Short Communication

Identification of envelope protein pORF81 of koi herpesvirus

Daniela Rosenkranz,[1] Barbara G. Klupp,[1] Jens P. Teifke,[2] Harald Granzow,[2] Dieter Fichtner,[2] Thomas C. Mettenleiter[1] and Walter Fuchs[1]

[1]Institute of Molecular Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, 17493 Greifswald-Insel Riems, Germany

[2]Institute of Infectology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, 17493 Greifswald-Insel Riems, Germany

Correspondence

Walter Fuchs

walter.fuchs@fli.bund.de

Koi herpesvirus (KHV), an emerging pathogen causing mass mortality in koi and common carp, possesses the largest known herpesvirus genome of 295 kbp predicted to encode 156 different proteins. However, none of them has been identified or functionally characterized up to now. In this study, a rabbit antiserum was prepared against a bacterial fusion protein that permitted detection of the predicted type III membrane protein encoded by ORF81 of KHV. In Western blot analyses, the abundant ORF81 gene product of KHV exhibited an apparent mass of 26 kDa and appeared to be non-glycosylated. It could be localized in the cytoplasm of infected cells and in virion envelopes by indirect immunofluorescence and immunoelectron microscopy, respectively. The antiserum was also suitable for the detection of pORF81 in sections of gills, kidneys, hepatopancreas and skin of KHV-infected carp by immunohistochemistry.

In the late 1990s, a previously unknown infectious disease, leading to mass mortality of common carp (*Cyprinus carpio*) and koi carp (*Cyprinus carpio koi*), was first observed in Israel and Western Europe, but has meanwhile spread over the majority of the world (Bretzinger *et al.*, 1999; Hedrick *et al.*, 2000; Neukirch *et al.*, 1999). Since the causative agent exhibited a herpesvirus-like morphology it had been designated koi herpesvirus (KHV; Hedrick *et al.*, 2000). Alternatively, it has been described as carp nephritis and gill necrosis virus (CNGV) because of the typical pathological effects (Ronen *et al.*, 2003).

The virus possesses an unusually large double-stranded DNA genome of approximately 295 kbp including 22 kbp direct repeat sequences present at both genome ends (Hutoran *et al.*, 2005). Complete sequence analysis of three different isolates revealed the presence of 156 common, presumably protein-encoding open reading frames (Aoki *et al.*, 2007). Many of the predicted genes of KHV possess significantly conserved homologues in other herpesviruses of fish and amphibians, such as channel catfish virus (ictalurid herpesvirus 1, IcHV-1) and ranid herpesviruses 1 and 2 (Davison, 1992; Davison *et al.*, 2006). In particular, the deduced amino acid sequences of selected genes of two previously described pathogens of cyprinids, carp pox herpesvirus (cyprinid herpesvirus 1) and haematopoietic herpesvirus of goldfish (cyprinid herpesvirus 2) exhibit high degrees of identity with the corresponding gene products of KHV, which has therefore been designated cyprinid herpesvirus 3 (Waltzek *et al.*, 2005). In contrast, detectable sequence homologies to mammalian herpesviruses are essentially limited to genes encoding enzymes involved in DNA replication, which are conserved in all organisms. Therefore, it has been proposed by the *Herpesviridae* Study Group to assign the herpesviruses of fish and amphibians to a new family *Alloherpesviridae*, which should be grouped together with herpesviruses of shellfish (*Malacoherpesviridae*), and herpesviruses of mammals, birds and reptiles (*Herpesviridae*) in the new order *Herpesvirales* (McGeoch *et al.*, 2006).

The lack of sequence identity to herpesviruses of humans and other mammals precludes predictions about the functions of most KHV genes. However, this information is urgently needed to obtain better tools for the diagnosis and combat of an important fish disease. In particular, it would be interesting to ascertain which of the predicted 27 membrane proteins of KHV (Aoki *et al.*, 2007) are major components of the virion envelope and relevant for virus–host cell interactions, as well as for the host immune response. However, up to now, no gene product of KHV has been characterized or even identified.

One of the putative membrane proteins of KHV is encoded by ORF81. This gene is a positional homologue of ORF59 of IcHV-1, which is considered to encode the major envelope glycoprotein of this virus (Aoki *et al.*, 2007; Davison & Davison, 1995; Kucuktas *et al.*, 1998). The deduced ORF81 gene product of KHV consists of 256 aa

and, like pORF59 of IcHV-1, specifies four predicted *trans*-membrane helices (Fig. 1c; Hirokawa *et al.*, 1998). The C-terminal domain of the type III membrane protein is very hydrophilic (Fig. 1c), correlating with a high predicted antigenic index (Jameson & Wolf, 1988). Therefore, in the present study ORF81 was cloned, its product (pORF81) was expressed in pro- and eukaryotic cells, and a monospecific antiserum was prepared and tested for its suitability for the detection of KHV in infected cells and carp tissues.

The koi herpesvirus used in our experiments had been isolated in Israel in 1998 (Hedrick *et al.*, 2000). It was propagated in common carp brain (CCB) cells (Neukirch *et al.*, 1999) and grown at 25 °C in minimum essential medium (MEM) supplemented with 10 % fetal calf serum (both purchased from Invitrogen). After complete cytolysis of infected cells, KHV virions were purified by consecutive centrifugation through a 35 % sucrose cushion, and a discontinuous (30, 40 and 50 %) sucrose gradient as described for pseudorabies virus (PrV; Böttcher *et al.*, 2007). Virion DNA was prepared (Fuchs & Mettenleiter, 1996), and viral DNA fragments generated by digestion with *Bam*HI were cloned into the plasmid vector pBluescript SK(-) (Stratagene). The complete ORF81 was recloned from pBl-KB4455 containing a genomic 4455 bp *Bam*HI fragment of KHV, as a 1109 bp *Psh*AI/*Xho*I fragment into the eukaryotic expression vector pcDNA3 (Invitrogen; Fig. 1b). Furthermore, a 555 bp *Bsa*I/*Xho*I subfragment of pBl-KB4455 was recloned in vector pGEX-4T-3 (Amersham; Fig. 1b). The resulting construct permitted abundant expression of a 36 kDa fusion protein consisting of glutathione *S*-transferase (GST) and the hydrophilic C-terminal part of pORF81 (aa 177–257; Fig. 1c) in *Escherichia coli*. The GST–pORF81 fusion protein was purified and used for immunization of a rabbit as described previously (Fuchs *et al.*, 2002).

The rabbit serum obtained after five immunizations with 100 µg each of the fusion protein was used for Western blot analyses of CCB cells harvested 2 days after infection with KHV at a multiplicity of 1 p.f.u. per cell, or after transfection (TransFectin lipid reagent; Bio-Rad) with pcDNA-KO81 (Fig. 1b). Lysates of 10^4 cells, or 2 µg purified virion proteins per lane were separated, transferred to membranes, and consecutively incubated with the anti-pORF81 serum (dilution 1 : 50 000) and horseradish peroxidase-conjugated secondary antibodies as described previously (Fuchs & Mettenleiter, 1999). The antiserum specifically reacted with a viral protein exhibiting an apparent molecular mass of 26 kDa (Fig. 2a), which was not found in uninfected CCB cells (Fig. 2a), and also was not detected by the preimmune serum (Fig. 2b). Thus, the size of the ORF81 protein of KHV closely matches the predicted molecular mass of 28.25 kDa (Aoki *et al.*, 2007). The marginally lower apparent mass of pORF81 is presumably not caused by the removal of an N-terminal signal peptide, since no corresponding consensus sequences (von Heijne, 1986) are present in the deduced translation product. Similar, 26 kDa proteins could be detected in cells transfected with pcDNA-KO81 (Fig. 2a),
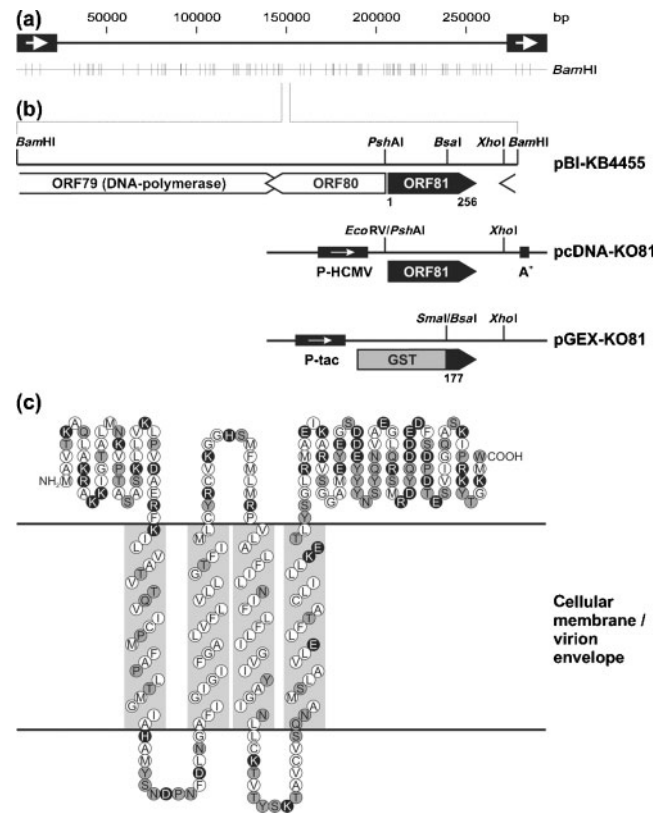

Fig. 1. (a) Map of the KHV genome containing direct terminal repeat sequences (black rectangles) with nucleotide numbers and positions of *Bam*HI restriction sites. (b) A detailed section showing the genomic *Bam*HI fragment cloned into pBl-KB4455. Open reading frames (pointed rectangles), codon numbers of the investigated gene and relevant restriction sites are indicated. The eukaryotic expression plasmid pcDNA-KO81 contains the complete ORF81 of KHV flanked by a herpesviral promoter (P-HCMV) and a polyadenylation signal (A^+). The 3′-terminal part of ORF81 could be expressed in *E. coli* from pGEX-KO81 as a fusion protein with GST under the control of a bacterial promoter (P-tac). (c) Sequence and predicted topology of pORF81 as determined by the computer program SOSUI (Hirokawa *et al.*, 1998). Polar and charged amino acids are indicated in grey and black, respectively. Four *trans*-membrane helices are highlighted.

and also after *in vitro* transcription and translation of this plasmid (data not shown). These findings indicate the absence of extensive post-translational modifications of pORF81, which is in agreement with the absence of putative *N*-glycosylation sites (Kornfeld & Kornfeld, 1985) in the deduced amino acid sequence (Fig. 1c). Western blot analysis of purified KHV particles indicated that pORF81 is efficiently incorporated into virions (Fig. 2a).

For indirect immunofluorescence tests, CCB cells were fixed 2 days after infection with KHV, using a 1 : 1 mixture of methanol, and acetone, and incubated with the pORF81-specific antiserum (dilution 1 : 200) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) according
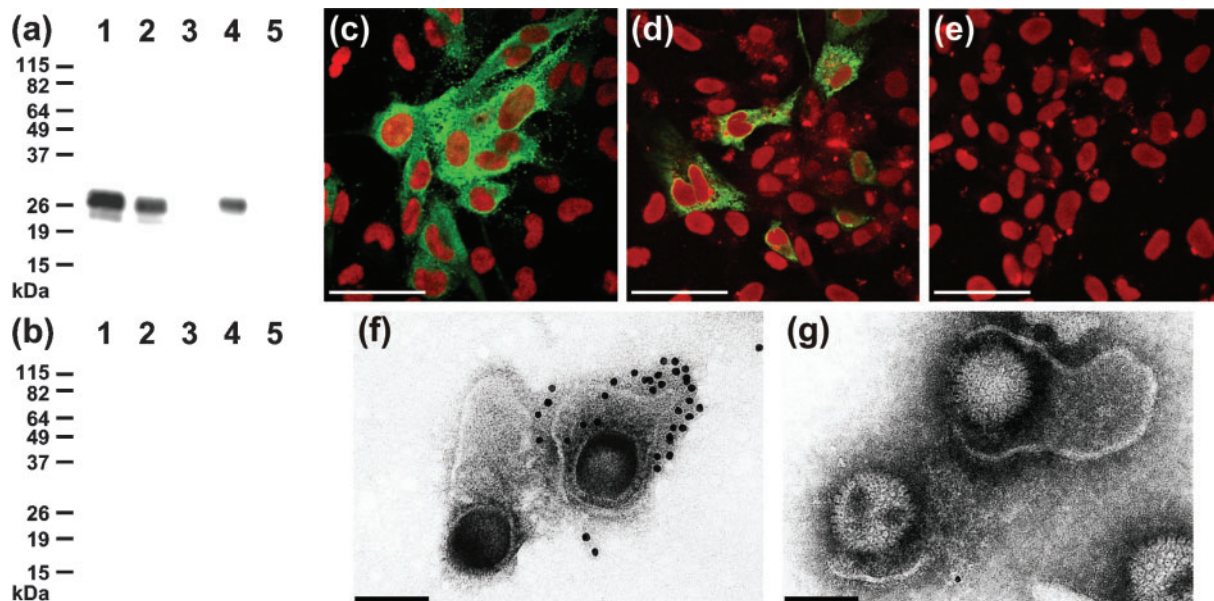
Fig. 2. Identification and localization of pORF81 of KHV, using a monospecific rabbit antiserum. Western blot analysis of cells harvested 2 days after infection with KHV (lane 1) or transfection with pcDNA-KO81 (lane 2), of untreated CCB cells (lane 3), and of purified KHV (lane 4) or PrV (lane 5) virions was performed with the anti-pORF81 serum (a) and the respective preimmune serum (b). Molecular mass markers are indicated to the left. For confocal microscopy, CCB cells were fixed 2 days after infection with KHV (c), or after transfection with pcDNA-KO81 (d) or an irrelevant control plasmid (e). Reaction of the anti-pORF81 serum was detected with Alexa Fluor 488-conjugated secondary antibodies (green) and chromatin was stained with propidium iodide (red). Bars, 50 μm. For immunoelectron microscopy, purified KHV (f) or PrV (g) particles were incubated with the anti-pORF81 serum. Antibody binding was detected with 10 nm gold-tagged secondary antibodies. Bars, 100 nm.

to established protocols (Fuchs *et al.*, 2002). Confocal laser scan microscopy (Zeiss LSM510) revealed a granular cytoplasmic fluorescence of infected cells (Fig. 2c), indicating the expected association of the predicted membrane-spanning protein with cytoplasmic organelles, such as endoplasmic reticulum or Golgi apparatus. A similar, predominantly cytoplasmic localization of pORF81 was detectable in cells transfected with pcDNA-KO81 (Fig. 2d), but not in cells transfected with a control plasmid (Fig. 2e). Although no pronounced accumulation of pORF81 at cytoplasmic membranes could be observed (Fig. 2c, d), the antiserum raised against the C-terminal part also showed weak, but clearly specific reactions with non-permeabilized cells that had been fixed with 3 % paraformaldehyde after infection or transfection (data not shown). Thus, although according to topology predictions (Krogh *et al.*, 2001) the location of the C terminus of pORF81 inside the cytosol is more likely, our experimental results suggest that this protein part might represent an ecto-domain.

To determine the location of pORF81 in virions, native KHV particles were prepared for immunoelectron microscopy as described previously (Veits *et al.*, 2003). After incubation with the diluted anti-pORF81 serum, specific binding could be detected using goat anti-rabbit antibodies tagged with 10 nm gold particles (British BioCell Int.). Examination with an electron microscope (Tecnai 12,

Philips) revealed the accumulation of gold particles only along the surface of intact KHV particles (Fig. 2f). In contrast, no label was found after incubation of KHV virions with irrelevant antisera (data not shown), or after incubation of similarly prepared PrV particles with the anti-pORF81 serum (Fig. 2g). Thus, pORF81 is incorporated into the viral envelope, and the antigenic C-terminal domain is presumably localized at the outside of the particle.

For *in vivo* studies, juvenile common carp (body mass approx. 5 g) were infected by immersion in water containing 10^5 p.f.u. l^{-1} KHV for 1 h and kept at 22 °C. All animals developed typical clinical signs of KHV infections like apathy, inappetence, dyspnoea, uncoordinated movement, gill necroses and skin lesions (Haenen *et al.*, 2004; Pikarsky *et al.*, 2004), and died between 7 and 11 days after infection. At necropsy the infected carp demonstrated mild to moderate branchial necrosis, haemorrhages of the gills and mild focal cutaneous ulcerations. For further investigations, organs were fixed for 24–48 h in 4 % neutral buffered formaldehyde and embedded in paraffin.

Histopathological investigation of haematoxylin and eosin (H&E) stained 2 μm sections revealed a partial loss of architecture of the gills with blunting of lamellae, and moderate to severe mixed cellular interstitial inflammatory infiltration in a large number of the filaments (Fig. 3a).
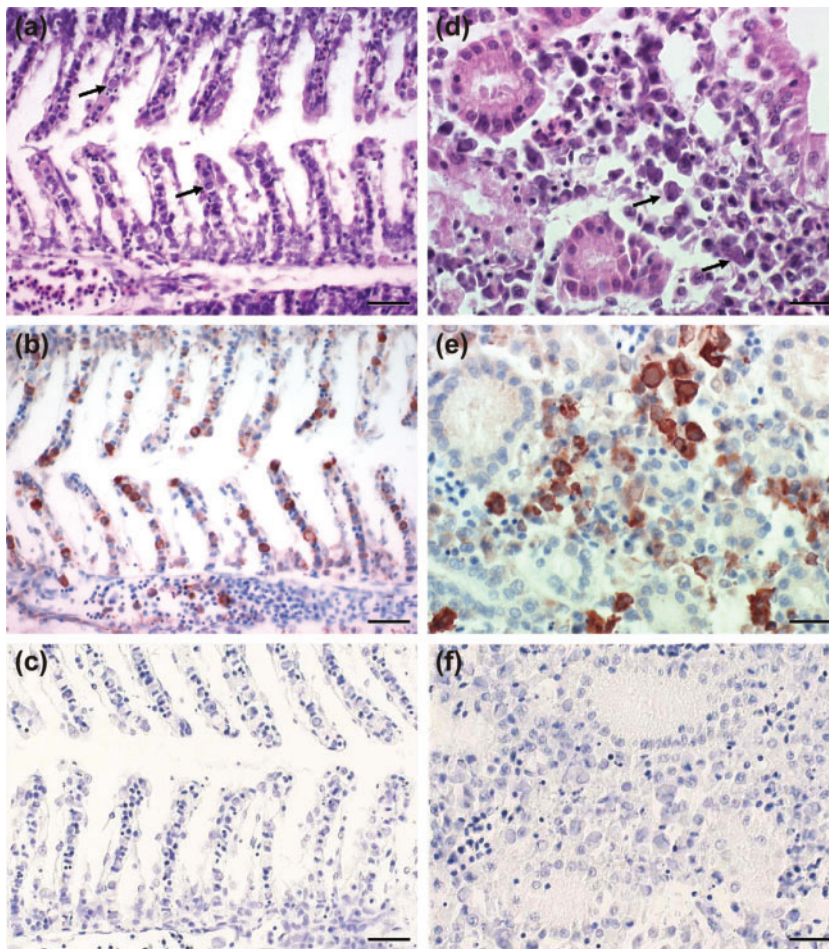
Fig. 3. Histopathology and immmunohisto-chemistry. Gill (a, b, c) and kidney (d, e, f) sections of a carp that died 8 days after infection with KHV. H&E staining (a, d) revealed an inflammatory infiltrate mainly composed of lymphocytes and large oval to polygonal baso-philic cells containing large intranuclear inclusion bodies (arrows). KHV antigen could be detected in leukocytes within capillaries and cells infiltrating the branchial (b) and renal interstitium (e) by immmunohistochemistry with the anti-pORF81 serum, using the ABC method (red staining). The respective preimmune serum showed no specific reactions (c, f). Bars, 75 μm (a, b, c) and 45 μm (d, e, f).

Congestion of the gills' central sinusoids was also present as multifocal detachment of the gill epithelium. Numerous large ovoid to polygonal basophilic cells with large pale basophilic intranuclear inclusion bodies (arrows) were scattered throughout the capillaries of the filaments or within larger vessels. The kidneys (Fig. 3d) showed moderate to severe diffuse interstitial infiltration with lymphocytes and numerous large polygonal to round cells with abundant basophilic cytoplasm and large pale basophilic nuclei with marginated chromatin and intra-nuclear inclusions (arrows). Similar large 'karyomegalic' cells were seen in the hepatopancreas within areas of degenerate and necrotic pancreatic acinar cells. A compar-able inflammatory infiltrate was also present multifocally in the skin and subcutis (data not shown).

For immunohistochemistry, fixed 3 μm sections were mounted on positively charged microscope slides, de-paraffinized in xylene and rehydrated through a series of graded *n*-propanol solutions. The sections were irradiated in a microwave for 10 min at 500 W in 10 mM citric acid buffer (pH 6.0), and incubated for 1 h with the rabbit anti-pORF81 serum diluted at 1:3000 in TBS (0.1 M Tris, 0.9 % NaCl, pH 7.6) at room temperature. Antibody binding was detected using biotinylated goat anti-rabbit

IgG1 diluted 1:200 in TBS, and an immunoperoxidase kit (Vectastain Elite ABC kit; Vector), which produced a bright red signal from the substrate, 3-amino-9-ethylcarb-azole (DAKO AEC substrate-chromogen system; Dako). By microscopy performed after counterstaining with Mayer's haematoxylin and sealing with aqueous medium (Aquatex; Merck), pORF81 could be abundantly detected in the cytoplasm and, with lower intensity within the nuclei of large polygonal cells in capillaries and interstitium of the gills (Fig. 3c), in cells infiltrating the renal interstitium (Fig. 3e), the submucosa of the intestine, in dermis and subcutis, spleen, periosteum and pancreas. In addition, few acinar cells of the exocrine pancreas and singular or groups of neurons in spinal cord and brain were also positive for KHV antigen. No specific reactivity was observed in tissues of non-infected carp (data not shown), or of infected carp incubated with the respective preimmune serum (Fig. 3c, f).

Thus, the presence of pORF81 correlates with the observed lesions in KHV-infected animals and is in general agreement with the distribution of viral antigens described in earlier pathogenesis studies (Pikarsky *et al.*, 2004). Since the anti-pORF81 serum showed no detectable cross-reactivity with cellular proteins, precise investigations of

organ-tropism of KHV and kinetics of virus spread might be further facilitated. If pORF81 is sufficiently immunogenic in naturally infected carp, the recombinant ORF81 proteins expressed in eukaryotic cells or bacteria might also be used as diagnostic antigens for the detection of KHV-specific serum antibodies by immunofluorescence tests or ELISAs. Furthermore, the ORF81 expression constructs might be suitable for the development of subunit- or DNA-vaccines against KHV.

In summary, we describe here the identification of the first KHV protein, using an antiserum of known specificity. The monospecific rabbit antiserum against the envelope protein pORF81 is suitable for sensitive detection of KHV infection and KHV particles *in vitro* and *in vivo*.

Acknowledgements

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