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ORIGINAL ARTICLE

Identification of structural proteins of koi herpesvirus

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Abstract As a prerequisite for development of improved vaccines and diagnostic tools for control of the fish pathogen koi herpesvirus, or cyprinid herpesvirus 3 (CyHV-3), we have started to identify putative viral envelope and capsid proteins. The complete or partial CyHV-3 open reading frames ORF25, ORF65, ORF92, ORF99, ORF136, ORF138, ORF146, ORF148, and ORF149 were expressed as bacterial fusion proteins, which were then used for preparation of monospecific rabbit antisera. All of the sera that were obtained detected their target proteins in cells transfected with the corresponding eukaryotic expression plasmids. However, only the type I membrane proteins pORF25, pORF65, pORF99, pORF136 and pORF149 and the major capsid protein pORF92 were sufficiently abundant and immunogenic to permit unambiguous detection in CyHV-3-infected cells. In indirect immunofluorescence tests (IIFT), sera from naturally or experimentally CyHV-3-infected carp and koi predominantly reacted with cells transfected with expression plasmids encoding pORF25, pORF65, pORF148, and pORF149, which represent a family of related CyHV-3 membrane proteins. Moreover, several neutralizing monoclonal antibodies raised against CyHV-3 virions proved to be specific for pORF149 in IIFT of transfected cells and in immunoelectron microscopic analysis of CyHV-3 particles. Since pORF149 appears to be an immunorelevant envelope protein of CyHV-3, a recombinant baculovirus was generated for its expression in insect cells, and pORF149 was shown to be incorporated into pseudotyped baculovirus particles, which might be suitable as diagnostic tools or subunit vaccines.

Introduction

Since the late 1990s, a virus disease leading to mass mortality of common carp (*Cyprinus carpio*) and koi (*Cyprinus carpio koi*) has spread over major parts of the world [7, 20, 28]. The causative agent was designated koi herpesvirus, or cyprinid herpesvirus 3 (CyHV-3), and classified as a member of the genus *Cyprinivirus* within the family *Alloherpesviridae* of the order *Herpesvirales* [19, 23, 27]. CyHV-3 possesses a double-stranded DNA genome of ca. 295 kbp, including 22-kbp direct repeat sequences at both ends of the genome. Complete genomic sequence analysis of three different isolates revealed the presence of 156 conserved open reading frames (ORFs) that are likely to encode proteins [2].

Mass spectrometric analysis of purified CyHV-3 particles detected 40 of the encoded gene products, including 13 predicted envelope proteins [26]. However, up to now, only a few CyHV-3 proteins have been identified and

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characterized using specific antisera or monoclonal antibodies (mAbs). These include the myosin-related virion protein pORF68 [3], the capsid triplex protein pORF72 [36], and the integral membrane proteins pORF65 and pORF81 [33, 40]. Detection and localization of additional gene products of CyHV-3 would be an important prerequisite for analysis of protein function, in particular, since the considerable phylogenetic distance of the *Alloherpesviridae* from well-characterized mammalian and avian herpesviruses [9, 10] precludes conclusions by analogy in most cases. Moreover, the identification of relevant immunogenic proteins of CyHV-3 should help in the development of improved vaccines and diagnostic tests.

Up to now, only conventionally attenuated live virus vaccines have been in use for the prevention of CyHV-3 infections in carp and koi [30–32]. Since these vaccines bear the risk of reversion to a more virulent phenotype, and since the protective efficacy of inactivated CyHV-3 preparations was unsatisfactory [30], CyHV-3 mutants with defined deletions of virulence-determining genes have been genetically engineered [8, 16]. Safer alternatives, such as potential subunit, or vectored vaccines containing selected CyHV-3 genes or proteins have not yet been described, but first candidate DNA vaccines were evaluated recently [42, 43].

Diagnosis of CyHV-3 infections is still largely limited to classical or real-time PCR detection of viral DNA [4, 5, 17, 18], since the specificity and/or sensitivity of serological tests for CyHV-3-specific antibodies such as ELISA and neutralization tests [1, 31, 34] is unsatisfactory. Most likely, ELISA specificity could be improved by substitution of CyHV-3 particles from infected cell cultures, which are presently used as antigens, by defined virion proteins produced in recombinant expression systems. Furthermore, monospecific antibodies might be utilized in competitive assays and also used for direct protein detection in tissues of CyHV-3-infected fish as shown previously with pORF65- and pORF81-specific antibodies [33, 40].

As promising tools for improved diagnostics and new vaccine development, we expressed nine CyHV-3 proteins in prokaryotic and mammalian and expression systems, and one of them in a baculovirus system. Since viral envelope glycoproteins usually represent major antigens for the humoral immune response, the ORFs 25, 65, 99, 136, 138, 146, 148 and 149 of CyHV-3 were chosen, which are all predicted to encode glycosylated type I integral membrane proteins [2]. ORF25, 65, 148, and 149 are members of a conserved family of distantly related cyprinid herpesvirus genes that might have evolved by duplication of a common ancestor [2, 10]. The corresponding four proteins, as well as pORF99 and pORF136, have been identified in CyHV-3 particles by mass spectrometry [26]. Furthermore, we have expressed and characterized the predicted major capsid protein encoded by ORF92 [2], which represents one of the most abundant virion components [26]. Our expression constructs were used for production of monospecific rabbit antisera, as well as for detection of the target proteins of CyHV-3-specific mAbs and serum antibodies from CyHV-3-infected fish.

Materials and methods

Viruses and cells

CyHV-3 strain Israel (CyHV-3-I) [20] was propagated in common carp brain (CCB) cells [28] grown at 25 °C in minimum essential medium (MEM) with 10 % fetal bovine serum (FBS) (Life Technologies). For preparation of CyHV-3 virus stocks, infected cells were harvested together with the supernatant when complete cytolysis occurred, and stored at −70 °C. Virions and viral DNA were prepared as described previously [14, 17, 33]. For plaque assays, CCB cell monolayers were incubated with serial virus dilutions for 2 h. Then, the inoculum was replaced by MEM containing 5 % FBS and 6 g of methylcellulose per liter. Because of their higher transfection efficiency rate, rabbit kidney (RK13) cells were used for transient expression studies of individual CyHV-3 genes after plasmid cloning (see below). These cells were grown and maintained at 37 °C in MEM containing 10 % or 5 % FBS. For propagation of baculoviruses, insect (Sf9) cells were grown in Grace's insect medium (Serva) with 10 % FBS at 27 °C.

Construction of expression plasmids

For cloning into prokaryotic and eukaryotic expression vectors, the complete ORFs 25, 65, 92, 99, 136, 138, 146,148 and 149 were amplified from genomic CyHV-3 DNA by PCR using Pfx DNA polymerase (Life Technologies). Amplification primers were designed based on the genome sequence of strain CyHV-3-I [2] and extended by adding artificial sequences containing unique *Eco*RI and *Xba*I or *Xho*I restriction sites for convenient cloning (Supplementary Table 1). After cleavage, the PCR products were inserted into the correspondingly digested plasmid vector pcDNA3 (Life Technologies), which permits protein expression in eukaryotic cells under control of the human cytomegalovirus (HCMV) immediate early promoter (Supplementary Figure 1). The DNA sequences of the complete insert fragments of the resulting plasmids were determined using the BigDye Terminator v1.1 Cycle Sequencing Kit and a 3130 Genetic Analyzer (Applied Biosystems).

For prokaryotic expression, the *Eco*RI/*Xho*I or *Eco*RI/*Xba*I insert fragments of the pcDNA3 constructs were recloned into plasmid pGEX-4T-1 (GE Healthcare), which

had been doubly digested with *Eco*RI and *Xho*I, or *Eco*RI and *Sma*I. Non-compatible fragment ends were blunted by treatment with Klenow polymerase prior to ligation. Since several of the originally obtained plasmids permitted only weak expression of the bacterial fusion proteins with glutathione S-transferase (GST) of *Shistosoma japonicum*, the insert fragments were 5'- and/or 3'-terminally truncated by double digestions with suitable restriction enzymes and subsequent religation (Supplementary Figure 1). The *Eco*RI/*Xho*I-fragment-containing ORF148 was recloned into the *Eco*RI/*Sal*I-digested expression vector pMAL-p2X (New England Biolabs), and the resulting plasmid had to be shortened by subsequent double digestions with *Eco*RI and *Not*I, and with *Ale*I and *Sbf*I, to achieve satisfactory expression of a fusion protein with the maltose-binding protein (MalE) of *E. coli* (Supplementary Figure 1).

For baculovirus expression, the *Eco*RI/*Xho*I-insert of pcDNA-KO149 was recloned into an *Eco*RI/*Kpn*I-digested derivative of transfer vector pFastBac Dual (Life Technologies), which had been modified by insertion of an expression cassette for green fluorescent protein to permit convenient selection of virus recombinants [22]. The baculovirus recombinant BV-KO149 was generated by site-specific transposition using the Bac-to-Bac system (Life Technologies).

Preparation of monospecific rabbit antisera, CyHV-3-specific mAbs, and carp sera

The prokaryotic expression plasmids pGEX-KO25, -KO65, -KO92, -KO99, -KO136, -KO138, -KO146, -KO149, and pMAL-KO148 were propagated in *E. coli* XL1-Blue MRF' (Agilent Technologies), and expression of the GST or MalE fusion proteins was induced for 3 h in LB medium containing 1 mM isopropyl β-D-1-thiogalactopyranoside. The proteins were purified as described [15], and adult rabbits were immunized by subcutaneous application of 100 µg of the purified fusion proteins emulsified in mineral oil four times at four-week intervals. Sera collected before and four weeks after the last immunization were analyzed.

Monoclonal antibodies against purified CyHV-3 particles [17] were prepared and characterized essentially as described [11]. Briefly, three female BALB/c mice were immunized with purified virions five times at five-week intervals, and fusion was performed 3 days after the last immunization. Hybridoma supernatants were screened for positive reactions in indirect immunofluorescence (IIF) tests on CyHV-3-infected cells and further analyzed for IIF reactions with RK13 cells transfected with expression plasmids encoding the investigated CyHV-3 proteins.

Sera from koi that had survived natural CyHV-3 infections were kindly provided by D. W. Kleingeld, Lower Saxony State Office for Consumer Protection and Food Safety, Hannover, Germany. Sera from one-year-old common carp were prepared prior to as well as 2 months after primary intraperitoneal infection with 10^3 PFU of CyHV-3-I, and another 2 months after secondary infection with the same virus.

Indirect immunofluorescence tests and confocal microscopy

For IIF tests, cells were grown in 24-well or 6-well plates that contained glass cover slips for subsequent confocal microscopy analysis. CyHV-3-infected CCB cells were incubated for 3 days under plaque assay conditions, Sf9 cells were incubated for 2 days after baculovirus infection at an m.o.i. of 0.1, and RK13 cells were used 2 days after transfection (XtremeGENE HP DNA Transfection Reagent, Roche) with expression plasmids. Cells were fixed with methanol and acetone (1:1) for 30 min at −20 °C, dried, blocked with 10 % FBS in PBS, and incubated with monospecific rabbit antisera (dilutions 1:100), hybridoma supernatants of CyHV-3-specific mAb (dilutions 1:10), or sera from CyHV-3-infected carp or koi (dilutions 1:50 to 1:500) for 1 h each. Binding reactions were detected by incubation for an additional hour with Alexa Fluor 488– or Alexa Fluor 568–conjugated anti-rabbit or anti-mouse antibodies (Life Technologies), or by subsequent incubation with an anti-carp IgM mAb (Aquatic Diagnostics) and conjugated anti-mouse antibodies. Secondary antibodies were diluted in PBS according to the manufacturer's instructions. In some experiments, chromatin was additionally stained with 500 ng/ml bisbenzimide H 33258 (Serva) in PBS for 10 min. The cells were repeatedly washed with PBS after each incubation step and preserved by treatment with a 9:1 mixture of glycerol and PBS containing 25 mg 1,4-diazabicyclooctane per ml and, optionally, 1 µg propidium iodide per ml for chromatin staining. Samples were analyzed either by conventional fluorescence microscopy (Nikon Eclipse Ti-S) or by confocal laser scanning microscopy (Leica SP5).

Western blot analysis

CCB cells were infected with CyHV-3-I at an m.o.i. of 0.1 and harvested after 3 days. Lysates of ca. 10^4 infected and control cells were separated by discontinuous SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibodies as described [29]. The monospecific rabbit sera were used at dilutions of 1:10,000 to 1:20,000. Chemiluminescence reactions of peroxidase-conjugated secondary antibodies were recorded (VersaDoc 4000 MP, Bio-Rad).

Neutralization tests

Cell-free virions of a CyHV-3 mutant expressing green fluorescent protein (KHV-TKG) [16] were diluted in MEM containing 10 % FBS to approx. 10^3 plaque-forming units (PFU)/ml and incubated for 2 h at 25 °C with and without 5 % hybridoma supernatants of CyHV-3-specific mAb, or 5 % of the rabbit-antisera described in this study. Rabbit preimmune sera and a mAb against glycoprotein gJ of avian infectious laryngotracheitis virus [38] served as negative controls. Residual virus titers were determined by plaque assays on CCB cells and evaluated by fluorescence microscopy. Mean titer reductions compared to negative controls were calculated from six experiments, and statistical significance was shown by Student's t-test.

Immunoelectron microscopy

Native virions of CyHV-3-I were prepared as described [33]. Baculovirus particles were purified from clarified (3000 × g, 20 min) supernatants of infected Sf9 cells by ultracentrifugation through cushions of 7.5 ml (per 25 ml supernatant) 25 % sucrose in PBS for 90 min at 25,000 rpm and 4 °C (SW32, Beckman) and resuspended in a small volume of PBS. The virions were adsorbed to Formvar-coated 400-mesh grids for 7 min. After blocking of the surface with 1 % coldwater fish gelatin, 20 mM glycine and 1 % BSA in PBS, the virions were incubated for 1 h with hybridoma supernatants of CyHV-3-specific mAb diluted 1:10 in PBS. After washing, diluted 10-nm-diameter gold-particle-tagged goat anti-mouse antibodies (BBI Solutions) were added for another 1 h. The virus particles were contrasted with 2 % phosphotungstic acid (pH 7.4) and examined using an electron microscope (Philips Tecnai 12).

Results and discussion

Cloning and expression of CyHV-3 genes encoding structural proteins

In the present study, the ORFs encoding eight predicted membrane proteins and the major capsid protein (ORF92) were cloned in prokaryotic and eukaryotic expression vectors (Supplementary Figure 1). The ORFs were amplified from genomic DNA of CyHV-3-I, or of an East Asian–origin CyHV-3 isolate. The deduced amino acid sequences of the cloned ORFs exhibited full identity to the published [2] sequences of CyHV-3-I (ORF25, ORF65, ORF92, ORF138, ORF148, ORF149; GenBank accession no. DQ177346) and the CyHV-3 strain TUMST1 (ORF99, ORF136; GenBank accession no. AP008984), respectively.

Monospecific rabbit antisera were raised against bacterial fusion proteins of the CyHV-3 gene products. Whereas the pORF136-, pORF138-, and pORF149 expression constructs contained the entire viral coding sequences, only truncated constructs containing codons 118 to 437 of ORF25, codons 35 to 351 of ORF65, codons 1 to 252 of ORF92, codons 81 to 255 of ORF99, codons 81 to 255 of ORF146, and codons 69 to 247 of pORF148 could be expressed at amounts sufficient for successful rabbit immunization.

All of the antisera that were obtained detected their target proteins in indirect IIF and Western blot analyses of rabbit kidney (RK13) cells transfected with eukaryotic expression plasmids encoding the corresponding CyHV-3 genes under the control of the human HCMV immediate-early promoter (Fig. 1, Table 1). Only moderate nonspecific background reactions were observed with untransfected cells or cells transfected with heterologous expression constructs (Fig. 1 and not shown). Furthermore, all pre-immune sera proved to be negative (results not shown).

Detection of virion proteins in CyHV-3 infected cells

Although all antisera showed strong specific reactions with transiently expressed target proteins in both Western and IIF analyses, none of the sera exhibited specific virus-neutralizing activities (not shown), and some of the Western blot or IIF reactions with CyHV-3-infected CCB cells were weak, or even undetectable. Only the rabbit sera raised against the major capsid protein pORF92 and the predicted membrane protein pORF149 showed strong reactions with virus-infected cells in IIF tests (Table 1). Weak IIF signals were also obtained with the pORF25- and pORF136-specific sera, whereas the other antisera showed no reactions beyond the background level (Table 1). Although pORF65 and pORF99 were undetectable in IIF analysis of infected cells, Western blot analysis demonstrated the presence of these proteins and confirmed viral expression of pORF25, pORF92, pORF136, and pORF149 (Fig. 2). In contrast, the ORF138, ORF146, and ORF148 gene products of CyHV-3 were neither detectable in Western blot nor in IIF analysis of infected cells (Table 1). These results indicated either that the amounts of these proteins were below the detection limit or that the epitopes detected by the antisera were hidden by conformations or modifications of the viral proteins that were different from those present in the products of the prokaryotic and eukaryotic expression constructs. Furthermore, it cannot be completely excluded that ORF138 and ORF146 are non-expressed pseudogenes of CyHV-3, since their products also had not been identified by mass spectrometric analysis of virus particles [26].

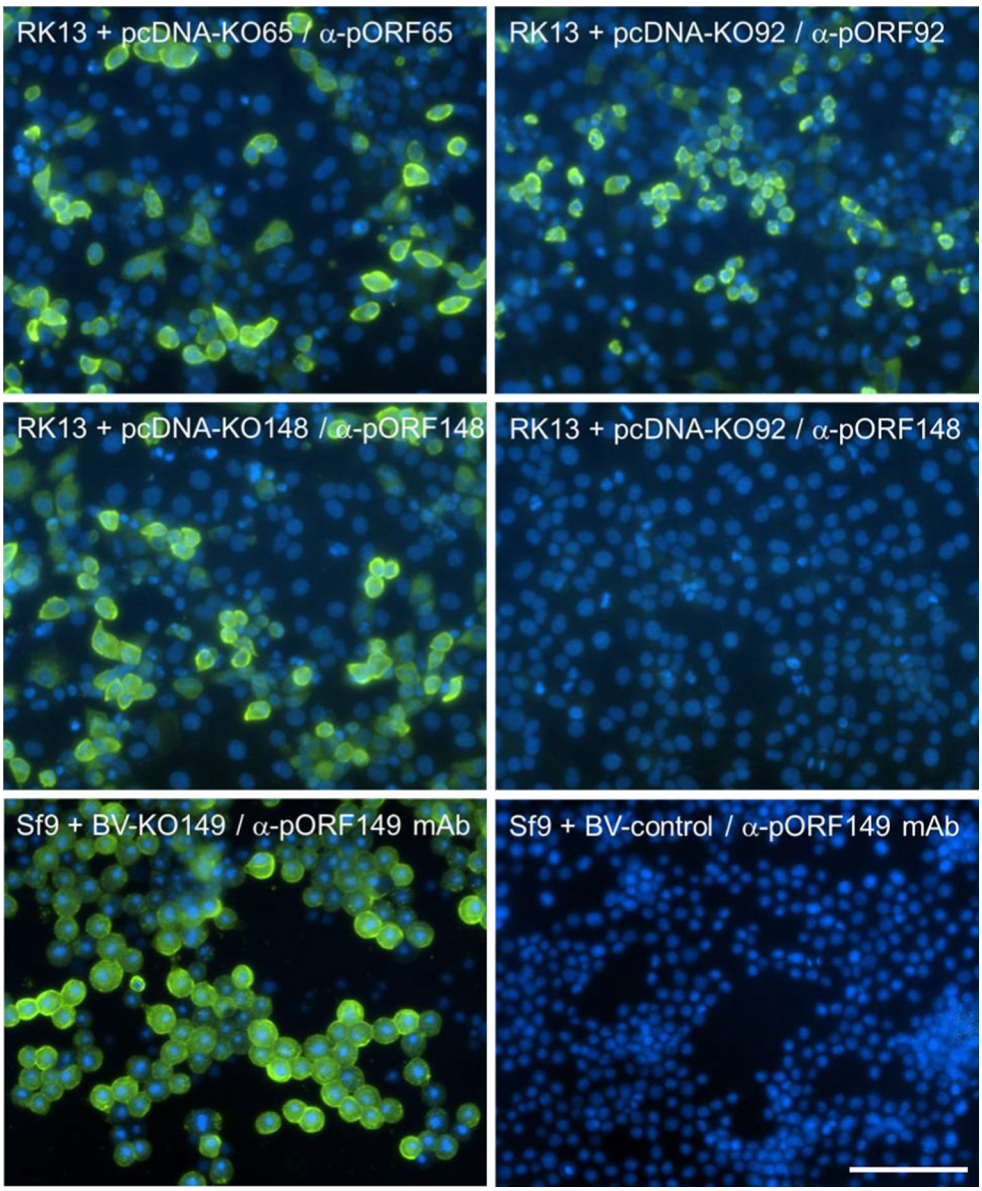

Fig. 1 IIF reactions of monospecific antibodies with recombinant CyHV-3 proteins. Rabbit kidney (RK13) cells transfected with eukaryotic expression plasmids for CyHV-3 proteins and baculovirus-infected insect cells (Sf9) were fixed after 48 h. Binding of rabbit antisera raised against bacterial fusion proteins, or of a CyHV-3-specific mAb (α-pORF149 mAb), was visualized using Alexa Fluor 488–conjugated secondary antibodies (green), and DNA was counterstained with Hoechst 33258 (blue). The bar represents 100 μm

In several instances, different processing was also indicated by comparison of the apparent molecular masses of the proteins detected in plasmid-transfected RK13 cells and CyHV-3-infected CCB cells (Table 1). Thus, expression of the proteins in fish cells and/or in the viral context might entail different glycosylation or the formation of stable protein complexes or aggregates that were not formed after expression of the individual proteins in mammalian cells. In addition to the effects of virus- or cell-type-specific enzymes, the structure and processing of the proteins might also have been influenced by the different incubation temperatures of the mammalian (37 °C) and fish cell cultures (25 °C) used in our studies.

Since all analyzed membrane proteins contain at least one putative N-glycosylation site (Asn-X-Ser/Thr) [24], it is likely that asparagine-linked carbohydrate chains contribute to the increased apparent molecular masses of the viral proteins compared to the calculated masses of the primary translation products (Fig. 2, Table 1). This was supported by tunicamycin-mediated inhibition of this modification [13] in cells transfected with expression plasmids but could not be investigated in CyHV-3-infected

Table 1 Properties of the investigated CyHV-3 proteins

Protein	Size (aa)[1]	Molecular mass (kDa)[2]			IIF detection[3]			
		Predicted	Apparent		Monospecific Ab		Sera from infected fish	
	(w/o SP)	(w/o SP)	Transfected	Infected	Transfected	Infected	Experimental	Natural
pORF25	601 (577)	65.4 (62.9)	85	125	++	+	+++	+++
pORF65	595 (574)	62.6 (60.9)	160	160	++	−	++	++
pORF92	1268	139.5	150	150	++	++	+	−
pORF99	1541 (1517)	166.8 (164.3)	190	80	++	−	++	−
pORF136	157 (140)	16.9 (15.1)	17	17	++	+	−	−
pORF138	392 (374)	45.2 (43.2)	70	−	++	−	−	−
pORF146	332 (309)	37.5 (35)	45	−	++	−	−	−
pORF148	609 (588)	63.9 (61.8)	95	−	++	−	+	++
pORF149	686 (659)	70.6 (67.8)	95	160	++	+++	+++	++

[1] Sizes and calculated [2] molecular masses of the proteins are given with and without predicted signal peptides [39]. The apparent masses of the most abundant proteins detected in Western blot analysis of plasmid-transfected and CyHV-3-infected cells are also indicated. [3]IIF reactions of monospecific antibodies with transfected and CyHV-3-infected cells and of sera from experimentally or naturally infected fish with expression plasmid-transfected cells were roughly quantified (no reaction: −, weak reaction: +, clear reaction: ++, strong reaction +++)

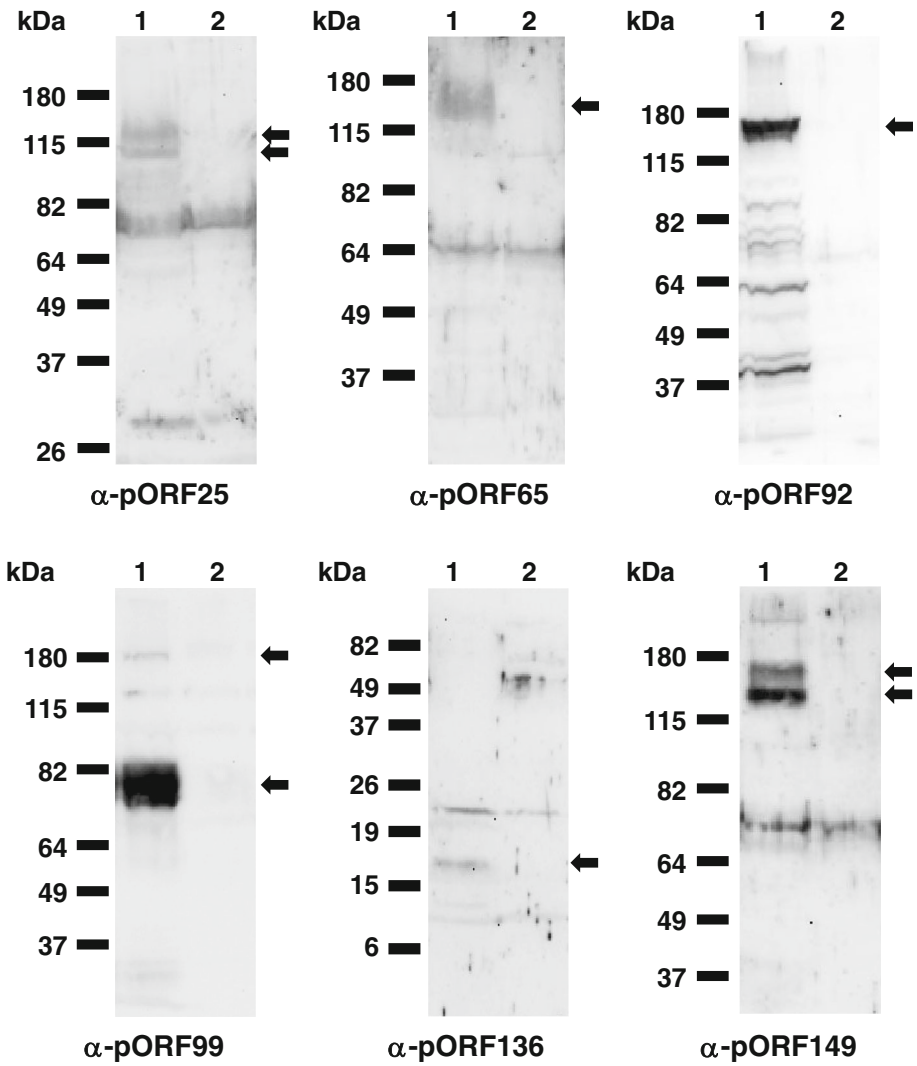

Fig. 2 Western blot detection of CyHV-3 proteins. Lysates of CyHV-3-infected (1) and uninfected (2) CCB cells were analyzed with monospecific rabbit antisera against pORF25, pORF65, pORF92, pORF99, pORF136, and pORF149. The masses of marker proteins are given, and specifically detected viral gene products are indicated by arrows

Table 2 Properties of CyHV-3 pORF149-specific mAbs

Antibody	Mouse	Subclass	IIF[1]	IEM[2]	WB[3]	Neutralization[4]
9F5	1	IgG 1	pcDNA-KO149, NT	CyHV-3, BV-KO149	-	63.9 % (±9.25)
10A9	1	IgG 1	pcDNA-KO149, BV-KO149	CyHV-3, BV-KO149	-	73.7 % (±8.95)
11A11	1	NT	pcDNA-KO149, NT	NT	-	63.5 % (±11.43)
17A9	2	IgG 2a	pcDNA-KO149, NT	NT	-	75.5 % (±9.63)

[1] Specific IIF reactions were observed with RK13 cells transfected with pcDNA-KO149 and with Sf9 cells infected with baculovirus BV-KO149. [2] Immunoelectron microscopy (IEM) was performed with purified CyHV-3 or BV-KO149 particles. [3] None of the mAbs showed positive Western blot (WB) reactions with transfected cells or with CyHV-3- or BV-KO149-infected cells. [4] All mAbs showed significant ($p < 0.001$) reactivity in virus neutralization tests. Mean titer reductions and standard deviations (in parentheses) of six independent experiments are given. NT: not tested

cells due to the deleterious effects of this drug on virus replication (results not shown). However, the small membrane protein pORF136, despite possessing five putative N-glycosylation sites in its ectodomain, was apparently not modified in this manner and exhibited approximately the calculated mass (Fig. 2, Table 1).

Interestingly, the majority of the viral ORF99 protein appeared significantly smaller than the transiently expressed gene product, and smaller than calculated (Fig. 2, Table 1). This strongly suggests proteolytic cleavage of the primary translation product, perhaps by a virus-encoded enzyme, since the deduced products of several CyHV-3 genes (ORF62, ORF78, ORF94) possess characteristics of proteases [2].

The envelope glycoprotein pORF149 is recognized by CyHV-3-specific monoclonal antibodies

To obtain monoclonal antibodies against structural components of CyHV-3, three mice were immunized with purified virions, and, after fusion of spleen cells, 30 hybridoma cell clones were shown to produce antibodies that reacted with CyHV-3-infected cell cultures in IIF tests (results not shown). Seventeen of these mAb were further characterized and tested for specific reactions with the nine CyHV-3 proteins investigated in this study, and with pORF81 [33]. Four of the mAb originating from two different mice and representing two different IgG subclasses showed strong specific IIF reactions with RK13-cells transfected with pcDNA-KO149 (Fig. 1, Table 2).

The reaction of one of these mAb (17A9) with CyHV-3-infected CCB cells was studied in more detail by confocal fluorescence microscopy, which revealed granular structures in the cytoplasm and in the extracellular space, but no pronounced accumulation at plasma membranes (Fig. 3). These findings were in line with the expected synthesis of pORF149 at the endoplasmic reticulum, followed by maturation and virion incorporation in the Golgi network. As, expected, pORF149 was not found within cell nuclei, whereas the reaction of the pUL92-specific rabbit serum demonstrated nuclear accumulation of the major capsid protein of CyHV-3 (Fig. 3).

Two of the pORF149-specific mAbs were further used for immunoelectron microscopy. Both antibodies detected their target protein in purified CyHV-3 particles (Fig. 4B, Table 2), but showed no reaction with pseudorabies virus (suid herpesvirus 1) particles (not shown). Thus, like pORF81 (Fig. 4A) [33], pORF149 is an abundant virion component, which confirms previous mass-spectrometric analyses [26]. Furthermore, we were able to demonstrate that all pORF149-specific mAb possess significant complement-independent virus-neutralizing activity (Table 2). This might indicate that pORF149 plays an important role during virus attachment to, or penetration of, host cells.

Despite strong positive reactions in IIF tests and immunoelectron microscopy, none of the four mAbs detected any CyHV-3-specific proteins in Western blot analysis of infected or pcDNA-KO149-transfected cells (Table 2). This indicates that the recognized epitopes might be discontinuous, or at least structure-dependent, and therefore no longer present after denaturation of pORF149. The viral target proteins of 13 other CyHV-3-specific mAbs remained unknown, although several of them showed strong Western blot reactions with distinct proteins in CyHV-3-infected cells (results not shown). Most likely, these mAb detect viral gene products other than those analyzed here (including pORF81), since no specific reactions with cells transfected with any of the available expression constructs were observed.

Reactions of sera from CyHV-3-infected carp with individual virion proteins

Although expression of individual CyHV-3 virion proteins in a heterologous system might affect their antigenic properties, it was tested whether they were detectable using serum antibodies from naturally or experimentally infected carp and koi. To this end, RK13 cells grown in microtiter plates were transfected with pcDNA plasmids encoding all of the investigated CyHV-3 proteins, including pORF81,

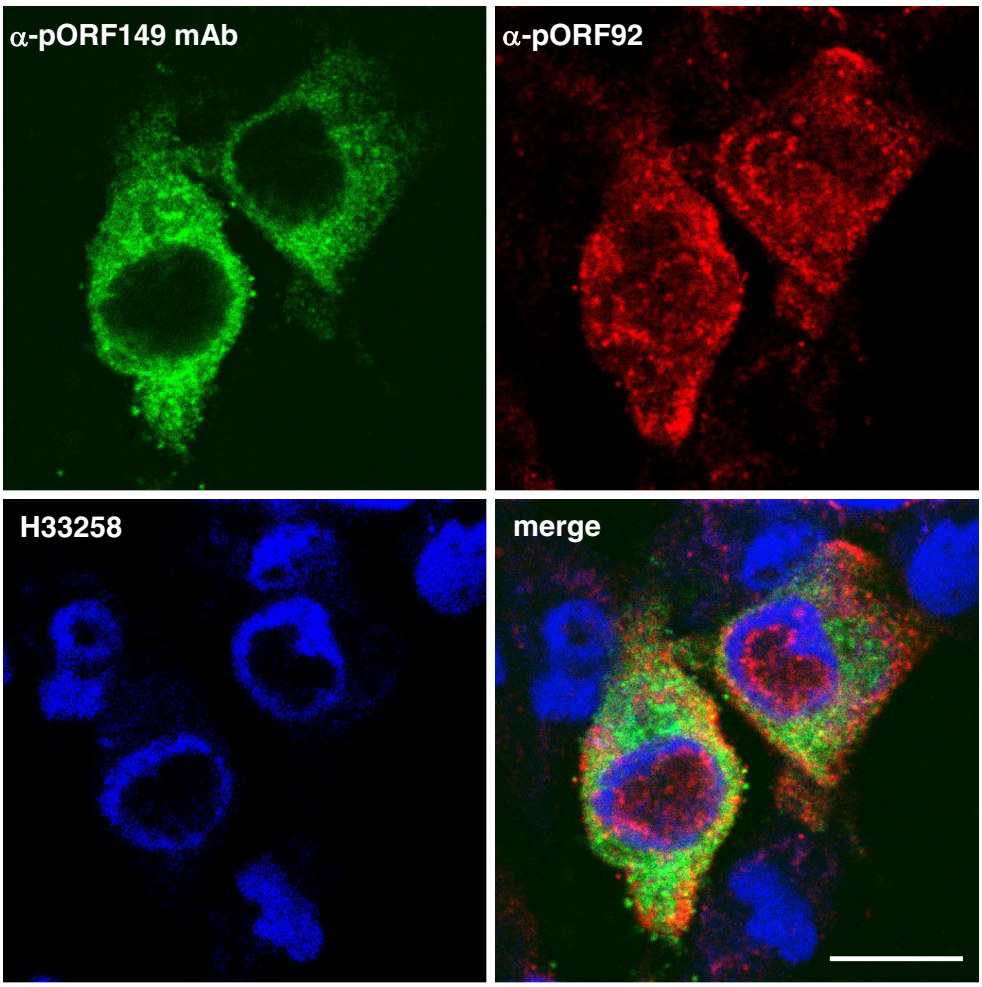

Fig. 3 IIF reactions of monospecific antibodies with CyHV-3-infected cells. Carp cells (CCB) were infected with CyHV-3 under plaque assay conditions and fixed after 3 days. Binding of a pORF149-specific mAb (α-pORF149 mAb) and of a pORF92-specific rabbit serum (α-pORF92) was visualized using Alexa Fluor 488– and Alexa Fluor 568–conjugated secondary antibodies, respectively. DNA was counterstained with Hoechst 33258. Fluorescence was analysed by confocal laser scanning microscopy. The bar represents 10 μm, and the size of the shown Z-section is 0.29 μm

and their abundant expression was confirmed by incubation of single wells with the corresponding monospecific rabbit antisera. Other wells were incubated with two sera each from uninfected carp, and from carp infected once or twice with CyHV-3. Furthermore, a pool of five sera from naturally infected koi that showed clear IIF reactions with CyHV-3-infected CCB cells was tested. At a dilution of 1:50, the control sera were negative as expected, whereas all other investigated fish sera showed strong specific IIF reactions with transiently expressed pORF25 and pORF149, and somewhat weaker reactions with pORF65 and pORF148 (Fig. 5, Table 1). Notably, these four gene products are members of a related family of type I membrane proteins of CyHV-3 [2, 10]. Thus, although the amino acid sequence identities were moderate, ranging from 28 % (pORF25-pORF149) to 44 % (pORF148-pORF149), it is conceivable that several carp antibodies might react with epitopes that are present in more than one of the proteins. However, the corresponding monospecific rabbit antisera and mAbs prepared during this study showed no cross-reactions in IIF tests or Western blot analysis (not shown). In addition, several of the analyzed carp sera detected the abundant [26] viral envelope protein pORF99 and the major capsid protein pORF92 (Fig. 5, Table 1). Surprisingly, reactions with pORF81 were not observed, although it has also been identified as an antigenic abundant virion protein of CyHV-3 [33] and as candidate gene product for DNA vaccines [43]. Possibly, the small ectodomain of this multiply membrane-spanning type III envelope protein is not sufficiently exposed in the viral context to induce a pronounced humoral immune response. The other investigated viral membrane proteins (pORF136, pORF138, pORF146) were also not detected by the analyzed fish sera, and this might be due to low

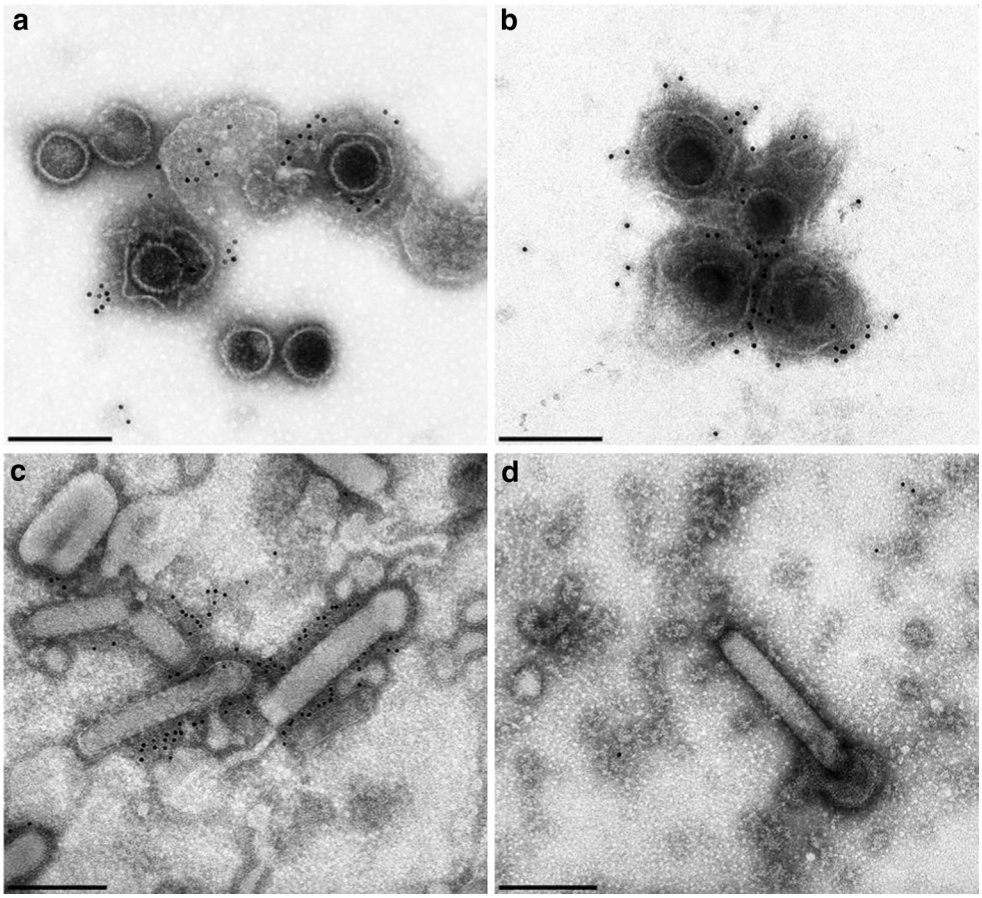

Fig. 4 Immunoelectron microscopy of CyHV-3 and recombinant baculovirus particles. Purified virions of CyHV-3 (**a**, **b**), a pORF149-expressing baculovirus recombinant (**c**), or a negative control baculovirus (**d**) were incubated with a pORF81-specific rabbit serum (**a**) or with a pORF149-specific mAb (**b**-**d**) and gold-tagged secondary antibodies. Bars represent 200 nm

expression of these proteins in CyHV-3-infected cells. Thus, the CyHV-3 envelope glycoproteins of the "ORF25 family" (pORF25, pORF65, pORF148, pORF149) and pORF99, either individually or in combination, might be proper target proteins for improved diagnostic tests, and they are possibly also suitable as components of subunit or vectored vaccines against CyHV-3 infection.

Baculovirus expression of CyHV-3 pORF149

Baculoviruses are well-proven vectors for large-scale expression of heterologous eukaryotic proteins that permit more-authentic modifications (e.g., glycosylation) than bacterial expression systems. In a first baculovirus recombinant, we inserted the authentic ORF149 of CyHV-3 under the control of the strong baculovirus p10 promoter (Supplementary Figure 1). Abundant expression of pORF149 was shown by IIF analysis of insect cells infected with the resulting virus BV-KO149 using the available mAb (Fig. 1) as well as the monospecific rabbit antiserum (not shown). The latter serum also specifically detected ORF149 products in Western blot analysis of proteins from purified BV-KO149 particles, which were not present in lysates of an irrelevant baculovirus recombinant (results not shown). Interestingly, the largest (ca. 160-kDa) forms of CyHV-3- and baculovirus-expressed pORF149 appeared quite similar, which might indicate congeneric post-translational modifications. Immunoelectron microscopy of BV-KO149 particles with a pORF149-specific mAb confirmed the presence of this protein in viral envelopes (Fig. 4C). We will now attempt to prepare corresponding pseudotyped baculovirus recombinants for the other identified immunorelevant proteins of CyHV-3, i.e., pORF25, pORF65, and pORF148. Because of the structural similarities of these "ORF25 family" proteins, they are likely to be incorporated into the insect virus particles as efficiently as pORF149.

In summary, the expression constructs generated in this study enabled us to prepare and evaluate monospecific antisera against nine predicted gene products of CyHV-3 and to unambiguously identify the major capsid protein pORF92 and the five membrane (glyco)proteins pORF25,

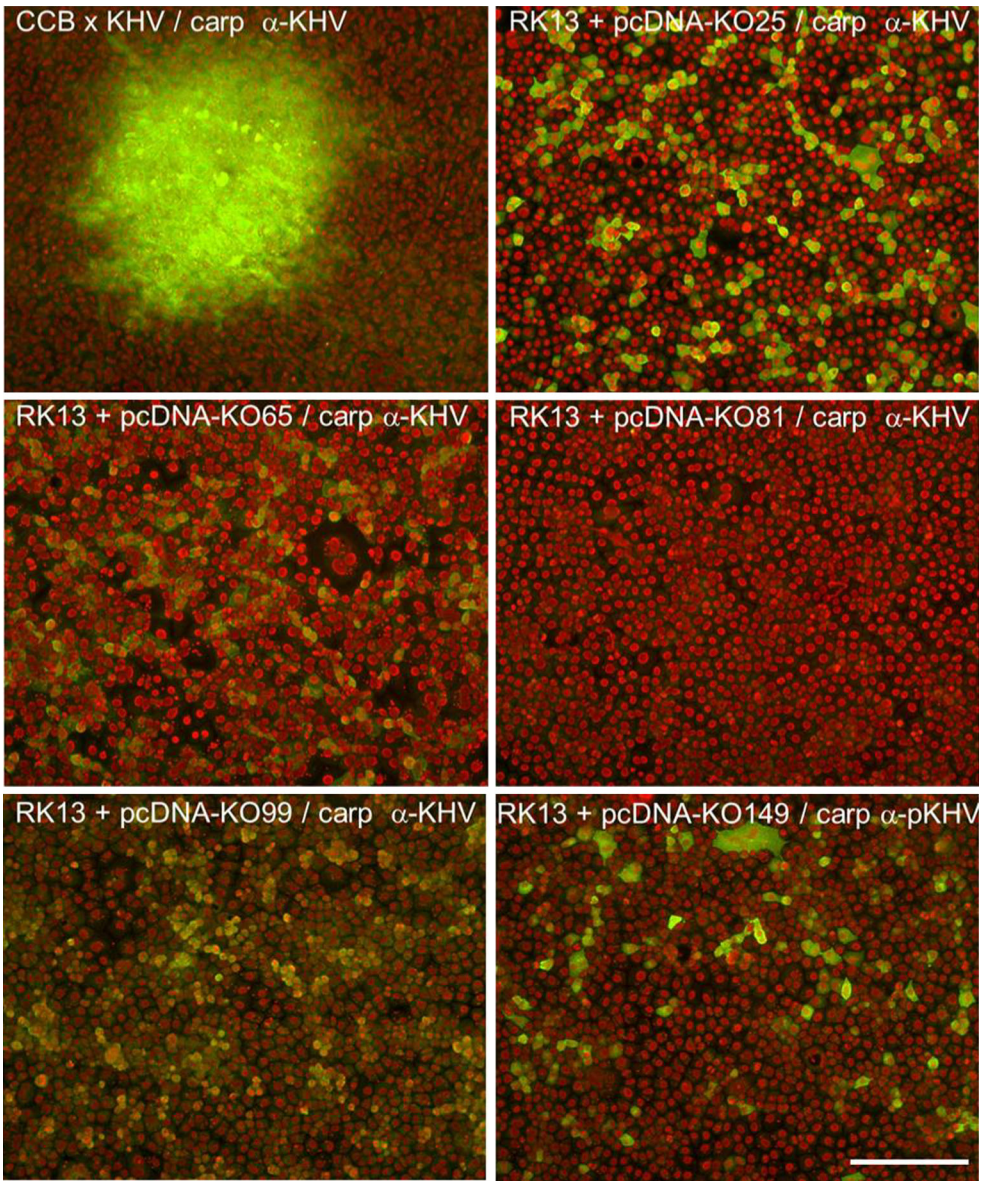

Fig. 5 IIF reactions of sera from CyHV-3-infected carp. CyHV-3-infected CCB cells and RK13 cells transfected with expression plasmids for individual CyHV-3 proteins were fixed after 2 days and incubated with a serum prepared from an experimentally CyHV-3-infected carp (2 months p.i.). Antibody binding was detected using a carp IgM-specific mAb and Alexa Fluor 488–conjugated anti-mouse antibodies (green). DNA was counterstained with propidium iodide (red). The bar represents 200 μm

pORF65, pORF99, pORF136, and pORF149 by IIF or Western blot analysis in virus-infected carp cells. The failure to detect the membrane proteins pORF138 and pORF146 correlates with their absence in mass spectrometric analysis of CyHV-3 particles [26] and might be explained by low expression levels. In contrast, the lack of reactivity of the rabbit serum raised against pORF148 was most likely due to low specific antibody titers, since abundant expression of this glycoprotein has been shown previously [26], and was confirmed here by detection of pORF148-specific antibodies in sera from CyHV-3-infected fish. Furthermore, the testing of fish sera with cells transfected with expression plasmids revealed that glycoproteins pORF25, pORF65 and pORF149 represent major targets of the CyHV-3-specific antibody response of carp and koi. Thus, the four products of phylogenetically related virus genes (OR25 family) [2] should be useful targets for the development of novel serological tests, e.g., of ELISAs using baculovirus-expressed antigens. Such assays might be further improved by introduction of the competitive anti-pORF149 mAb identified in this study and are likely to become more specific, and possibly also more sensitive, than the currently used ELISA tests based on crudely purified CyHV-3 particles [1, 31].

The pORF149-specifc mAb might be also suitable for detection of CyHV-3 antigen in samples from infected fish, as was previously described for antibodies against pORF65 and pORF81 [33, 40]. Furthermore, the anti-pORF149 mAbs may become valuable tools for investigation of CyHV-3 replication in cultured cells by confocal fluorescence microscopy or immunoelectron microscopy.

On the other hand, the available attenuated CyHV-3 mutants evaluated as putative live-virus vaccines [8, 16, 31, 32] still exhibit some residual virulence, which might further increase during animal passage, either by reversion, compensatory mutations or recombination between different CyHV-3 mutants. In particular for precious ornamental koi, subunit or DNA vaccines might be more expensive but safer alternatives. The efficacy of such recombinant vaccines against different viral and nonviral fish pathogens has been reported (reviewed in Ref. [6]), including baculovirus-expressed antigens and pseudotyped baculoviruses [25, 35, 41] and DNA vaccines utilizing the HCMV immediate-early promoter for expression of the viral antigens in immunized carp [12, 21]. Quite recently, it has been shown that DNA vaccination of koi with a pORF25 expression plasmid significantly reduced mortality after CyHV-3 challenge [42]. Thus, our baculovirus-expressed CyHV-3-proteins, as well as different combinations of the eukaryotic expression plasmid DNAs encoding the investigated CyHV-3 proteins also have to be evaluated as putative vaccines in animal experiments. If protective, these vaccines would also permit easy differentiation of infected from vaccinated animals (DIVA strategy) [37] by testing for the presence or absence of serum antibodies against defined glycoproteins encoded by the ORF25 gene family that are not contained in the vaccine.

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