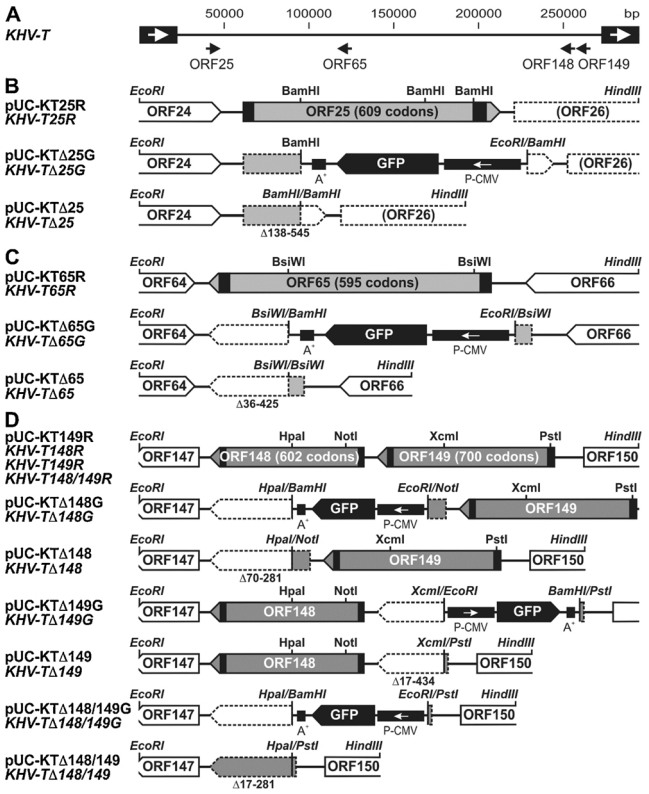

Fig. 1. Construction of CyHV-3 recombinants. **(A)** Map of the KHV-T genome with localizations of ORF25, ORF65, ORF148, ORF149 and direct terminal repeat sequences (rectangles). Enlarged maps of the **(B)** ORF25, **(C)** ORF65, and **(D)** ORF148/ORF149 gene regions illustrate cloning and mutagenesis. Viral ORFs (pointed rectangles) and relevant restriction sites are shown. Non-functional, fragmented or frameshifted ORFs are indicated by dotted lines and different shading. Engineered restriction sites are printed in Italics. Black rectangles indicate encoded signal peptides and transmembrane domains of the CyHV-3 glycoprotein genes. Major parts of them were either replaced by a GFP expression cassette containing the human cytomegalovirus immediate early promoter (P-CMV), and a polyadenylation signal (A^+), or deleted without substitution. The deleted codon ranges are given. Designations of plasmids and resulting virus recombinants (printed in Italics) are given at the right.

were further characterized by PCR and sequencing using the specific primers described here (Table 1), and previously (Fuchs et al., 2014).

2.3. In vitro replication studies and indirect immunofluorescence (IIF) tests

For determination of replication kinetics CCB cells were grown in 24-well plates and infected with KHV-T and the generated virus recombinants at a multiplicity (MOI) of 0.1 at 4 °C to permit virus adsorption. After 1 h, pre-warmed medium was added and incubation was continued for 2 h at 25 °C to allow penetration. Subsequently, non-penetrated virus was inactivated by treatment with citric acid-buffered saline (pH 3.0) (Mettenleiter, 1989), and incubation at 25 °C was continued under fresh medium. At different times after the temperature shift, individual plates were frozen at −80 °C. For determination of progeny virus titers, the plates were thawed at 25 °C, and serial dilutions of the cell lysates were analyzed by plaque assays on CCB cells. After 4 d at 25 °C, the cells were washed with phosphate-buffered saline (PBS), and fixed with 50% methanol / 50% acetone for 30 min at -20 °C. The fixed cells were incubated with a monospecific rabbit antiserum detecting the envelope protein pORF81 of CyHV-3, and fluorochrome-conjugated secondary antibodies as described (Rosenkranz et al., 2008), and investigated by fluorescence microscopy (Eclipse Ti-S with software NIS-elements, version 4, Nikon). Mean titers of four replicates per virus mutant were calculated. Furthermore, the areas of 30 plaques per virus were measured, and average sizes as well as standard deviations were calculated as percentages of the mean plaque areas induced by KHV-T. Unpaired two-sided Student's t-tests were applied to evaluate the statistical significance of differences between plaque sizes and titers of the CyHV-3 mutants.

2.4. In vitro penetration kinetics

CCB cells grown in 6-well plates were infected with approximately 250 plaque-forming units (PFU) of the GFP-expressing virus mutants KHV-TΔ148G, KHV-TΔ149G, KHV-TΔ148/149G, KHV-TΔ25G, and KHV-TΔ65 G, or, as wild-type like control, KHV-TΔDUTGFP (Schröder et al., 2018). After 1 h at 4 °C, prewarmed medium was added, and incubation was continued at 25 °C. Before and 10, 20, 30, 60 and 120 min after the temperature shift, remaining extracellular virus particles were inactivated by low pH treatment (Mettenleiter, 1989). Subsequently, the cells were washed with PBS, overlaid with semisolid medium, and incubated for 3 d at 25 °C. The plaques of the penetrated viruses after different times were counted by fluorescence microscopy, and compared to plaque numbers obtained in wells which were not acid-treated, and overlaid with semisolid medium after 120 min at 25 °C (Vallbracht et al., 2017). Mean percentage values, and standard deviations were determined in three independent experiments. Unpaired two-sided Student's t-tests were applied to evaluate the statistical significance of differences between penetration rates of the CyHV-3 mutants.

2.5. Western blot analyses

CCB cells were infected with KHV-T or the generated virus recombinants at an MOI of 1, and incubated for two days at 25 °C. The cells were lysed and proteins were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer to nitrocellulose membranes, and subsequent incubation of the blots was done as described (Pavlova et al., 2009). Monospecific rabbit antisera against CyHV-3 pORF25, pORF149, and the major capsid protein pORF92 (Fuchs et al., 2014) were used at dilutions of 1 : 10,000. Binding of peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) was detected and recorded (Clarity Western ECL Substrate, and VersaDoc 4000 M P, Bio-Rad).

2.6. Ultrastructural analyses

CCB cells were infected with KHV-T or the generated virus recombinants at an MOI of 1, and incubated for 20 h at 25 °C. The samples were fixed and processed for transmission electron microscopy as described previously (Klupp et al., 2000), and examined using a Tecnai Spirit electron microscope (FEI) and an acceleration voltage of 80 kV.

2.7. Sequence analyses

The inserts of transfer plasmids, and the modified genome regions of virus recombinants were analyzed by sequencing using the BigDye Terminator v1.1 cycle sequencing kit, and a 3130 Genetic Analyzer (Applied Biosystems). Results were evaluated with the Geneious™ software package in version 10.2.3 (Biomatters). Furthermore, the full genome sequence of KHV-TΔ148/149 was determined as described recently (Höper et al., 2015; Schröder et al., 2018).

2.8. Animal experiments

In vivo attenuation and protective efficacy of the generated ORF148 and ORF149 mutants of KHV-T was tested using five groups of carp, each consisting of 40 nine months-old (approximately 8 g / 5–7 cm) and 5 two years-old (approximately 200 g / 20–23 cm) fish (Fischzucht Traßdorf 1967 e.V., Traßdorf, Germany). The animals were kept at a

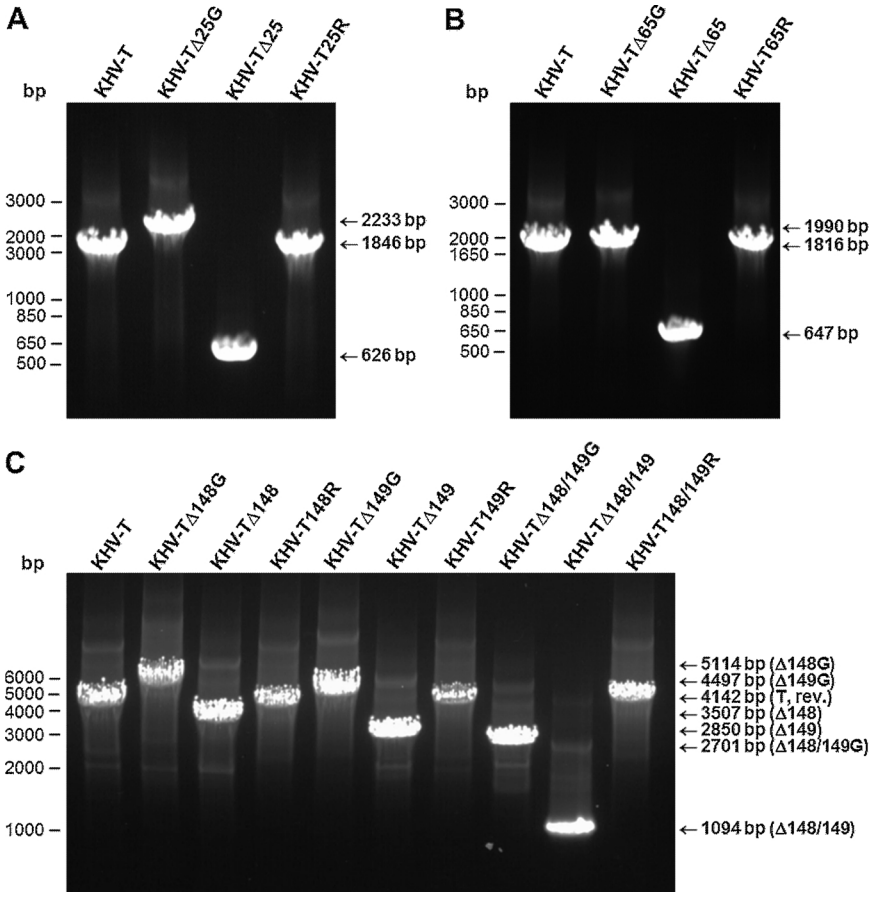

Fig. 2. PCR products of the authentic or mutated CyHV-3 genes **(A)** ORF25, **(B)** ORF65, and **(C)** ORF148/ORF149 amplified from genomic DNA of the generated recombinants obtained with primer pairs CyHV-3O25-F/R, CyHV-3O65-F/R, or CyHV-3O148-R and CyHV-3O149-F (Fuchs et al., 2014). Marker DNAs (1 kb Plus DNA ladder, Thermo Fisher Scientific), and calculated product sizes are indicated at left and right, respectively.

Table 2
Sequence analysis KHV-TΔ148/149 compared to KHV-T.

Position in KHV-T	ORF	Alteration
37986 - 38007	–	Δ GTGTGTGTGTGTGTGTGTGT
75907 - 75915	45	Δ AGGAGGTGG (ΔGGG at codon 336)
91174/91175	52	+ TAACCACCGAGGAAACCACCGAGGAAACCACC GAGGAAACCACCGAGGAAACCACCGAGGTAAC CACCGAGGAAACCACCGAGG (+ VTTEETTEETT EETTEETTEVTTEETTE at codon 133)
91222/91223	52	+ AAACCACCGAGG (+ ETTE at codon 149)
91247/91248	52	+ AACCACCGAGGA (+ TTEE at codon 158)
102598	–	+ GGGG
138261	72	A → C (K → N at codon 103)
177575 - 177594	–	Δ AGAGAGAGAGAGAGAGAGAG
216478/216479	–	+ CTTCAGCACCTTCAGCACCTTCAGCACCTTCAG CACCTTCAGCACCTTCAGCACCTTCAGCACCTT CAGCACCTTCAGCACCTTCAGCAC
254891 - 257899	**148/149**	**Δ ORF149 / ORF148 (from codon 17 to 281)**
257991	–	T → C
270671 - 270685	–	Δ CGACACAGACTACAG

Nucleotide positions of alterations in KHV-TΔ148/149 refer to the published genome sequence of the CyHV-3 strain KHV-T (GenBank accession number # MG925491) (Gao et al., 2018). Deleted nucleotide rages are indicated, and the two flanking nucleotides are provided at insertion sites. Designations of affected ORFs, nucleotide sequences of insertions (+) or deletions (Δ), and consequences on deduced amino acid sequences including codon positions are given. The targeted mutation unique to KHV-TΔ148/149 is printed in bold letters.

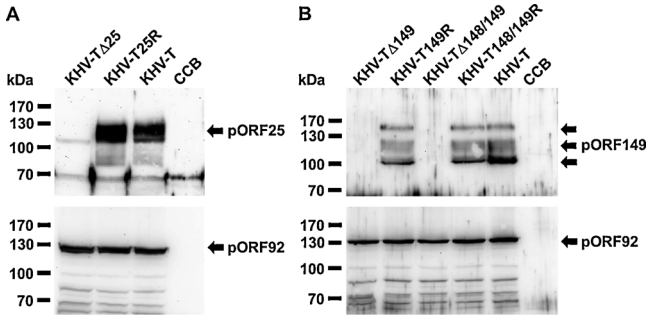

Fig. 3. Western blot analyses of CCB cells infected with the indicated CyHV-3 recombinants (MOI 1, 2 d p.i.). Blots of infected and uninfected cells were probed with monospecific rabbit antisera against **(A)** pORF25, **(B)** pORF149, and the major capsid protein pORF92 (lower panels). Marker proteins (PageRuler Plus Prestained Protein Ladder, Thermo Fisher Scientific), and specifically detected viral proteins are indicated at the left and right, respectively.

constant temperature of 20 °C, and infected by immersion for 1 h in 5 l water containing 2.5×10^7 PFU of either KHV-T, KHV-TΔ148, KHV-TΔ149, KHV-TΔ148/149, or cell culture medium. After 35 d, all carp that had survived primary CyHV-3 infection were challenged by immersion for 1 h in 5 l water containing 2.5×10^7 PFU of KHV-T. The fish were examined for additional 28 days for clinical symptoms. Blood samples and gill swabs were taken from the two years-old carp before, and two and four weeks after primary infection, as well as after challenge. The young carps were considered for evaluation of the mortality rates. Statistical significance of differences between the groups was evaluated using two-sided Fisher's exact tests (Agresti (1992)).

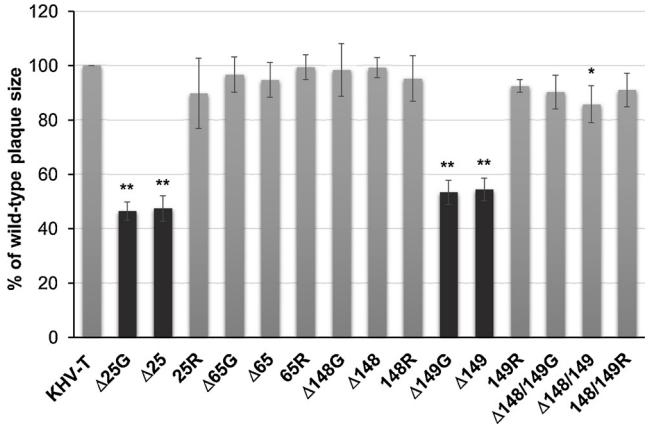

Fig. 4. Plaque sizes of CyHV-3 recombinants on CCB cells. Areas of 30 plaques per virus were determined after 4 days at 25 °C, and compared to plaques of parental KHV-T, which were set as 100%. Mean sizes and standard deviations are shown. Asterisks indicate significant (* $p < 0.05\%$, ** $p < 0005$) reduction compared to wild-type plaques sizes.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Blood samples from infected and uninfected carp were centrifuged at 600 x g for 15 min and the supernatant was used in the ELISA. NUNC POLYSORP immuno-plates (Thermo Fisher Scientific) were coated with sucrose-gradient purified KHV-T particles, blocked, incubated with the 1 : 300 diluted sera in two replicates. An anti-carp IgM monoclonal antibody (Aquatic diagnostics), and horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch) were added as described (Bergmann et al., 2017). Substrate reactions were measured in an Infinite F200 microplate reader (Tecan), and OD_{450nm} values > 0.2 were considered as positive.

3. Results and discussion

3.1. Generation and genetic characterization of glycoprotein deletion mutants of KHV-T

Previous studies have shown that sera from naturally or experimentally CyHV-3 infected carp and koi specifically reacted with cells transfected with expression plasmids encoding pORF25, pORF65, pORF148, and pORF149, which are distantly related members of a family of type I envelope glycoproteins (Fuchs et al., 2014). Despite their immunogenicity, the four glycoproteins have recently been shown to be dispensable for *in vitro* replication of a highly virulent CyHV-3 isolate from Belgium, which has been cloned and engineered as a bacterial chromosome (Costes et al., 2008; Vancsok et al., 2017). In the present study, we generated similar gene deletion mutants of the cell culture-adapted strain KHV-T (Mletzko et al., 2017).

Like in our previous studies isolation of the gene deletion mutants, and of corresponding revertants was facilitated by transient insertion of a GFP reporter cassette (Schröder et al., 2018). The deletion of ORF25 (609 codons, Fig. 1B) encompassed codons 138 to 545, whereas from ORF65 (595 codons, Fig. 1C), codons 36 to 425 were removed, and the remaining 5′ and 3′ ends were fused out-of-frame in the final deletion mutants KHV-TΔ25 and KHV-TΔ65. The single deletions of ORF148 (602 codons) and ORF149 (700 codons), removed codons 70 to 281 and 17 to 434, respectively (Fig. 1D), and frameshifted the retained parts of the ORFs in KHV-TΔ148 and KHV-TΔ149. Consequently, the double deletion ranged from codon 17 of ORF149 to codon 281 of ORF148, but in this case the deletion was in frame. However, expression of a chimeric membrane protein by KHV-TΔ148/149 is unlikely, since the predicted signal peptide of pORF149 was affected by the deletion (Fig. 1D).

Genomic DNA of all generated CyHV-3 recombinants was analyzed by restriction enzyme digestions and Southern blot hybridizations, which confirmed the desired, and indicated no unwanted alterations (see supplementary figure S1). Furthermore, the mutated genes were amplified from viral DNA by PCR and subsequently sequenced using the primer pairs CyHV-3O25-F and -R, CyHV-3O65-F and -R, or CyHV-3O148-R and CyHV3-O149-F, respectively (Fuchs et al., 2014). The PCR products of all CyHV-3 mutants exhibited the expected sizes (Fig. 2) and sequences. Analysis of the complete genome sequence was performed only for the recombinant KHV-TΔ148/149. As expected, this sequence was very similar to that of the parental virus strain KHV-T (Gao et al., 2018), except the deletion within ORFs 148 and 149 (Table 2). Nine of the 11 other observed alterations affected homopolymeric nucleotide stretches or tandem repetitions of short motifs which tend to be highly variable. Although few of these regions were localized in ORFs 45 and 52, no frameshifts or stop codons were introduced (Table 2). We found similar differences to the published sequence, including two single base substitutions, and a considerable elongation of ORF52 also in another recently sequenced recombinant of KHV-T which lacked the viral thymidine kinase and dUTPase genes (Schröder et al., 2018). Thus, these alterations are presumably present in the KHV-T variants used in different labs, and not due to accidental mutations which occurred during preparation of the virus recombinants.

3.2. Protein expression of the CyHV-3 mutants

Protein expression of the generated glycoprotein gene deletion mutants of KHV-T was analyzed using available antisera (Fuchs et al., 2014). CCB cells were harvested for Western blotting 2 d after infection at an MOI of 1 with the respective CyHV-3 mutants. Glycoprotein pORF25 was detectable in cells infected with KHV-T or the revertant KHV-T25R, but not in cells infected with KHV-TΔ25 (Fig. 3A). Like the corresponding protein of KHV-I (Fuchs et al., 2014), pPORF25 of KHV-T (calculated mass of the primary translation product of 66.1 kDa), exhibited an apparent molecular mass of approximately 125 kDa, indicating presence of several carbohydrate chains, i.a. at 5 predicted N-linked glycosylation sites (asparagine-X-serine/threonine). In contrast, pORF149 (calculated mass 72.0 kDa) contains only one potential N-glycosylation site, but three protein bands of approximately 100, 120 and 160 kDa were detected by the antiserum (Fig. 3B). The specificity of these reactions was confirmed by the absence of these signals in cells infected with the deletion mutants KHV-TΔ149 and KHV-TΔ148/149, and their reappearance in ORF149 rescuants (Fig. 3B). It remains to be elucidated whether the large forms of pORF149, which contains 74 serine (10.6%) and 143 threonine (20.5%) residues, are due to O-linked glycosylation, other post-translational modifications, or stable protein-protein interactions. An antiserum against the major capsid protein pORF92 of CyHV-3 (Fuchs et al., 2014) served as control, and similar amounts of the approximately 140 kDa gene product (calculated mass 139.6 kDa) were detected in all infected samples (Fig. 3, lower panels). Our available pORF65- and pORF148-specific sera (Fuchs et al., 2014) did not permit unambiguous detection of their target proteins by Western blot or IF analyses of CyHV-3 infected cells, but expression of pORF25 and pORF149 was demonstrated to be unaffected in ORF65 and ORF148 mutants of KHV-T (results not shown).

Although we cannot identify pORF148 directly, previous studies showed that its expression in another CyHV-3 strain was not affected by deletion of the upstream ORF149 (Vancsok et al., 2017), and, therefore, it appears likely that ORF148 is also expressed by KHV-TΔ149. This assumption is supported by the different phenotypes of our ORF148, ORF149, and double deletion mutants (see below).

The absence of pORF65 from cell lysates infected with KHV-TΔ65 or KHV-Δ65G, as well as its rescue in KHV-T65R, could be demonstrated by lateral flow tests (FASTest® KOI HV, Megacor) based on pORF65-specifc mAb (Vrancken et al., 2013) (see supplementary figure S2). This

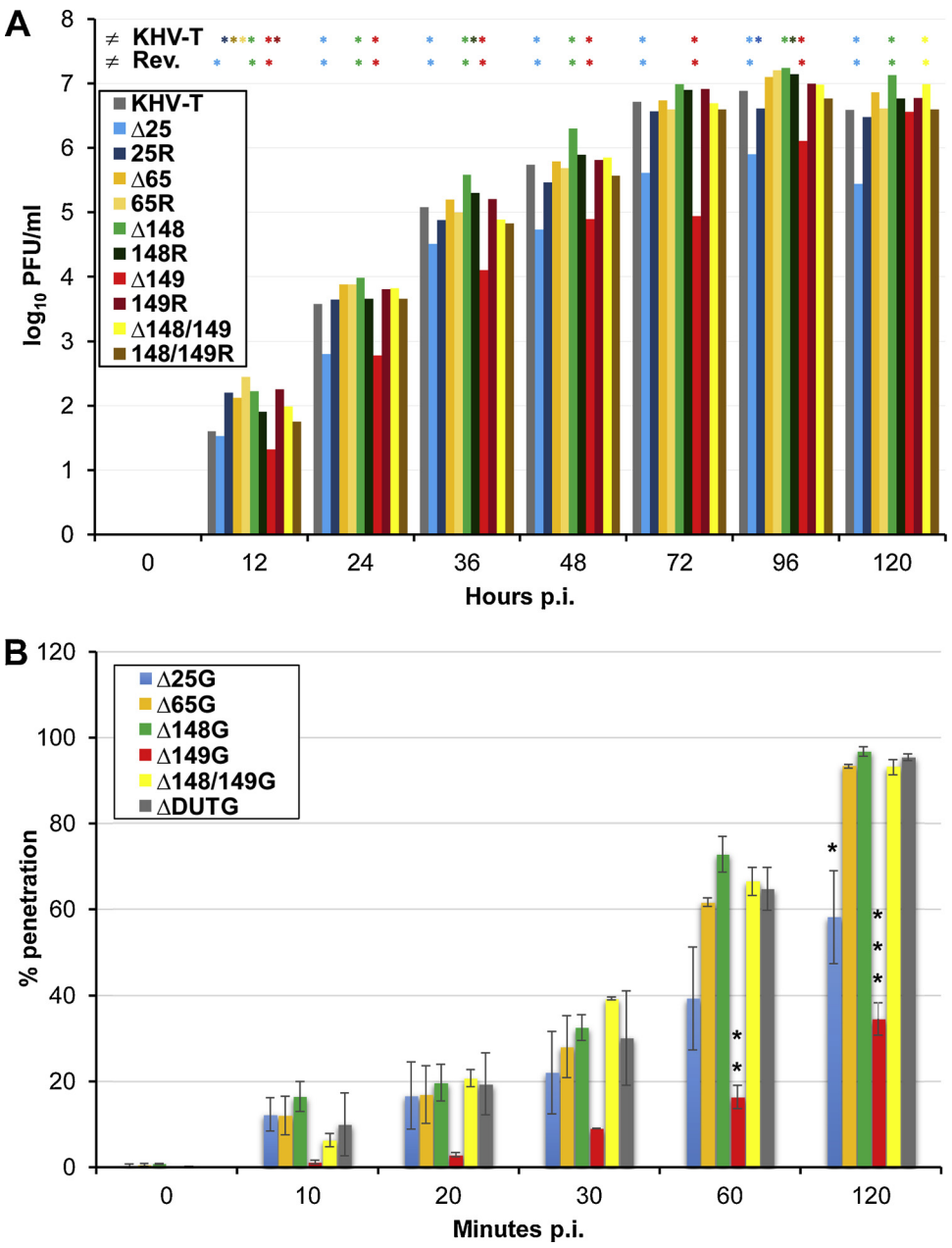

Fig. 5. Replication and penetration kinetics of CyHV-3 recombinants on CCB cells **(A)** For determination of replication kinetics the cells were infected at a MOI of 0.1, and incubated for the indicated times at 25 °C. Total progeny virus titers were determined by plaque assays on CCB cells. Shown are the mean results of four experiments. Asterisks indicate significant ($p < 0.05$) titer differences compared to wild-type virus (≠ KHV-T), and to the corresponding rescue mutants (≠ Rev.) at the same time after infection. **(B)** For determination of penetration kinetics CCB cells were infected with approximately 250 PFU of the GFP-expressing KHV-T recombinants, using the described GFP-expressing dUTPase-negative mutant KHV-TΔDUTGFP (Schröder et al., 2018) as wild-type like control. After adsorption at 4 °C, the cells were incubated for indicated times at 25 °C prior to acid inactivation of non-penetrated virus. Then incubation at 25 °C was continued under plaque assay conditions for 3 d. Numbers of plaques were compared to those obtained without acid inactivation of the same virus mutant. Mean percentages of three independent experiments and standard deviations are shown. Asterisks indicate significantly reduced (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.005$) penetration rates.

test might also permit fast differentiation of potential CyHV-3 vaccines lacking ORF65 from wild-type virus in carp.

3.3. In vitro replication properties of the CyHV-3 mutants

Cell-to-cell spread of KHV-T and the derived mutants was compared by plaque assays on CCB cells, which were evaluated 4 d after infection (Fig. 4). Infected cells were identified by IIF tests and plaque areas were determined microscopically. The plaque sizes of the ORF65 and ORF148 mutants ranged between 95% and 99% of wild-type levels, which was statistically not significant. In contrast, plaque sizes of KHV-TΔ25G, KHV-TΔ25, KHV-TΔ149G, and KHV-TΔ149 were significantly ($p < 0.005$) reduced by approximately 50% compared to wild-type KHV-T. These spreading defects were corrected in the rescue mutants KHV-T25R and KHV-T149R. Interestingly, the double gene deletions in KHV-TΔ148/149G and KHV-TΔ148/149 also resulted in almost wild-type sized plaques (Fig. 4), indicating that the adverse effect of ORF149 deletion is compensated by the additional removal of ORF148. Although plaques of KHV-TΔ148/149 appeared significantly ($p < 0.05$) smaller than those of KHV-T, no significant size differences were observed between the deletion mutant and the rescuant KHV-T148/149R.

Growth kinetics analyzed after synchronized infection at a MOI of 0.1 confirmed that ORF25, ORF65, ORF148 and ORF149 are dispensable for productive replication of CyHV-3 in CCB cells (Fig. 5A). Single deletions of ORF25 (blue bars) or ORF149 (red bars) resulted in delayed replication compared to wild-type KHV-T and the corresponding revertants. The observed differences were statistically significant ($p < 0.05$) at nearly all times after infection. The ORF25-deleted, but not the ORF149-deleted virus also exhibited a significant, approximately tenfold reduction of final titers. Whereas deletion of ORF65 (orange bars), and ORF148/149 (yellow bars) had no distinct effect, single deletion of ORF148 (green bars) slightly enhanced replication. This enhancement was significant ($p < 0.05$) at 6 of the 7 analyzed times after infection when compared to wild-type KHV-T, and at 5 time points when compared to the revertant KHV-T148R (Fig. 5A). Apart from few outliers at single times, all revertant viruses exhibited wild-type like growth properties (Fig. 5A). The GFP-expressing gene deletion mutants exhibited similar replication kinetics as the

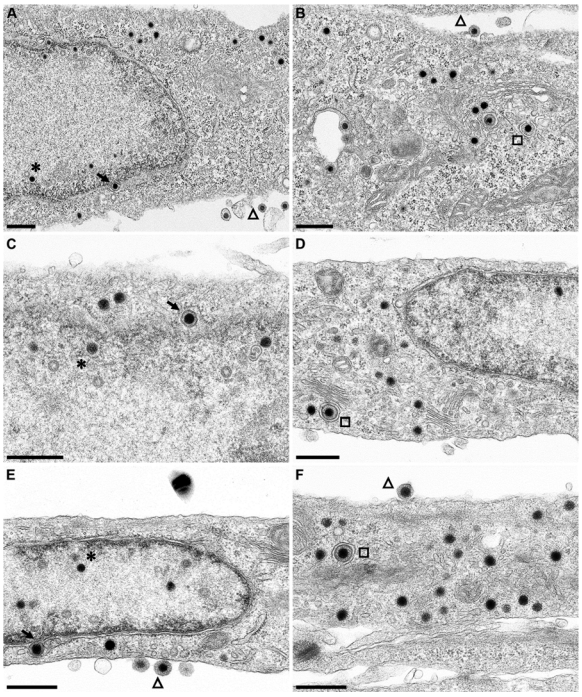

Fig. 6. Virion morphogenesis of CyHV-3 glycoprotein mutants. CCB cells were infected with KHV-T (**A, B**), KHV-TΔ149 (**C, D**) or KHV-TΔ25 (**E, F**) at an MOI of 1, and fixed and processed for transmission electron microscopy after 20 h at 25 °C. Empty capsids and nucleocapsids in the nucleus (asterisks), nuclear egress (arrows), final envelopment in the cytoplasm (squares), and extracellular virus particles (triangles) are indicated. Bars represent 500 nm.

corresponding reporter-less mutants (results not shown), and were omitted to reduce complexity of the diagram. However, to confirm the effects of ORF148 and ORF149 deletion, the corresponding subset of CyHV-3 recombinants was reanalyzed in a second series of growth kinetic studies at lower MOI (0.01) (see supplementary figure S3). These studies confirmed that, independent of presence or absence of a GFP reporter gene, the ORF149 deletion delayed, and the ORF148 deletion enhanced replication of KHV-T. Again the effects appeared additive, resulting in wild-type like growth of the double deletion mutants.

In general, these results are in line with those of previous investigations of similar single gene deletion mutants generated by mutagenesis of a BAC-cloned CyHV-3 isolate from Belgium (Vancsok et al., 2017). These studies also showed wild-type like growth of the ORF65-deleted virus, but delayed replication and cell-to-cell spread of ORF25- and ORF149-deleted mutants on CCB cells, as well as reduced final titers of ORF25-negative virus. However, unlike in our experiments a beneficial effect of ORF148 deletion was not observed, and ORF148/149 double mutants were not investigated in the previous study (Vancsok et al., 2017).

To further elucidate the functions of the ORF25 gene family members of CyHV-3, we analyzed *in vitro* penetration kinetics of the different deletion mutants using acid-inactivation protocols established for another herpesvirus, Pseudorabies virus (PrV, *Suid alphaherpesvirus 1*) (Mettenleiter, 1989; Vallbracht et al., 2017). To facilitate evaluation by direct fluorescence microscopy, the GFP-expressing CyHV-3 glycoprotein deletion mutants were used for these experiments, and a GFP-expressing dUTPase-deletion mutant of KHV-T (KHV-TΔDUTGFP) served as "wild type" control. This virus had been shown to exhibit wild type-like replication properties in CCB cells (Schröder et al., 2018). After synchronized adsorption to CCB cells on ice, infectious particles of KHV-TΔDUTGFP, KHV-TΔ65G and KHV-TΔ148G were shown to enter cells almost completely within two hours at 25 °C (Fig. 5B). In contrast, only 58% of the KHV-TΔ25G and 36% of the KHV-TΔ149G particles became insensitive to acid treatment within this time, and also at earlier times lower proportions of these two mutants had penetrated into the cells (Fig. 5B). For ORF149-deleted CyHV-3 the observed penetration defect is statistically highly significant ($p < 0.005$). These findings explain the delayed replication (Fig. 5A), and the reduced plaque sizes (Fig. 4) of ORF25 and ORF149-deleted CyHV-3. Interestingly, the double mutant KHV-TΔ148/149 G exhibited wild type-like penetration kinetics (Fig. 5B).

To exclude relevance of the envelope glycoproteins pORF25, pORF65, pORF148, and pORF149 for virion morphogenesis, CCB cells were analyzed by electron microscopy 20 h after infection with KHV-T or the single and double mutants at a MOI of 1 (Fig. 6). As exemplarily shown for KHV-TΔ25 and KHV-TΔ149, all stages of morphogenesis, including nucleocapsid formation in the nucleus (Fig. 6A, C, E, asterisks), nuclear egress by transit through the nuclear membrane (Fig. 6A, C, E, arrows), final envelopment of nucleocapsids in the cytoplasm (Fig. 6B, D, F, squares), and released mature virus particles (Fig. 6A, B, E, F, triangles) were found in cells infected with either virus. Thus, we found no evidence for a role of the investigated glycoproteins in maturation or egress of CyHV-3 virions, although it cannot be excluded considering a potential functional redundancy within the paralogous ORF25 family proteins.

However, based on our results the ORF25 family members pORF25 and pORF149 play an accessory role during virus entry into host cells. This process and the involved proteins have been extensively investigated for mammalian alphaherpesviruses like herpes simplex virus type 1 (HSV-1, *Human alphaherpesvirus 1*) (Eisenberg et al., 2012; Sathiyamoorthy et al., 2017), whereas little is known about the corresponding gene products of alloherpesviruses. In most alphaherpesviruses four envelope glycoproteins (gB, gD, gH, gL) are required for specific receptor binding and subsequent fusion of the viral envelope with the host cell plasma membrane. However, except gD, the former proteins are conserved throughout the *Herpesviridae*, whereas the ORF25 family seems to be restricted to the *Cyprinivirus* genus of the *Alloherpesviridae* (Aoki et al., 2007; Davison et al., 2013). Moreover, at least the core fusion protein gB, and gH are absolutely essential for productive replication of the *Herpesviridae*, whereas the ORF25 family members of CyHV-3 are dispensable (Vancsok et al., 2017; this study). Therefore, the proteins investigated in this study are presumably not required for the membrane fusion process per se. However, it appears conceivable that the structurally related gene products of the ORF25 family represent redundant receptor binding proteins of CyHV-3, which are able to bind the same or different host cell membrane proteins, and to activate the hitherto unknown alloherpesvirus fusion protein(s). Therefore, it has to be tested whether combined deletion of all ORF25 family members from the CyHV-3 genome, *i.e.* of ORF25, ORF65, ORF148, ORF149, and the often spontaneously deleted or frameshifted ORFs 26 and 27, still results in a replication competent virus.

An interesting finding was the positive effect of ORF148 deletion, which compensated the adverse effects of ORF149 deletion in the double deletion mutants. Although KHV-TΔ148/149 might express a chimeric membrane protein containing the C-terminal part of pORF148, and a modified N-terminal signal sequence originating from pORF149, this was not possible in KHV-TΔ148/149 G, where the two gene remnants were separated by the GFP cassette (Fig. 1D). Nevertheless both double mutants exhibited similar, almost wild type like *in vitro* replication properties, which were clearly improved compared to those of the ORF149 single deletion mutants (Figs. 4 and 5). Furthermore, full genome sequencing of KHV-TΔ148/149 revealed no accidental mutations compared to KHV-T which could compensate for ORF149 deletion. However, pORF148 and pORF149 exhibit an amino acid sequence identity of 37.5%, suggesting related "functions". Possibly, pORF148 represents a nonfunctional "copy" of pORF149, which is expressed and able to bind cellular receptors, but not to trigger subsequent virus entry. Thus, absence of pORF148 might facilitate receptor binding of pORF149, which seems to be the most relevant

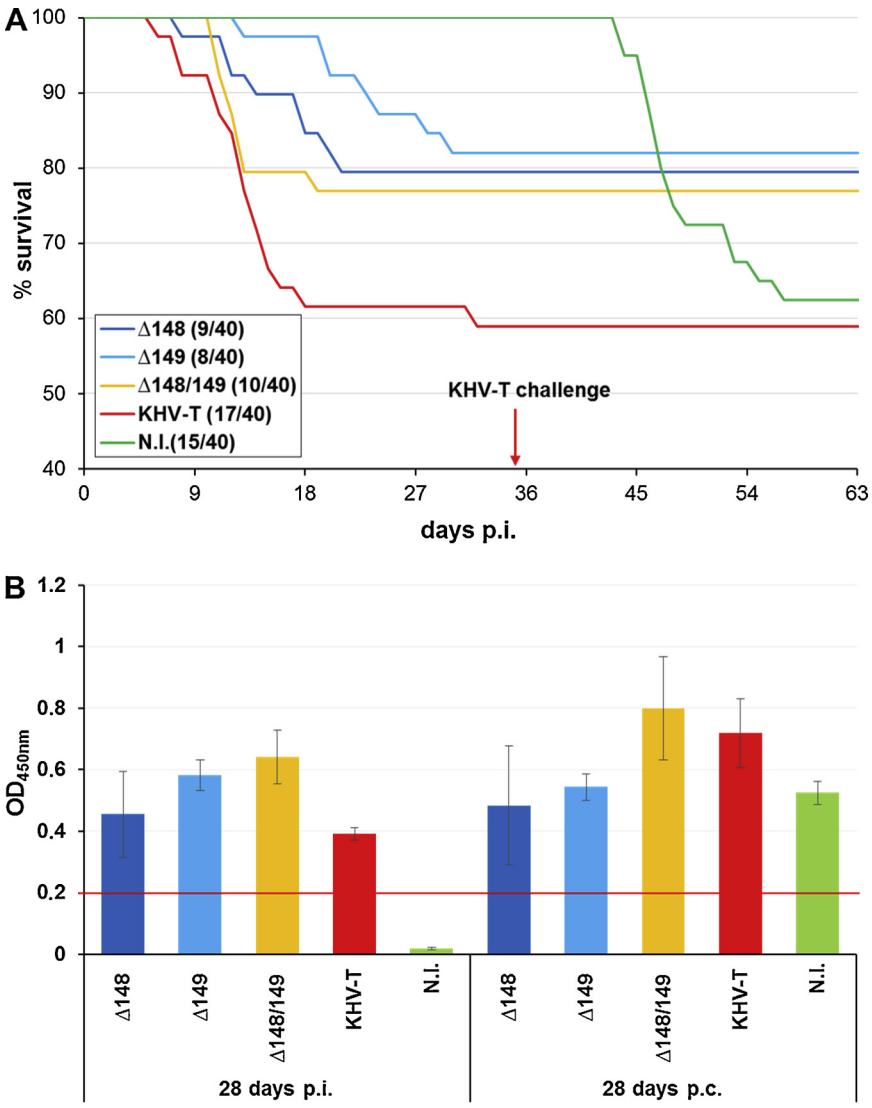

Fig. 7. Attenuation and protective efficacy of CyHV-3 recombinants. Nine months and two years-old carp were infected with KHV-T or the indicated virus mutants by immersion in 5 l water containing 5×10^3 PFU/ml. After 35 d all surviving fish and mock-infected control animals (N.I.) were challenged with the same dose of KHV-T. (A) Shown are the time courses of survival rates (%) of the nine months-old fish, and total numbers of deceased and infected animals in each group. **(B)** Sera of two years-old carp collected 28 days after primary infection (p.i.) with the indicated viruses or mock infection (N.I.), as well as 28 days after challenge (p.c.) with KHV-T were tested for KHV-specific antibodies by an ELISA (Bergmann et al., 2017). The mean substrate reaction values (OD_{450nm}) of two replicas each of sera from four animals per group and standard deviations are indicated. Samples showing an OD_{450nm} of > 0.2 (red line) were considered as positive (For improved interpretation, the reader is referred to the coloured web version of this figure).

ORF25 family member for efficient entry (Fig. 5). In the absence of both proteins, other receptor binding proteins, *e.g.* pORF25 or pORF65, might be able to compensate. This hypothesis is supported by the moderately delayed penetration of ORF25-deleted CyHV-3 (Fig. 5).

3.4. Virulence and protective efficacy of ORF148 and ORF149 gene deleted CyHV-3 mutants in carp

Previous experiments have shown that deletion of ORF25 leads to attenuation of CyHV-3, but interferes with protection against subsequent wild type challenge (Vancsok et al., 2017). This might have been either due to the relevance of pORF25-specific immune reactions for protection (Zhou et al., 2014b), or to an inefficient *in vivo* replication of the deletion mutant. In contrast, ORF65, ORF148, and ORF149 single gene deletion mutants of the BAC-cloned Belgian CyHV-3 isolate were not sufficiently attenuated (Vancsok et al., 2017). However, previously we have shown that combined deletion of the TK and dUTPase genes of CyHV-3 leads to an improved attenuation compared to the corresponding single deletions without affecting protective efficacy of the candidate vaccine (Fuchs et al., 2011; Schröder et al., 2018). Therefore, we now examined the virulence of, and the immune responses induced by the double mutant KHV-TΔ148/149. The corresponding single mutants KHV-TΔ148 or KHV-TΔ149 were also evaluated, and compared to the parental strain KHV-T, which has been considered to be much less virulent than the virus used for previous *in vivo* testing of glycoprotein deletion mutants of CyHV-3 (Gao et al., 2018). In the present animal trial, five groups consisting of 40 nine months- and 5 two years-old carp, were infected by immersion in 5 l water containing 2.5×10^7 PFU of the virus mutants, KHV-T or cell culture medium. Between day 6 and 31 after wild-type infection, 17 of the juvenile carp died, resulting in a total mortality rate of 42.5% (Fig. 7A). This rate was twice as high as observed by others using the corresponding virus strain (Gao et al., 2018), but in line with the results of previous animal experiments with KHV-T in our institute (Schröder et al., 2018). It remains to be elucidated whether this difference is due to different susceptibilities of the used carp breeds, different virus doses or water temperatures, or to the few determined sequence alterations between the KHV-T preparations used in the different laboratories (Table 2) (Schröder et al., 2018).

Infection with KHV-TΔ148 or KHV-TΔ149 led to loss of 9 (22.5%) or 8 (20%) animals, respectively (Fig. 7A). Mortality caused by the double deletion mutant KHV-TΔ148/149 was slightly higher (10 of 40 carp or 25%). According to Fisher's exact tests the observed reductions of mortality rates compared to wild type infection were not significant ($p > 0.05$), and the animals of all infected groups developed one or more signs of CyHV-3 infection like fatigue, skin lesion and excess mucus production. This applied also to the two years-old fish, which, however, survived infection with wild-type or mutant KHV-T. Thus, in

line with previous results (Vancsok et al., 2017) deletion of ORF148 and ORF149 did not sufficiently attenuate CyHV-3 for use as a live vaccine. Unlike the TK and dUTPase double deletions (Schröder et al., 2018), combined removal of ORF148 and ORF149 did not improve *in vivo* attenuation of the virus, correlating with the enhanced *in vitro* growth of KHV-TΔ148/149 compared to KHV-TΔ149 (Figs. 4 and 5).

Five weeks after primary infection all surviving fish and naïve control animals were challenged by immersion in 5 l water containing 2.5×10^7 PFU of wild-type KHV-T. The mock-immunized carp developed severe clinical signs, and 15 of them (37.5%) died (Fig. 7A). In contrast, no symptoms were observed in any of the immunized carp, and all of them survived challenge infection (Fig. 7A). Although responses to heterologous, more virulent, challenge virus strains remain to be evaluated, the present results indicate that even combined deletion of the highly immunogenic envelope glycoproteins pORF148 and pORF149 (Fuchs et al., 2014) did not substantially affect protective efficacy of CyHV-3. In line with this, an ELISA using plates coated with purified CyHV-3 virions (Bergmann et al., 2017), indicated similar or even higher titers of specific serum antibodies in animals infected with the deletion mutants than in wild type infected carp (Fig. 7B). We have shown previously that several other CyHV-3 proteins, including glycoproteins pORF25, pORF65, pORF99, and the major capsid protein pORF92 also induce humoral immune responses in carp (Fuchs et al., 2014), which might have covered the lack of pORF148 and/or pORF149 specific antibodies in the present animal experiments. Since all glycoproteins encoded by the ORF25 gene family of CyHV-3 are obviously dispensable for *in vitro* virus replication (Vancsok et al., 2017, and this study), their deletion might support the development of DIVA vaccines (van Oirschot, 1999) which permit serological differentiation between immunized and wild type-infected fish.

Conflicts of interest

The authors declare that there are no conflicts of interest

Ethical statement

The carp used in this study were kept in accordance with the German protection of animal act. The experiments were approved by the animal experiment commission of the federal state Mecklenburg-West Pomerania. Suffering of animals was minimized.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2018.12.004.

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