Piscine reovirus encodes a cytotoxic, non-fusogenic, integral membrane protein and previously unrecognized virion outer-capsid proteins

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Piscine reovirus (PRV) is a tentative new member of the family Reoviridae and has been linked to heart and skeletal muscle inflammation in farmed Atlantic salmon (Salmo salar L.). Recent sequence-based evidence suggests that PRV is about equally related to members of the genera Orthoreovirus and Aquareovirus. Sequence similarities have also suggested that PRV might encode a fusion-associated small transmembrane (FAST) protein, which in turn suggests that PRV might be the prototype of a new genus with syncytium-inducing potential. In previous support of this designation has been the absence of identifiable PRV-encoded homologues of either the virion outer-clamp protein of ortho- and aquareoviruses or the virion outer-fibre protein of most orthoreoviruses. In the current report, we have provided experimental evidence that the putative p13 FAST protein of PRV lacks the defining feature of the FAST protein family – the ability to induce syncytium formation. Instead, p13 is the first example of a cytosolic, integral membrane protein encoded by ortho- or aquareoviruses, and induces cytotoxicity in the absence of cell–cell fusion. Sequence analysis also identified signature motifs of the outer-clamp and outer-fibre proteins of other reoviruses in two of the predicted PRV gene products. Based on these findings, we conclude that PRV does not encode a FAST protein and is therefore unlikely to be a new fusogenic reovirus. The presence of a novel integral membrane protein and two previously unrecognized, essential outer-capsid proteins has important implications for the biology, evolution and taxonomic classification of this virus.

INTRODUCTION

Palacios et al. (2010) reported the full-length cDNA sequences of all ten genome segments of an apparent reovirus isolated from farmed Atlantic salmon (Salmo salar L.). This virus, designated piscine reovirus (PRV), has been linked to heart and skeletal muscle inflammation in this host, a serious disease with associated economic losses (Kongtorp et al., 2006; Palacios et al., 2010). Other fish reoviruses, which are grouped in the genus Aquareovirus, have 11 genome segments (Attoui et al., 2011). Other fish reoviruses, which are grouped in the genus Aquareovirus, have 11 genome segments (Attoui et al., 2011). By having ten segments, PRV is instead like members of the genus Orthoreovirus, which are isolated from a diverse range of mammals, birds and reptiles (Day, 2009). Sequence-based phylogenetic comparisons have indicated that PRV is about equally related to members of the genera Orthoreovirus and Aquareovirus, suggesting that it might be the prototype of a new genus in the subfamily Spinareovirinae, family Reoviridae (Palacios et al., 2010).

The ten genomic positive-sense strands of PRV (GenBank accession nos GU994013–GU994022) encompass 11 long ORFs (Palacios et al., 2010). Segments L1, L2, L3, M1, M2, M3, S2 and S3 are monocistronic, and encode homologues of the mammalian reovirus (MRV) l3, l2, l1, m2, m1, mNS, s2 and sNS proteins. The homologous proteins are named lB, lC, lA, mA, mB, mNS, sA, and sNS, respectively, in avian reoviruses (ARVs) and VP2, VP1, VP3, VP5, VP4, NS1, VP6 and NS2, respectively, in aquareoviruses (Attoui et al., 2011). Notably, no PRV homologues have been identified for the outer-clamp protein of ortho- and aquareoviruses (s3, sB and VP7 in MRV, ARV and
aquareoviruses, respectively), which stabilizes the outer capsid, or the outer-fibre protein of orthoreoviruses (σ1 and σC in MRV and ARV, respectively), which is involved in cell attachment. In addition, the monocistronic S4 segment of PRV encodes a 315 aa predicted protein that has no identified homologue in ortho- or aquareoviruses (Palacios et al., 2010). Lastly, the S1 segment is bicistronic, encompassing two overlapping ORFs. S1 ORF1 (nt 29–1021) encodes a predicted 330 aa protein that has no reported homology to translation products of the bi- or tricistronic genome segments of other ortho- and aquareoviruses (Palacios et al., 2010). S1 ORF2 (nt 108–482) is embedded within ORF1 in a different reading frame and potentially encodes a 124 aa protein, designated p13. This predicted protein shares certain similarities with the fusion-associated small transmembrane (FAST) proteins of fusogenic ortho- and aquareoviruses, which are responsible for cell–cell fusion and syncytium formation (Corcoran and Duncan, 2004; Dawe & Duncan, 2002; Racine et al., 2009; Shmulevitz & Duncan, 2000; Thalmann et al., 2010), suggesting that PRV might be a new fusogenic reovirus (Palacios et al., 2010).

The sequence diversity, host range, apparent absence of two important structural proteins, presence of a potential FAST protein and presence of two additional PRV proteins with no known homologues in ortho- or aquareoviruses all suggest that PRV might define a new genus in the family Reoviridae (Palacios et al., 2010). To investigate this proposal, we reanalysed the PRV sequences and examined the functional properties of its predicted FAST protein. The results indicated that the S1 ORF2 product p13 is not a FAST protein but is instead a cytosolic, integral membrane protein that induces cytotoxicity in the absence of cell–cell fusion. PRV is therefore distinct from recognized ortho- and aquareoviruses in encoding a demonstrated integral membrane protein that is not a FAST protein, and thus represents the only second non-fusogenic species of these viruses. We also found that the predicted S1 ORF1 product has a signature zinc-binding motif of the outer-clamp protein of ortho- and aquareoviruses, and the predicted S4 product has a signature coiled-coil motif of the outer-fibre protein of most orthoreoviruses. The new findings thus identify several unusual features of this virus and highlight certain difficulties in assigning PRV to a particular genus in the family Reoviridae.

RESULTS

PRV p13 is cytotoxic but not syncytigenic

The 124 aa predicted translation product of PRV S1 ORF2, p13, contains a predicted transmembrane domain (TMD; aa 28–52) and two additional, moderately hydrophobic regions (aa 13–24 and 65–80), both features of the FAST proteins that are responsible for cell–cell fusion induced by fusogenic ortho- and aquareoviruses (Boutilier & Duncan, 2011). However, most FAST proteins are highly basic (isoelectric point (pI) >8.5) and contain a membrane-proximal cluster of basic residues important for fusion activity, whereas we noted that p13 was predicted to be an acidic protein (pI=4.8) that lacks a polybasic motif. To directly assess the fusogenic activity of PRV p13, Vero and QM5 cells were transfected with plasmids expressing p13. These two cell lines are highly susceptible to syncytium formation induced by most known FAST proteins, including p22 of aquareovirus A (Racine et al., 2009). ARV p10, which has the slowest kinetics of syncytiogenesis of any FAST protein (Salsman et al., 2005), was used as a positive control for syncytium formation in these experiments. Light microscopy of Giemsa-stained monolayers fixed at 24 h post-transfection (p.t.) revealed extensive formation of multinucleated syncytia in p10-transfected monolayers, whilst there was no evidence of syncytia in p13-transfected monolayers (Fig. 1). Even following prolonged incubation of cells for 72 h p.t., p13 failed to induce syncytia. The absence of cell–cell fusion was not due to a failure to express p13, as shown by immunoblotting of lysates from cells transfected with a FLAG-tagged version (Fig. 2c). PRV p13 therefore lacks the defining attribute of a FAST protein – the ability to induce cell–cell fusion and syncytium formation.

Both Vero and QM5 monolayers expressing p13 had reduced cell numbers compared with vector-transfected cells, and QM5 cells expressing p13 also displayed altered morphology and clumping (Fig. 1). An MTT cell-proliferation assay revealed that p13 expression induced a dramatic lag or reduction in cell numbers over time relative to plasmid-transfected control cells (Fig. 2a). A FACS-based propidium iodide cell-viability assay, where increased fluorescence indicates loss of membrane integrity, determined that the decreased cell numbers observed in the proliferation assay were the result of p13-induced cytotoxicity (Fig. 2b), which could arise via apoptosis or necrosis. The gradual increase in cell numbers over time (Fig. 2a) and the decrease in the fraction of dead cells from 16 to 24 h p.t. (Fig. 2b) presumably reflected the transfection efficiency of QM5 cells, which was determined to be ~22%, based on FACS analysis of cells transfected with a plasmid expressing EGFP (data not shown). As a result, non-transfected cells would not be subject to p13-induced cytotoxicity and would continue to proliferate. In contrast, expression of N-terminally FLAG-tagged ARV p10 did not inhibit cell proliferation (Fig. 2a) and did not induce cytotoxicity (Fig. 2b). This ARV p10 construct has greatly reduced cell–cell fusion activity but is still expressed as an integral membrane protein on the surface of cells, suggesting that the effects of p13 are not due to mere overexpression of a small, integral membrane protein. Based on the syncytiogenic and cell-viability assays, we concluded that PRV p13 is non-fusogenic but is cytotoxic when expressed ectopically in cells.

PRV p13 is a cytoplasmic, integral membrane protein

To describe further functional attributes of PRV p13, we examined the p13 distribution in cells. Transfected QM5
cells were lysed and separated into soluble, integral membrane and peripheral membrane fractions by high-pH salt extractions and ultracentrifugation, and the distribution of p13 in these fractions was assessed by immunoblotting. Lysates from cells expressing GFP or FLAG-tagged ARV p10 were used as positive controls for soluble and integral membrane proteins, respectively, whilst lysates probed for protein disulfide isomerase (PDI), an ER luminal protein, confirmed separation of peripheral or luminal proteins from integral membrane proteins (Fig. 2c). Immunoblotting detected two species of p13, both of which existed as integral membrane proteins, implying that the predicted TMD in p13, which spans aa ~28–52, functions as an internal signal anchor to direct p13 into a bitopic membrane topology. The origin of the two p13 polypeptides was not determined, but we noted a single N-linked glycosylation consensus sequence near the N terminus of p13 (Asn-Ile-Thr, aa 4–6). The addition of a high-mannose oligosaccharide at this location would add ~2.5 kDa to p13, which closely approximates the estimated 3 kDa difference in molecular mass of the two p13 polypeptides based on their electrophoretic mobility, suggesting that p13 may be glycosylated. If so, then p13 would assume an N external/C internal membrane topology.

When examined by immunofluorescence microscopy, N-terminally FLAG-tagged ARV p10 displayed a punctate staining pattern, concentrated in the perinuclear region and radiating out to the periphery, consistent with trafficking through the secretory pathway to the plasma membrane (Shmulevitz & Duncan, 2000). FACS analysis of immunostained, non-permeabilized cells with anti-FLAG antibody detected N-terminally tagged but not C-terminally tagged ARV p10 on the cell surface (Fig. 3b, left panel), indicating that p10 assumed the correct N external/C internal membrane topology. In contrast, although p13 was also localized in the perinuclear region and throughout the cytoplasm in a punctate staining pattern, there was no apparent localization to the plasma membrane (Fig. 3a). This staining pattern applied to both N-terminally FLAG-tagged p13 (Fig. 3a) and to a C-terminally tagged construct. FACS analysis of permeabilized cells, using both N- and C-terminally FLAG-tagged versions of p13, clearly detected intracellular p13 expression. However, similar analysis of live cells failed to detect any p13 on the surface of cells in either an N external or C external membrane topology (Fig. 3b, right panel). PRV p13 is therefore an integral membrane protein that localizes to one or more cytoplasmic membrane compartments but does not traffic to the plasma membrane.

**The PRV S1 ORF1 product has a signature motif of reovirus outer-clamp proteins**

The absence of an outer-clamp protein homologue in PRV was surprising (Palacios et al., 2010), as it is found in all other known ortho- and aquareoviruses and is thought to form an essential intermediate for outer-capsid assembly by complexing with the T=13 outer-shell protein (Chandran et al., 1999; Liemann et al., 2002). Upon
was subjected to a high-pH wash to strip proteins peri-pherally associated with the membrane and to release luminal contents and was repelleted. The total lysate (L) and soluble (S), peripheral/ luminal (P) and integral membrane (I) fractions were resolved by SDS-PAGE and visualized with immunoblotting using anti-FLAG, anti-GFP or anti-PDI antibodies.

inspecting the deduced amino acid sequence of S1 ORF1 (330 aa, calculated molecular mass 37.0 kDa), we noted a CxxC-n_{16}-HxC putative zinc-binding motif at aa 53–75 (Fig. 4). This motif is strongly reminiscent, in both sequence and relative position, of the CxxC-n_{18}-HxC known zinc-binding motif (Olland et al., 2001; Schiff et al., 1988) in MRV outer-clamp protein \( \sigma_3 \), the CxxC-n_{18}-HxC motif in ARV outer-clamp protein \( \sigma_B \) and an essentially identical motif in the outer-clamp proteins of other orthoreoviruses (Fig. 4). Moreover, an essentially identical motif is also shared by the VP7 outer-clamp protein of aquareoviruses (Fig. 4). At least one main function of this zinc-binding region in \( \sigma_3/\sigma_B/\text{VP7} \) is in organizing a protein-interaction domain that allows the outer-clamp protein to interact in a stabilizing complex with (i.e. to ‘clamp’) the trimeric \( T=13 \) outer-shell protein of each virus (\( \mu_1 \) in MRV, \( \mu_B \) in ARV and VP4 in aquareoviruses) (Cheng et al., 2010; Dryden et al., 1993; Liemann et al., 2002; Nason et al., 2000; Zhang et al., 2005a, b). Notably, several structure-based homology-search programs also identified either MRV \( \sigma_3 \) (pGen THREADER, FUGUE) or aquareovirus VP7 (HHpred) as the top-scoring hit for likeness to the PRV S1 ORF1 product. We therefore concluded that the S1 ORF1 product is the outer-clamp protein of PRV.

The PRV S4 product has a signature motif of orthoreovirus outer-fibre proteins

No homologues for the predicted PRV S4 ORF translation product were identified previously (Palacios et al., 2010). Upon inspecting its deduced amino acid sequence (315 aa, calculated molecular mass 34.6 kDa), we noted that a long region in its N-terminal half receives high coiled-coil prediction scores from several programs (Fig. 5). As indicated by these programs and also evident from visual inspection, this region of the PRV S4 product was notable for having hydrophobic residues in a regular heptad-repeat pattern (\( a-b-c-d-e-f-g \)).

Fig. 2. PRV p13 is a cytotoxic, integral membrane protein. (a) The increase in cell numbers over time of QM5 cells transfected with p13 (shaded squares), N-terminally FLAG-tagged ARV p10 (filled squares) or empty vector (open squares) was quantified at the indicated times p.t. using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Results are reported as absorbance readings at 570 nm \( (A_{570}) \). Non-linear regression was used to fit the data to sigmoidal curves. The results are shown as means \( \pm \) SD from six experiments each conducted in triplicate. (b) QM5 cells transfected with p13 (shaded columns), N-terminally FLAG-tagged ARV p10 (filled columns) or empty vector were incubated with propidium iodide at the indicated times post-transfection. Fluorescence measurements were obtained via flow cytometry and cell death was quantified as the percentage positive fluorescence compared with cells transfected with empty vector calculated via Overton subtraction of the histograms. The results are shown as means \( \pm \) SD from six experiments each conducted in triplicate. (c) QM5 cells transfected with N-terminally FLAG-tagged PRV p13 or ARV p10 or with GFP were disrupted and separated into soluble and insoluble membrane fractions by ultracentrifugation. The membrane pellet
two Pro residues that are otherwise uncommon in predicted coiled-coil regions (Fig. 5b). Such stutters have been noted in other coiled-coil proteins and are thought to cause underwinding of the supercoil in that region (Brown et al., 1996). The N-terminal coiled-coil region forms a major portion of the fibre region of σ1/σC, which is involved in projecting the more C-terminal cell-adhesion domains away from the capsid surface (Bassel-Duby et al., 1985; Chappell et al., 2002; Furlong et al., 1988; Guardado-Calvo et al., 2009). Based on the presence, length and relative position of the heptad-repeat pattern, we concluded that the S4 product is the outer-fibre protein homologue of PRV.

**DISCUSSION**

**Relating the new findings to PRV disease in fish**

As the causative agent of heart and skeletal muscle inflammation in Atlantic salmon, PRV is an economically important pathogen for salmon aquaculture (Kongtorp et al., 2004a; Palacios et al., 2010). Although we still have much to learn about the pathogenesis of this disease, the results described in this report identified two proteins that seem likely to play key roles, p13 and the predicted outer-fibre protein, p35. The predicted outer-fibre protein seems likely to be a receptor-binding protein and a determinant of virus spread and tropism within fish, whilst the cytotoxicity of p13 in cell cultures may translate to tissue injury within fish. The newly proposed coding assignments for two essential outer-capsid proteins of PRV (S1 ORF1 and S4, respectively) also provide two excellent targets for PRV-directed vaccines, passive immunization or small-molecule inhibitors. In the case of MRV, the outer-clamp and fibre proteins both contain important epitopes to which neutralizing or protective antibodies and other host responses are directed (Virgin et al., 1998). We propose that the same may hold true for the predicted p37 outer-clamp and p35 outer-fibre proteins of PRV. Furthermore, assuming that the outer-clamp protein of

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Fig. 3. Subcellular distribution of the p13 protein. (a) QM5 cells transfected with N-terminally FLAG-tagged versions of ARV p10 or PRV p13 were fixed and stained at 24 h p.t. with anti-FLAG antibody and Alexa Fluor 488-conjugated secondary antibody. The left panels are the fluorescent images, captured under identical parameters, and the right panels are fluorescent images overlaid on differential interference contrast images. (b) QM5 cells transfected with plasmids expressing N-terminally (left panel) or C-terminally (right panel) FLAG-tagged constructs of p13 (green), p10 (red) or pcDNA3 empty vector (shaded histogram) were immunostained with anti-FLAG primary antibody and Alexa Fluor 488-conjugated secondary antibody, before fixation and analysis by flow cytometry. The results are presented as the relative fluorescence intensity of 10 000 individual cells.
PRV must be degraded by host proteases in order for cell entry to proceed to membrane penetration, as is the case for MRV (Chandran & Nibert, 2003; Danthi et al., 2010), then targeting this essential step with small-molecule inhibitors might be another effective strategy against PRV in fish.

Important features of PRV integral membrane protein p13

PRV p13 is the first directly demonstrated example of an integral membrane protein in the ortho- or aquareoviruses that is not a FAST protein. Comparisons of the deduced amino acid sequences of PRV p13 and the FAST proteins of other reoviruses led us to suspect that p13 might not be a FAST protein. In addition to being acidic and lacking a polybasic motif, p13 lacks a myristoylation consensus sequence at its N terminus, as present in most known FAST proteins (Boutilier & Duncan, 2011). The PRV p13 ORF (S1 ORF1) is also nested within an encompassing ORF (S1 ORF1), suggesting that it co-evolved with this other product (see below) and not independently, as is the case with FAST proteins, whose ORFs lie outside the co-cistronic ORF. Most importantly, p13 was non-fusogenic in two cell lines that are highly permissive to fusion by most known FAST proteins. Furthermore, whilst p13 is indeed an integral membrane protein (as shown by cell fractionation), it did not traffic to the plasma membrane as do the FAST proteins (as shown by immunofluorescence microscopy and cell-surface FACs analysis) and therefore cannot function to induce plasma membrane fusion and syncytium formation. This latter point also makes it highly unlikely that p13 might mediate temperature-sensitive syncytium formation (i.e. only at temperatures consistent with the body temperature of its natural host). Consistent with our results showing that p13 lacks cell–cell fusion activity, there has been no reported evidence for syncytium formation in histopathological specimens of fish infected with PRV (Kongtorp & Taksdal, 2009; Kongtorp et al., 2004b, 2006; Palacios et al., 2010). We therefore conclude that PRV, unlike currently recognized ortho- and aquareoviruses except for MRV, is non-fusogenic.

If not a FAST protein, then what is the function of p13 in PRV infection? The features of p13 described in this report indicate that it is a new member of the class of small ortho- and aquareovirus non-structural proteins. Aside from the FAST proteins, all recognized ortho- and aquareoviruses encode one small, non-structural protein involved in RNA binding and an additional one (orthoreoviruses) or two (aquareoviruses) such proteins of variable or undetermined function (Table 1). There are no defined functions of the two smallest, non-FAST aquareovirus non-structural proteins. However, they are twice the size of PRV p13 and are devoid of a TMD consistently identified by all prediction programs, implying they are not p13 homologues. In the case of MRV, the s1s protein mediates G2/M cell-cycle arrest and is not essential for replication in culture but impacts on pathogenesis in animals (Hoyt et al., 2005). Unlike PRV p13, s1s is not a membrane protein and shows strong nuclear localization. As with MRV s1s, ARV p17 also inhibits cell proliferation by inducing G2/M cell-cycle arrest but also contributes to shut-off of host
translation induced by ARV, both properties that correlate with increased levels of virus replication (Chulu et al., 2010; Liu et al., 2005). ARV p17 was originally proposed to be a membrane protein (Bodelo´n et al., 2001). However, more recent studies indicate that p17 is a nucleocytoplasmic protein (Costas et al., 2005), not an integral membrane protein, and that cell-cycle arrest does not induce cytotoxicity (Chulu et al., 2010), two key attributes of PRV p13. Thus, p13 represents a new addition to the diverse group of small, non-structural ortho- and aquareovirus proteins. The membrane-localized, cytotoxic properties of p13 are similar to viroporins, a diverse group of small, membrane-interactive viral proteins that promote cytotoxicity and the associated assembly or release of both enveloped and non-enveloped viruses (Nieva et al., 2012). Further studies are needed to understand the effects of p13 on cells and PRV replication, and the evolutionary origins of this novel protein.

Fig. 5. The N-terminal coiled-coil motif of orthoreovirus outer-fibre proteins also present in the predicted S4 translation product of PRV. Coiled-coil predictions for the figures were made using Paircoil2 (McDonnell et al., 2006) with an averaging window of 21. (a) Probability scores at each amino acid position for the different proteins analysed are shown graphically, using the output from Paircoil2 (a lower score represents a greater likelihood of forming a coiled-coil). (b) Full-length sequences are shown for each protein analysed on the left. The sequence region encompassed by coiled-coil predictions from Paircoil2 is in bold, and shown with underlining in either black (probability threshold 0.05) or grey (probability threshold 0.15) according to the results in (a). Residues in the $a$ and $d$ positions of the heptad repeats of the predicted coil-coil regions are indicated in black. The NCBI Protein accession numbers for the outer-fibre protein sequences analysed are: MRV-T3D, P03528; ARV-176, AAF45153; NBV, AAF45159; RRV, AAP03135; PRV, GU994021.
Table 1. Protein lengths and pI values of PRV and representative ortho- and aquareoviruses

Strain details: PRV, Salmo/GP-2010/NOR; MRV, type 1 Lang; ARV, 176; AqRVC, golden shiner reovirus; AqRVG, American grass carp reovirus. Results are shown as protein length (aa), pI. pI values were calculated using the Compute pi/Mw tool at SIB-ExPASy.

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<th>Protein</th>
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<th>ARV</th>
<th>AqRVC</th>
<th>AqRVG</th>
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*Protein nomenclature suggested by Yan et al., (2011) to standardize names between species and genera. NS, Non-structural (not in virions); RdRp, RNA-dependent RNA polymerase.

†These are putative FAST proteins based on sequence features and genome-coding position comparable with the proven FAST protein of aquareovirus A (Racine et al., 2009).

Unique coding arrangements in the PRV genome

The present results revealed several unique coding arrangements in the PRV genome. Firstly, PRV appears to represent the first ortho- or aquareovirus in which the outer-clamp protein is identified to be encoded on a polycistronic (bi- or tricistronic) genome segment. Secondly, PRV appears to represent the first ortho- or aquareovirus in which the polycistronic genome segment is identified to encode neither the outer-fibre protein nor the FAST protein. In recognized orthoreoviruses, the polycistronic genome segment encodes the FAST protein and a non-structural protein of unclear function (MRV); the outer-fibre protein and the FAST protein (RRV); the outer-fibre protein, the FAST protein and a non-structural protein of unclear function (ARV and NBV); or the FAST protein and a non-structural protein of unclear function (BRV and BroV) (Corcoran and Thalmann, 2009; Shmulevitz et al., 2002; Thalmann et al., 2010). In recognized aquareoviruses, the polycistronic genome segment encodes the FAST protein and a non-structural protein of unclear function (Attoui et al., 2002; Ke et al., 2011; Mohd Jaafar et al., 2008; Racine et al., 2009), similarly to BRV and BroV. Thirdly, by predicting that the monocistronic S4 genome segment of PRV encodes the outer-fibre protein, we have identified PRV as apparently the first orthoreovirus in which the outer-fibre protein, when present, is not encoded on a polycistronic genome segment.

Taxonomic assignment of PRV

Previous results have suggested that PRV represents a new genus in the family Reoviridae (Palacios et al., 2010). This proposed taxonomic assignment reflects sequence-based phylogenetic comparisons (which indicated PRV branching off the root of the genera Aquareovirus and Orthoreovirus), the apparent absence of the outer-fibre protein characteristic of most orthoreoviruses, the presence of ten genome segments (not 11 as in all recognized aquareoviruses) and the isolation of PRV from a piscine host (in the genus Orthoreovirus, it would be the sole fish-derived member to date). However, sequence-based phylogenetic comparisons have revealed that most PRV translation products cluster somewhat more closely with those of orthoreoviruses (e.g. the core RdRp protein, core NTPase protein and outer-shell protein). By concatenating the sequences of the nine homologous proteins (seven structural and two non-structural proteins) shared by PRV and representative ortho- and aquareoviruses, we were able to generate new trees that showed PRV clustering more closely with recognized orthoreoviruses than with recognized aquareoviruses (Fig. 6). This analysis included two tentative novel aquareoviruses (GenBank accession nos HQ231198–HQ231208 and JN967629–JN967639), GCRVs GD108 (Ye et al., 2012) and 104 (Y. D. Fan, L. B. Zeng, S. J. Rao & J. Ma, unpublished), both of which have 11 genome segments. These new phylogenetic comparisons therefore suggest that PRV is more closely related to the orthoreoviruses. All currently recognized members of the genus Aquareovirus lack homologues of the outer-fibre protein (α1/αC) present in most members of the genus Orthoreovirus (Table 1). The fact that PRV is now predicted to possess an outer-fibre protein further argues for assigning it to the genus Orthoreovirus. Also in favour of assigning PRV to the genus Orthoreovirus is
its possession of ten genome segments, not 11 as in all recognized aquareoviruses. Based on the preponderance of evidence to date, we therefore conclude that PRV would probably be best classified at present as a new species in the genus *Orthoreovirus*. Moreover, according to the expanded genus boundaries suggested in Fig. 6, genome segment number would continue to discriminate the two genera, with orthoreoviruses having ten segments and aquareoviruses having 11.

**METHODS**

**Cells and plasmids.** Vero and QM5 cells were cultured in Medium 199 as described previously (Corcoran & Duncan, 2004). The PRV p13 ORF was synthesized by GenScript and cloned into the pUC57 vector. This construct was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen) as either an untagged version or with an N- or C-terminal triple-FLAG tag. The untagged ARV p10 clone has been described previously (Shmulevitz & Duncan, 2000) and was also recloned with an N- or C-terminal triple-FLAG tag. The sequences of all constructs were confirmed prior to transfection.

**Transfection and immunoblotting.** All cells were transfected with plasmid DNA using polyethylenimine at a 1:3 ratio (w/w), and at 4–6 h p.t.) the transfection mix was replaced with fresh growth medium. Cell lysates were subjected to SDS-PAGE (15% acrylamide) and transferred to PVDF membranes. The membranes were probed with dilutions of primary antibody (1:20 000 mouse anti-FLAG, 1:10 000 rabbit anti-GFP or 1:1000 mouse anti-PDI), followed by a 1:10 000 dilution of HRP-conjugated secondary antibody. Western blots were developed with ECL Plus reagent (GE Healthcare) and visualized on a Typhoon 9410 Variable Imager (Amersham Biosciences).

**Syncytial indexing.** Twelve-well plates of Vero or QM5 cells were transfected using 0.5 μg of the indicated plasmid DNA per well, and at the indicated times, the cells were fixed with methanol and stained with Wright–Giemsa stain (Siemens Healthcare Diagnostics). The stained monolayers were examined by light microscopy under 200× magnification to determine the presence or absence of syncytia.

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Fig. 6. Phylogenetic comparison of concatenated protein sequences. For each indicated virus, sequences of the core shell ($T=1$), core turret, core RdRp, core NTPase, core clamp, outer-shell ($T=13$), outer-clamp, NS factory and NS RNA proteins (see Table 1) were joined end to end. The concatenated sequences were then aligned using CLUSTAL Omega, and the alignment was used for maximum-likelihood phylogeny analysis (PhyML version 3.0 aLRT) at http://www.phylogeny.fr/. The tree presented here is an unrooted radial phylogram. Bar, number of substitutions per amino acid position. All branch support values were $\geq 85\%$. The extents of the current genera *Aquareovirus* and *Orthoreovirus* as they encompass formally recognized species in the tree are indicated by dark-grey shading. The proposed extents of these genera after expansion to encompass PRV and the recent GCRV isolates GD108 (Ye et al., 2012) and 104 (Y. D. Fan, L. B. Zeng, S. J. Rao & J. Ma, unpublished) are indicated by light-grey shading. The respective outer-clamp, NS factory and NS RNA proteins of GCRVs GD108 and 104 were newly identified as such for generating the concatenated sequences. The demarcation of isolates containing ten or 11 genome segments is indicated. AqRVA-SmReV, aquareovirus *A,a Scophthalmus maximus* (turbot) reovirus.
Membrane fractionation. QM5 cells in 10 cm dishes were transfected with 6 μg of the indicated plasmid DNA per dish. At 24 h.p.t., the cells were harvested in cold PBS containing 50 mM EDTA. The cells were disrupted by syringing ten times with a 27-gauge needle. Cellular debris and nuclei were pelleted by centrifugation at 500 g, and the membrane fraction was obtained by centrifugation at 100 000 g for 30 min. The membrane pellet was treated with 100 mM Na2CO3 (pH 11.4) for 30 min on ice to extract peripheral membrane-associated proteins and luminal proteins from the intracellular membrane compartments, followed by centrifugation at 100 000 g for 30 min to recover the membrane fraction and associated integral membrane proteins.

Cell-proliferation assay. Twelve-well plates of QM5 cells were transfected as described above, and at the indicated times, 50 μl MTT (Sigma-Aldrich) was added. The cells were incubated for 2 h at 37 °C, after which 50 μl MTT solubilization solution (4 mM HCl, 10% Triton X-100 in anhydrous 2-propanol; Sigma-Aldrich) was added and the plates were incubated until the formazan crystals were completely dissolved. Absorbance and reference readings were taken at 570 and 690 nm, respectively, using a Varian spectrophotometer. Background readings were obtained by following the above protocol in wells containing no cells. Experiments were performed in three replicates, each in triplicate, and results were determined as means ± SD from each experiment.

Cell-viability assay. Twelve-well plates of QM5 cells were transfected as described above, and at the indicated times cells were resuspended in 1 ml 0.05% trypsin/EDTA (Gibco) for 5 min at 37 °C. The cell suspension was pooled with the removed growth medium and centrifuged at 500 g for 5 min. The cell pellets were washed with 1 ml PBS and resuspended (in the dark) in 100 μl 0.5 μg propidium iodide ml−1 in labelling solution [10 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM CaCl2]. After a 15 min incubation at room temperature, 450 μl labelling solution was added to each tube and the samples were immediately analysed by flow cytometry using a Becton Dickinson FACSCalibur. The fluorescence intensity of 10 000 cells from each sample was measured using the FL2 channel and population fluorescence was quantified with FCS Express 2.0 (DeNovo Software) using Overton histogram subtraction versus empty vector-transfected cells. Experiments were performed in three replicates, each in triplicate, and results were determined as means ± SD from each experiment.

Fluorescence microscopy. QM5 cells grown on coverslips were transfected as described above, and at 24 h.p.t., the cells were fixed with acetone for 5 min. Cells were blocked with 1% BSA in PBS (PBS/BSA) for 30 min, incubated with a 1:1000 dilution of mouse anti-FLAG primary antibody in PBS/BSA, washed with PBS and incubated with a 1:500 dilution of Alexa Fluor 488-conjugated goat anti-mouse secondary antibody in PBS/BSA. All incubations were carried out in a humidified chamber for 1 h at room temperature. The coverslips were mounted and sealed using Prolong Gold Antifade Reagent (Invitrogen) and imaged using an Axiovert 200M inverted microscope (Zeiss).

FACS analysis of cell-surface expression. Twenty-four-well plates of QM5 cells were transfected as described above and at 24 h.p.t., the cells were blocked in staining buffer (PBS/BSA + 0.02% NaN3) at 4 °C for 30 min, incubated in a 1:1000 dilution of mouse anti-FLAG primary antibody in staining buffer for 1 h, washed, incubated with a 1:2000 dilution of Alexa Fluor 647-conjugated goat anti-rabbit secondary antibody in staining buffer for 1 h, washed with staining buffer and ice-cold PBS, and resuspended in PBS containing 50 mM EDTA for 1 min at room temperature. Resuspended cells in 150 μl cold PBS were added to an equal volume of 4% formaldehyde in PBS, and 10 000 cells were analysed on a FACSCalibur flow cytometer using FCS Express 2.0 (DeNovo Software).

Sequence analyses. All analysed PRV genes are those from strain Salmo/GP-2010/NOR. Sequence analyses were performed on PRV, orthoreovirus and aquareovirus deduced amino acid sequences using resources compiled at EMBL-EBI (http://www.efb.ac.uk/Tools/) and SIB-ExPaSy (http://www.expasy.org/protomics). For coiled-coil predictions, we used Paircoil2 (McDonnell et al., 2006; http://groups.canl.mit.edu/cb/paircoil2/) as indicated in Fig. 5 and also coils version 2.2 (Lupas, 1996). Other analyses included multiple sequence alignments for Table 4 and calculations of masses and pl for Table 1. TMD-prediction programs (HMMPK, Memsat3, PRED-TMR, SOSUI, SpliR, TMHMM and TMpred) and sequence-based homology-search programs (Pufgue, HHpred and pGenTHREADER) were also used. For the phylogenetic comparisons in Fig. 6, concatenated sequences were aligned using Clustal Omega and then submitted for phylogeny analyses with default settings as implemented at http://www.phylogeny.fr/ (Dereeper et al., 2008).

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