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Restricted growth of U-type infectious haematopoietic necrosis virus (IHNV) in rainbow trout cells may be linked to casein kinase II activity

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Abstract

Previously, we demonstrated that a representative M genogroup type strain of infectious haematopoietic necrosis virus (IHNV) from rainbow trout grows well in rainbow trout-derived RTG-2 cells, but a U genogroup type strain from sockeye salmon has restricted growth, associated with reduced genome replication and mRNA transcription. Here, we analysed further the mechanisms for this growth restriction of U-type IHNV in RTG-2 cells, using strategies that assessed differences in viral genes, host immune regulation and phosphorylation. To determine whether the viral glycoprotein (G) or non-virion (NV) protein was responsible for the growth restriction, four recombinant IHNV viruses were generated in which the G gene of an infectious IHNV clone was replaced by the G gene of U- or M-type IHNV and the NV gene was replaced by NV of U- or M-type IHNV. There was no significant difference in the growth of these recombinants in RTG-2 cells, indicating that G and NV proteins are not major factors responsible for the differential growth of the U- and M-type strains. Poly I:C pretreatment of RTG-2 cells suppressed the growth of both U- and M-type IHNV, although the M virus continued to replicate at a reduced level. Both viruses induced type 1 interferon (IFN1) and the IFN1 stimulated gene Mx1, but the expression levels in M-infected cells were significantly higher than in U-infected cells and an inhibitor of the IFN1-inducible protein kinase PKR, 2-aminopurine (2-AP), did not affect the growth of U- or M-type IHNV in RTG-2 cells. These data did not indicate a role for the IFN1 system in the restricted growth of U-type IHNV in RTG-2 cells. Prediction of kinase-specific phosphorylation sites in the viral phosphoprotein (P) using the NetPhosK program revealed differences between U- and M-type P genes at five phosphorylation sites. Pretreatment of RTG-2 cells with a PKC inhibitor or a p38MAPK inhibitor did not affect the growth of the U- and M-type viruses. However, 100 μM of the casein kinase II (CKII) inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), reduced the titre of the U type 8.3-fold at 24 h post-infection. In contrast, 100 μM of the CKII inhibitor reduced the titre of the M type only 1.3-fold at 48 h post-infection. Our data suggest that the different growth of U- and M-type IHNV in RTG-2 cells may be linked to a differential requirement for cellular protein kinases such as CKII for their growth.

Keywords: casein kinase II, IHNV, RTG-2 cells, U-type growth.

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Introduction

Infectious haematopoietic necrosis virus (IHNV) is a rhabdovirus with a linear single-stranded, negative-sense RNA genome of approximately 11,000 nucleotides. The IHNV genome encodes a non-virion

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protein (NV) and five structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase protein (L) (Kurath, Ahern, Pearson & Leong 1985; Morzunov, Winton & Nichol 1995). IHNV causes an acute disease in wild and hatchery-reared salmonid fish in North America, Europe and Asia (Wolf 1988; Bootland & Leong 1999). Phylogenetic analysis of IHNV glycoprotein (G) sequences differentiated North American IHNV isolates into three genogroups, denoted as U, M and L (Kurath, Garver, Troyer, Emmenegger, Einer-Jensen & Anderson 2003). There are general host preferences associated with the U and M genogroups. Most of the U genogroup viruses were isolated from sockeye salmon, *Oncorhynchus nerka* (Walbaum), and the M genogroup viruses were mostly from rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Kurath *et al.* 2003). In addition, the U and M genogroup viruses show host-specific virulence *in vivo* (LaPatra, Fryer & Rohovec 1993; Garver, Batts & Kurath 2006). While the U genogroup viruses are highly virulent to sockeye salmon but not rainbow trout, the M genogroup viruses are highly virulent to rainbow trout and not to sockeye salmon. Detailed studies comparing U- and M-type strain virus infections in rainbow trout (Peñaranda, Purcell & Kurath 2009) and sockeye salmon (Purcell, Garver, Conway, Elliott & Kurath 2009) revealed that in each host, higher virulence was associated with more rapid virus replication to higher viral load and persistence, despite a strong host innate interferon (IFN1) response. However, the molecular basis for the host-specific virulence difference of the U and M genogroups is not known.

Based on numerous observations from phylogenetic studies and historical literature on IHNV (Kurath *et al.* 2003), we have previously hypothesized that the U genogroup in sockeye salmon represents the ancestral IHNV host–virus association, and the M genogroup arose following a host jump into rainbow trout. A viral pathogen must overcome many hurdles to replicate successfully in a novel host. It must enter the host cell, replicate with the assistance of host factors and evade inhibitory host products (Webby, Hoffmann & Webster 2004). Each of these stages may require adaptive changes in the pathogen. Entry of a virus particle into a cell is initially mediated by the interaction of a virus protein with its corresponding host cell receptors. Different viruses use various host molecules as receptors, some of which are more globally conserved than others. For example, arenaviruses, the causative agents of several haemorrhagic fevers, use the widely conserved α-dystroglycan protein as a receptor (Meyer, de la Torre & Southern 2002). Coronaviruses, by contrast, generally show a restricted host range that is mediated by specific interaction of the viral spike protein with glycoproteins on the host cell surface (Holmes, Zelus, Schickli & Weiss 2001). For rhabdoviruses, the viral G protein is the only viral protein on the external surface of virions, and it mediates attachment to cellular receptors. In the well-studied mammalian rhabdovirus vesicular stomatitis virus (VSV), two major serotypes, New Jersey (VSNJV) and Indiana (VSIV), have been described based on neutralizing antibodies to the surface glycoprotein (G) (Cartwright & Brown 1972), and these serotypes differ in virulence for swine (Bridges, McCluskey, Salman, Hurd & Dick 1997). By using recombinant VSV viruses that differed only in their G genes, Martinez, Rodriguez, Jimenez, Pauszek & Wertz (2003) demonstrated that the G protein was a determinant of VSV virulence in this natural host.

Once inside a cell, the virus has to produce copies of its genome and virion components. With the exception of some larger DNA viruses, most viruses with limited coding capacity have to rely on host functions for virus replication. Within host cells, VSV transcribes viral mRNAs and replicates viral RNA genomes by using an RNA polymerase complex consisting of the viral L, P (Emerson & Yu 1975) and N proteins (Qanungo, Shaji, Mathur & Banerjee 2004). It is reported that phosphorylation of the VSV P protein by a host casein kinase II (CKII) is required for regulating RNA synthesis (Barik & Banerjee 1992) and that mutations altering the phosphorylation of the P protein affect RNA transcription or RNA genome replication (Das & Pattnaik 2004). Besides CKII, protein kinase B (PKB) is reported to play a critical role in viral RNA synthesis of nonsegmented negative-stranded RNA viruses by phosphorylation of P protein (Sun, Fuentes, Timani, Sun, Murphy, Lin, August, Teng & He 2008).

Virus-infected cells synthesize and secrete type I interferons (IFNs), which are considered the first line of host defence against virus infection (Garcia-Sastre & Biron 2006; Haller, Kochs & Weber 2006; Sadler & Williams 2008). Secreted IFNs stimulate susceptible cells to express more than 300 IFN-stimulated genes (ISGs), whose concerted action leads to limiting further viral growth and

spread (Biron & Sen 2001; Sadler & Williams 2008). The myxovirus resistance gene Mx, the PKR protein kinase stimulated by dsRNA and the 2′-5′ oligoadenylate synthetase (OAS) are among the best characterized ISGs with antiviral activity (Biron & Sen 2001). Viruses would not replicate successfully in a host if they had not evolved efficient escape strategies allowing them to evade the host IFN response. It is becoming increasingly clear that IFN antagonistic properties can determine viral virulence in a given host (Webby *et al.* 2004). Strategies used by viruses to suppress IFN production, to down-modulate IFN signalling and to block the action of antiviral effector proteins have been reported (Haller *et al.* 2006; Haller, Kochs & Weber 2007). In the case of VSV, virally produced dsRNA plays a crucial role in inducing an antiviral state in virus-infected cells (Ostertag, Hoblitzell-Ostertag & Perrault 2007). VSV M protein is reported to suppress the IFN gene expression through inactivation of transcription factor II D (Ahmed, McKenzie, Puckett, Hojnacki, Poliquin & Lyles 2003). However, once IFN signalling establishes an antiviral state, VSV replication is inhibited by IFNs at multiple stages of infection (Trottier, Palian & Reiss 2005). VSV grows readily to high titres in PKR-null cultured cells, which suggest that protein kinase R (PKR) plays a critical role in limiting VSV replication (Balachandran, Roberts, Brown, Truong, Pattnaik, Archer & Barber 2000).

We previously reported that while a representative M genogroup type strain (referred to hereafter as M-type) from rainbow trout grows well in rainbow trout gonad-2 (RTG-2) cells, the growth of a representative U genogroup strain (U-type) from sockeye salmon is restricted in the same cells (Park, Moon, Wargo, Purcell & Kurath 2010). The growth difference between the U- and M-type IHNV viruses was correlated with a difference in viral RNA synthesis in RTG-2 cells. In this study, we further investigated the molecular basis for the growth difference between the U- and M-type viruses in RTG-2 cells. Our data suggest that a specific host protein kinase is required for IHNV replication, possibly for viral RNA synthesis. We hypothesize that during the adaptation to rainbow trout as a new host, the M genogroup virus developed a requirement for different host protein kinases to achieve optimal growth, and different host protein kinase requirements are at least partially responsible for the different growth of U- and M-type IHNV in RTG-2 cells.

Materials and methods

Cells and viruses

Rainbow trout gonad 2 (RTG-2) cells and cyprinid *epithelioma papulosum cyprini* (EPC) cells were grown at 25 °C in Eagle's minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS). The RTG-P1 cell line, a transgenic RTG-2 derivative stably transfected with a luciferase reporter vector controlled by a rainbow trout Mx1 promoter (Collet, Boudinot, Benmansour & Secombes 2004), was purchased from ATCC (CRL-2829) and cultivated in Eagle's MEM supplemented with 10% FBS and 200 μg mL^{-1} neomycin (G418; Sigma). The BLk94 and 220-90 IHNV strains (Garver *et al.* 2006) were used as type strains to represent the U and M genogroups, respectively. Viruses were propagated in EPC cells at 15 °C and were quantified in terms of plaque forming units (PFU mL^{-1}) by standard plaque assay (Batts & Winton 1989).

Effect of poly I:C on IHNV growth

To determine the effect of pretreatment of cells with poly I:C on the growth of IHNV, a 25-cm^2 flask of RTG-2 cells was inoculated with poly I:C (25 μg mL^{-1}) (Sigma) and incubated for 24 h. Then, the medium was removed and replaced by fresh medium without poly I:C, and the cells were challenged with IHNV at a multiplicity of infection (MOI) of 1 PFU per cell. Samples of the supernatant were collected at 0, 24, 48, 72, 120 and 192 h after virus infection and stored at −80 °C until use.

To assess the effect of poly I:C treatment after virus infection, RTG-2 cells were challenged with IHNV at an MOI of 1 PFU per cell and incubated for 24 h at 15 °C. The medium was removed and replaced by fresh medium with or without poly I:C (25 μg mL^{-1}). After further incubation for 24 h, supernatant samples were collected and stored at −80 °C until use.

Mx1 promoter-reporter assay

To confirm the presence of, and to quantify IFN in culture supernatants, we used the transgenic RTG-P1 cell line (Collet *et al.* 2004). One mL of culture supernatant sample from RTG-2 cells was added to each of three wells in a 24-well plate containing the transgenic RTG-P1 cells and incubated for 24 h. As

a positive control, RTG-P1 cells were treated with poly I:C (25 μg mL^{-1}) for 24 h. Cells were then rinsed with MEM without FBS and trypsinized. The detached cells were collected by centrifugation, and the pellet stored at −80 °C. To assay luciferase activity cells were resuspended in luciferase lysis buffer (Promega) and luciferase substrate (Promega), and emission of light was measured in triplicate wells of a 96-well plate with a Luminoskan Ascent luminometer (Labsystem).

Plasmid construction and recovery of recombinant virus

A full-length antigenomic IHNV cDNA construct plasmid (pIHNV) derived from a French IHNV strain (IHNV 32/87) (Biacchesi, Thoulouze, Béarzotti, Yu & Brémont 2000) was used to generate recombinant IHNVs. Recombinant viral progeny of the full-length clone pIHNV was designated rIHNV. To generate chimeric recombinant viruses, coding regions (open reading frames, ORFs) of the G and NV genes of the U- and M-type IHNV strains were amplified by RT-PCR from RNA extracted from infected EPC cells. RT-PCR was performed with primers as follows: G_U: GAGCAG AGGTGAAGACTAGA, GATAAGTAACTTGC CACTGC; G_M: CACGTAAAGTACCAGGTC ATC, ACACACCCACAGTATCACT; NV_U: AG TGAGCAGGAAGACTC, GGTAGGAGCTATG GCGACT; NV_M: AGTGAGCAGGAAGACTC, GGTAGGAGCTATGGCGACT. Unique *Spe*I and *Sma*I restriction enzyme sites surrounding the G or NV ORFs of pIHNV were created by site-directed mutagenesis with the Quick Change kit (Stratagene). The entire G ORF of pIHNV was deleted by *Spe*I–*Sma*I digestion and replaced with amplified DNA fragments containing the G_U or G_M ORFs to generate pIHNV-G_U or pIHNV-G_M. Similarly, the entire NV ORF of pIHNV was deleted and replaced with DNA fragments containing the NV_U or NV_M ORFs to generate pIHNV-NV_U or pIHNV-NV_M, respectively (Fig. 1a). The presence and position of the inserted genes were confirmed by RT-PCR and restriction enzyme analysis, and sequencing of the inserted sites in the plasmids. Recombinant viruses were recovered from EPC cells as previously described (Biacchesi *et al.* 2000). Briefly, EPC cells were infected with the vaccinia virus recombinant that expresses T7 RNA polymerase (vTF7-3) (Fuerst, Niles, Studier & Moss 1986) and transfected with the full-length cDNA clones and three support plasmids that expressed the N, P and L proteins required for RNA encapsidation and replication. Infectious viruses were recovered from the supernatant medium and amplified by passage on EPC cells at low MOI. Recombinant viral progeny from cells transfected with the clones pIHNV-G_U, pIHNV-G_M, pIHNV-NV_U or pIHNV-NV_M were designated rIHNV-G_U, rIHNV-G_M, rIHNV-NV_U or rIHNV-NV_M, respectively. Viral titres of recombinant virus stocks were quantified by plaque assay.

Real-time RT-PCR for Mx1 and IFN1 expression in RTG-2 cells

Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen) following the manufacturer's protocol. The synthesis of cDNA was performed using the Taqman RT kit (Applied Biosystems) at a final reaction volume of 25 μL. First, 9.125 μL RNA and 2.5 μM oligod(T)16 (final concentration) were mixed and denatured at 70 °C for 10 min. Denaturing reactions were placed on ice and mixed with 1× reverse transcriptase buffer, 5.5 mM $MgCl_2$, 2 mM dNTPs, 0.4 U RNase inhibitor and 1.25 U Multiscribe reverse transcriptase. Reactions were incubated at 48 °C for 90 min.

The Taqman primers and probes were designed to detect the genes encoding the rainbow trout type I IFN1 gene (Zou, Tafalla, Truckle & Secombes 2007) and rainbow trout Mx1 (Trobridge & Leong 1995). The acidic ribophosphate protein P0 (ARP) was used as a normalizing gene (Purcell, Kurath, Garver, Herwig & Winton 2004). Primers and dual-labelled probes (5′ 6-FAM and 3′ TAMRA) were purchased from Integrated DNA Technologies (IDT) and are shown in Table 1. Calibration was performed on 10-fold serial dilutions of plasmid containing the target sequence.

One microlitre of first-strand cDNA synthesis reaction was added to 10 μL of Taqman 2× PCR mix (ABI), 900 nM forward and reverse primer, and 250 nM probe in a 20-μL reaction. The reactions were pipetted into individual wells of a 96-well optical plate (ABI) and analysed in an ABI-Prism model 7000 sequence detector. Polymerase chain reaction conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 50 cycles consisting of 15 s at 95 °C followed by 1 min at 60 °C. C_t values were calculated by the SDS software algo-

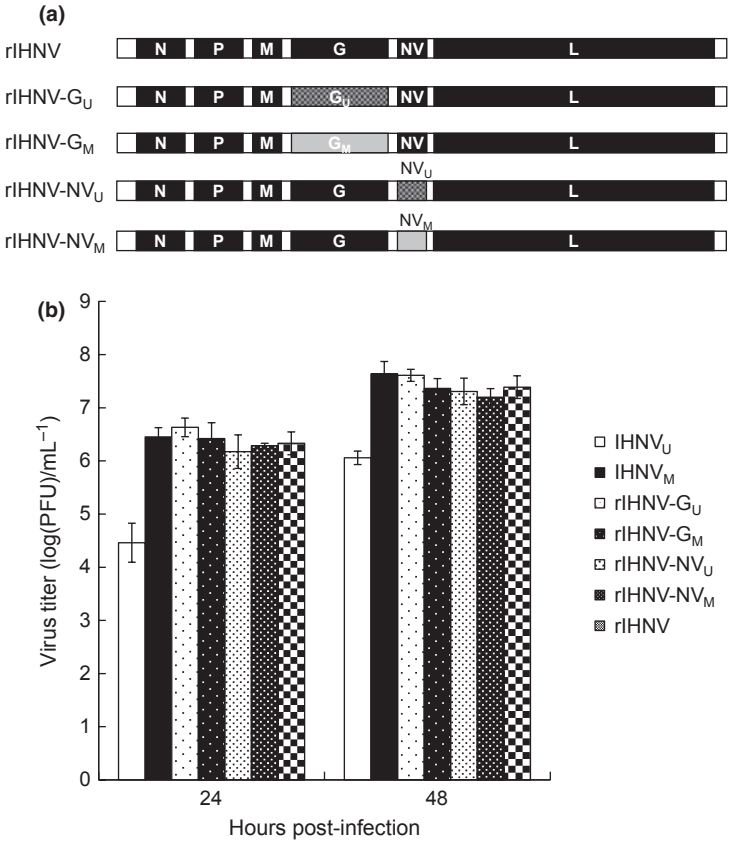

Figure 1 (a) Schematic representation of recombinant IHNV genomic RNAs indicating gene order and expressed proteins. G_U and G_M refer to the glycoprotein genes from the IHNV U-type and M-type strains, respectively. NV_U and NV_M refer to the non-virion genes from the IHNV U-type and M-type strains, respectively. (b) Comparative growth of IHNVs and rIHNVs in RTG-2 cells. The cells were infected at a multiplicity of infection of 1 PFU per cell, and samples of the supernatant medium were collected at the indicated time points. The samples were titrated in duplicate by plaque assay on EPC cells. The results are presented as the means ± SD of three independent experiments. IHNV, infectious haematopoietic necrosis virus; EPC, *epithelioma papulosum cyprini*.

Table 1 Primers and probes used in this study for real-time RT-PCR

Transcript	Primer	Sequence (5′–3′)
Rainbow trout IFN1	Rt-IFN1-F	gcgaaacaaactgctatttacaatgtata
	Rt-IFN1-R	tcacagcaatgacacacgctc
	Rt-IFN1-probe	6′FAM-cagagctggagttgtatttttcttattatttgcagtatgc-TAMRA
Rainbow trout Mx1	Rt-Mx1-F	ggttgtgccatgcaacgtt
	Rt-Mx1-R	ggcttggtcaggatgcctaat
	Rt-Mx1-probe	6′FAM-aagatggcacaagaggtggaccctgaag-TAMRA
Rainbow trout ARP	Rt-ARP-F	gaaaatcatccaattgctggatg
	Rt-ARP-R	cttcccacgcaaggacaga
	Rt-ARP-probe	6′FAM-ctatcccaaatgtttcattgtcggcgc-TAMRA
IHNV-G	IHNV-G-F	gcgcacgccgagataatatcaa
	IHNV-G-R	tcccgtgatagatggagcctttt
	IHNV-G-probe	6′FAM-cgatctccacatcccggaataaatgacgtct-TAMRA

IHNV, infectious haematopoietic necrosis virus; ARP, acidic ribophosphate protein P0.

rithms (ABI) and converted into equivalent target amount (ETA) using the statistical standard curves established using the calibrator standards. The expression level of a given gene was expressed relative to the ARP control by calculating the ratio ETA gene/ETA ARP.

Phosphorylation site prediction

The potential for phosphorylation in IHNV P protein was predicted by NetPhos (Blom, Sicheritz-Ponten, Gupta, Gammeltoft & Brunak 2004). The nucleotide and amino acid sequences of P proteins of IHNV BLk94 and IHNV 220-90 were deposited in GenBank (GenBank accession numbers: IHNV BLk94 P, [HQ189136]; IHNV 220-90 P, [HQ189137]).

Protein kinase inhibitors

Inhibitors against the host kinases CKII (5,6-dichlorobenzimidazole riboside, DRB), protein kinase C (PKC) (bisindolylmaleimide XI hydrochloride) and p38MAPK (SB203580) were purchased from Sigma. The compounds were dissolved in dimethyl sulphoxide and used at the concentrations indicated in the figure legends. RTG-2 cells were pretreated with protein kinase inhibitors at the concentrations indicated for 1 h and then infected with IHNV at an MOI of 1. The samples of the supernatant medium were collected at the indicated time points and titrated in duplicate by plaque assay on EPC cells.

Statistical analysis

Differences in the expression of IFN1 and Mx1 and differences in growth of M-type and U-type viruses were evaluated by Student's *t*-test or one-way ANOVA. A P value < 0.05 was considered to indicate statistical significance.

Results

The viral glycoprotein is not responsible for the growth restriction of U-type IHNV in RTG-2 cells

To determine whether the viral G protein is responsible for the differential growth of U- and M-type IHNV in RTG-2 cells, we generated two recombinant IHNVs based on an infectious clone of the French IHNV 32/87 strain (Biacchesi *et al.* 2000), in which the homologous $G_{32/87}$ ORF was replaced by the G_U or G_M as shown schematically in Fig. 1a. The RNA genomes of viruses recovered from cDNA were characterized by sequence analysis to confirm that the anticipated sequences were present after the recovery process. To determine whether the replacement of G protein affected the growth of the recombinant viruses, rIHNV-G_M (with the G ORF of M-type virus) and rIHNV-G_U (with the G ORF of U-type virus) were compared for growth in RTG-2 cells (Fig. 1b). Control viruses replicated as expected, with significantly less growth of the U-type than the M-type parental strain, and growth of the original recombinant rIHNV was similar to the M-type virus (Fig. 1b). There were no significant differences in growth among the rIHNV, rIHNV-G_M or rIHNV-G_U recombinant viruses (Fig. 1b).

NV is not responsible for the growth restriction of U-type IHNV in RTG-2 cells

To determine whether IHNV NV protein was responsible for the differential growth between U- and M-type viruses in RTG-2 cells, two recombinant IHNVs based on the genomic background of rIHNV 32/87 were generated by deleting the NV ORF and replacing it with the NV ORF of M-type virus (rIHNV-NV_M) or U-type virus (rIHNV-NV_U) (Fig. 1a). As shown in Fig. 1b, the growth of these recombinants in RTG-2 cells was similar to the original rIHNV, and there was no significant difference among them.

Pretreatment with poly I:C reduces the growth of M-type IHNV but completely blocks the growth of U-type IHNV for 72 h p.i.

To test whether the U- and M-type viruses differ in ability to evade the IFN system of RTG-2 cells, cells were pretreated with 25 μg mL^{-1} of poly I:C, which has been reported to induce the IFN system in RTG-2 cells (Trobridge, Chiou & Leong 1997; Collet & Secombes 2002). As shown in Table 2, poly I:C treatment significantly induced the expression of both IFN1 and the Mx1 gene by 6 h post-treatment in RTG-2 cells, and their induced expression continued through 24 h. At 24 h after poly I:C treatment, cells were infected with U- or M-type viruses at an MOI of 1 PFU per cell, and

Table 2 Expression of IFN1 and Mx1 gene in poly I:C-treated RTG-2 cells determined by real-time PCR

Time after poly-IC treatment (h)	IFN1	Mx1
6	41×[a]	316×
24	45×	2879×

[a]Fold relative to untreated control.

infectious titres in the culture supernatants were determined over time (Fig. 2a). Compared with mock pretreated cells, the poly I:C pretreatment significantly blocked the growth of both U- and M-type viruses. The growth of U-type virus was almost completely blocked for 72 h post-infection (p.i.) and then growth resumed. In contrast, the M-type virus grew steadily, albeit more slowly, in the presence of the poly I:C-induced IFN1 response, and its yield at 72 h p.i. was only 10-fold less than in untreated control infections (Fig. 2a).

To determine whether poly I:C treatment after virus infection can block the growth of the U- and M-type viruses, RTG-2 cells were infected with U- or M-type virus at an MOI of 1 PFU per cell and then treated 24 h p.i. with 25 μg mL^{-1} of poly I:C. The culture supernatants were collected at 24 h after poly I:C treatment, and infectious titres in the culture supernatants were determined by plaque assay. As shown in Fig. 2b, poly I:C treatment after virus infection did not exert significant change in the growth of either U- or M-type virus.

Different growth of U- and M-type viruses is not correlated with ability to block induction of the IFN1 system

If the differential growth of U- and M-type IHNV in RTG-2 cells was based on a different ability to block interferon responses, we would predict that the more restricted U virus would be less able to block these host responses, and thus should induce

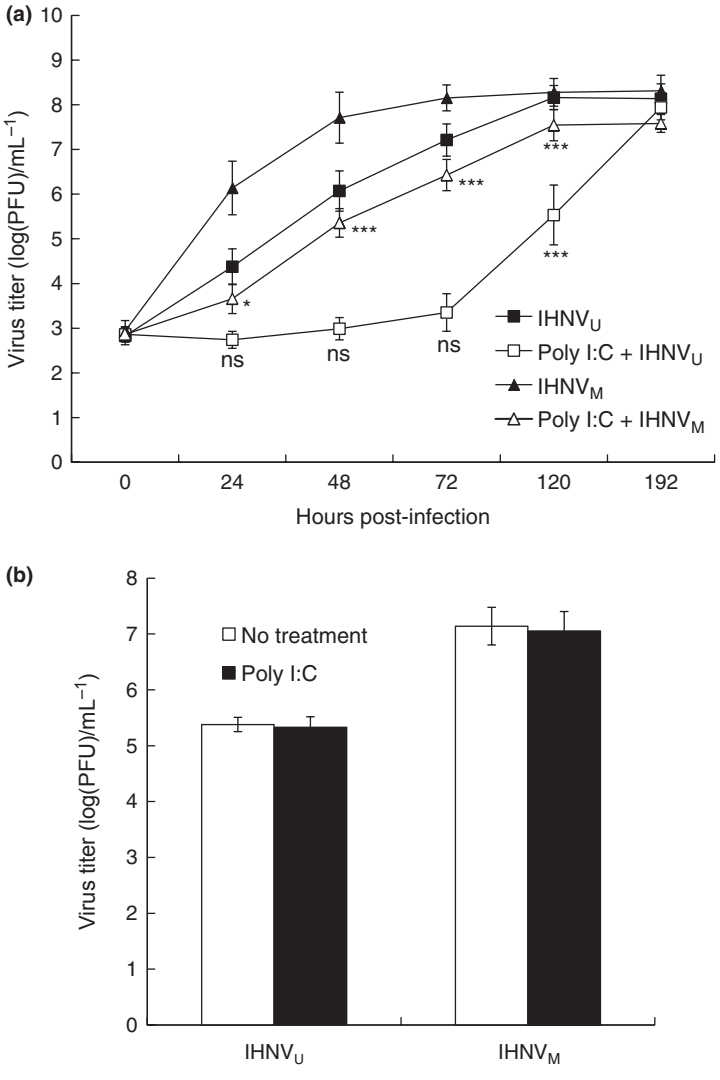

Figure 2 (a) Effect of poly I:C pretreatment on the growth of U- and M-type IHNV in RTG-2 cells. Cells were pre-incubated with poly I:C at 25 μg mL^{-1} for 24 h. Then, cells were infected with IHNV U- or M-type virus at a multiplicity of infection (MOI) of 1 PFU per cell, and samples of the supernatant medium were collected at the indicated time points. The samples were titrated in duplicate by plaque assay on EPC cells. The results are presented as the means ± SD of three independent experiments; ns, not significant, $^{*}P < 0.05$, $^{***}P < 0.05$ vs. virus titre at 0 h. (b) Effect of poly I:C treatment after virus infection. RTG-2 cells were infected with IHNV U- or M-type strains at an MOI of 1 PFU per cell for 24 h prior to poly I:C treatment. After further incubation for 24 h, samples of the supernatant medium were collected and titrated in duplicate by plaque assay on EPC cells. The results are presented as the means ± SD of three independent experiments. IHNV, infectious haematopoietic necrosis virus; EPC, *epithelioma papulosum cyprini*.

higher levels of IFN1 and ISGs than the M-type virus. To determine whether U- and M-type viruses induced different levels of IFN1, RTG-2 cells were infected at an MOI of 1 PFU per cell, total RNA was extracted at 6 and 24 h p.i., and the induction of IFN1 gene expression was analysed by real-time PCR. Relative to mock-infected control cells, U-type IHNV did not induce detectable IFN1 at either time point, but M-type IHNV induced IFN1 expression by 24 h p.i. (Fig. 3a). Relative to the U-type virus the M-type IHNV induced 1.5- and 3.1-fold higher levels of IFN1 gene expression at 6 h and 24 h p.i., respectively, and the difference at 24 h was significant (Fig. 3a).

To determine whether the two IHNV strains differed in their release of functional IFN1, supernatants were collected from U- and M-type IHNV-infected RTG-2 cells, as well as uninfected cells as a control, and IFN1 activities in the supernatants were assayed using RTG-P1 cells. RTG-P1 cells are RTG-2 derivatives stably transfected with a luciferase reporter vector controlled by a rainbow trout Mx1 promoter (Collet *et al.* 2004). RTG-P1 cells were treated with supernatants from IHNV-infected RTG-2 cells and after incubation for 48 h, activation of the Mx1 promoter was measured by luciferase production. As a control for background levels of luciferase activity induced by IHNV in the supernatant, RTG-P1 cells were incubated for 48 h with supernatant harvested from IHNV-infected EPC cells, which do not appear to have a homologous IFN1 response but support IHNV replication to levels comparable to the RTG-2 cells. RTG-P1 cells treated with 25 μg mL^{-1} of poly I:C were used as a positive control. The supernatants of M- and U-type-infected RTG-2 cells showed 5- and 2.2-fold increases in luciferase activity, respectively, compared with that of mock-infected RTG-2 cells (Fig. 3b). RTG-P1 cells treated with supernatants from U- and M-type virus-infected EPC cells showed 1.5- and 1.4-fold increases in luciferase activity, respectively, compared with mock-infected RTG-P1 cells (Fig. 3b). The EPC cell control data indicated that most of the luciferase activity of RTG-P1 cells induced by supernatant from IHNV-infected RTG-2 cells was not induced by virus in the supernatant, but rather it was directly from interferon activity in the supernatant.

IFN can provide signal to induce ISGs with antiviral activity such as Mx, PKR and OAS (Biron & Sen 2001). Even though U- and M-type IHNV both induce IFN1 expression, they may differ in the inhibition of IFN1 signalling pathways and, thus, they may differ in the induction of ISGs. If this is the case, we would predict that the more restricted U-type virus would be less able to inhibit signalling, and thus should induce higher levels of ISGs. To test this, we analysed the expression level of the Mx1 gene, one of the ISGs, in the IHNV-infected RTG-2 cells by real-time PCR. In RTG-2 cells, both M- and U-type strains induced Mx1 gene expression relative to mock-infected controls (Fig. 3a). However, the M-type induced significantly more, with 3.5-fold more Mx1 gene expression than the U-type IHNV at 24 h p.i.

U–type growth restriction does not require PKR activation

To test the possibility that PKR activation might play a role in the U-type IHNV restriction, we made use of a well-characterized inhibitor of PKR activity, 2-AP. Figure 3c shows the effects of 2-AP on infectious virus yields from the M- and U-type-infected RTG-2 cells. There was no significant change in the growth of either the U- or M-type IHNV in 2-AP-treated RTG-2 cells.

Casein kinase II inhibitor reduces the growth of U-type but not M-type IHNV

To investigate whether the difference in growth of U- and M-type IHNV in RTG-2 cells may be owing to a difference in P protein phosphorylation, we determined the P gene sequences of U-and M-type IHNV and then used the NetPhosK server (Blom *et al.* 2004) to identify predicted kinase-specific phosphorylation sites in the P proteins. The results showed five potential predicted phosphorylation sites for PKC, CKII or p38MAPK (Thr 53, Ser 149, Thr 200, Thr 213 and Thr 221) that differed between the U and M viruses (Fig. 4a). This result led us to hypothesize that differential phosphorylation at one or more of these sites by PKC, CKII or p38MAPK kinases may result in different levels of viral RNA synthesis and thus different growth between U- and M-type viruses in RTG-2 cells.

To determine whether any of these kinases exert effect on the growth of U- and/or M-type strains in RTG-2 cells, cells were incubated with inhibitors for each of these three kinases and then infected with the IHNV strains at an MOI of 1. Twenty-four and 48 h p.i., the supernatants were collected

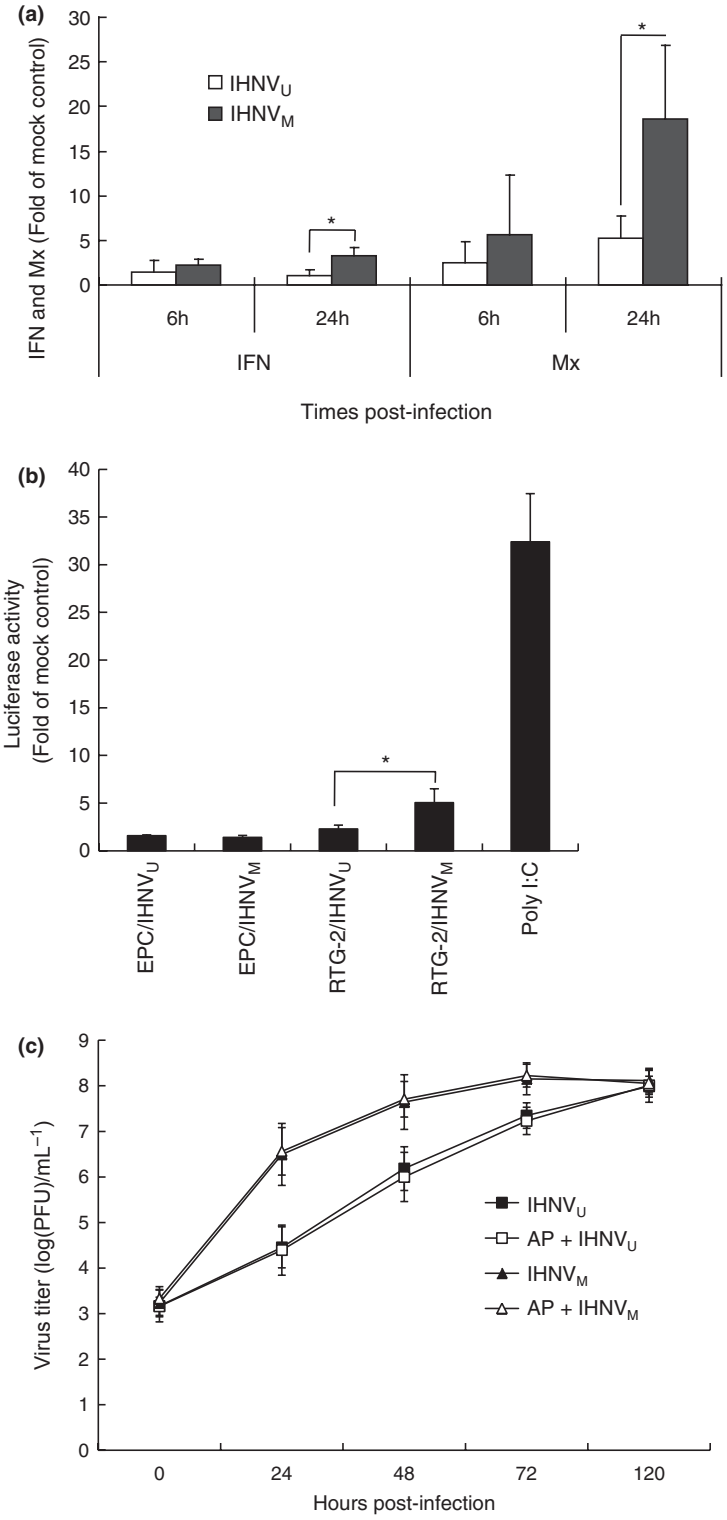

Figure 3 Analysis of interferon (IFN)-related responses in RTG-2 cells infected with U-type and M-type IHNV. (a) IFN1 and Mx1 expression in IHNV-infected RTG-2 cells. RTG-2 cells were harvested at indicated times after U- or M-type IHNV infection, or mock infection, and total RNA was analysed with real-time PCR for IFN1 and Mx1. The expression level of IFN1 and Mx1 in mock-infected cells at 0 h was defined as one. The results are presented as the means ± SD of three independent experiments. (b) Assay for functional IFN1 by luciferase activity in RTG-P1 cells after incubation for 24 h with supernatants from mock-infected or virus-infected fish cells (RTG-2 or EPC cell lines). The positive control is RTG-2 cells stimulated with 25 μg mL^{-1} poly I:C. The results are presented as the means ± SD of three independent experiments. (c) The effect of PKR inhibitor on the growth of U- and M-type IHNV. RTG-2 cells were infected with IHNV U- or M-type strains at a multiplicity of infection of 1 PFU per cell in the presence or absence of the PKR inhibitor, 2-AP (1.5 mM). The samples of the supernatant medium were collected at the indicated time points and titrated in duplicate by plaque assay on EPC cells. The results are presented as the means ± SD of three independent experiments, $^*P < 0.05$. IHNV, infectious haematopoietic necrosis virus; EPC, *epithelioma papulosum cyprini*.

for plaque assays to determine the viral titres. As shown in Fig. 4b,c, the addition of inhibitors of PKC or p38MAPK did not affect the growth of either the U- or M-type viruses at any of the inhibitor concentrations tested. However, 100 μM of the CKII inhibitor effectively blocked the

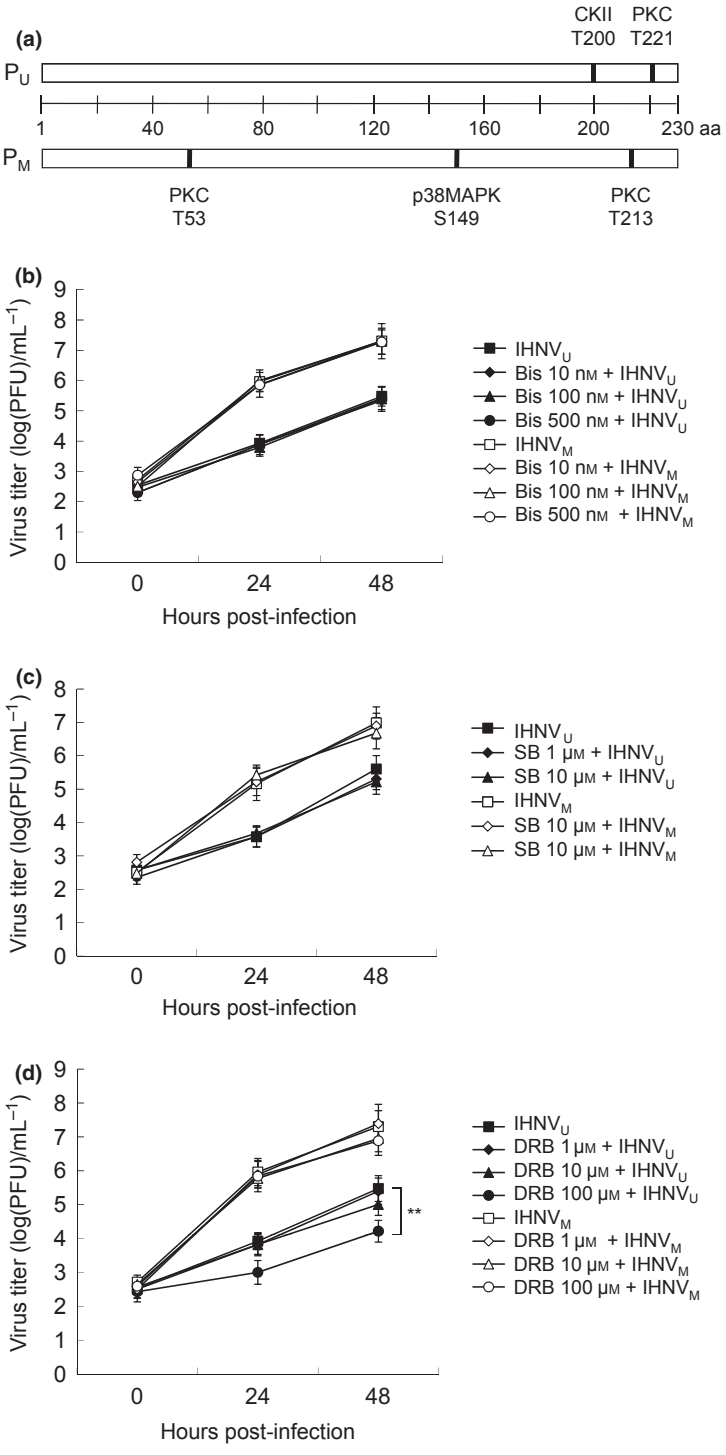

Figure 4 (a) Schematic representation of P genes showing five potential predicted phosphorylation sites, which are different between U- and M-type viruses. (b,c,d) Effects of inhibitors against the three host kinases PKC, p38MAPK, and casein kinase II (CKII) on the growth of U- and M-type IHNV strains. RTG-2 cells were pretreated with (b) PKC inhibitor (bisindolylmaleimide XI hydrochloride), (c) p38MAPK inhibitor (SB203580) or (d) CKII inhibitor (5,6-dichlorobenzimidazole riboside DRB) at the concentrations indicated for 1 h and then infected with IHNV at a multiplicity of infection of 1. The samples of the supernatant medium were collected at the indicated time points and titrated in duplicate by plaque assay on EPC cells. The results are presented as the means ± SD of three independent experiments, $^{**}P < 0.005$. IHNV, infectious haematopoietic necrosis virus; EPC, *epithelioma papulosum cyprini*.

cytopathic effect induced by U-type infection (data not shown) and reduced the titre of the U type 8.3- and 18-fold at 24 h and 48 h p.i., respectively (Fig. 4d). In contrast, 100 μM of CKII inhibitor reduced the titre of the M-type IHNV only 1.3- and 2.6-fold at 24 and 48 h p.i., respectively. The decrease in virus production caused by the CKII inhibitor was statistically significant for the U-type,

but not for the M-type virus. No cell death caused by the inhibitor treatment at the inhibitory concentrations was observed by counting trypan blue-stained cells (data not shown).

Discussion

One of the interesting features of IHNV is that IHNV isolates vary in virulence for different salmonid species (LaPatra *et al.* 1993); M genogroup viruses isolated from rainbow trout are highly virulent to rainbow trout, but U genogroup viruses isolated from sockeye salmon show low virulence to rainbow trout (Garver *et al.* 2006). We previously observed that while the M-type strain grows well in rainbow trout-derived RTG-2 cells, the growth of U-type IHNV is restricted in the same cells (Park *et al.* 2010). The purpose of this work was to investigate the basis of the difference in growth between U- and M-type IHNV in RTG-2 cells. Several factors suggested the hypothesis that the viral G protein may be responsible for this difference in growth: (1) IHNV genogroups are based on G nucleotide sequences (Kurath *et al.* 2003); (2) rhabdovirus G protein is responsible for the first steps of infection, attachment and fusion (Matlin, Reggio, Helenius & Simons 1982); (3) G protein plays an essential role in the pathogenesis of rhabdoviruses including rabies virus and viral haemorrhagic septicaemia virus (VHSV) (Dietzschold, Wunner, Wiktor, Lopes, Lafon, Smith & Koprowski 1983; Gaudin, de Kinkelin & Benmansour 1999; Ito, Takayama, Yamada, Sugiyama & Minamoto 2001); (4) G protein of the rhabdovirus VSV activates a specific antiviral TLR4-dependent pathway (Georgel, Jiang, Kunz, Janssen, Mols, Hoebe, Bahram, Oldstone & Beutler 2007); and (5) VHSV G induces type 1 interferon expression in neighbouring cells (Acosta, Collet, Lorenzen & Ellis 2006). In this study, to assess a role of G protein in the differential growth of U- and M-type strains, we generated recombinant IHNVs that expressed the G protein of representative U- or M-type viruses. Both rIHNVs grew equally well in RTG-2 cells, indicating that the growth difference between the U- and M-type IHNV in these cells was not a result of differences in G.

The non-structural proteins of RNA viruses have often been reported to be implicated in down-regulation of the host innate immune response (Garcia-Sastre & Biron 2006; Haller *et al.* 2006). Thoulouze, Bouguyon, Carpentier & Brémont (2004) previously reported that although an NV deletion mutant of rIHNV is able to replicate, the NV protein is essential for the efficient growth of IHNV in cell culture and also for pathogenicity in rainbow trout. Although the function of the IHNV NV protein is not clearly understood, it is possible that the NV proteins from the U- and M-type viruses possess different capabilities for down-regulation of the host immune system, leading to their different growth in RTG-2 cells. Here, we tested whether NV is responsible for the different growth between U- and M-type viruses in RTG-2 cells by using chimeric recombinant IHNVs that express NV of U-type or M-type within the same genomic background. However, the growth of these two recombinant viruses was not significantly different in RTG-2 cells, which means that NV does not play a key role in the difference in growth between U- and M-type IHNVs.

Type I IFNs are induced by viral infection (Garcia-Sastre & Biron 2006) and lead to the production of ISGs with antiviral activity (Biron & Sen 2001; Sadler & Williams 2008). Viruses, in turn, have evolved multiple strategies to block or evade the induction of interferon and also ISGs, which would otherwise suppress virus growth early in infection (Haller *et al.* 2006). Recently, IFN genes have been identified in a number of species of teleost fish including rainbow trout (Robertsen 2006). To determine whether antiviral activities induced by the IFN system can block the growth of the U- and M-type viruses differently, cells were treated with poly I:C, a dsRNA homologue that induces a strong IFN response in rainbow trout. Poly I:C pretreatment of cells 24 h before infection induced the expression of the IFN and Mx1 genes and blocked the growth of both U- and M-type viruses in RTG-2 cells. However, the inhibitory effects of poly I:C pretreatment on the growth of U- or M-type viruses were different; while the growth of U type was completely blocked by poly I:C pretreatment until 72 h p.i., the M type grew steadily, although more slowly, and reached a titre that was only 10-fold less than that of the untreated control infections at 72 h p.i. Thus, the antiviral activities induced by poly I:C blocked the growth of both U- and M-type viruses at the early stage of virus infection, but the U type was more severely restricted. This suggests that the M type, but not the U type, has some mechanisms to withstand the

antiviral activities induced by poly I:C and can continue to grow in IFN1-induced RTG-2 cells. This may be reflective of the higher titres reached by M-type virus in the absence of poly I:C stimulation, and/or it could be that the slower growth of the U-type virus allows the host more time to mount an effective antiviral response. After 72 h, the U type began to grow, presumably owing to waning of the strong poly I:C-induced IFN1 response. Eventually, the U type reached a titre equivalent to the M-type virus at 192 h p.i., probably due to extensive cell death in the M-type infections at that time as reported previously (Park *et al.* 2010).

It is possible that the different growth between U- and M-type viruses in RTG-2 cells results from different capabilities in blocking of the induction of IFNs and ISGs. If this is true, we would expect the M-type virus to more effectively block the IFN1 response, facilitating its higher growth. However, several lines of evidence showed that the growth restriction of the U type was not caused by induction of a higher level of IFN or ISGs: (1) M-type virus induced more up-regulation of IFN1 and Mx1 gene expression than U-type virus in RTG-2 cells; (2) M-type-infected cells produced a higher level of IFN1 activity in cell culture supernatant relative to U-type-infected cells, as indicated using RTG-P1 cells; (3) the PKR inhibitor did not exert any effects on the growth of either the U or M type. These results were somewhat unexpected, as IFNs, Mx and PKR are well known to have antiviral activities and suppress the growth of viruses, including the fish rhabdovirus VHSV (Haller *et al.* 2006; Tafalla, Sanchez, Lorenzen, DeWitte-Orr & Bols 2008). Our data revealed that poly I:C treatment before virus infection induced a high level of IFN1 and Mx1 and limited production of both U- and M-type viruses. However, when cells were treated with poly I:C after virus infection, there was no effect on the growth of either of the U-or M-type strains. Given that IFN1 is usually induced after virus infection, any effect of the poly I:C treatment post-infection would have been in addition to the IFN1 induced by the viruses themselves. Nevertheless, conventional antiviral activity induced by IFN1 does not appear to be a major factor responsible for the difference in growth between U- and M-type viruses in RTG-2 cells.

What then is the factor responsible for the different growth of the U- and M-type IHNVs in RTG-2 cells? Previously, we reported that expression levels of viral mRNA and genome RNA of the U-type virus were significantly reduced compared with those of M type in RTG-2 cells (Park *et al.* 2010). Until now, regulation mechanisms of IHNV RNA synthesis have not been well studied. However, work with the mammalian rhabdovirus VSV revealed that viral RNA synthesis is carried out by two RNA polymerase complexes; a complex of the viral L and P proteins for transcription and a complex of viral L, P and N proteins for replication (Gupta, Shaji & Banerjee 2003; Qanungo *et al.* 2004). Both viral transcription and replication are affected by phosphorylation of viral P protein by host kinases (Barik & Banerjee 1992; Das & Pattnaik 2004; Sun *et al.* 2008). If P proteins of the U and M genogroups differ in phosphorylation sites and, thus, require different protein kinases for their activation, the growth of U and M strains could be controlled by specific protein kinases of host cells. Using the NetPhosK server (Blom *et al.* 2004), we predicted differences in five kinase-specific phosphorylation sites in the P proteins of the U- and M-type IHNV strains; three PKC, one CKII and one p38MAPK phosphorylation sites. To determine whether any of these protein kinases are important for the differential growth of U- and M-type viruses, RTG-2 cells were pretreated with specific inhibitors against these protein kinases and viral growth was analysed. It is not certain whether protein kinase inhibitors used in this study are effective against kinases of RTG-2 cells. However, several reports have confirmed the inhibitory effects of protein kinase inhibitors, including the p38MAPK inhibitor used here, in trout and salmon (Lacroix & Hontela 2001; Ebner, Fiechtner, Pelster & Krumschnabel 2006; Hansen & Jørgensen 2007). This suggests that protein kinase inhibitors are likely to inhibit enzymes in RTG-2 cells. We found that the PKC and p38MAPK inhibitors did not exert effects on the growth of either the U or M types, but the CKII inhibitor specifically blocked the growth of the U-type virus. Although we did not determine the phosphorylation of P protein after treatment of inhibitors, the specific block of growth of the U-type suggests that it requires CKII activity for its growth in RTG-2 cells, but the M-type may use a different protein kinase than CKII. We therefore hypothesize that P proteins of U- and M-type IHNV are phosphorylated by different host protein kinases that have different activities in virus-infected RTG-2 cells. This could cause a difference in RNA polymerase activities and result in different growth between U- and M-types.

If this is the case, the observation of equal growth of U- and M-types IHNV in EPC cells (Park *et al.* 2010) would suggest that the U-virus P protein is able to interact with an EPC cell kinase that is either not present in RTG-2 cells or the RTG-2 kinase is sufficiently different from the EPC cell kinase such that it cannot recognize the U-type virus P protein.

In conclusion, our results using recombinant viruses to assess roles of individual viral proteins showed that the G and NV proteins were not responsible for the growth difference between U- and M-type strains in RTG-2 cells. Studies of host innate immune responses showed that even though the growth of both U- and M-type IHNV was suppressed by antiviral activities induced by poly I:C, the U-type virus restriction was not caused by the induction of higher levels of IFN1 or ISGs, and the IFN1 system did not appear to be a major determinant of the growth difference. Finally, kinase inhibitor studies showed that a CKII kinase inhibitor significantly reduced the growth of U-type, but not M-type virus, in RTG-2 cells. These data suggest that host protein kinase is required for IHNV replication, possibly for viral RNA synthesis. We hypothesize that during the adaptation to rainbow trout as a new host, the M genogroup viruses developed a requirement for a different host protein kinase(s) to achieve optimal growth, and differences in host protein kinase requirements are at least partially responsible for the differential growth of U- and M-type IHNV in RTG-2 cells. At present, it is not known whether the CKII inhibitor blocks the growth of U-type through modulation of viral RNA synthesis and whether CKII activity is IFN1 dependent or not. These possibilities will be examined in future studies.

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