The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions

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Koi herpesvirus, also known as cyprinid herpesvirus 3 (CyHV-3), is the aetiological agent of an emerging and mortal disease in common and koi carp. CyHV-3 virions present the characteristic morphology of other members of the order Iridovirales, being composed of an envelope, a capsid containing the genome and a tegument. This study identified CyHV-3 structural proteins and the corresponding encoding genes using liquid chromatography tandem mass spectrometry-based proteomic approaches. In addition, exponentially modified protein abundance index analyses were used to estimate the relative abundance of the identified proteins in CyHV-3 virions. These analyses resulted in the identification of 40 structural proteins, which were classified based on bioinformatic analyses as capsid (three), envelope (13), tegument (two) and unclassified (22) structural proteins. Finally, a search for host proteins in purified CyHV-3 virions indicated the potential incorporation of up to 18 distinct cellular proteins. The identification of the proteins incorporated into CyHV-3 virions and determination of the viral genes encoding these proteins are key milestones for further fundamental and applied research on this virus.

INTRODUCTION

Koi herpesvirus, also known as cyprinid herpesvirus 3 (CyHV-3; species Cyprinid herpesvirus 3, genus Cyprinivirus, family Alloherpesviridae, order Iridovirales), is the aetiologic agent of an emerging and mortal disease in common and koi carp (Cyprinus carpio koi) carp (Bretzinger et al., 1999; Hedrick et al., 1999, 2000). Since its emergence in the late 1990s, this highly contagious and dreadful disease has caused severe economic losses in both common and koi carp culture industries worldwide (Haenen et al., 2004; Hedrick, 1996).

The genome of CyHV-3 comprises a linear, double-stranded DNA sequence of ~295 kb (Aoki et al., 2007), similar to that of CyHV-1 (Waltzek et al., 2005), but larger than those of other members of the order Iridovirales, which generally range from 125 to 240 kb. Phylogenetic analysis of the CyHV-3 genome sequence led to its classification in the new family Alloherpesviridae, encompassing herpesviruses of fish and amphibians (Davison et al., 2009; McGeoch et al., 2006). The CyHV-3 genome contains 156 potential protein-coding open reading frames (ORFs), some of which have relatives in other herpesviruses, and a few of which have relatives in poxviruses, iridoviruses and other large DNA viruses (Aoki et al., 2007; Ilouze et al., 2006; Waltzek et al., 2005).

Like all members of the order Iridovirales, CyHV-3 virions are composed of an icosahedral capsid containing the genome, a lipid envelope bearing viral glycoproteins, and an amorphous layer of proteins termed the tegument, which resides between the capsid and the envelope (Miyazaki et al., 2008). CyHV-3 genome analyses performed by Aoki et al. (2007), which depended on the experimental findings of Davison & Davison (1995) for ictalurid herpesvirus 1 (IcHV-1), identified 27 predicted membrane proteins, putative components of the viral envelope and only three predicted capsid proteins. Electrophoretic protein analysis of CyHV-3 virions revealed the presence of 31 polypeptides (Gilad et al., 2002). The genes encoding these proteins have not been identified, with the exception of ORF81, which encodes a type 3 membrane protein expressed on the CyHV-3 envelope (Rosenkranz et al., 2008). The expression product of ORF81 (pORF81) is thought to be one of the most immunogenic (major) membrane proteins of CyHV-3 (Rosenkranz et al., 2008).

Identification of the protein constituents of the virion and determination of the viral genes encoding these proteins
represent key milestones for fundamental and applied research on CyHV-3. Indeed, this knowledge is crucial for understanding the biology and pathogenesis of CyHV-3 infection (including virus–cell interactions) and for facilitating the development of efficacious protein-based diagnostic methods and vaccine candidates. The goal of the present study was to identify CyHV-3 structural proteins and to determine the genes encoding these proteins. To reach this goal, two complementary approaches based on liquid chromatography tandem mass spectrometry (LC-MS/MS) were applied to various protein extracts. In addition, exponentially modified protein abundance index (emPAI) analyses (Ishihama et al., 2005) were used to estimate the relative abundance of these proteins in CyHV-3 virions. These approaches led to the identification of 40 virally encoded structural proteins and indicated the potential incorporation of up to 18 cellular proteins in mature virions.

RESULTS AND DISCUSSION

The goal of the present study was to identify CyHV-3 structural proteins and to determine the genes encoding these proteins. The analysis consisted of purifying CyHV-3 virions, separating the proteins by gel electrophoresis and identifying peptides in the proteins by MS.

Purification of CyHV-3 virions

Sample purity is crucial for analysing the protein content of viral particles. To monitor the purity of purified virions, they were analysed by electron microscopy (EM). As shown in Fig. 1, the viral preparation was free of detectable cell debris and most of the viral particles presented the characteristic morphology of mature virions, bearing an intact envelope surrounding the nucleocapsid. To test the purity of the particles further, they were analysed by gel electrophoresis and silver staining. Cell supernatant and cell lysate of mock-infected and CyHV-3-infected cells were used as controls. On a silver-stained gel, comparison of mock-infected and infected cell lysate bands suggested the presence of at least 15 bands specific to or increased in the viral samples (Fig. 2, lane 2, black dots). In the cell supernatant, only a few bands were identified as being specific to CyHV-3 infection (Fig. 2, compare lanes 3 and 4). Comparison of mock-infected and infected cell supernatant revealed that several bands present in the former were of reduced intensity or even absent in the latter (Fig. 2, compare lanes 3 and 4). This observation suggested that CyHV-3 infection might induce down-regulation of host cell protein expression. Analysis of purified virions revealed a strong enrichment of bands specific to the virus: at least 25 bands were identified (Fig. 2, lane 5, black dots). Importantly, viral purification led to the lack of detectability of cellular proteins, supporting the efficacy of the purification process. Taken together, these results suggested that the purified CyHV-3 preparation was relatively pure.

Fig. 1. Analysis of purified CyHV-3 virions by EM. Purified virions were negatively stained and observed by EM. Bar, 50 nm.

Fig. 2. Protein content analysis of purified CyHV-3 virions. Purified virions (0.1 µg), cell culture supernatant (1 µg) and cell lysate (1 µg) were loaded onto 4–12 % Bis–Tris polyacrylamide gels and silver stained. Black dots indicate bands that were found exclusively or that were more intense in infected conditions. Molecular masses in kDa are indicated on the left.
Identification of CyHV-3 viral proteins

In order to optimize the characterization of CyHV-3 proteins, two complementary MS strategies were used (Fig. 3a, b). Both approaches were accomplished in technical replicates.

The first strategy, termed 1D gel/nanoLC-MS/MS, consisted of extracting and separating CyHV-3 virion proteins by SDS-PAGE, followed by excision of 17 contiguous sections of the gel along the migration path. These sections were then subjected to ‘in-gel’ trypsin digestion followed by nanoLC-MS/MS analysis (Figs 3 and 4). This strategy was carried out with different protein extracts. The first extract, produced with Laemmli buffer (LB extract) led to the identification of 20 different proteins, a number that was greater than the number of visible bands (Fig. 4). In order to increase the proteome coverage, a sequential extraction procedure was performed on virions, using three successive detergent treatments (Fig. 3). Analysis of the Triton X-100 extract (TX extract) led to the identification of 13 additional proteins compared with the LB extract. The SDS extract revealed only one additional protein that was undetected in the former extracts (Fig. 4). Finally, purified virions were treated with different glycosidases (Deglyco extract) (Fig. 3a, b). This extraction led to the identification of only one additional protein that was undetected previously (Fig. 4).

A second MS approach, termed 2D nanoLC-MS/MS or ‘in-solution’ trypsin digest, was carried out as a complement to the gel-based approach. Indeed, omission of a gel separation step has been shown to enhance the recovery of peptides derived from proteins that are prone to aggregation, contain hydrophobic peptides or are present in low abundance. This strategy consisted of a shotgun method in which proteins were extracted by guanidine chloride (GC extract) (Fig. 3). After the extraction process, proteins were subjected to trypsin digestion, followed by analysis by 2D nanoLC-MS/MS. This complementary procedure led to the identification of five additional proteins that were not detected by the gel-based approach. Taken together, the procedures described above led to the identification of 40 virally encoded proteins in CyHV-3 virion particles (Table 1). This number is consistent with the numbers reported previously for other members of the family Herpesviridae (Johannsen et al., 2004; Kattenhorn et al., 2004; Loret et al., 2008; O’Connor & Kedes, 2006).

Among the 40 identified proteins, only nine (pORF62, -68, -72, -78, -81, -90, -99 and -123) exhibited homologies with expression products encoded by IcHV-1 and/or ranid herpesvirus 1 (RaHV-1) and RaHV-2 (members of the Herpesvirales). Two proteins (pORF32 and -123) presented homology with iridovirus and/or poxvirus expression products. Surprisingly, most of the CyHV-3 structural proteins presented no homology with members of the family Herpesviridae.

The 40 proteins identified in CyHV-3 virions are listed in Table 1 according to their putative localization in the viral particles. Their allocation to virion subcomponents was made (i) on the basis of previous predictions for CyHV-3 proteins by Aoki et al. (2007), which depended on the experimental findings of Davison & Davison (1995) for IcHV-1, (ii) on the presence of hydrophobic domains in some of the detected proteins (for envelope proteins) or (iii) not in any direct way from the results presented here. Proteins for which data were insufficient to suggest localization were classified as ‘unclassified proteins’.

**Fig. 3.** Schematic representation of the CyHV-3 protein extraction procedure (a) and the MS strategies (b) used in this study. SCX, Strong cation exchange; RPC, reverse-phase chromatography.
Three capsid, 13 envelope, two tegument and 22 unclassified structural proteins were identified. The relative abundance of these proteins in CyHV-3 virions was estimated by emPAI (Table 1, Fig. 5).

Capsid proteins. Capsids of herpesviruses are known to contain five conserved proteins (Mettenleiter et al., 2009; Steven et al., 2005). Here, we identified three orthologues of alloherpesvirus capsid proteins in CyHV-3 virions. The product of ORF92 encoding the orthologue of the major capsid protein of IcHV-1, RaHV-1 and RaHV-2 was found to be the most abundant protein in CyHV-3 virions with the highest emPAI. pORF72 was also detected as an abundant constituent of CyHV-3 virions, and is the orthologue of the capsid triplex protein 2 of IcHV-1, RaHV-1 and RaHV-2 (Table 1). The capsid maturation protease (pORF78) was found in low abundance in CyHV-3 particles and was identified by five peptides corresponding to the N-terminal part of ORF78 expression product. This protein may represent the functional homologue of human herpesvirus 1 (HHV-1) maturation protease VP24 (Homa & Brown, 1997).

Envelope proteins. Thirteen of the proteins identified in CyHV-3 virions were classified as putative envelope proteins (Table 1). Most of these proteins were predicted to be glycosylated by bioinformatics analysis. Despite extensive searches, no similarity was found between CyHV-3 and mammalian herpesvirus proteins, and few of the CyHV-3 proteins (pORF81 and -99) exhibited similarity with IcHV-1 gene products. These observations are consistent with the proposed evolution of CyHV-3, which is thought, based on host/virus co-speciation, to have diverged from mammalian herpesviruses and IcHV-1 around 450 and 250 million years ago, respectively (Briggs, 2005; Davison, 2002; Kumazawa et al., 1999). pORF81 is the only CyHV-3 envelope protein that has been described to date (Rosenkranz et al., 2008). It is a multi-spanning protein thought to be the immunodominant CyHV-3 envelope protein (Rosenkranz et al., 2008). Consistent with the results of Rosenkranz et al. (2008), we detected pORF81 in gel slices corresponding to 25–32 kDa. pORF99 was detected in CyHV-3 virions as a large (170.7 kDa) and relatively abundant (emPAI of 1.22) protein. Despite the absence of a transmembrane domain predicted using the usual prediction software, this protein was classified as a putative membrane protein on the basis of previous predictions for CyHV-3 proteins by Aoki et al. (2007), which depended on the experimental findings of Davison & Davison (1995) for IcHV-1. It is possible that this protein associates with another membrane protein to form a complex exposed on the viral envelope, as observed for glycoprotein L of mammalian herpesviruses (Reske et al., 2007). A family of four paralogous proteins was detected in CyHV-3 virions (pORF25, -65, -148 and -149; Table 1). These proteins are type 1 membrane proteins bearing numerous glycosylation sites.

Based on the CyHV-3 genome sequence, 30 proteins were predicted to possess a transmembrane domain (Aoki et al., 2007). In the present study, only 13 of these proteins were detected in virions of the CyHV-3 FL strain and are proposed as envelope proteins (Table 1). Several hypotheses can explain the discrepancy between these numbers. Firstly, five ORFs of the CyHV-3 FL strain (listed in Table 2) bore mutations incompatible with gene product expression. This observation is consistent with data published previously by Aoki et al. (2007) for other strains of CyHV-3. Secondly, some of the proteins could be expressed at a concentration lower than the detection level of the present approach. Thirdly, some proteins could be undetected as a consequence of their resistance to trypsin digestion. Finally, some proteins may remain anchored exclusively in cellular membranes.

Tegument proteins. Two of the proteins identified in CyHV-3 virions were predicted to be tegument proteins: pORF62 and pORF123. ORF62 encodes an ovarian tumor
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*All homologies were determined using BLAST and BioEdit software (e-value <1e-5), and compared with Aoki et al. (2007) (GenBank accession no. DQ657948). Genes sequences for RaHV-1 and -2 are from Davison et al. (2006) (GenBank accession nos NC_008211 and NC_008210, respectively). Gene sequences for IcHV-1 are from Davison et al. (1992) (GenBank accession no. NC_001493). Unless specified (by underlined type), all homologies found with RaHVs represent indirect homology to IcHV-1.
†emPAI values were determined for proteins identified in the GC extract.
‡Properties are given for envelope proteins only.
§Envelope protein predictions were examined using TMHMM, SignalP, NetNGlyc and NetOGlyc server from the CBS website.
(OTU)-like cysteine protease domain and is the largest protein (442.2 kDa) detected in CyHV-3 virions. These observations suggest that it could be the homologue of the ‘large tegument protein’ conserved in all mammalian and avian herpesviruses (Johannsen et al., 2004; Kattenhorn et al., 2004; Kunec et al., 2009; Loret et al., 2008). pORF123 is a small protein (29.5 kDa) that is predicted to be a dUTPase and is found in the tegument of several herpesviruses (Delhon et al., 2003; O’Connor & Kedes, 2006; Varnum et al., 2004). CyHV-3 thymidine kinase (TK), encoded by ORF55, was not detected in virions (Table 1). This contrasts with other herpesviruses where TK is expressed as a tegument protein (Bechtel et al., 2005; Dry et al., 2008; Johannsen et al., 2004; Loret et al., 2008; O’Connor & Kedes, 2006; Zhu et al., 2005).

Unclassified proteins. Twenty-two proteins were unclassified due to the lack of information on their putative locations in virions. Three of these proteins, pORF43, -57 and -66, were among the most abundant proteins detected in CyHV-3 virions. Based on the results presented above, a schematic representation of CyHV-3 virions was built (Fig. 5). This figure also illustrates the relative abundance of the proteins.

Host proteins associated with CyHV-3 virions

During assembly of viral particles, cellular proteins may be packaged into virions. The host protein content of CyHV-3 virions was determined by searching for peptides from a bony vertebrate database. A total of 18 host cell proteins was identified (Table 3). This list included cytoskeleton proteins (β-actin, cofilin-like protein and α-tubulin; Ichetovkin et al., 2000), signal transduction proteins (Ras-related protein and caseine kinase α; Wennerberg et al., 2005), proteins involved in vesicular trafficking...

**Table 2. Undetected predicted membrane proteins in CyHV-3 virions**

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<td>144</td>
<td>Predicted membrane glycoprotein</td>
<td></td>
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<tr>
<td>145</td>
<td>Predicted multiple transmembrane protein</td>
<td></td>
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<tr>
<td>146</td>
<td>Predicted membrane glycoprotein</td>
<td></td>
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<tr>
<td>147</td>
<td>Predicted multiple transmembrane protein</td>
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<tr>
<td>148</td>
<td>Predicted membrane glycoprotein</td>
<td></td>
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<tr>
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<td>Predicted multiple transmembrane protein</td>
<td></td>
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<tr>
<td>150</td>
<td>Predicted membrane glycoprotein</td>
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<tr>
<td>151</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>153</td>
<td>Predicted multiple transmembrane protein</td>
<td></td>
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</tbody>
</table>

*All descriptions are from the paper by Aoki et al. (2007) (GenBank accession no. DQ657948), and were confirmed using TMHMM server predictions from the CBS website.

†All alignments were realized using multiple sequence alignment (CLUSTAL W) comparing CyHV-3 I (GenBank accession no. DQ177346), CyHV-3 J (GenBank accession no. AP008984), CyHV-3 U (GenBank accession no. DQ657948) strains and our own sequenced CyHV-3 FL (unpublished data) strain.
through the endoplasmic reticulum and Golgi (Rab proteins, annexin and ADP ribosylation factor; Gerke & Moss, 2002; Stenmark & Olkkonen, 2001), proteins involved in translational control (elongation factor; Nilsson & Nissen, 2005), in stress response (heat-shock protein; De Maio, 1999), in immnosuppression (PPlase and FK506-binding protein; Göthel’ & Marahiel, 1999) and in cell-signalling regulation (14-3-3 protein; Mhawech, 2005). The identification of cellular proteins in CyHV-3 virions relied on the detection of only one to three peptides/proteins. The low emPAI observed for the cellular proteins suggested that these proteins are either minor components of the virions or were cellular protein contaminants of the virion preparation. Several speculative arguments are in favour of the first hypothesis. Firstly, most of the cellular proteins detected in viral particles were not major cellular proteins. Secondly, these proteins were not specifically secreted in supernatant that had been harvested to recover viral particles. Thirdly, most of the cellular proteins listed above have been found previously in herpesvirus virions (Chung et al., 2006; Dry et al., 2008; Johannsen et al., 2004; Kattenhorn et al., 2004; Loret et al., 2008; Resch et al., 2007; Varnum et al., 2004; Zhu et al., 2005).

Validation of the proteomic approach

Validation of the data generated above is difficult for several reasons. Firstly, to date only one protein has been identified as a CyHV-3 component (Rosenkranz et al., 2008). Secondly, very few CyHV-3 proteins present similarities to other known viral proteins. Finally, no monoclonal antibodies directed against specific CyHV-3 virion proteins are available. However, several observations suggest the accuracy of our proteomic approach: (i) the number of proteins detected in the present study is comparable to the numbers described for other herpesviruses (Johannsen et al., 2004; Kattenhorn et al., 2004; Loret et al., 2008; O’Connor & Kedes, 2006); (ii) our analysis detected pORF81, identified as an envelope protein by Rosenkranz et al. (2008); (iii) non-structural proteins such as proteins involved in DNA replication [primase (ORF46), DNA helicase (ORF71), DNA polymerase (ORF79)], DNA packaging [terminase (ORF33)] or secreted proteins [tumour necrosis factor receptor (ORF4, ORF12)] were not detected in CyHV-3 virions; and (iv) at least one protein specific for mature virions was detected (the N-terminal part of pORF78).

METHODOLOGY

Cells and virus. C. carpio brain (CCB) cells (Neukirch et al., 1999) were cultured in minimum essential medium (Invitrogen) containing 4.5 g glucose (D-glucose monohydrate; Merck) l−1 and 10 % fetal calf serum. Cells were cultured at 25 °C in a humid atmosphere containing 5 % CO2. The CyHV-3 FL strain was isolated from the kidney of a carp that had died from CyHV-3 infection (Costes et al., 2008).

Table 3. Cellular proteins associated with purified CyHV-3 virions

<table>
<thead>
<tr>
<th>Host protein*</th>
<th>Species origin†</th>
<th>NCBI ID</th>
<th>Predicted MM (kDa)</th>
<th>No. of peptides</th>
<th>Coverage (%)</th>
<th>Mascot score</th>
<th>emPAI‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab6A</td>
<td>Danio rerio</td>
<td>47087033</td>
<td>24</td>
<td>3</td>
<td>17</td>
<td>225</td>
<td>ND</td>
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<tr>
<td>Annexin A2a</td>
<td>D. rerio</td>
<td>32308153</td>
<td>38</td>
<td>2</td>
<td>8</td>
<td>166</td>
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<tr>
<td>Rab11A</td>
<td>D. rerio</td>
<td>55925277</td>
<td>24</td>
<td>3</td>
<td>21</td>
<td>155</td>
<td>ND</td>
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<tr>
<td>Heat-shock protein 8</td>
<td>Salmo salar</td>
<td>213514058</td>
<td>71</td>
<td>3</td>
<td>6</td>
<td>142</td>
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<tr>
<td>α-Tubulin</td>
<td>D. rerio</td>
<td>35902919</td>
<td>51</td>
<td>1</td>
<td>4</td>
<td>121</td>
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<tr>
<td>Rab5A</td>
<td>D. rerio</td>
<td>41393127</td>
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<td>1</td>
<td>5</td>
<td>84</td>
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</tr>
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<td>β-Actin</td>
<td>D. rerio</td>
<td>18858335</td>
<td>42</td>
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<td>14</td>
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<td>PPlase</td>
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<td>77</td>
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<tr>
<td>Ras-related protein</td>
<td>S. salar</td>
<td>213514648</td>
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<td>2</td>
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<td>Lactate dehydrogenase</td>
<td>D. rerio</td>
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<td>3</td>
<td>74</td>
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<tr>
<td>Rab5C</td>
<td>D. rerio</td>
<td>41393159</td>
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<td>1</td>
<td>5</td>
<td>70</td>
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<tr>
<td>ADP ribosylation factor</td>
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<tr>
<td>Carbonyl reductase 1-like protein</td>
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<td>30</td>
<td>1</td>
<td>3</td>
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<td>Caseine kinase 2</td>
<td>D. rerio</td>
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<td>3</td>
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<td>50</td>
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<tr>
<td>Elongation factor</td>
<td>Cyprinus carpio</td>
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<td>59</td>
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<td>FK506-binding protein 1A</td>
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<td>12</td>
<td>2</td>
<td>26</td>
<td>54</td>
<td>0.67</td>
</tr>
</tbody>
</table>

*All homologies were determined using BLAST search software in the NCBI bony vertebrate database.
†As a complete C. carpio sequence was not available, homologies were found in other species.
‡emPAI values were determined for proteins identified in the GC extract.
Production and purification of CyHV-3 virions. CCB cells were infected with the CyHV-3 FL strain at an m.o.i. of 0.02 p.f.u. per cell (Costes et al., 2009). To reduce cellular contaminants, the supernatant was harvested at the beginning of viral release when cell lysis was minimal (4 days post-infection). Viral particles were purified from the cell supernatant as described previously (Vanderplaschens et al., 1993). Briefly, viral particles from the cell supernatant were pelleted by centrifugation through a sucrose cushion and banded on potassium tartrate gradients. Purified virions were resuspended in PBS and stored at −80 °C.

Negative staining and EM. Copper grids covered by a thin film of Formvar (400 mesh; Agar Scientific) were incubated for 10 min with 1% Alcian blue 8G solution (Gurr Microscopy Materials, BHD) to add positive charges. After washing, purified virions (106 p.f.u. ml−1) were adsorbed to the grids for 10 min. Virions were then stained for contrast by incubation in 2% uranyl acetate solution for 10 s (Agar Scientific). Samples were observed using a transmission electron microscope (FEI Tecnai Spirit).

Production of cell supernatant and cell lystate for electrophoresis. CCB cells were mock-infected or infected with the CyHV-3 FL strain at an m.o.i. of 1 in serum-free medium (UltraMDCK medium; Lonza). The cell supernatant was collected at 48 h post-inoculation, centrifuged at 2000 g for 20 min at 4 °C and concentrating 25-fold by centrifugation (2000 g, 75 min, 4 °C) through an Amicon Ultra-15 centrifugal filter unit (3K NMWL; Millipore). For cell lystate production, cells were scraped, washed with PBS and lysed in PBS containing 1% SDS.

Gel electrophoresis. Samples were diluted in NuPAGE LDS sample buffer (Invitrogen) containing a reducing agent and heat-denatured for 10 min at 80 °C. The samples were electrophoresed on NuPAGE Novex 4–12% Pre-cast Bis-Tris polyacrylamide gels (Invitrogen), and purified virions as presented in Fig. 3(a). The GC extract was obtained in 1% (w/v) SDS buffer. Finally, undissolved viral proteins were extracted in 1% (w/v) OGP. Finally, undissolved viral proteins were extracted in 1% (w/v) SDS buffer. The protein concentrations of the six extracts were measured using a BCA protein assay kit (Pierce).

1D gel/nanoLC-MS/MS approach. All protein extracts (except the GC extract) were resuspended in Laemmli buffer, sonicated for 5 min and incubated for 30 min at room temperature (Fig. 3b). After centrifugation (18 000 g, 15 min, room temperature), protein (15 µg) was separated by SDS-PAGE on 4–20% acrylamide short gels (7 cm long; Invitrogen) and detected by Coomassie blue staining (Imperial Protein Stain; Roche). The entire lanes of the gel were cut into 17 homogeneous slices independent of the presence of visible bands, as shown in Fig. 4. The gel slides were washed separately and digested with modified sequencing-grade trypsin (Promega), and the resulting peptides were extracted as described elsewhere (Leroy et al., 2007). The peptides were analysed by nanoLC-MS/MS as described previously (Mastrolo et al., 2009).

In-solution trypsin digestion and 2D nanoLC-MS/MS approach. The GC extract resuspended in 50 mM Tris/HCl (pH 8), 2 M urea was digested overnight at 37 °C with trypsin (enzyme : substrate ratio of 1 : 50) (Fig. 3b). Tryptic peptides were then cleaned using spin tips (Thermoscientific) following the manufacturer’s instructions. Proteins (25 µg) were analysed by 2D (strong cation exchange, reverse-phase) chromatography and online MS/MS, as described by Mastrolo et al. (2009), except that only three successive salt plugs of 5, 50 and 1000 mM NaCl were used.

MS/MS analysis. Peptides were analysed using the ‘peptide scan’ option of the HCT Ultra ion trap (Bruker), consisting of a full-scan MS and MS/MS scan spectrum acquisitions in ultrasonic mode (26 000 m/z s−1). Peptide fragment mass spectra were acquired in data-dependent AutoMS(2) mode with a scan range of 100–2800 m/z, three means and five precursor ions selected from the MS scan 300–1500 m/z. Precursors were actively excluded within a 0.5 min window, and all singly charged ions were excluded. Peptide peaks were detected and deconvoluted automatically using Data Analysis 2.4 software (Bruker). Mass lists in the form of Mascot Generic Files were created automatically and used as the input for Mascot MS/MS ion searches of the NCBI nr database release 20080704 using an in-house Mascot 2.2 server (Matrix Science). The default search parameters used were: Taxonomy=Virus; Enzyme=Trypsin; Maximum missed cleavages=1; Fixed modifications=Carbamidomethyl (C); Variable modifications= Oxidation (M); Peptide tolerance ± 1.5 Da; MS/MS tolerance ± 0.5 Da; Peptide charge = 2+ and 3+; Instrument=ESI-TRAP. All data were also searched against the NCBI non vertebrate database in order to detect host proteins. Only sequences identified with a Mascot Score greater than 50 were considered, and single peptide identification was systematically evaluated manually. In addition, the emPAI (Ishihama et al., 2005) was calculated to estimate protein relative abundance in the GC extract only.

Bioinformatic protein analyses. Proteins potentially containing CyHV-3 peptides were searched using two approaches. Firstly, protein sequences were submitted to a BLAST search using the extended available database, and the results were compared with the CyHV-3 genome annotation made previously (see Table 1 in the paper by Aoki et al., 2007). Secondly, amino acid sequences were analysed with the BioEdit program using a protein database containing three alloherpesviruses (IChV-1, RaHV-1 and -2), five alphaherpesviruses (HHV-1 and -3, gullid herpesvirus 1 and 2, psittacid herpesvirus 1), five betaherpesviruses (HHV-6B and -7, macacine herpesvirus 3, murid herpesvirus 1 and 2), five gammaherpesviruses (alcelaphine herpesvirus 1, callitrichine herpesvirus 3, equine herpesvirus 2, HHV-4, murid herpesvirus 4), one mallaco-herpesvirus (ostreid herpesvirus 1), five iridoviruses (Ambystoma et al.
tigrinum virus, frog virus 3, infectious spleen and kidney necrosis virus, lymphocystis disease virus 1, Singapore grouper iridovirus) and five poxviruses (Amastia moorei entomopoxvirus, cowpox virus, deerpox virus, molluscum contagiosum virus, myxoma virus).

The amino acid sequences of putative CyHV-3 structural proteins were analysed using bioinformatic tools from the CBS website (http://www.cbs.dtu.dk/index.shtml) to identify potential transmembrane domains (TMHMM), signal peptides (SignalP) and glycosylation sites (NetNGlyc and NetOGlyc) (Bendtsen et al., 2004; Julenius et al., 2005).

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