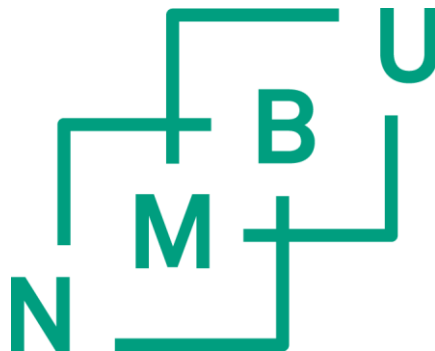# The interplay between IPNV and host cell machinery: Possible strategies for immune evasion

Philosophiae Doctor (PhD) Thesis

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LIST OF ABBREVIATIONS

AGK   - Asian grouper strain K cells

Aim2   - absent in melanoma 2

ALRs   - Aim2 like receptors

ASC   - apoptosis associated speck-like protein containing a CARD

ATP   - adenosine tri-phosphate

CARD   - caspase activation and recruitment domain

CPE   - cytopathogenic effect.

DAI   - DNA-dependent activator of IRFs

DISC   - death inducing signaling complex

DNA   - deoxyribonucleic acid

dsDNA   - double stranded DNA

eIF   - eukaryotic initiation factor

ER   - endoplasmic reticulum

FADD   - Fas/Apo-1 associated death domain protein

GCN2   - general control nondepressible 2

HRI   - hem-regulated inhibitor

IFN   - interferon

IkB   - inhibitor of NFκB

IKK   - IkB kinase

IL-1   - interleukin-1

IPN   - Infectious pancreatic necrosis

IPS-1   - IFNB-promoter stimulator 1

IRAK   - IL-1 receptor associated kinase

IRF   - interferon regulatory factor

JNK   - c-JUN N‑terminal kinase

LGP2   - laboratory of genetic and physiology 2

LRR   - leucine rich repeats

MAPK   - mitogen-activated protein kinase

MAPKK   - MAPK kinase;

MDA5   - melanoma differentiation-associated gene 5

MOI   - multiplicity of infection

MyD88   - myeloid differentiation factor 88

Mx   - myxovirus resistance protein.

NFκB   - Nuclear factor-κB

NLR   - Nod-like receptor, nucleotide-binding domain LRR-containing family

NOD   - nucleotide-binding oligomerization domain

OAS   - oligo adenylate synthase

ORF   - open reading frame

PACT   - PKR activating protein

PAMP   - pathogen associated molecular pattern

PERK   - PKR-like endoplasmic reticulum kinase

PKR   - dsRNA activated protein kinase R

PI   - propidium iodide

Poly I:C   - polyinosinic-polycytidylic acid

PRR   - pattern recognition receptor

PS   - phosphatidylserines

RD   - repressor domain

RdRp   - RNA-dependent RNA polymerase

RIG-I   - retinoic acid inducible gene I

RIP   - receptor-interacting protein

RLR   - RIG-1 like receptor

RNA   - ribonucleic acid

RTG   - rainbow trout gonads cells

ssRNA   - single stranded RNA

TAB1/TAB2   - Tak binding proteins

TAK1   - TGF-b activated kinase

TBK   - TANK-binding kinase

TCID   - tissue culture infective dose

TIR   - Toll/IL-1 receptor

TLR   - Toll like receptor

TIRAP   - TIR domain-containing adaptor protein, a.k.a. MAL (MyD88 adaptor like)

TNF   - tumor necrosis factor

TRAF   - TNF-receptor-associated factor

TRAM   - TRIF-related adaptor molecule

TRIF   - TIR-domain-containing adaptor protein inducing IFNβ

uORF   - upstream ORF

UPR   - unfolded protein response

VP   -virus protein

ZBP   - Z-DNA binding protein

SUMMARY

The aquatic birnaviruses cause diseases in different aquatic species worldwide. Infectious pancreatic necrosis (IPN), caused by IPN virus (IPNV) in salmonids, has arguably been the most economically important disease caused by these viruses. Although the number of cases and subsequently the impact of the disease has been reduced in recent years, concomitant with the use of genetically resistant fish, the ubiquitous nature of the virus and the lack of knowledge about reservoirs and interspecies transmission paves the way for disease reemergence in future. Understanding the virus interaction with host cell and the interplay with the immune system is crucial for development of viral therapies. Information about IPNV interaction with the host cells and immune system has been either lacking or inconsistent.

In this thesis, we have attempted to understand some aspects of virus interaction with the host cell with the aim to unravel possible mechanisms used for immune evasion or subversion. Three studies that were conducted *in vitro* are reported. First, in paper I, we compared responses induced by different IPNV isolates. The study revealed that infection with different strains of IPNV results in different cytopathogenicity and cytokine profiles. Notably, The Sp serotype of IPNV had a higher capacity to suppress the expression of type I IFN and to inhibit protein synthesis. Consequently, in paper II and III, we focused our studies on a virulent Sp isolate. We selected common responses induced by all the isolates, namely inhibition of protein synthesis and apoptosis, and investigated the underlying mechanism and their impact on IPNV replication. The study reported in paper II was directed at understanding the roles these responses play during IPNV infection and their effect on virus replication. The findings indicate that inhibition of protein synthesis might be used to prevent the production of type I IFN and its antiviral effectors such as Mx. In addition, the mechanisms by which protein synthesis is inhibited was investigated partly paper II and III, and the involvement of eIF2α and PKR was demonstrated. However, paper III was mainly performed to address the interplay between IPNV and PKR using chemical inhibition. The data reported in this study shows that PKR activation is beneficial to the virus. Although, the detailed mechanisms involved could not be elucidated we speculate and provide suggestion about the possible underlying mechanisms.

Collectively, the findings reported in this thesis provide new insights into IPNV interaction with host cells and suggest that IPNV is able to hijack some of the crucial antiviral responses and use it to evade type I IFN responses as well as to increase virus replication and facilitate its release from infected cells.

SUMMARY IN NORWEGIAN

Birnavirus er årsaken til sykdommer i forskjellige akvatiske arter over hele verden. Infeksiøs pankreasnekrose (IPN), forårsaket av IPN viruset (IPNV) hos laksefisk, har uten tvil vært den økonomisk mest viktige sykdommen relatert til disse virusene. Selv om antall sykdomstilfeller og betydningen av dem har blitt redusert i de siste årene, samtidig som genetisk motstandsdyktig fisk er tatt i bruk, så vil virusets utbredte natur kombinert med mangel på kunnskap om reservoarer og overføring mellom arter bane vei for reintroduksjon av sykdommen i fremtiden. Forståelse av virusets interaksjon med vertscellen og samspillet med immunsystemet er viktig for utvikling av virale behandlingsmetoder. Informasjon om IPNV sitt samspill med vertsceller og immunforsvaret har enten vært mangelfull eller inkonsistent.

I denne oppgaven har vi forsøkt å forstå noen aspekter ved samspillet mellom virus og vertscelle med sikte på å avdekke mulige mekanismer viruset bruker for å unngå en immunreaksjon. Tre *in vitro*-baserte studier beskrives. I første artikkel (Paper I), sammenlignet vi immunresponser indusert av forskjellige akvatiske birnavirus-isolater. Studien viste at IPNV infeksjon resulterer i ulike cytopatogenitets- og cytokin-profiler. Særlig IPNV serotype Sp hadde en høyere kapasitet til å dempe ekspresjon av type I IFN og hemme proteinsyntese. Som en følge av dette, fokuserte vi våre studier på et virulent Sp isolat i de påfølgende artiklene (Paper II og III). Vi valgte vanlige responser indusert av alle isolater, dvs. hemming av proteinsyntesen og apoptose, og undersøkte underliggende mekanismer og deres innvirkning på IPNV replikering. Studien beskrevet i artikkel II var rettet mot å forstå responsenes roller i IPNV infeksjoner og deres effekt på virusreplikasjon. Funnene indikerer at inhibering av proteinsyntese kan bli brukt til å forhindre produksjon av type I IFN og dets antivirale effektorer som for eksempel Mx. I tillegg ble de mekanismene som fører til hemming av proteinsyntesen delvis undersøkt i artikkel II og III, hvor involvering av eIF2α og PKR ble demonstrert. Artikkel III var imidlertid hovedsakelig utført for å undersøke samspillet mellom IPNV og PKR ved bruk av kjemisk hemming. Dataene beskrevet i denne studien viser at PKR aktivering er gunstig for viruset. De detaljerte mekanismene som er involvert kunne ikke beskrives, men vi spekulerer og gi forslag om mulige underliggende mekanismer.

Samlet gir resultatene som er beskrevet i denne oppgaven ny innsikt i IPNV sitt samspill med vertscellene og indikerer at IPNV er i stand til å kapre noen av de viktige antivirale

responsene og bruke dette til å unngå type I IFN responser, samt til å øke virusreplikasjon og lette

frigivelsen fra infiserte celler.

LIST OF PAPERS

Paper I:

Gamil AAA, Evensen Ø, Mutoloki S. **Infection profiles of selected aquabirnavirus isolates in CHSE cells.**

PLoS One. 2015 Aug 11;10(8):e0134173. doi: 10.1371/journal.pone.0134173. eCollection 2015.

Paper II:

Gamil AAA, Mutoloki S, Evensen Ø. **A piscine birnavirus induces inhibition of protein synthesis in CHSE-214 cells primarily through the induction of eIF2α phosphorylation.**

Viruses. 2015 Apr 15;7(4):1987-2005. doi: 10.3390/v7041987.

Paper III:

Gamil AAA, Xu C, Mutoloki S, Evensen Ø. **PKR activation favors infectious pancreatic necrosis virus replication in infected cells**

Submitted: Viruses.

INTRODUCTION

The innate immune system and virus invasion:

The innate immune system plays an indispensable role in defending the body against virus invasions. At the early stages of virus infections, innate immune sensors recognize the virus antigens or nucleic acid and alert the body against the invasion. Pathogen recognition by these sensors additionally induces different signaling cascade that generate important innate antiviral responses. These responses are crucial to control the virus infection and limit the spread within the organism (1). In addition, the innate immune system also participates in generating specific antiviral adaptive responses (2, 3).

Recognition of virus infection:
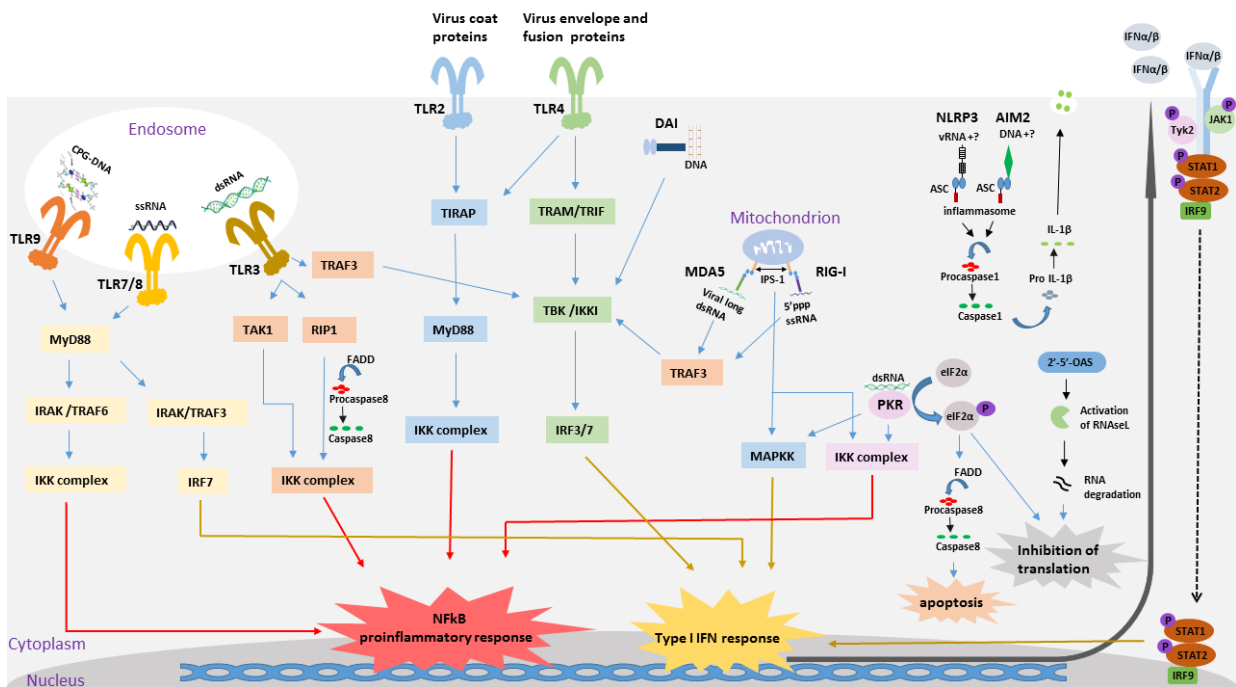
Figure1. Sensors of virus infection. TLRs localized at the cell surface (TLR2 and TLR4) senses surface virus structures such as coat proteins while those localized in the endosome (TLR 3, 8/7 and 9) senses virus genome. Virus nucleic acid present in the cytoplasm is sensed by different cytoplasmic sensors including RLRs, NLRs, ALRs, PKR, DAI, and 2'-5'OAS. Signaling from TLRs signal through the adaptor proteins MyD88 (TLR2, TLR4, TLR7/8, and TLR9) and TRIF (TLR3 and TLR4) while IPS-1 mediates the signaling through Rig-I and MDA5. Responses generated by the different signaling cascades include type I IFN responses, NFκB mediated proinflammatory responses, inhibition of protein synthesis and apoptosis. The produced IFN in turn binds its receptor on the cell surface and induce signaling cascade through JAK/STAT pathway leading to the transcription of several antiviral genes. Made by the author.

A diverse set of sensors are involved in the recognition of virus pathogens. The majority of these sensors sense virus nucleic acids although some detect other virus structures such as virus coat and enveloped proteins (Fig. 1). Most of the sensors are classified under groups of receptors that recognize conserved pathogen associated molecular patterns (PAMPs), thus called pattern recognition receptors (PRR), while the few remaining are considered part of the type I interferon (IFN) induced responses (4). Each of the sensors has specific signaling cascades that generate responses that are pertinent to the nature of the invading pathogen. Figure 1 summarizes the different viral PAMPs as well as the corresponding sensors and their involvement in generating different antiviral responses.

Toll like receptors (TLRs):

TLRs are the most extensively studied PRR. Since the identification of their critical role in antifungal responses in fruit fly (5), they have been characterized in several mammalian and non-mammalian species (6, 7). They are type I membrane glycoproteins consisting of extracellular leucine rich repeats (LRRs), and cytoplasmic Toll/Interleukin-1 receptor (TIR) domain (8, 9). TLRs recognize only PAMPs presented at the cell surface or within the endosomal compartment. Of the different types of TLRs, TLR2, 3, 4, 7/8, and 9 are the ones identified to mediate the antiviral responses in mice (4). The viral PAMPs recognized by each of the receptor are shown in Fig. 1. Recognition of the viral PAMPs triggers intracellular signaling cascades that ultimately lead to generation of the antiviral responses. Except for TLR3, all the above-mentioned receptors signal through MyD88-dependent signaling pathway that ultimately leads to NFκB activation and induction of inflammatory responses. TLR3 can also induce NFκB mediated inflammatory responses but through a MyD88-independent pathway. TLR 7/8 and 9 use MyD88 dependent signaling pathways to activate IRF7 and consequently induce type I IFN responses whereas TLR 3 and 4 use a MyD88-independent pathway which induce Type I IFN responses through either IRF3 or IRF7 (10).

TLRs in fish:

Fish TLRs share the main structural features and the general signaling molecules/domains with their mammalian counterparts (11). However, they also have distinct features. Several non-mammalian TLRs have been identified in different fish species while some of the mammalian TLRs, such as TLR4 (with few exceptions), 6, and10 are lacking (12). With regard to sensing

virus infections, the indications are that the roles of TLR3, 7, 8, and 9 in sensing and recognizing viral PAMPs, as well as the downstream signalling induced are conserved (11). In addition, some of the fish specific TLRs were also shown to be involved in sensing viral PAMPs. For example, it was shown that a teleost specific TLR, termed TLR21/22, mediates the induction of type I IFN production following stimulation with polyI:C (13), suggesting differences in the PRR repertoires between fish and mammals.

The cytoplasmic sensors:

RIG-I-like receptors (RLRs):

These are a group of cytoplasmic RNA helicases that consist of three members: retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (14). All the three have DExD/H-box RNA helicase domain with ATPase activity (15). RIG-I and MDA5 have additionally two N-terminal caspase activation and recruitment domains (CARDs) which are crucial for signaling (15). The C-terminus of RIG-I and LGP2 contains a repressor domain (RD) that keeps the molecules in an inactive forms (16). RIG-I recognizes the 5'triphosphate (5'ppp) RNAs while MDA5 is believed to recognizes long dsRNAs (17, 18). LGP2 is thought to function as a negative regulator of RIG-I and MDA5 by sequestering the RNA (19) but the recent finding that it can potentiate IFN production during virus infection (20) indicates the need for more studies to understand its true function. Following RNA binding RIGI-I and MDA5 interact with the mitochondria-associated adaptor protein IPS-1 (also known as MAVS, VISA and Cardif) through their CARD domain and trigger signaling cascade that ultimately lead to phosphorylation and activation of the transcription factors IRF3 and IRF7 and the induction of type I IFN responses. Alternatively, activation of MAPKs p38 and JNK can also be involved in inducing type I IFN responses (21). IPS-1 can also activate NFκB through FADD and Caspase 8 or Caspase10 (22).

dsRNA activated protein kinase R (PKR).

PKR is a sensor of virus dsRNA that is produced during virus replication. Nevertheless, it also plays other physiological and developmental roles (23). PKR is constitutively expressed in almost all cells but its expression is upregulated by type I IFNs (24). Structural wise, PKR consists of an amino-terminal regulatory domain containing the dsRNA binding motif (dsRBM)

and carboxy-terminal catalytic domain containing eleven kinase subdomains (25, 26). Recognition and binding of the dsRNA to its binding motif in PKR triggers a series of autophosphorylation events that ultimately lead to PKR activation (27). In addition to dsRNA, PKR can also be activated by TLRs, several cytokines and growth factors as well as by the PKR-associated activator (PACT) during stress responses (23). Activation of PKR triggers a signaling cascade that can ultimately lead to apoptosis, type I IFN production and induction of NFκB mediated proinflammatory response (23). The classical and probably most important function of activated PKR is inhibition of global protein synthesis through phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2α) (27) leading to inhibition or block of virus replication. The detailed mechanisms by which PKR induces inhibition of protein synthesis will be discussed later.

DNA-dependent activator of IFN regulatory factor (DAI):

DAI, previously named DLM-1 and also Z-DNA binding protein (ZBP), is an IFN inducible gene that encodes a protein that recognize and bind to B-form DNA. Signaling through DAI results in induction of IFN response through IRF3 pathway (28).

Nucleotide-binding domain LRR containing receptors (NLRs):

NLRs are cytosolic sensors for bacterial and virus nucleic acid. They consist of three domains: C-terminal LRR domain responsible for ligand binding, central nucleotide-binding oligomerization domain (NACHT or NOD) which facilitate self-oligomerization and activation and N-terminal effector domain (29). NLRs have been divided into 5 subfamilies based on the type of effector domains: NLRAs with acidic activation domain, NLRB, with baculovirus IAP repeat (BIR) domain, NLRCs with CARD domain, NLRPs (also NALPs and NLRs) with Pyrin domain and NLRXs with effector domain that have no strong homology to any of the other subfamilies (30). Once activated, most of the NLRs assemble into large multimeric protein complexes termed 'inflammasomes'. The inflammasomes contribute to the processing and release of pro-inflammatory cytokines such as IL-1β and IL-18 as well as pyroptosis (31, 32). This requires interaction between the inflammasomes and procaspase-1 that is facilitated by the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC) (31, 33). Some of the inflammasomes appear to be involved in sensing and combating infections with some RNA and DNA viruses (33, 34). In addition, some of the NLRs, NOD2 for example, can

modulate innate and adaptive responses through inflammasome-independent mechanisms. These mechanisms involve translocation to the plasma membrane and interaction with RIP2 (receptor-interacting protein 2) that subsequently trigger the activation of NFκB and p38 MAPK-dependent signaling pathway (35).

Absent in melanoma 2 (AIM2)-like receptors (ALRs):

They are structurally similar to NLRPs except they have HIN 200 domain instead of the NACHT domain. Their mode of action and signaling is also similar to NLRs (33). Unlike NLRs, however, the ligand of ALRs has been identified as the dsDNA and direct interaction between dsDNA and the HIN 200 domain have been demonstrated (36). The family contains two members AIM2 and IFI16. AIM2 is a cytosolic dsDNA sensor while IFI16 senses the foreign dsDNA in the nucleus (33).

Cytoplasmic sensors of virus infection in fish:

Orthologues of the three members of the RLR system are present in fish. Interestingly, however, RIG I orthologues has so far only been identified in certain species, namely salmonids and cyprinids, while LPG2 and MDA5 were identified in all the species tested (37). Fish RLRs appear to play similar roles during virus infections as in higher vertebrates (38). Similarly, fish possess orthologues that are structurally similar to many of the mammalian NLRs in addition to a unique group that is only present in fish (37, 39). Limited functional data are available but the upregulation of NLRs genes during virus infection or after polyI:C stimulation suggest a conserved role during virus infections (40, 41). PKR homologs has also been identified in several fish species (42). In contrast, neither DAI nor ALRs orthologues have been identified in fish to date.

Innate responses against virus infection:

Inhibition of protein synthesis:

The ultimate aim of virus infections is to use the cell machinery to produce their progeny. One of the important defense mechanism used by cells therefore is inhibiting global protein synthesis in order to prevent virus protein synthesis and formation of progeny. There are two main mechanisms by which global inhibition of protein synthesis occurs:

2',5' oligoadenylate synthase (OAS)/RNaseL system:

The OAS/RNAseL system is one of the antiviral responses upregulated by type I IFN system and play crucial role in blocking virus replication. This pathway is activated when the OAS encounters and recognizes viral dsRNA produced during virus replication (43). An active form of the OAS is then produced which in turn activates the inactive RNAseL monomer to produce an active dimer with potent RNAse activity (44-47). As a result, viral and cellular RNAs are degraded and protein synthesis is consequently inhibited (44, 48). Depending on the RNA substrate, cleaving of the RNA can also help to induce Type I IFN responses or eliminate infected cells by apoptosis (49, 50).

Inhibition of translation:

Cap-dependent translational initiation is the major mechanism for the translation of the vast majority of eukaryotic mRNAs. The process starts with the recognition and binding of the cap structure by the eIF4F cap-binding complex (composed of eIF4A, eIF4E, and eIF4G) through interaction with eIF4E, while eIF4G is a scaffolding protein that binds eIF4E, eIF4A and the mRNA (51). Meanwhile the 80S ribosomes dissociate into 60S and 40S ribosomal subunits and the latter binds the ternary complex consisting of eIF2, a hetero-trimer of α, β and γ (or a, b, and g) subunits, methionyl-initiator tRNA (Met-tRNAi) and GTP to form the 43S pre-initiation complex (52). The assembled 43S pre-initiation complex then binds the mRNA at the cap structure and scans along the mRNA until an AUG start codon is encountered (53, 54). Protein synthesis commences when the 60S ribosomal subunit joins the 40S subunit, mediated by initiation factor eIF5B and GTP hydrolysis, with release of eIF2–GDP (55). To secure rounds of initiation, GDP on eIF2 is recycled to GTP under the regulation of the GTP exchange factor eIF2B (56). Figure 2 details the different steps and molecules involved in initiation of translation in eukaryotes.
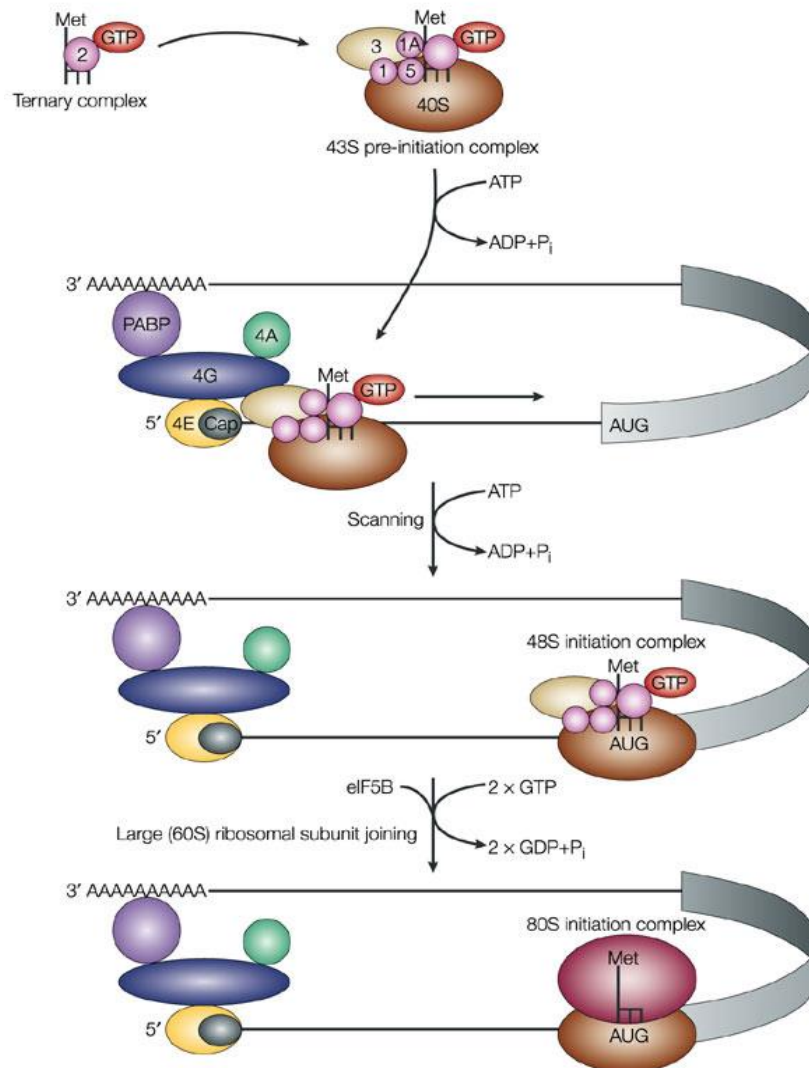
Figure 2. Cap-mediated translation initiation. Eukaryotic initiation factors (eIFs) are depicted as coloured, numbered shapes in the figure. The methionine-loaded initiator tRNA (L-shaped symbol) binds to GTP-coupled eIF2, to yield the ternary complex. This complex then binds to the small (40S) ribosomal subunit, eIF3 and other initiation factors to form the 43S pre-initiation complex. The pre-initiation complex recognizes the mRNA by the binding of eIF3 to the eIF4G subunit of the cap-binding complex. In addition to eIF4G, the cap-binding complex contains eIF4E, which directly binds to the cap, and eIF4A, an RNA helicase that unwinds secondary structure during the subsequent step of scanning. eIF4G also contacts the poly(A)-binding protein (PABP) and this interaction is thought to circularize the mRNA. The 43S pre-initiation complex scans the mRNA in a 5'→3' direction until it identifies the initiator codon AUG. Scanning is assisted by the factors eIF1 and eIF1A. Stable binding of the 43S pre-initiation complex to the AUG codon yields the 48S initiation complex. Subsequent joining of the large (60S) ribosomal subunit results in the formation of the 80S initiation complex. Both AUG recognition and joining of the large ribosomal subunit trigger GTP hydrolysis on eIF2 and eIF5B, respectively. Subsequently, the 80S complex is competent to catalyze the formation of the first peptide bond. P_i, inorganic phosphate. Reprinted with permission from (57).

The primary mechanism by which translation initiation is regulated in eukaryotes is the phosphorylation of Ser (S) residues in the α-subunit of eIF2. When eIF2α is phosphorylated the exchange of GDP for GTP by eIF2B is inhibited and protein translation is attenuated because eIF2α-GDP becomes a competitive inhibitor of eIF2B, as eIF2B has a significantly higher affinity to phosphorylated than unphosphorylated eIF2α–GDP (58). Regulation of translational initiation via phosphorylation of eIF2α by different kinases occurs under various conditions. Four different kinases have been identified so far: the haem-regulated inhibitor (HRI) is induced by haem depletion; general control non-depressible 2 (GCN2) is mainly activated by amino acid starvation; the double-stranded RNA activated protein kinase (PKR) is stimulated in response to viral infection; PKR-like endoplasmic reticulum kinase (PERK) is activated during endoplasmatic reticulum (ER) stress and the unfolded protein response (UPR) which can also be initiated due to virus infections (59).

Apoptosis:

Apoptosis or programmed cell death is a physiological process that play important roles during development and can also be induced in response to cell injuries as well as virus infections. Apoptotic cells exhibit distinct morphological features including shrinking, plasma membrane blebbing, chromatin condensation and DNA fragmentation (60). These features are used to distinguish apoptosis from necrosis, the second form of cell death. It is noteworthy that the differences between the two death pathways are not always distinct and consequently a new form of cell death named necroptosis has recently emerged (61). Apoptosis can be induced via two pathways, the mitochondrial and cell death receptor pathways, and in both cases it is executed by a family of cysteine proteases called Caspases. The mitochondrial pathway is initiated due to changes in mitochondrial membrane permeability in response to different apoptotic signals. Members of the Bcl-2 protein family are important regulators of this pathway with some of the members such as Bcl-2 acting to promote cell survival while others such as Bax are proapoptotic (62). The death receptor pathway, on the other hand is initiated through type I transmembrane receptors belonging to the tumor necrosis factor (TNF) receptor gene family. Initiation of this pathway requires the interaction between the death receptors and their ligands resulting in the recruitment of the adaptor molecule fas-associated protein with death domain (FADD), and Caspase 8 to form a death inducing signaling complex (DISC) and subsequently activation of the

apoptotic process (62). During virus infections, apoptosis is induced as an innate defense mechanism to contain the infection and prevent virus spread (63).

Interferon responses:

Type I interferon (IFNα/β) responses are among the most important and probably the most studied innate antiviral responses. Their initiation requires stimulation of IFNα/β production that can be achieved through different pathways (64). IFNα/β then binds its receptor on the cell surface and triggers signaling cascades through the JAK/STAT pathway (Fig. 1). Consequently, the transcription of several antiviral IFN stimulated genes (ISGs) is increased and an antiviral state is established. The myxovirus resistance protein (Mx), the dsRNA activated protein kinase R (PKR) and OAS are among the best studies IFN stimulated genes (65). PKR and OAS mediate responses that aim towards inhibiting production of new virus progenies at translational or mRNA levels respectively and as already described above. In addition to translational control, the antiviral roles of PKR includes induction of apoptosis, formation of stress granules (SGs) and IFN-induced cellular necrosis (66-68). The PKR-mediated apoptosis can be initiated via the death receptor, caspase 8 mediated or mitochondrial, caspase 9 mediated pathways; and involves both NFκB activation and eIF2α phosphorylation (66, 69). IFN-induced necrosis requires IFN as well as interaction between PKR and RIP1, to trigger necrosome formation, and is licensed by FADD and caspases (68) while the involvement of PKR in SG formation is not clear but it includes eIF2α phosphorylation (70). Mx protein, on the other hand inhibits virus replication at transcriptional or post transcriptional level (71) by physically interacting with virus vius proteins or cellular components essential for virus replication such as some cellular helicases (72-74).

Innate antiviral responses in fish:

The OAS detection system is yet to be identified in fish but many of the other innate antiviral components are present. Inhibition of protein synthesis during virus infections has been demonstrated (75). The involvement of eIF2α phosphorylation (75) in addition to the identification of PKR genes in several fish species (42) suggests similar underlying mechanisms of translational control during virus infections. Interferon genes have also been identified in several fish species. In contrast to the mammalian IFN genes, that do not contain introns, fish type I IFNs have 4 introns (76). Despite so, the biological functions were shown to be similar. For example, Atlantic salmon IFNa was shown to induce Mx expression and protect the cells

against virus induced CPE (75, 77). Based on the numbers of cysteine residues type I IFNs of cyprinids and salmonids have been classified into two subgroups, I and II containing 2 and 4 cysteine residues, respectively (76). The different type I IFNs of salmonids were shown to respond differently to poly I:C stimulation as well as to the virus infection (76, 77).

Virus pathogenicity and host responses:

Viruses must overcome the innate antiviral responses before they can establish a successful infection and induce pathology. To do that many viruses use "evade or invade" concept. Some viruses develop strategies that enable them to circumvent recognition by the innate antiviral sensors or specific antiviral responses generated after recognition (78). Others, on the other hand, target key components of the innate antiviral responses for disruption or modulation and possibly further use it to their own favor (78). A good example for this is the interplay between different viruses and the host translation machinery. Some viruses avoid recognition by PKR by either binding the dsRNA binding domain on PKR thereby competitively inhibiting dsRNA binding (79) or by encoding proteins that binds dsRNA, masking it from recognition by PKR (80). Physical interaction between virus proteins and PKR can also lead to inhibition of PKR activation (81). On the contrary, other viruses target certain components of the translation initiation machinery for disruption leading the disruption of cap-dependent protein translation and inhibition of protein synthesis (82). However, because they depends on their host's machinery to produce their own proteins, viruses that disrupt the cap-dependent translation employ alternative strategies that enables them to use only part of the translation machinery to produce their progeny (82, 83). The most common of these strategies is possessing an internal ribosome entry site (IRES) that directly interact with ribosomes and initiate translation in cap-independent manner (84). Interestingly, some viruses further benefits from the inhibition of protein synthesis resulting from the disruption of the translation machinery to block the production of IFN and other antiviral proteins (85, 86).

A second example is the interplay between viruses and type I IFN responses. The ability for the virus to downplay interferon responses, among other innate responses, might be crucial for establishing successful virus infection and pathology (87). This can be achieved by disrupting the recognition mechanisms and/or the signaling cascade that lead to IFN production, using inhibition of protein synthesis to prevent IFN production and type I IFN effectors, as discussed

above, blocking induction of the responses by targeting different aspects of the signaling cascade generated by binding of type I IFNs to their receptors or by targeting specific ISGs and inhibit their antiviral effect (88). On the contrary, some viruses uses IFN responses, and other immune responses, to induce pathology and help virus spread (89).

A third and final example is the interplay between viruses and the apoptotic process. Although employed by the cells as survival strategies as explained above, viruses can also manipulate the process to their advantage. Some viruses use apoptosis to be released from infected cells (90). However, they employ strategies that enables them to delay the apoptotic process in order to prevent premature cell death and maximize virus replication (91). In contrast, viruses that possess alternative release strategies block the apoptotic process to avoid the detrimental effect on virus replication (92). The strategies used to block or delay apoptosis are diverse and induced at different levels. It can be by 1) inhibiting responses that lead to apoptosis such as type I IFN and TNFα responses; 2) ability of virus products to inhibit caspases; or 3) possessing Bcl-2 homologs. A more detailed description of these mechanisms can be found elsewhere (91).

Aquatic birnaviruses:

Structure and genome organization:

The aquatic birnaviruses, as indicated by the name, are member of the family *Birnaviridae* and are classified under distinct genus called Aquabirnavirus. They are a non-enveloped, double stranded, icosahedral RNA virus that is about 60nm in size (93). The viral proteins are encoded by two genomic segments A and B (94). Segment A comprises two open reading frames (ORFs): the long one encoding a 107KDa polyprotein that is post translationally cleaved to produce the structural virus proteins VP2 and VP3 as well as the non-structural protein VP4 (95-98). The short ORF, on the other hand, overlaps the first longer ORF and encodes a non-structural protein VP5 that can only be detected in infected cell lysates but not in purified viruses (98, 99). In contrast to segment A, segment B consists of a single ORF that encodes the virus polymerase VP1 (100).
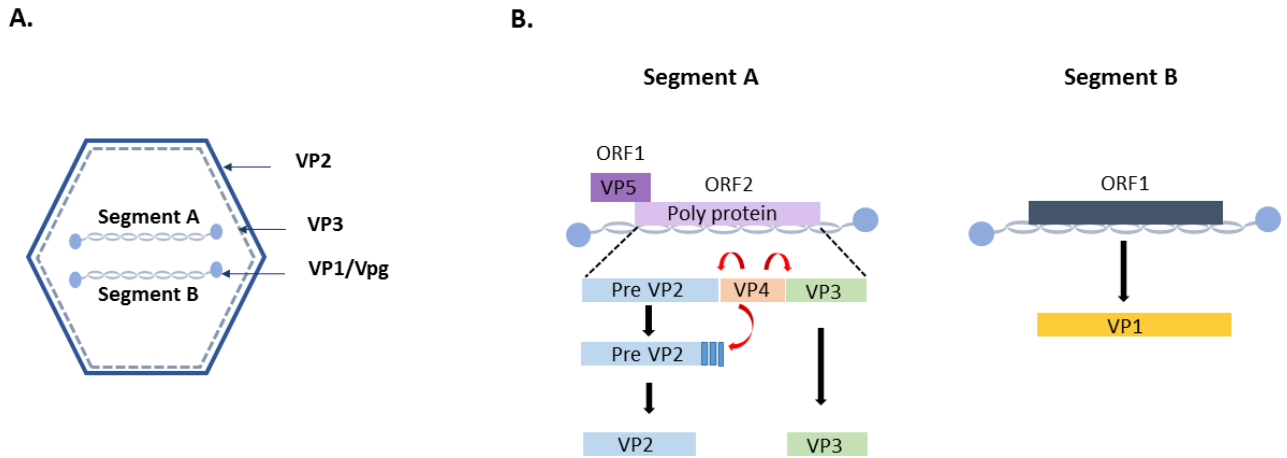
Figure 3. IPNV structure and genome organization. **A.** Schematic presentation of the virion structure. **B.** The genome organization of segment A and B, their encoded proteins and the cleavage sites in segment A are illustrated. Modified by the author from (101)

Diseases

Aquatic birnaviruses have been isolated from several aquatic vertebrate and invertebrate species that inhabit fresh, brackish and seawater (102). The type species of the genus is infectious pancreatic necrosis virus (IPNV) the causative agent of infectious pancreatic necrosis (IPN) in salmonid fish (101, 102). The disease has been described since the 1920s as "whirling sickness" or "octomitiasis" affecting different species of trout fingerlings (103). The first proper description of the disease was made in 1941 by M'Gonigle who accordingly changed the name to "acute catarrhal enteritis" (103). It was not until 1960, however, when the virus agent was isolated (104). To date, IPN is well known as a disease of salmonid fish and is probably the most economically important disease cause by the aquatic birnaviruses. The disease affects all age groups with different degrees of susceptibility (105, 106). Infections are associated with necrosis of the exocrine pancreas, catarrhal enteritis and multifocal hepatic necrosis (107). Mortalities range from negligible to almost 100% (108) and survivors become persistently infected and shed the virus into the environment (109-111). It is worth mentioning, however that impact of the disease on the salmonid industry, particularly in Norway, have decreased (Fig. 4) mainly due to the selective breeding of resistant fish (112).
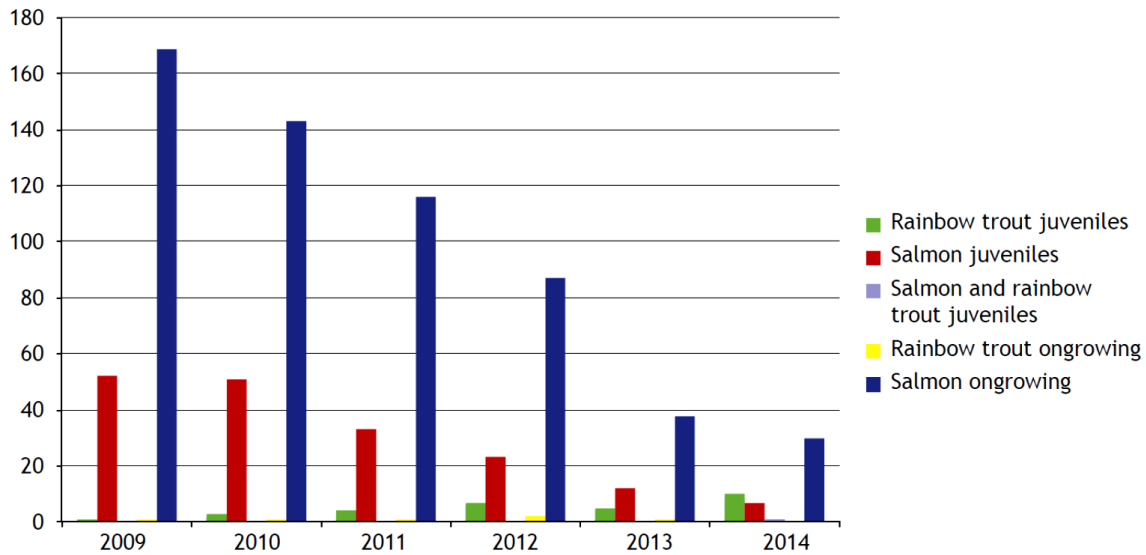
Figure 4. Number of sites with IPN, distribution in salmon and rainbow trout hatcheries and on growing sites 2009-2014. Rapid and steady decline in the sites reported with IPN in the recent year. Adapted from (113).

The clinical picture in other species is very different from that of IPN in salmonids and less characterized. In turbot (*Scopthalmus maximus*), the virus infection is associated with hematopoietic and renal necrosis (114). Infection with the virus is also associated with the spinning disease in Atlantic menhaden fish (*Brevoortia tryrranus*) (115) while characterized by ascites in yellowtail (*Seriola quinqueradiata*) (116). A disease characterized by ascites and cranial hemorrhage is also caused is by the virus in Japanese flounder (*Paralichthys olivaceus*) (117). In Japanese eel (*Anguilla japonica*), the disease is associated with branchionephritis (118). In addition, the virus has also been isolated from ulcerative lesions in different fish species (119) and from clams with darkened gills and gill necrosis (120). As already stated, the pathological conditions caused in other species not as well defined as IPN although more detailed description of some of these diseases and the lesions associated can be found elsewhere (102).

Nomenclature:

The wide host and geographical range has resulted in confusion regarding the nomenclature of the different isolates. It was suggested that isolates causing overt IPN disease in salmonids are the only one to be called IPNV while the rest of the isolates are to be called aquabirnaviruses (102, 121). In addition, the term marine aquabirnaviruses (MABV) was used to

describe viruses that have been isolated from non-salmonid aquatic vertebrate and invertebrate species particularly in Asia (122, 123). This group includes the Yellowtail Ascites Virus (YTAV), first isolated from yellowtail fingerlings in Japan (116) and Tellina virus (TV) (124) initially isolated from molluscs. It is noteworthy however that these nomenclatures has not been universally accepted as some investigators still describes isolates infecting non-salmonid species as IPNV (125). In this thesis I will follow the most recent classification provided by the International Committee on Taxonomy of Viruses (101) in which all the isolates, except YTAV and TV are referred to as IPNV.

Classification

Attempts to classify aquatic birnaviruses has started since the early 1980s. Initially, three reference serotypes, namely VR-299, Sp and Ab, were identified by cross-neutralization tests (126, 127). Later on, by using well standardized reciprocal cross neutralization approach and more than 200 isolates, Hill and Way have reclassified the viruses into two serogroups A and B (128). Serogroup A consists of nine serotypes: West Buxton (WB), Sp, Ab, He, Te, Canada (Can.) 1, Can. 2, Can. 3, and Jasper also designated A1– A9, respectively. With the exception of A4 and A5 serotypes, isolated from molluscs and pike respectively, all of the serotypes were initially isolated from either Europe or North America in association with IPN (128). Serogroup B, on the other hand, contains only one serotype TV-1 (B1) and less than 10 isolates from the UK (128). In addition to the serotypic classification, Blake and coworkers have recently classified the different isolates into six genogroups using nucleotide and deduced amino acid sequences of segment A or the entire VP2 gene (129). A good agreement between serological grouping and genogrouping was found, with serogroups A1 and A9 corresponds with genogroup 1; A3, A2 and A4 corresponding with genogroups 3, 5 and 6, respectively; A5 and A6 correspond with with genogroup 4, while serogroups A7 and A8 correspond with genogroup 2 (129). More recently, by sequencing 310bp fragment representing the VP2/NS region from 96 isolates, Nishizawa and coworkers reproduced a similar genotypic classification and further suggested a seventh genogroup containing Japanese strains isolated from marine fish and molluscan shellfish (130).

Pathogenicity and host responses to IPNV.

Although, IPNV is one of the best studied fish viruses with regards to host-virus interactions, many aspects of the virus infection, pathogenicity and interplay with the immune

system are still not known. For example, although virulence factors was mapped to certain amino acid positions, namely 217, 221 and 247, of the VP2 (108), the detail interaction between these motifs and cells is yet to be illustrated. Moreover, neither the mode of virus entry into the cells nor the mechanism of virus replication and translation of virus proteins are currently known.

Pathogenicity:

Virus-host interactions are better studied *in vivo*. Mortality rates during IPNV vary according to the temperature, stress, virus strain and host species (105, 114, 131, 132). Susceptibility also varies within species (132) and genetic QTLs responsible for disease resistance has been identified (133). As indicated above, three motifs in the VP2 region are determinants of virus virulence (108) for the Sp isolates but whether this apply to other isolates or not remained to be answered. The gills, skin and intestine are thought to be the ports of entry into the host while blood may play important role in virus dissemination (134). Within 2-3 days after IP injection of the virus, virus replication can be detected in the headkidney and virus titres as high as 10^{10} TCID$_{50}$ can be obtained from kidney tissues (135, 136). Detection of virus in the hepatic tissue can be considered a correlate of pathology (137).

Replication:

IPNV replicates in a variety of cell lines from teleost fishes at temperatures below 24°C (94). The low temperature requirement for replication is an intrinsic property of the virus (94). A single cycle of replication in RTG-2 cells took16 hours at 24°C and 24 hours at 15°C when cells were infected with 6.7 and 8.5 MOI respectively (138). The rate at which the virus replicates is dependent on different factors including:

a) Temperature: The optimal temperatures varies between the different strains and Temperatures higher than or below optimal will result in compromised replication, resulting in lower virus titres (138). The optimal growth temperature can also vary between the different cell lines (139).

b) MOI: Higher MOIs are usually associated with high replication rates resulting in higher end titres and more rapid CPE(138).

c) The ability of the cell line to produce IFN: It was previously shown that the virus yields obtained from RTG-2 cells that are known to produce high IFN and mount

strong IFN responses are >10 folds higher than those obtained from CHSE cells which is inferior in producing IFN and mounting IFN reponses (140).

As previously mentioned, the detailed mechanisms involved in virus entry and replication inside the cell are currently obscure. How the virus gains entry into the cells remained an unanswered question. Following entry, replication ssRNA intermediates that are associated with polysomes and that can also hybridize with the denatured virus genome are detected (141). These ssRNA intermediates resembles those of the reoviruses which was shown to function as mRNAs (142, 143). It was therefore suggested that the early events of IPNV replication is similar to reoviruses (94). For reoviruses, entry is followed by partial uncoating, which take place in the endosomes, to produce core-like particles and subsequently penetration to the cytoplasm where the replication occurs. The partial uncoating activates the virion transcrptases and production of viral mRNAs. These mRNAs are then used for transcription in order to produce the negative strand as well as to translate and initiate the synthesis of the virus progeny (144, 145). It is noteworthy that the transcription occurs inside the core-like particles (142) making the viral genome unexposed and helping the virus to escape recognition by the innate sensors . Thusfar, a similar events are thought to occur during early stages of IPNV replication. The finding that the virion-associated RdRp is active without any proteolytic pretreatment of the virus (146) suggests that uncoating is not necessary for IPNV replication and would further support the similarities between the early events of IPNV and reoviruses replication. Nevertheless, direct experimental evidence is needed to prove or disprove this assumption.

In contrast to the early replication events some information are available about the assembly and maturation. It is believed that immediately after dsRNA synthesis has been initiated, the newly synthesized dsRNA assembles into immature particles. Maturation of these particles occur in the cytoplasm with the involvement of VP4 leading to the production of new infectious virions (93). However, the detailed mechanisms of virus assembly and maturation are yet to be elucidated.

Virus interplay with immune responses:

There are indications, although in few cases, from field observations that the immune system can clear IPNV infection (102), but the underlying mechanisms remained obscure. This observation, however, highlights the important of understanding immune protective mechanisms for effective virus control. Between the two arms of the immune responses, the adaptive responses, particularly humoral responses, are better studied than the innate responses due to its direct connection to evaluation of vaccines. Anti IPNV antibodies has long been detected following IPNV infection (147). Recently, the protective roles of antibodies in vaccinated individuals has been demonstrated and it was shown that, when they are above a certain threshold, the antibody levels can be used as correlates of protection (137). In contrast, the cell mediated immune responses to IPNV are not well studied although it was recently shown that induction of the expression of genes associated with CD4 and CD8 T cells responses correlates with protection in vaccinated fish (148).

Many aspects of innate immune responses to IPNV are not known and basic information such as how is the virus recognized, how does it interact with cell translation machinery, what type of immune responses are crucial against the virus and how does the virus evade or subvert the immune responses are still lacking. Interferon responses and apoptosis are probably the best two studied innate responses to IPNV. *In vitro*, interferon pre-treatment of cells (149, 150) and overexpression of Mx protein (151, 152) result in inhibition of virus replication suggesting that type I IFN responses are important in controlling IPNV replication. However, at least some virus strains are able to replicate under high levels of interferon and Mx gene expression both *in vivo* and *in vitro* (137, 153, 154). The latter findings point towards IPNV being able to counteract type I IFN responses and is supported by reports showing that concentrations as high as 200U/ml of type I IFNs does not block but only able to reduce virus replication and prevent CPE (77). It appears that the virus suppresses type I IFN signaling with possible involvement of VP4 and VP5 (150, 155) but the detailed mechanisms are still unknown. It is worth noting that the interplay between IPNV and the type I IFN differs between cell lines obtained from different species (154, 155). Whether this reflect an interspecies variation or it is due to the different cell types constituting the different cell lines is not yet known. Induction of apoptosis during IPNV, on the other hand is not clear. Some investigator reported atypical apoptosis preceeding the necrotic changes (156) while others found that infected cells are mostly necrotic (157). Because these data

were obtained using different isolates as well as different methods, they are difficult to compare and further investigations are needed to better understand cell death following infection with different IPNV isolates. In addition to apoptosis and type I interferon responses, there has also been some attempts to understand the interaction with translation machinery by assessing protein synthesis that has also yielded different results (158-161). These studies have similar limitations as those of the apoptotic studies.

OBJECTIVES

The overall objective of the current study was to characterize the interaction between IPNV and host cell machinery with the aim to unravel possible mechanisms for immune evasion. The objective was sub-divided into the following sub-objectives:

- To characterize the differences between the pathogenic mechanisms of different IPNV isolates *in vitro* by comparing the profile of cellular responses induced by isolates belonging to different sero-/genogroups.
- To study and characterize the interplay between virus and the host's translation machinery.
- To unravel the mechanisms of cell death during IPNV infections.
- To investigate the role of PKR during IPNV infections.

SUMMARY OF THE PAPERS

Paper I:

Infection profiles of selected aquabirnavirus isolates in CHSE cells.

Gamil AAA, Evensen Ø, Mutoloki S.

IPNV has wide host range and geographical distribution. Consequently, different isolates with distinct genotypic and serotypic characteristics are present. The pathogenic mechanisms employed by the different isolates and their interaction with hot cells has not been studied in details. In this study we have compared the replication levels and responses induced by the different isolates in vitro using CHSE-214 cells. The isolates used were the Asian E1S isolates from the A3 serotype, the VR-299 from the A1 serotype and highly virulent (TA) as well as low virulent (PT) isolates from the A2 serotype. The data shows clear differences between the isolates. In term of replication the E1S isolate showed the highest ability to replicate and had the highest end titre followed by the PT, TA and finally the VR299. Interestingly, the least ability of VR299 to replicate was linked with high ability to induce type I IFN responses and inhibition of protein synthesis. The E1S isolate induced high type I IFN expression but the downstream responses was not induced. The most prominent finding for the two Sp strains was their low ability to induce type I IFN response combined with high ability to induce TNFα gene expression. Only the E1S was able to induce high apoptosis while the rest of the isolates failed to do so. These findings suggest that different pathogenic mechanisms are employed by the different isolates.

A piscine birnavirus induces inhibition of protein synthesis in CHSE-214 cells primarily through the induction of eIF2α phosphorylation.

Gamil AAA, Mutoloki S, Evensen Ø.

Induction of protein synthesis and the mechanisms of cell death employed by birnaviruses, including IPNV has not been known. In this study, we focused on understanding the mechanisms employed by a virulent strain of the Sp serotype of IPNV to inhibit protein synthesis and induce cell death as well as the impact of these responses on virus replication. We show that IPNV is able to inhibit protein synthesis but this inhibition has no impact on virus replication. The mechanism employed in inhibiting protein synthesis was found to be the translational arrest induced through phosphorylation of eIF2α. Using different methods, we found that necrosis is the predominant form of cell death induced by IPNV while apoptosis was only induced at the terminal stages. Although the expression of type I IFN was induced, no induction of Mx or PKR gene expression was observed. We therefore suggested that IPNV uses the inhibition of protein synthesis to evade type I IFN responses and block the production of the downsteam effectors.

PKR activation favors infectious pancreatic necrosis virus replication in infected cells

Gamil AAA, Xu C, Mutoloki S, Evensen Ø.

The role of PKR during IPNV infection has not been clear. In this study we used chemical inhibition of PKR, using the specific PKR inhibitor C16, as well custom-made antibodies to investigate the interplay between PKR and IPNV. The data presented shows that no PKR upregulation was detected in IPNV infected cells. Despite so, IPNV induced eIF2α phosphorylation was inhibited in cells treated with the PKR inhibitor. Furthermore, inhibition of PKR resulted in decreased IPNV titres and was also associated with decreased ability to induce necrosis. The decreased titres is presumably due to direct effect on virus replication since it was detected both intracellular and in the supernatant. Collectively, the data shows that PKR is the kinase responsible for eIF2α phosphorylation during IPNV infection and its activation benefits virus replication.

METHODOLOGY

All the data reported in this thesis were obtained from *in vitro* experiments. A well characterized recombinant and virulent Sp strain of IPNV carrying the amino acids $T_{217}A_{221}T_{247}$ of VP2 (162) was the main isolate used although other Sp and other serotypes/genotypes of aquabirnavirus isolates were also included based on the objectives of individual studies. Table 1 lists the isolates used in each of the studies and provide summarized information about the origin of each isolate.

Table 1. Summary of the isolates of IPNV used in the individual studies.

Isolate	Source	Temperature	Geno-group	Sero-group	Reference	Used in
E1S	Isolated from eel	20°C	3	A3	(163)	Paper I
VR-299	Isolated from rainbow trout	15°C	1	A1	(147)	Paper I
Avirulent Sp ($P_{217}T_{221}A_{247}$)*	Template used isolated from Atlantic salmon	15°C	5	A2	(108, 162)	Paper I
Virulent Sp ($T_{217}A_{221}T_{247}$)*	Template used isolated from Atlantic salmon	15°C	5	A2	(108, 162)	Paper I, II and III

* Amino acid position in the VP2 protein of IPNV.

More than one method or cell line was used in the different studies and these are summarized in Figure 5.
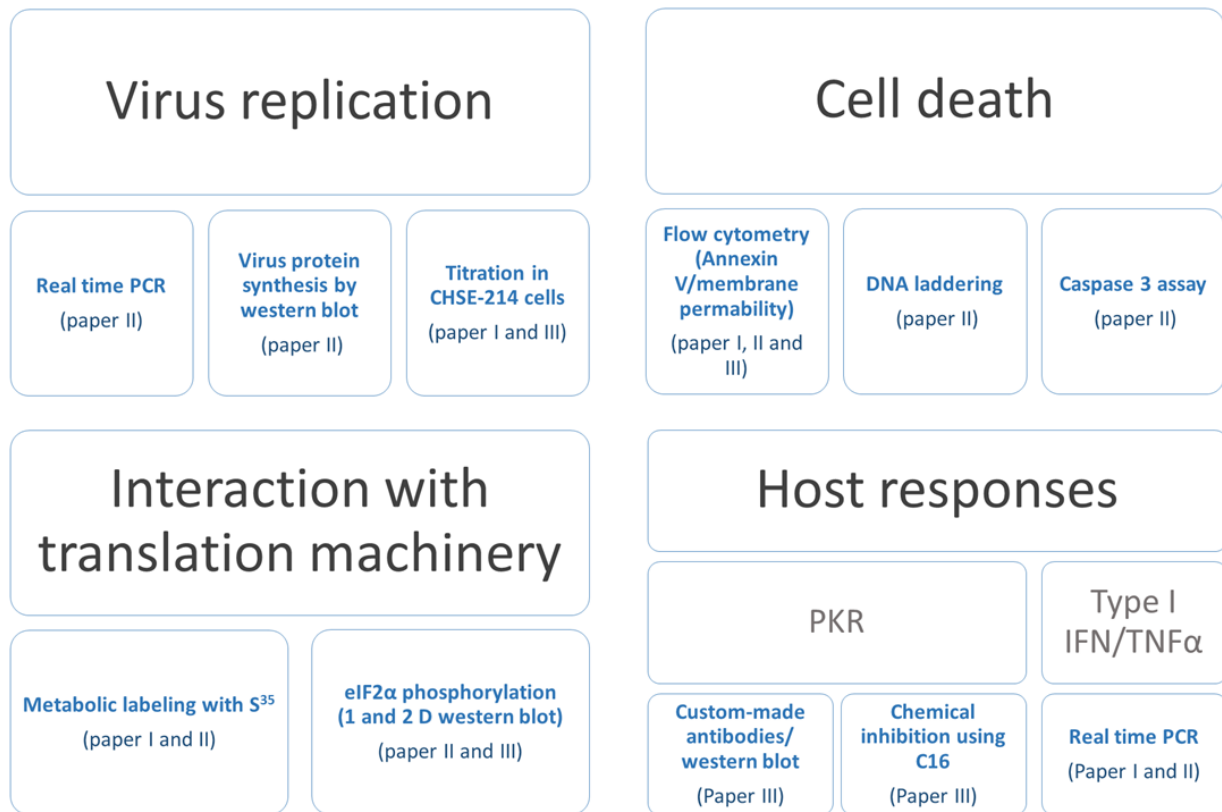
Figure 5. Summary of the different methodologies used to quantify and assess the effect of virus infection/cellular response in the different studies.

Cell lines, virus propagation and virus infection

Different cell lines were used to propagate the virus, namely RTG-2 (paper I) and AGK (paper II and III) cells. In the first study, the aim was to compare our findings to previous studies particularly on inhibition of protein synthesis and since RTG-2 cells were previously used in some of these studies (159-161), we opted to use this cell line instead of others. In ensuing studies however, the focus changed to obtaining high titres and we therefore used AGK cells.

Virus – cell experiments were all performed using CHSE cells. This cell line is permissive to all the virus isolates used in this study and had extensively been used to study the interplay between viruses and host responses. It was therefore logical to use the cell line in order to compare our results to the findings of others. However, in paper III we additionally used the TO cell line to test the universality of effect of the chemical inhibition of PKR.

Experimental conditions:

Cells were infected with 20 Pfu/cell with the aim to produce single cycle infections. This MOI was determined based on observations from preliminary studies. The incubation temperature was similar, 15°C in all except one study (Paper I, for E1S isolate).

Virus replication:

Different methods were used to evaluate virus replication. In papers I and III, the virus loads both intra- and extra-cellularly (cell culture supernatants) were evaluated using standard titration procedures. Use of the titration procedure is more suitable for these type of the studies since it allows measuring the impact of the generated immune responses on the production of viable virus. In paper II, the objective was to understand the role of the innate mechanism of translation control in limiting virus replication. For this purpose, virus replication was evaluated at mRNA level using real time PCR as well as at protein level using western blot.

Assessment of cell death:

Flow cytometric analysis of fluorescent labeled Annexin V staining combined with membrane permeable dyes has been widely used (164), and was the method of choice in the studies in this thesis. The principle lies in detecting the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane that occurs at early stages of apoptosis. The assay utilizes the detection of fluorescent labeled annexin V, which is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS (165). Since externalization of PS occurs in the earlier stages of apoptosis, the assay can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation (165). To distinguish between apoptotic and necrotic cells, membrane impermeable dyes that emit fluorescence upon DNA binding, such as propidium iodide (PI), were used. Accordingly and as illustrated in figure 6, the cells can be classified as viable (unstained), early apoptosis (Annexin V positive and membrane impermeable) and necrotic (Annexin V positive and membrane impermeable) (164). However, late apoptotic cells are also membrane permeable and the assay therefore does not distinguish late apoptotic and necrotic cells. Nevertheless, when apoptosis is measured over time, a pattern can be observed from early to late apoptotic stages and the movement of cells through these

stages suggests apoptosis while necrotic cells become membrane permeable without staining positive for Annexin V at any of the time points.

To understand the mechanism of cell death during IPNV infection, two other methods were used in addition the flowcytometry. First, we used the classical DNA fragmentation assay. Although the intranucleosomal DNA laddering is considered as hallmark of apoptosis, the same type of DNA laddering can occur in necrotic cells (166). We have therefore also used a highly sensitive caspase 3/7 activation assay that measure the bioluminescence generated upon the cleavage of luminogenic substrate by activated caspases.
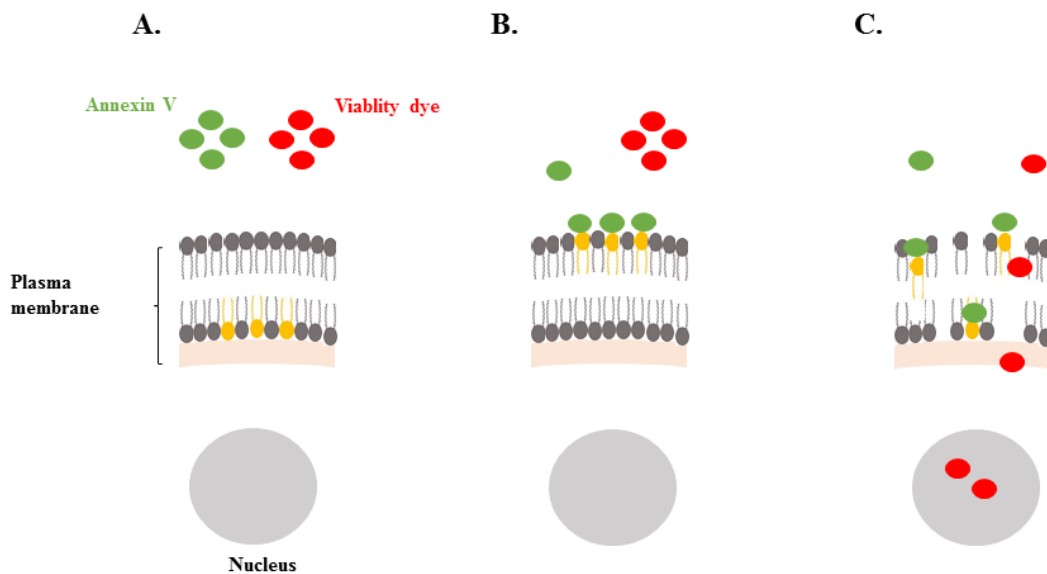
Figure 6. Schematic presentation of the principle of Annexin V/membrane permeability assay.. In normal cells (A), PS (yellow) is predominantly located on the inner leaflet of the plasma membrane. When cells undergo apoptosis (B), PS is rapidly translocated to the outer leaflet and binds to the labelled Annexin V (green) with high affinity. When the cell membrane is damaged as in necrotic and late apoptotic cells (C) Annexin V can also bind to PS. In addition, the membrane impermeable viability dye can also enter into the cell and binds the DNA in the nucleus. Modified by the author from http://www.flow-cytometry.us/index.php?page=apoptosis.

Evaluation of virus interaction with host's translation machinery:

We used metabolic labeling and western blot to understand the interaction with the translation machinery (Fig. 7). First metabolic labeling using S^{35} was used in paper I and II to evaluate global protein synthesis at specific time post infection. This method is widely used

although it can have adverse effects on cell proliferation and can lead to DNA fragmentation (167). These adverse effects occur after 1-4 hours exposure (167). In our experiments, we used 30min exposure to minimize the adverse effect although the anti-proliferative effect would have not interfered with our experiment since the metabolic labeling was an end step prior to cell lysis. After establishing that IPNV infection results in inhibition of protein synthesis, in paper II, we illustrated the involvement of eIF2α phosphorylation in inducing this inhibition using 1D western blot. For this purpose, commercially available antibodies raised against human eIF2α that was previously shown to interact with salmonid eIF2α (75) were used. In paper III, we wanted to understand the role of PKR in inducing this phosphorylation by comparing between eIF2α phosphorylation levels post IPNV infection in normal cells and cells treated with the PKR inhibitor C16. We encountered problem in quantifying the differences between C16 treated and untreated infected cells when using the previously used antibodies raised against the phosphorylated form, presumably due to reduced sensitivity or affinity to the salmonid eIF2α. An alternative approach was therefore to use the mobility shift induced by protein phosphorylation using 2D western blot.
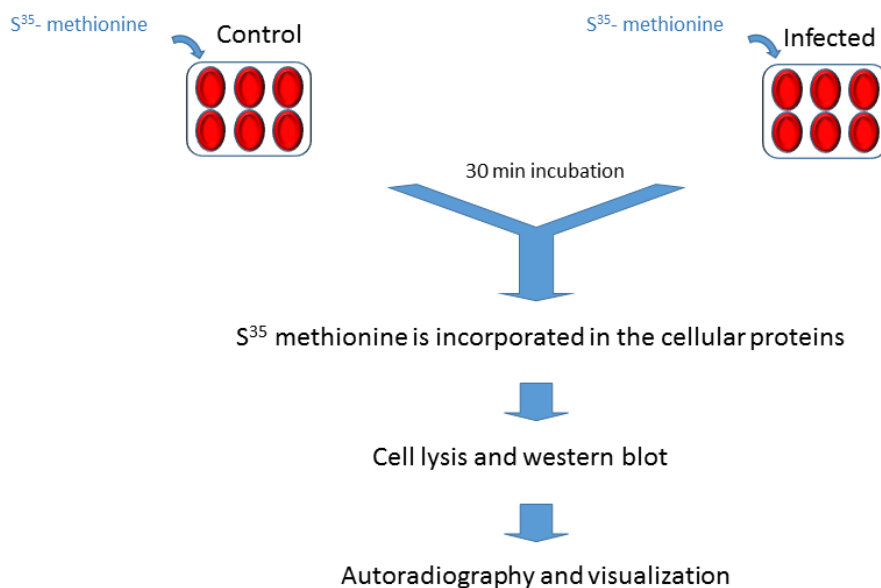

Figure 7. Schematic presentation of the method employed to assay protein synthesis. First, infected and control cells are incubated with S35 labeled methionine and incubated for 30 minutes in order to allow

the cells to incorporate it into the newly synthesized protein. Cells are then lysed and lysates are electrophorised and blotted onto PVDF membrane. Finally the membranes are autoradiographed and the signal is read using typhoon. Made by the author.

Assaying host immune responses:

Cellular responses can be measured using assays that detect changes in protein levels of immune effectors using several techniques including flowcytometry and immunoassays; or measuring changes in gene expression by assaying mRNA levels using techniques such as real time PCR, microarray and RNAseq (168, 169). Both flowcytometry and immunoassays make the use of antibodies generated against the target molecules and provide more insights into the functional aspects of these molecules. Gene expression based techniques, on the other hand, are used to quantify the mRNA levels of the target molecule using gene specific primers. The main two limitations of these techniques are:1) mRNA levels do not always correspond to protein levels (170), and 2) they are often not designed to detect the post transcriptional changes that can affect the immune function (171). The lack of commercially available antibodies and optimized immunoassays resulted in gene expression techniques to be the chief techniques used to evaluate immune responses in the field of fish research. Of these techniques, real-time PCR has been the most used because it is easy and rapid to perform, relatively cheap, and sensitive (172). The real-time PCR was therefore the method used to evaluate host responses. We used SYBRgreen based realtime PCR assay due to the sensitivity and low cost. The drawback of this method is the lack of specificity because the dye binds nonspecifically to the dsDNA (172). This limitation was overcome, by 1) using DNase treatment during RNA extraction to get rid of the genomic DNA contamination and 2) using optimally designed and validated primers and, 3) performing melting curve analysis to ensure that a single product is generated. It is noteworthy that primers for IFN used in paper I and II were designed in a conserved region for all the three type I IFN genes, IFNa, b and c because different patterns of expression have been reported in different salmonid species (76, 77). On the other hand, primers for TNF alpha 2 genes were used because they were previously identified to be more responsive in cell cultures (173).

RESULTS AND GENERAL DISCUSSION

In general, the studies constituting this thesis were targeted at understanding the pathogenicity and interaction of IPNV with host cells *in vitro*. Although the development of genetically disease-resistant fish has reduced the incidence of IPNV and consequently its impact on the salmonid industry in Norway, it is not unlikely that the virus can re-adapt and pose a fresh challenge. Studying the detailed mechanism used by the virus and its interaction with the host serves as a model for understanding the virus-host interaction for other viruses and will also provide knowledge that can be used to develop antiviral therapies. From the studies done here, the following are apparent:

Pathogenicity differs between different isolates of IPNV *in vitro*

At the beginning of these studies, there were conflicting reports regarding the induction of host responses, particularly apoptosis and host protein synthesis (156-158, 161) following infection with IPNV. A careful review of these conflicting reports suggested that the different findings could be attributed to the use of different virus isolates in the different studies. To test this thought, a study of host responses to selected IPNV isolates was done (Paper I). This study showed that isolates belonging to different geno-/serogroups induce different host responses. Since these isolates have adapted to different ecological niches, these findings indicate that evolution in different environments may be one of the important determinants of virus pathogenicity, and the origin of the virus isolate should therefore be considered when studying pathogenicity and vaccine efficacy. The latter point is of significant importance to the development of successful vaccines particularly in a places like Chile where isolates of a mixed origins are found (174). Furthermore, differences between genetically similar isolates of Sp that differ only in their virulent motifs reflect the importance of these motifs in interaction with host cells although the detailed molecular mechanisms is yet to be revealed. In addition, our findings show that functional differences do not always correlates with the differences in the virus genome. It is noteworthy that the low virulent strains has higher replication level in cell culture than the highly virulent strains which is in conformity with the general understanding that replication fitness *in vitro* and *in vivo* are not necessary similar.

Another prominent finding of paper I is that the Asian E1S isolate and the North American VR299 isolates induced higher Type I IFN gene expression while the European Sp isolates induced significantly higher TNFα gene expression. These findings give a good example linking pathogenic profiles with virus evolution or adaptation to different ecological niches. It also exposes our lack of understanding to the pathogenic mechanisms used by the virus to induce disease. Both of these cytokines are potent inducers of inflammation; and although inflammatory responses are generally considered beneficial to the host, cytokine dysregulation can also promote pathology and can therefore be detrimental to the host (89). The role of these two cytokines, and others, in inducing or protecting the host from pathology during IPN disease has been overlooked and must be further explored.

IPNV infections inhibit protein synthesis through PKR activation and eIF2α phosphorylation

An important element that determines the fate of virus invasion is the successful utilization of the host cell's translation machinery to produce new progeny. In this context, the ability of the virus to escape the innate translation control mechanism is crucial. The nature of the virus and its replication mechanism is largely responsible of the interaction of the virus with the translation machinery. Some viruses simply escape the recognition while others use more sophisticated mechanisms as explained earlier (83). Most of the viruses however, have the ability to initiate translation of their proteins in cap independent manner. The main mechanisms employed by many viruses is the possession of IRES (175). Recently, the presence of uORF emerged as a new mechanism (176). In this case, phosphorylation of eIF2α promotes the initiation of translation of the main ORF positioned downstream to one or more uORFs. The data presented in paper I and II provide evidence that IPNV infections results in global inhibition of protein synthesis. This inhibition is not cell type or MOI specific as it has been detected in AGK, RTG-2 (158) and TO cells (Fig.8) infected with different MOI. The mechanisms involved was investigated in paper II and III; and we have shown that it involves eIF2α phosphorylation that is induced through PKR activation. We also investigated the effect of this inhibition on virus replication at mRNA and proteins levels (Paper II). The virus did not seem to bother with this inhibition as virus replication was not affected at either levels. In addition, in paper III, production of infectious virus particles decreased when eIF2α phosphorylation was reduced following chemical inhibition of PKR. These findings show that IPNV actually benefits from eIF2α

phosphorylation rather than negatively impacted, and implies that the translation of IPNV proteins is resistant to eIF2α phosphorylation. What remained unanswered, however, is the mechanism(s) employed by the virus to translate its protein when the global translation is inhibited. Whether this is due to uORF present upstream the main ORF in segment A or due to possessing an alternative mechanisms of translation initiation remained an unanswered question. Of particular interest is the possibility of translational control by the uORF specially that mutations within certain regions upstream the main ORF in segment A did not yield viable virus (177).
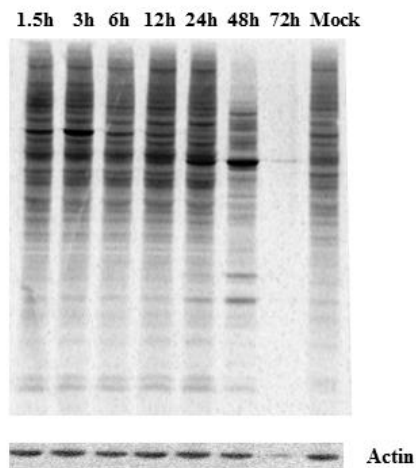
Figure 8. Global protein synthesis in TO cells following IPNV infection, made by the author. Cell were infected with 1 MOI and labeled with S35 30 min prior to sampling. Numbers represent time in hours post infection. Mock= uninfected cells

Inhibition of protein synthesis is a possible immune evasion strategy

One of the ways by which viruses benefit from inhibition of protein synthesis is the inhibition of the production of the antiviral effectors (85). In paper II, despite of the induction of type I IFN mRNA we found no up-regulation of Mx and PKR suggesting that the downstream type I IFN responses were not induced. Similarly, in paper III, no up-regulation of PKR protein was observed. Combined with the inhibition of protein synthesis observed in paper I and II, these results suggest that IPNV is using inhibition of protein synthesis as a means to inhibit the production of antiviral effectors such as type I IFN and its induced antiviral proteins.

Necrosis is the main cell death pathway following IPNV infection

As already indicated, cell death during IPNV infection has been a source of confusion with some investigators suggesting apoptotic cell death preceding atypical necrotic changes while

other reported only necrotic cell death (156, 157). Because the studies were performed using different isolates, in paper I we investigated Annexin V staining/PI staining following infection with different IPNV isolates. It was notable that for all isolates used changes in membrane permeability, indicated by PI staining, occurred prior to Annexin V staining, although significantly higher Annexin V staining was observed for the E1S isolate compared to the other isolates. The significantly higher Annexin staining points towards intrinsic properties that allow the E1S isolate to induce more apoptosis as indicated previously (156), although differences in the incubation temperature may also have played a role. A more thorough investigation of apoptotic changes were performed in paper II, but only for the Sp isolates. Again, we found that changes in membrane permeability occur prior to the apoptotic changes. It is noteworthy however that we detected caspase 3 activity in infected cell lysates at late time points indicating that apoptosis occurs later in the infection. Collectively, our findings indicate that necrotic cell death is the major cause of cell death in CHSE-214 cells while apoptosis comes late onto the scene. The timing of occurrence of the two cell death pathways may reflect a fight for survival between the virus and the host cell with the necrotic changes induced by the virus for release while apoptosis is induced by the cells to limit virus spread. Whether the virus plays an active role in delaying the apoptotic process is not yet clear.

PKR activation during IPNV infections is beneficial for the virus:

PKR is constitutively expressed in the cells but is upregulated by type I IFN. Even constitutive levels of PKR are sufficient to inhibit virus replication when activated (178). Some viruses, on the other hand, benefit from PKR activation mainly by using inhibition of protein synthesis to block the production of antiviral effectors (85, 86). The findings of paper II suggested that IPNV may be using the latter strategy to counteract IFN responses. We therefore decided to study the role of PKR activation during IPNV infection (paper III). Ideally, PKR activation is studied by detecting the increase in the expression of phosphorylated form using specific antibodies. Sometimes, it is also combined with using chemical inhibitors, such as C16, or siRNA (85). Studying PKR activation is difficult in fish due to lack of antibodies and optimized protocols. Some investigators used overexpression of PKR and/or dominant negative mutant of PKR (179). However, overexpression of PKR can be problematic since it can trigger apoptosis and other unspecific responses (personal observation). In paper III we used chemical inhibition of PKR to assess its role during IPNV infections. We demonstrated that inhibition of PKR has

negative impact on virus replication as it reduced virus yields in the supernatants as well as intracellular. We did not further explore the mechanisms involved. However, since PKR inhibition decreased eIF2α phosphorylation, we suggested that the translation of virus proteins is controlled by the uORF encoding VP5, and is enhanced when eIF2α is phosphorylated as indicated earlier. This interpretation remains however hypothetical and requires further investigation. Collectively, the findings of paper II and III how that PKR activation is advantageous to the virus in two ways: 1) blocking the production of antiviral effectors as a consequence for protein synthesis inhibition; and 2) promoting virus the replication by unknown mechanism. It will not be easy to determine which of the PKR induced responses are benivitial to IPNV due to the complexity of PKR signaling and functions. One candidate that worth investigation, however, is the NFκB activation. This is because NFκB activation was previously shown to have impact on the replication of several viruses (180) and it was also shown to occur following IPNV infection (181). Another important outcome is the possibility of C16 to be used as antiviral treatment against IPNV.

Model for IPNV interaction with the host cell

Based on the findings presented in this thesis we hypothesized the following model for IPNV interaction with host cells (Fig. 9):
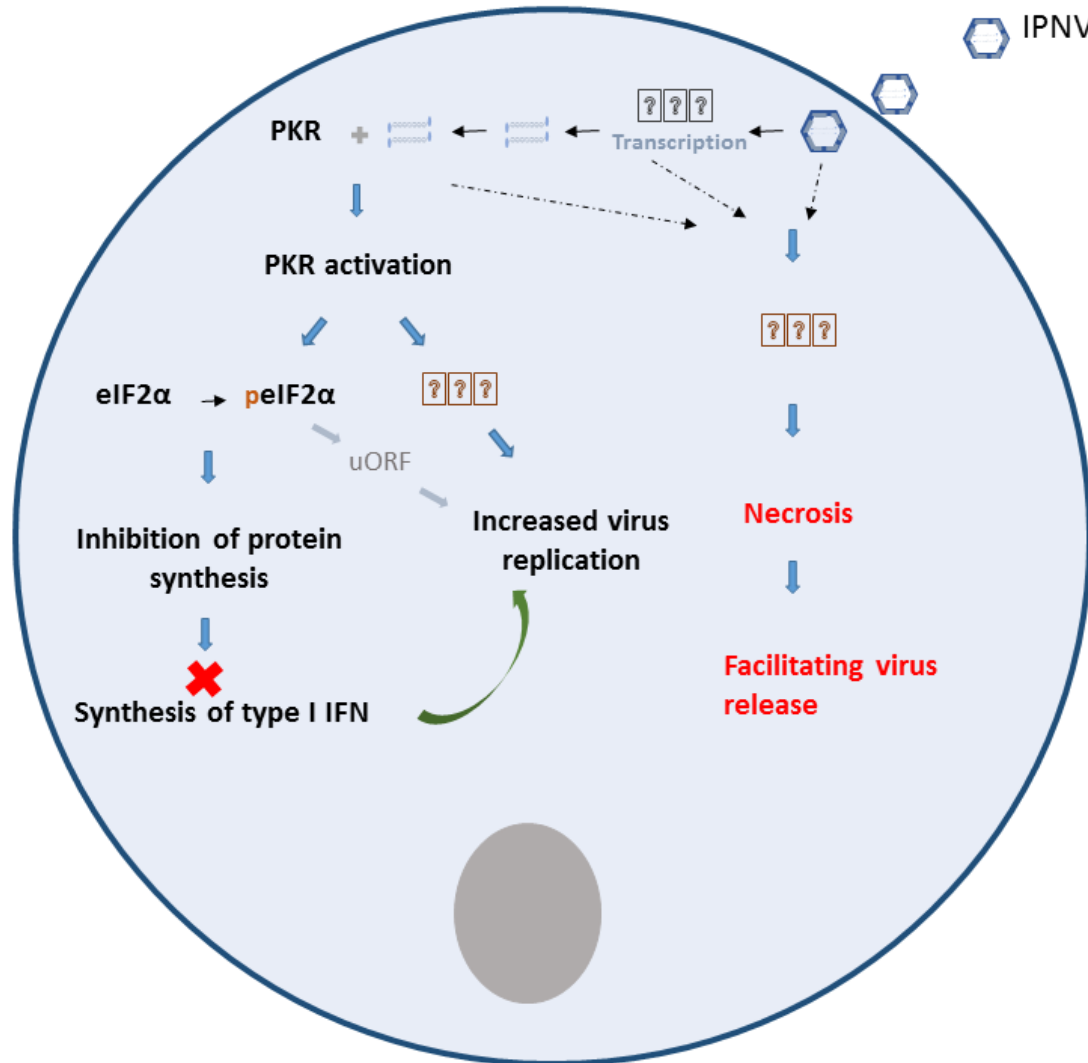
Figure 9. Suggested model for IPNV interaction with host cell protein. After attachment and entry of the virus into the cell, the virus nucleic acid is transcriped and dsRNA is produced. The produced dsRNA is then recognized by PKR in the cytoplasm of infected cells. The interaction between the virus nucleic acids and PKR lead to PKR activation which benefits virus replication in two ways: 1) Phosphorylation of eIF2α and subsequent inhibition of protein synthesis that blocks production of type I IFN and its effector proteins. There is also a possibility that the translation of virus proteins is enhanced under eIF2α phosphorylation due to the presence of uORF. 2) Unknown downstream signaling that facilitate virus replication. IPNV infection also results in necrosis that help virus spread but neither the initiation nor the execution mechanism are known. Made by the author.

CONCLUSIONS

Although the impact of IPNV on the salmonid industry is currently minimal, the ubiquitous nature of the virus and its persistence in the aquatic environment together with the high mutation rates of the RNA viruses pave the way to the re-emergence of the disease anytime in the future. This is particularly because the reduced disease occurrence is mainly due to changes in the host genetic for which the virus can adapt. Vaccination against IPNV has not been successful and has been hampered by the lack of understanding of the virus host interaction. Studies that generate knowledge about host virus interaction will therefore be useful for the future development of successful vaccines or antiviral therapies. The studies reported in this thesis provide new information about hot virus interaction that can be summarized as follows:

- Pathogenicity vary between different IPNV isolates based on virulence as well as adaptation and evolution in different ecological niches. Since differences in pathogenicity coincides with different cytokine profiles, the inflammatory cytokines might play roles in virus pathogenicity.
- Infection with all the IPNV isolates results in inhibition of protein synthesis. The inhibition correlates with limited ability to induce type I IFN responses for the Sp isolates but not the VR299 and E1S isolates.
- The mechanisms by which IPNV inhibits protein synthesis involves eIF2α phosphorylation that is induced through PKR activation.
- Necrosis is the main cell death pathway induced in IPNV infected cells while apoptosis induced late in the infection, probably as an attempt to contain the infection.
- PKR activation during IPNV infection is beneficial to the virus since it block the production of antiviral effectors and promote virus replication.
- PKR inhibitors might be a good anti IPNV drug candidates.

FUTURE PERSPECTIVES

The findings reported in this thesis open the door for new research possibilities. Some of the questions raised by the findings of the different studies are:

- What are the true roles of the inflammatory cytokines in virus pathogenicity?

  With this regards it is interesting to know whether a similar differences in cytokine profiles induced by different isolates occur *in vivo*. If so, studies should be conducted to understand the roles these cytokines play during infection with the different isolates and whether they promote or protect against pathology. Nevertheless, since these cytokines has immunomodulatory effect, it is also of interest to understand how it affect responses and susceptibility to other diseases especially that it is not uncommon for salmonids to be coinfected with different pathogen.

- What is the interplay between the VR299 isolate and IFN responses?

  One of the intriguing findings reported in this thesis is that ability of the VR299 isolate to induce high mRNA expressions of the downstream type I IFN responses. It is possible that this isolate use inhibition of translation to prevent the production of the different antiviral effectors. Even though, however, this does not explain why cells mount strong interferon responses against this isolate. This could be due to a different replication mechanisms possessed by this isolate but this require further investigation.

- What are the replication mechanisms and how does the virulence motifs interact with host cells?

  The previous point highlights our poor understanding of the replication mechanisms employed by the different isolates. It is therefore necessary to understand the details mechanisms of entry and intracellular replication in order to better understand the interaction with the host and innate immune defenses. At least for the Sp strains, the virulent motifs on VP2 seems to play important role in interaction with host cells since the responses induced by the virus infection differed between the two recombinant isolates that differs only at these motifs. Future studies should therefore be conducted to

better understand the interaction of these motifs with the host cell as well as the detailed machanisms of virus replication.

- What are the mechanisms involved in the translation of IPNV proteins?

  It is clear from the studies reported in this thesis that IPNV can efficiently translate its protein global protein synthesis is inhibited and under high levels of eIF2α phosphorylation. This indicate that IPNV processes an alternative mechanism or mechanisms of translation and further studies are warranted to understand these mechanisms. Of particular interest is the possibility of the translation control by the uORF present in seqment A.

- Can PKR inhibitors inhibit the replication of IPNV *in vivo*?

  The inhibition of IPNV replication by the PKR inhibitor is an interesting finding and open the door for it to be used as anti IPNV treatment. However, *in vi*vo studies should be first conducted to see if a similar effect can be observed.

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Infection Profiles of Selected Aquabirnavirus Isolates in CHSE Cells

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Abstract

The wide host range and antigenic diversity of aquabirnaviruses are reflected by the presence of a collection of isolates with different sero- and genotypic properties that have previously been classified as such. Differences in cytopathogenic mechanisms and host responses induced by these isolates have not been previously examined. In the present study, we investigated infection profiles induced by genetically and serologically closely related as well as distant isolates *in-vitro*. CHSE-214 cells were infected with either E1S (serotype A3, genogroup 3), VR-299 (serotype A1, genogroup 1), highly virulent Sp (TA) or avirulent Sp (PT) (serotype A2, genogroup 5). The experiments were performed at temperatures most optimum for each of the isolates namely 15°C for VR-299, TA and PT strains and 20°C for E1S. Differences in virus loads and ability to induce cytopathic effect, inhibition of protein synthesis, apoptosis, and induction of IFNa, Mx1, PKR or TNFα gene expression at different times post infection were examined. The results showed on one hand, E1S with the highest ability to replicate, induce apoptosis and IFNa gene expression while VR-299 inhibited protein synthesis and induced Mx1 and PKR gene expression the most. The two Sp isolates induced the highest TNFα gene expression but differed in their ability to replicate, inhibit protein synthesis, and induce gene expression, with TA being more superior. Collectively, these findings point towards the adaptation by different virus isolates to suit environments and hosts that they patronize. Furthermore, the results also suggest that genetic identity is not prerequisite to functional similarities thus results of one aquabirnavirus isolate cannot necessarily be extrapolated to another.

Introduction

Aquabirnaviruses are a genus in the family *Birnaviridae* that infect aquatic animals including fish, crustaceans and molluscs. They are non-enveloped, icosahedral double-stranded RNA viruses with genomes encoding two segments A and B [1]. Segment A comprises two overlapping open reading frames (ORFs), with the first (short) encoding a non-structural protein VP5 [2,3]. The second long ORF encodes structural proteins VP2 and VP3 as well as a non-structural protein VP4 [3–6]. Segment B has a single ORF that encodes the virus polymerase VP1 [7].

The type species of the genus aquabirnavirus is infectious pancreatic necrosis virus (IPNV) which is responsible for a highly contagious disease in salmonids. The genus has other members including Yellowtail Ascites Virus (YTAV), first isolated from yellowtail (*Seriola quinqueradiata*) in Japan [8] and Tellina virus (TV) [9] infecting other fish species. These viruses were tentatively called Marine birnaviruses (MABV) [10].

Aquabirnaviruses have high antigenic diversity and are classified into serogroups A and B. The former consists of nine serotypes designated A_1 to A_9; also known as West Buxton (WB), Sp, Ab, He, Te, Canada (Can.) 1, Can. 2, Can. 3 and Jasper, respectively [11]. Serogroup B has only one serotype, Tv-1. In addition to this classification, the isolates have also been grouped into six geno-groups, giving good agreement with the serological classification [12]. Accordingly, sero-groups A_1 and A_9 correspond with geno-group 1; A_3, A_2 and A_4 correspond with geno-groups 3, 5 and 6, respectively; A_7 and A_8 with geno-group 2; A_5 and A_6 with geno-group 4. Recently, a seventh group containing Japanese marine isolates obtained from fish and mulluscs has also been discovered [13].

The wide host range and antigenic diversity of aquabirnaviruses have been a source of confusion in terms of isolate nomenclature i.e. when a virus should be called IPNV and also to what extent findings of one isolate should be extrapolated to others. To harmonize the former, it was suggested that only isolates causing overt IPN in salmonids would be called IPNV [14]. All other isolates therefore were to be called aquabirnaviruses. This nomenclature however did not gain universal acceptance as some scientists continued making references to isolates infecting other species, for example E1S of eel, as IPNV [15]. Another problem associated with this nomenclature is that it did not take into account differences in virulence of identical isolates; a non-pathogenic isolate having an identical genetic sequence as a virulent one but differing only with two amino acids [16] would accordingly be excluded from list of IPNV. The purpose of the present study was to compare the mechanisms of cytopathogenesis between genetically and serologically identical and more distant isolates of aquabirnaviruses. Previous studies have shown contradictory findings related to protein shutdown and apoptosis in cells infected with IPNV [17–21], suggesting that pathogenic mechanisms are different. While infection profiles of different isolates of MABV and IPNV have been compared in different fish species and shown to differ in terms of mortalities induced in different species (i.e. in salmonids versus other fish species) [22,23], there is no documentation of the molecular basis of these differences. In this study, we specifically investigated the cell-pathogen interaction of IPNV isolates (TA & PT, serogroup A_2, geno-group 5) and (VR-299, A_1, geno-group 1) infecting salmonids on one hand and MABV (E1S, A3, geno-group 3) on the other. The results show differences in virus replication, protein synthesis inhibition, gene expression profiles and their ability to induce apoptosis.

Materials and Methods

Cells and Viruses

Rainbow trout gonad-2 (RTG-2) [24] and Chinook salmon embryonic (CHSE-214) cells [25] were grown in L-15 media with Glutamax (Gibco) supplemented with 10% and 5% FBS (Sigma Aldrich), respectively. Cells were cultured at 20°C until 90% confluence prior to virus infection.

Virus propagation

Four virus isolates from different sero- and geno-groups were first propagated in RTG-2 cells in order to scale up the virus to be used in comparison experiments in this study. Two recombinant high and low virulent isolates previously made by reverse genetic [26] were chosen to represent the Sp serotype (A_2). The high and low virulent isolates have the following virulence

motifs $T_{217}A_{221}$ (TA) and $P_{217}T_{221}$ (PT), respectively in the VP2 capsid protein [16]. The E1S isolate (Ab serotype (A_3)) originally from eel was provided by Professor Jen-Leih Wu (Academia Sinica, Taiwan). The VR-299 Isolate was kindly supplied by Professor Espen Rimstad (Norwegian University of Life Sciences). Table 1 lists virus isolates used in this study and the temperatures at which experiments were conducted in CHSE cells.

Virus infection and metabolic labeling

Six-well plates (Corning) containing approximately 90% confluent CHSE 214 were infected with MOI = 20 pfu/cell of each of the four isolates of IPNV. Infection was done in reverse order to result in the following time points 3, 12, 24 and 48 hours at the time of metabolic labeling. Metabolic labeling was done by washing the cells 3 times with PBS and incubation with Methionine-, Lysine- and L-glutamine-free Dulbecco's modified Eagle's medium (L-15 media, Sigma Aldrich) containing 20 μCi/ml S^{35} Methionine (Montebello), 1% L-glutamin (Sigma Aldrich) and 2% FBS for 30 minutes. After metabolic labeling, the cells were washed once with PBS, lysed using 250μl Cell M lysing reagent (Sigma Aldrich) and placed on a shaker for 15 minutes. The cells were then scraped to detach them and supernatants were collected in 1.5 ml eppendorf tubes followed by clearing by centrifugation at 11800 g for 5 minutes. Total protein in each lysate was estimated using Quick Start Bradford Protein Assay Kit (Biorad). To determine the amounts of labeled protein, equal amounts of total protein from each cell lysate was subjected to SDS-PAGE and blotted onto PVDF membrane. Finally, the membrane was incubated with a storage phosphor cassette overnight for autoradiography and the radioactivity was detected using the Typhoon (GE Health care). To quantify the levels of protein synthesis, ImageQuant software (GE Healthcare) was used to measure the density of a specific band common to all samples (infected and uninfected) at different time points post infection. The basis for selection of this band was that it had to be 1) prominent; 2) consistently expressed in infected and uninfected cells and 3) reflected or represented the general visual trend of protein expression of the samples. Host protein bands from infected cells and uninfected controls were expressed as percentages of infected relative to the uninfected controls.

Virus Replication assays

To monitor the dynamics of virus replication during the infection period, confluent monolayers of CHSE-214 cells in 24-well plates (Corning) were infected with the different IPNV isolates at 20 pfu/cell. After incubation for 1 hour, virus supernatants were removed, the cells were washed once with PBS and maintenance media (L-15 media containing 2% FBS and 50μg/ml gentamycin) was added to the wells. At 3, 12, 24 and 48 hours post infection (hpi) supernatants were harvested and kept at 4°C until titration. In addition, infected cell monolayers were trypsinized by adding 100μl Trypsin EDTA (Sigma Aldrich) per well for about 5 min. Subsequently 100μl maintenance media was added and cells were subjected to 3 rounds of freeze-thawing to release the virus into the supernatant. Supernatants containing cell debris and virus were then transferred to eppendorf tubes, cleared by centrifugation and subsequently titrated. Eight samples of virus supernatants and five of supernatants containing virus released from cells, were titrated and used to infect confluent CHSE-214 grown in 96 wells plates. $TCID_{50}$/ml was calculated using Karber's method [29].

Assessment of Apoptosis by flowcytometry

24-well plates containing confluent CHSE cells were used in a similar setup as described in the section above. In addition, parallel wells were treated with 1 μM Staurosporine for 12 hours as positive controls for apoptosis. At each sampling, the supernatant was transferred to a 2ml

Table 1. Isolates used in the study.

Isolate	Source	Temperature	Genogroup	Serogroup	Reference
E1S	Isolated from eel	20°C	3	A3	[27]
VR-299	Isolated from rainbow trout	15°C	1	A1	[28]
Sp (TA and PT)	Template used isolated from Atlantic salmon	15°C	5	A2	[16,26]

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centrifugation tube or 5ml polystyrene round-bottomed tube (BD Biosciences). Adherent cells were washed twice with PBS prior to trypsinization. Trypsinization was done as described above for 5 minutes and stopped by adding fresh media. The trypsinized cells were then pooled with the original supernatants and cells were pelleted by centrifugation at 300 x g for 10 minutes. The supernatant was removed and cells were re-suspended in 100µl Hepes buffer containing 2µl Fluoresceine conjugated Annexin-V staining reagent (Annexin-V-FLUOS Staining Kit, Roche). After incubating for 30 minutes, the volume was adjusted to 200µl. To differentiate between apoptotic and necrotic cells, membrane permeability was assessed by adding Propidium Iodide (PI, Sigma Aldrich) to a final concentration of 8 µg/ml just before analysis. Flowcytometry was performed for 5,000 events using a Guava easyCyte Flow Cytometer (Millipore) while data analysis was performed using InCyte software, version 0.2 (Merck Millipore). The following parameters were measured to identify apoptotic cells: 1) the area pulse of forward light scatter (FSC-A) versus side scatter (SSC-A), and 2) fluorescent intensities of FITC (filter 525/30) and PI (filter 690/50) upon excitation with 20 mW 488 nm laser. Cell aggregates and debris were identified and excluded by using the width pulse of FSC-A versus area width of SSC-A.

Quantitative real time PCR analysis

Real-time RT-qPCR was used to quantify the expression of IFNa, PKR, Mx1, and TNFα. Since no clear agreement on the nomenclature of fish IFN has been reached so far, it is noteworthy that the nomenclature used in this study was that suggested by Zou et al [30]. Cells were infected with the different isolates as described in the section on the protein shutdown with the exception that this time three parallels were used per treatment. At 3, 12, 24 and 48 hpi, cells were lysed using RLT buffer (RNeasy minikit, Qiagen) containing 10µl/ml β-mercaptoethanol. Total RNA was isolated by using the RNeasy Plus minikit according the manufacturer's instructions and the concentration of RNA was determined by using the Nanodrop ND1000 (NanoDrop technologies). 400 ng of total RNA from each sample was used for cDNA synthesis, using a Transcriptor first strand cDNA synthesis kit (Roche) according to the manufacturer's instructions. The cDNA was diluted five times and stored at -20°C until required.

Quantitative PCR was performed in 96 well plates using the LightCycler 480 system (Roche). For each reaction, 2µl cDNA was mixed with 10pmol gene specific primers and 10µl LightCycler 480 SYBR green I master mix. The final concentration was adjusted to 20µl using RNase free water. The sequences of primers used in the reactions are provided in Table 2. The cycling conditions for the PCR reactions were as follows: denaturation 94°C for 10 sec; annealing 60°C for 10 sec; elongation 72°C for 10 sec. The results were analyzed using the ΔΔCT relative quantification approach [31] with β-actin as a reference gene. Graphs were drawn with the help of GraphPad Prism 5.0 (GraphPad Software Inc.).

Statistics

To compare the gene expression and virus titre results, two way ANOVA analysis followed by Bonferroni test to compare the difference between isolates at each time point was performed using GraphPad Prism 5.0 (GraphPad Software Inc.).

Table 2. Primers used for PCR.

Gene	Accession no.	Direction	Sequence
B-actin	AF012125	F	CCAGTCCTGCTCACTGAGGC
		R	GGTCTCAAACATGATCTGGGTCA
Interferon α (IFNa)	NM_001123570.1	F	TGGGAGGAGATATCACAAAGC
		R	TCCCAGGTGACAGATTTCAT
Mx1 protein	U66475	F	TGCAACCACAGAGGCTTTGAA
		R	GGCTTGGTCAGGATGCCTAAT
Tumor necrosis factor α 2 (TNFα)	DQ787158	F	AGATATTTAGGCGAACATTCAGTT
		R	TGACTCAGAGGAGTGGTACG
PKR	EF523422.1	F	TGAACACAGCCAGAAGAACAA
		R	GACTACCGCCACATAACTCCA

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Results

In the present study, an MOI = 20 pfu/cell was used. While this is high, it would guarantee that all cells were infected with more than 1 virus particle (using Poisson's distribution), in principle ensuring a synconous 1-cycle infection kinetics. This was also in line with one of our recent publication [32].

When it comes to the temperatures used, E1S took 4 days to induce full CPE and to reach a virus titer of 10^7 TCID50/ml at 15°C in CHSE cells in a previous study [33]. The same result was achieved in only 2 days at 20°C. For NVI-15 (TA isolate), it took 7 days to obtain full CPE and a virus titer of 10^7 TCID50/ml in CHSE cells while at 20°C, the same number of days were required for CPE but only a virus concentration of 10^6 TCID50/ml could be achieved. Against this background, cells in the present study were infected at temperatures optimal for respective virus propagation, namely 20°C for E-1S and 15°C for the others.

Virus induced CPE

As a starting point, the temporal difference between the isolates' ability to induce CPE was assessed. All isolates induced CPE but at different time points following infection (Fig 1). E1S induced the earliest onset of CPE, seen at 12 hrs post infection (hpi), with rapid progression to 48 hpi. Onset of CPE by VR-299 and TA was at 24 hpi, however becoming only pronounced at 48 hpi, while the PT isolate showed the slowest induction, commencing at 48 hpi (Fig 1).

Kinetics of virus titers in infected cells and supernatants

Intracellular virus loads. Next, virus titers in the intracellular compartment were determined, starting at 3 hpi, all isolates were approximately the same level, but with E1S having a slightly higher intracellular titer than the others (Fig 2a; not statistically different). By 12 hpi, E1S had significantly higher titer (p<0.001) compared to other isolates, showing a 3.7 $\log_{10}$-fold increase, while for the other isolates, titer was 0.85 $\log_{10}$-fold to 1.3 $\log_{10}$ higher than at 3 hpi. By 24 hpi, E1S titers decreased by 0.7 $\log_{10}$ (compared to 12hpi), while for other strains there was an increase, about 1.76 $\log_{10}$, 1.36 $\log_{10}$ and 2.38 $\log_{10}$ for PT, TA and VR299, respectively. By 48 hpi, E1S showed a further decline of 0.9 $\log_{10}$ compared to 24 hpi, while VR-299 dropped by 0.6 $\log_{10}$ and PT by 0.5 $\log_{10}$. Only TA showed an increase, 0.3 $\log_{10}$ relative to the 24 hpi time point (Fig 2a).

Virus loads in supernatants. The kinetics of extracellular virus titers was different among the isolates and reflected intracellular events. E1S showed a 2.8 $\log_{10}$ increase from 3 to 12 hpi,
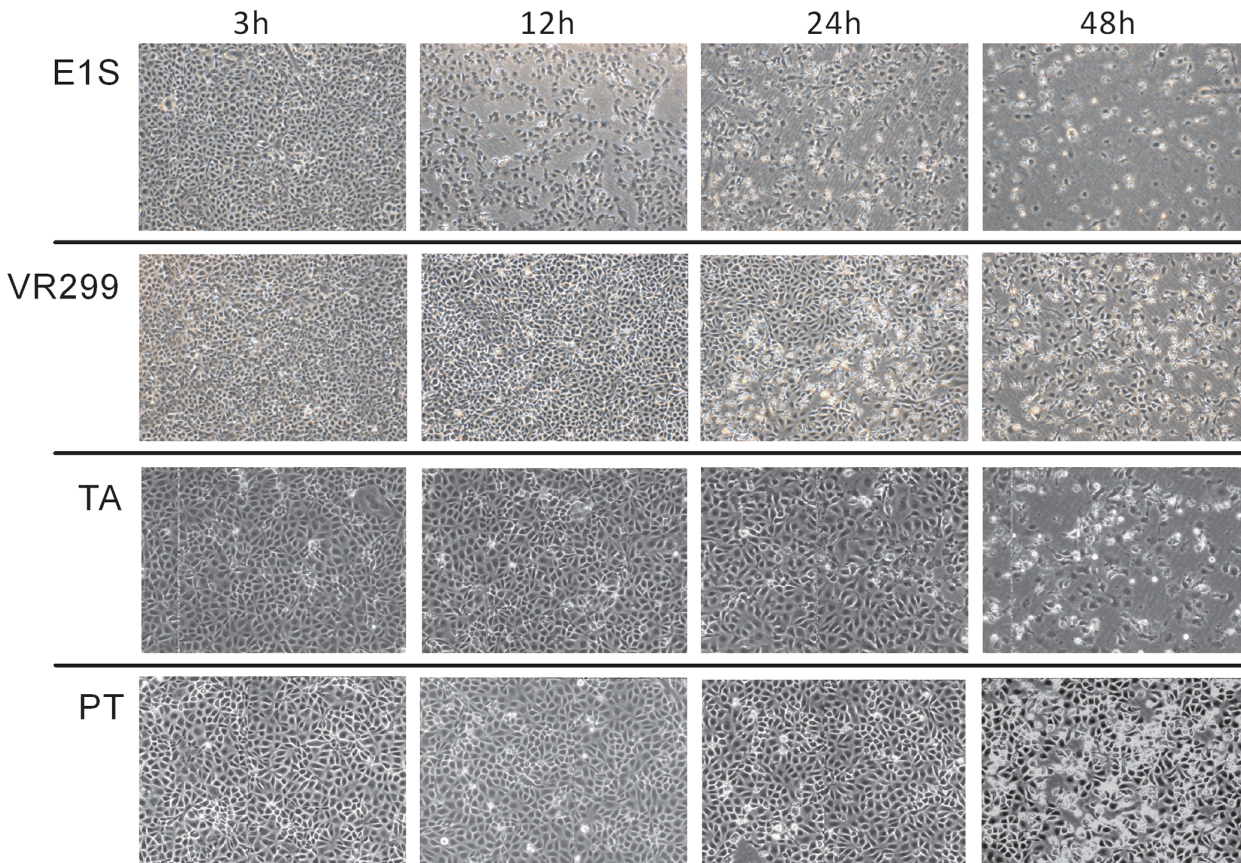
Fig 1. CPE development in CHSE-214 cells after infection with different aquabirnavirus isolates. Phase-contrast microscopical images at different times post infection.

while the other isolates ranged between $0.11 \log_{10}$ and $0.43 \log_{10}$ increase (Fig 2b). By 24 hpi, E1S leveled off ($0.88 \log_{10}$ increase) similar to VR-299 and TA over this time span (0.76 and $0.67 \log_{10}$, respectively). For PT there was a $1.32 \log_{10}$ increase. By 48 hpi, E1S remained at the same titer while the titre increase for the others ranged from about 1.2–$1.91 \log_{10}$, with the highest increase being for VR-299 (Fig 2b). In summary, E1S had generated about 60% of the total number of progeny over the incubation period by 24h post infection, while VR-299
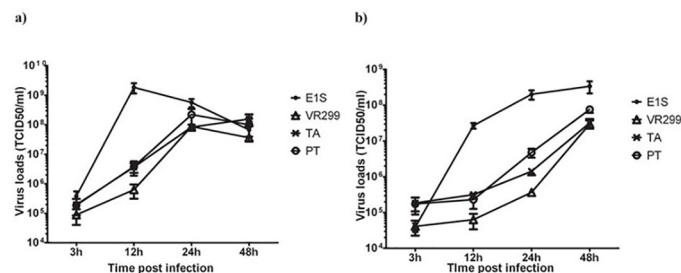

Fig 2. Replication curves for different aquabirnaviruses in CHSE-214 cells. Cell monolayers were infected with different IPNV isolates at 20pfu/cell. Amounts of virus in A) intracellular or B) supernatant at different time points post infections were determined by titration in CHSE-214 cells. The data are presented as mean ±S.E.M. $\log_{10}$ TCID50/ml of minimum of 5 replicated taken from at least two independent experiments.

produced the least at this time (below 2%). PT and TA on the other hand produced about 6 and 4% of the total progeny during the same period respectively. This means that VR-299, PT and TA generated more than 94% of the total virus particles over the last 24h of the incubation period (Fig 2b). On this basis, it can be assumed that the strategies explored by the virus to circumvent or counteract cellular antiviral responses would differ between the isolates.

Inhibition of protein synthesis

We first analyzed the extent to which the different isolates induced shut-down of protein synthesis, a strategy used by many viruses to prevent cellular anti-viral responses. Two patterns of responses were observed. Cells infected with VR-299 and TA isolates showed a rapid onset with considerable reduction in protein synthesis (Fig 3a and 3b) by 24 hpi. In contrast, E1S and PT exhibited a slow and moderate onset. By 48 hpi, CPE was so extensive in all infected cells (except for PT isolate) that quantification of protein synthesis by labeling was not possible (thus not shown). Interestingly, as the host protein synthesis decreased, virus protein bands gained strength as can be seen from the strong intensity (Fig 3a), especially in cells infected with E1S and TA isolates (data not shown).

Post-infection expression of selected genes

Selected genes associated with anti-viral responses were then analysed, IFNa, Mx1, and PKR, all by real-time PCR. E1S induced the highest increase in relative expression of IFNa from 12 to 24 hpi (Fig 4a), aligning with the highest production of viral progeny over this period. Interestingly, expression of Mx1 and PKR (Fig 4b and 4c) showed a very moderate increase over the same period, indicating that the virus inhibits down-stream induction of IFNa stimulated genes. VR-299 showed a less pronounced increase (25-fold up-regulation) of IFNa over the first 24h while the Mx1 and PKR induction were high already at 12 hpi (25-fold), increasing to more than 60-fold by 48 hpi (Fig 4b and 4c). The cellular responses to VR-299 were in strong contrast to that elicited by E1S, where the former replicated and produced more than 95% of its progeny under high expression of anti-viral factors. TA-infected cells showed slow onset of IFNa expression (5-fold at 24 hpi and 30-fold by 48h). Correspondingly, Mx1 and PKR also showed late up-regulation (20-fold at 48 hpi for Mx1 and less than 5-fold for PKR). This aligns with viral replication gaining momentum towards the end of the infection cycle and also with marked protein shut-down at 24 hpi (55% reduction). The ability of TA strains to replicate under high IFNa levels, has been shown before [34] and the fact that TA proliferates even when the cellular protein machinery is markedly lowered, would point towards the virus exploiting cap-independent initiation of translation.

For TNFα, the highest induction was after infection with TA and PT isolates (Fig 4d). E1S induced the weakest expression of TNFα while VR-299 only induced marked expression at 48 hpi.

Flowcytometry results

We used flowcytometry to assess the timing of apoptosis and necrosis induction in infected cultures. There was very little increase in Annexin V-positive (apoptotic cells) except for E1S where counts were significantly higher than other isolates at 24 and 48 hpi (Fig 5a). For the other virus strains, the percentage apoptotic cells remained below 5% throughout the study (Fig 5a).

PI positive or necrotic cells in the E1S infected cells peaked as early as 12 hpi, concomitant with high virus release to the supernatant (Figs 2a and 5b). For TA and PT, similar trends and correlates were seen; the highest percentage of PI-positive cells (Fig 5b) coincides with the
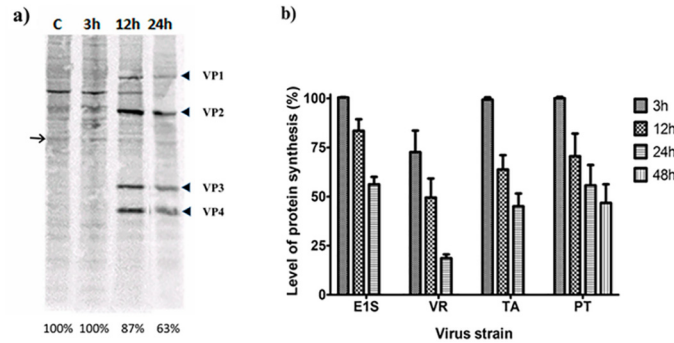
Fig 3. Impact of different aquabirnavirus isolates on global protein synthesis in CHSE-214 cells. Cells infected with either E1S, VR-299, TA or PT isolates collected at different times post infection were incubated with S35 Methionine for 30 min before lysis. Control cells were used in addition. Lysates (7μg total proteins) were subjected to SDS-PAGE, autoradiographed by incubation in storage phosphor cassettes before analysis by Typhoon. The ImageQuant software (GE Healthcare) was used to estimate the protein quantity by measuring the density of one band at different time points for each isolate. A) Autoradiograph image showing virus (VP1-4) and host protein (arrow) bands as an example. C = control sample at 48hrs post infection; Percentages = relative amounts of S35 Methionine-labeled host cell proteins calculated on the basis of the protein band shown as an arrow. B) Histogram of S35 Methionine-labeled host cell proteins of infected relative to uninfected cells as described above. Each entry represents three independent experiments and error bars are ± S.E.M. Key: VR = VR299.

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highest increase in virus titer in the supernatant (Figs 2b and 5b). The most puzzling finding is for VR-299. While virus proliferation in the intracellular compartment starts late compared to the others, mirrored by a late release to the extracellular compartment, the majority of virus release is over the last 24h of the infection period (Fig 2a and 2b). However the PI-positive cells remain low throughout, peaks at 24 hpi but only 10% of the cells are positive (Fig 5b). It thus seems like virus is released without the cells going into dissolution, which makes it difficult to explain the mechanisms by which the virus is released to the extracellular compartment.
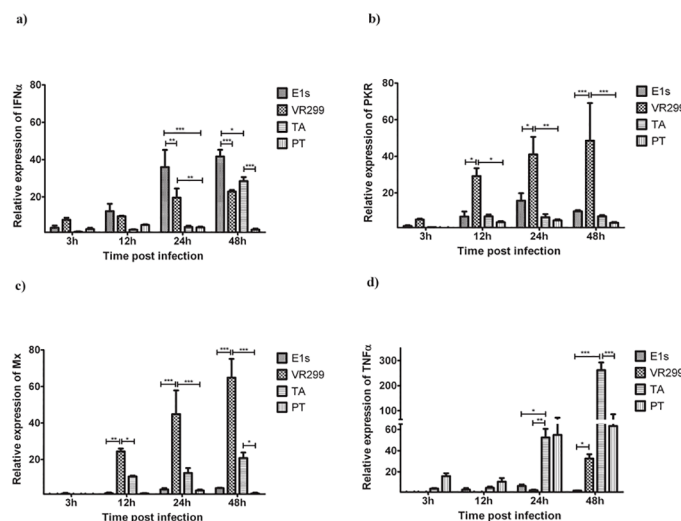

Fig 4. Type I IFN response induced by different isolates of aquabirnaviruses. Normalized gene expression of A) IFNα, B) PKR C) Mx1, and D) TNFα2 at different time points post infection ± S.E.M.. *P<0.05; **P<0.01; ***P<0.001. N = 3.

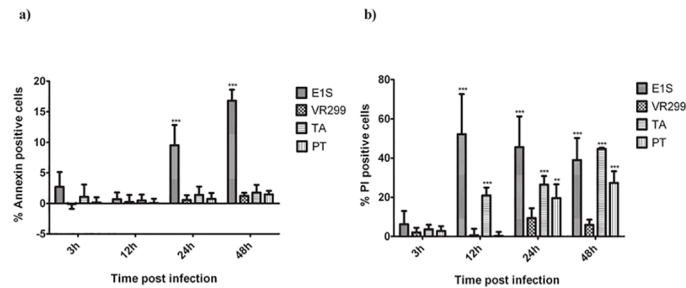doi:10.1371/journal.pone.0134173.g004

Fig 5. Cell death induced by different virus isolates of aquabirnaviruses. Flow cytometric analysis of FITC-Annexin (a) and PI (b) staining of cells infected with different isolates. The charts show the percentage of FITC-annexin stained cells (apoptosis) and PI stained cells (cells with compromised membranes) after subtracting the percentage of the FITC-annexin and PI positive percentages obtained from uninfected cells, respectively. The data represent the means of three independent experiments ±SD.

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Discussion

The main findings of this study can be summarized as follows: different aquabirnavirus isolates are associated with 1) differences in replication patterns over time; 2) consistent induction of IFNa relative to intracellular virus replication (apart from the PT strain); 3) differences in expression of anti-viral genes in infected cells after IFNa up-regulation. Furthermore, there was a remarkably low number of PI positive cells in VR-299 infected cells despite virus progeny being released to the supernatant.

E1S was propagated at a temperature (20°C) different from the other virus isolates (15°C), in line with optimal growth temperatures for the different strains. It is possible this influenced the findings since at higher temperatures, cells have higher metabolism and more rapid induction of immune responses, but for relevant comparison to be made, the viruses were propagated at their optimum temperatures. Nevertheless, the replication of aquabirnaviruses is sensitive to temperature and it is well established from previous studies that the replication of MABV and other isolates originally found at relatively high temperatures, is compromised at lower temperatures while the opposite is true for the cold water isolates [33,35–37]. A uniform temperature for all isolates would have therefore disadvantaged the replication/function of at least one of the isolates as previously demonstrated [33,37]. Temperature adaptation of aquabirnaviruses is likely one of the most prominent factors playing a role in determining the outcome of natural infections in different fish species. Similar findings have been reported for viruses like viral hemorrhagic septicemia virus [38]. Interestingly in this study, while E1S induced the highest IFNa gene expression, downstream responses like Mx1 and PKR were not induced suggesting that these isolates employ different strategies to evade host cell antiviral mechanisms.

CHSE cells were chosen to compare the different isolates in this study and this was done on the basis of 1) they were permissive to all isolates under study, 2) had previously been comparatively more extensively characterized than other salmonid cell lines, 3) availability. Although these cells can mount an innate response, it has been shown for IPNV that timing is important as further discussed below. The choice of cells is therefore unlikely to have contributed much to the outcome of this study. It is also noteworthy that CHSE cells themselves grow well at 20°C. At this temperature, the metabolism of the cells and likely the antivirus responses are higher than at 15°C. However, since some of the responses induced by E1S, e.g. Mx1 and PKR, were not more superior to other isolates, it is unlikely that temperature on cells per se plays an overarching effect on the growth of the viruses, rather, the different strains have adapted to the temperatures in their ecological niche.

E1S induced the quickest onset of CPE (Fig 1) coinciding with high virus multiplication in intracellular compartments and release to the supernatant compared to other isolates (Fig 2).

In line with this, there was early induction of necrosis (Fig 5) while the fraction of apoptotic cells was highest at the end of the replication cycle. This finding contrasts a previous study [20] which showed the opposite that apoptosis precedes necrosis. The reason for these contrasting findings is not easily explained.

The antiviral effects of IFNa and Mx1 against several fish viruses including IPNV have been previously demonstrated [39–42]. Whether cells are protected or not is an issue of timing [39,41]. IFNa expression followed increase in intracellular virus levels, apart from E1S, where IFN up-regulation was delayed relative to increase in virus titer (Figs 2a and 4a). The downstream responses to IFNa, i.e. induction of anti-viral genes (Mx1 and PKR) expression, was delayed and relatively down-played for all isolates except for VR-299 and the TA isolate, less pronounced for the latter. Despite the induction of antiviral genes, both VR-299 and TA produced progeny in intracellular compartments and with high release to the supernatant, i.e. more than 90% of virus released to the supernatant occurred between 24 and 48 hours post infection (Fig 2). The strategy of the PT isolate is puzzling; despite a vivid intracellular production of progeny between 12 and 48 hpi, there is literally no IFNa response and also no Mx1 or PKR responses seen in infected cells. Furthermore, CPE occurs late which coincides with PI-positive cells and release of virus to the supernatant. Obviously, the PT strain has an ability to evade the sensing mechanisms of the infected cell, which is surprising in light of the only difference between the TA and PT strains being 3 amino acid residues; P217T, T221A, and A247T [26]. The findings are in conformity with a previous report [42]. Further, the rapid induction of host cell protein shutdown, induction of CPE and increase in PI-positive cells by the TA isolate is probably a strategy for the virus to rapidly disperse from cell to cell prior to the onset of cellular, anti-viral mechanisms (Fig 4). These differences in mechanisms between identical isolates suggest that genetic identity is not prerequisite to functional similarities and therefore results of one isolate may not necessarily be extrapolated to others.

To overcome the drastic effect of IFN responses, many viruses employ different strategies to disrupt these responses. While some viruses disrupt Type I IFN induction, others antagonize the responses by disrupting the signaling cascade or, more specifically, target downstream genes and their products [43,44]. There are indications that aquatic birnaviruses, downplay IFN responses [42,45] but the underlying mechanisms remain obscure. In the present study, different IPNV isolates showed different abilities to induce IFNa or IFNa induced gene expression suggesting that different strategies are used by these isolates to overcome type I IFN responses. The weak ability of the Sp strains to induce IFNa and its downstream gene expression is remarkable and is in agreement with previous reports [42,45,46]. While the PT isolate suppressed the induction of these genes throughout the observation period, the TA isolate only delayed the induction until 48 hpi. The reason behind these findings is not clear although it is not unlikely that IFN responses may be used for the benefit of the virus, for example in the induction of necrosis/apoptosis and virus release. These findings bring to the fore the deficit in understanding the interplay between aquabirnaviruses and the IFN system and additional studies are required to understand the underlying strategies.

The percentage of PI-positive cells for the TA and E1S strains are in strong contrast to what was observed for VR-299, where they never exceeded 10% (Fig 5b). No doubt, the release of virus to the supernatant was delayed compared to the other strains (Fig 2b), but still the release of virus did not correlate with PI-positivity, particularly at 48 hpi. This contradiction is difficult to explain. One possibility is that VR299 is very efficient at inducing necrosis [19] so that lysed cells are discarded as debris when preparing cells for flow cytometry analysis. The possibility of an alternative release-mechanism has also to be considered but the general understanding is that IPN virus is released through induction of necrosis in the infected cell [19].

The differences in infection kinetics of the different isolates agree well with mortalities (or lack of) observed in previous studies where IPNV or MABV were used to infect salmonids or other species [22,23,47]. Collectively, these findings support the view that a distinction should be made between IPNV and MABV, where only isolates causing overt disease in salmonids should be referred to as IPNV [14] and vice-versa.

Contradicting reports of protein shutdown by IPNV in RTG-2 cells have previously been observed, with some suggesting induction [18] and others the lack of it [17]. Although CHSE cells were used in the present study instead of RTG-2 cells (in previous studies), the findings here in general support reports [18,32,42] that propose that different isolates of aquabirnaviruses induce different onsets/rates of host protein shut down. These results are however at variance with the findings of others [17] who found no shut down. While the same isolate VR299 was used in both studies, the results of the present study show that this virus induced the quickest and most pronounced protein shutdown. A possible explanation of the contradiction between these results is most likely methodological.

TNFα plays different roles with respect to viral infection. It may either inhibit or promote virus replication in infected cells [48,49]. In the current study, different isolates induced different levels of TNFα expression. Induction of TNFα expression by the E1S isolate has previously been studied using zebra fish embryonic cell line where it was suggested that it is involved in regulation of cell death [15,50]. In that study, the induction of TNFα started as early as 6 hpi and was detected up to 24 hpi, in contrast to results of the present study (Fig 4d). This discrepancy is likely due to different host cells used suggesting that differences in host cells should be taken into account when drawing conclusions about host-virus interaction. Following extensive cell death proceeding infection, most of the remaining cells become persistently infected but survive, forming a monolayer. It is therefore not unlikely that TNFα can promote cell survival and also play a role in virus persistence. The fact that PT in this study showed the least ability to induce CPE as well as the highest ability to induce TNFα at early time points (3 and 12 hpi, Fig 4d) supports this argument. Nevertheless, further studies are needed to clarify this.

Apoptosis refers to programmed cell death and is sometimes elevated during virus infections. It may also be induced by viruses to facilitate their release from cells [51], while in other cases it is induced as an attempt by the cells to commit suicide and therefore arrest the replication and spread of viruses [52]. Induction of apoptosis following infection with aquabirnaviruses has been a subject of several studies with conflicting results [19–21]. The findings of the present study demonstrate that only E1S induced significant apoptosis (p<0.001) (Fig 5a) while for the other strains, the number of apoptotic cells remained at a minimum.

In conclusion, we show that different isolates of aquabirnaviruses induce different patterns of host responses. These differences may be explained by adaptation to a wide variety of hosts.

Acknowledgments

Author Contributions

Conceived and designed the experiments: AAAG ØE SM. Performed the experiments: AAAG. Analyzed the data: AAAG ØE SM. Contributed reagents/materials/analysis tools: ØE. Wrote the paper: AAAG ØE SM.

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Article

A Piscine Birnavirus Induces Inhibition of Protein Synthesis in CHSE-214 Cells Primarily through the Induction of eIF2α Phosphorylation

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Abstract: Inhibition of protein synthesis represents one of the antiviral mechanisms employed by cells and it is also used by viruses for their own propagation. To what extent members of the *Birnaviridae* family employ such strategies is not well understood. Here we use a type-strain of the Aquabirnavirus, infectious pancreatic necrosis virus (IPNV), to investigate this phenomenon *in vitro*. CHSE-214 cells were infected with IPNV and at 3, 12, 24, and 48 hours post infection (hpi) before the cells were harvested and labeled with S^{35} methionine to assess protein synthesis. eIF2α phosphorylation was examined by Western blot while RT-qPCR was used to assess virus replication and the expression levels of IFN-α, Mx1 and PKR. Cellular responses to IPNV infection were assessed by DNA laddering, Caspase-3 assays and flow cytometry. The results show that the onset and kinetics of eIF2α phosphorylation was similar to that of protein synthesis inhibition as shown by metabolic labeling. Increased virus replication and virus protein formation was observed by 12 hpi, peaking at 24 hpi. Apoptosis was induced in a small fraction (1−2%) of IPNV-infected CHSE cells from 24 hpi while necrotic/late apoptotic cells increased from 10% by 24 hpi to 59% at 48 hpi, as shown by flow cytometry. These results were in accordance with a small decline in cell viability by 24hpi, dropping below 50% by 48 hpi. IPNV induced IFN-α mRNA upregulation by 24 hpi while no change was observed in the expression of Mx1 and PKR mRNA. Collectively, these findings show that IPNV induces inhibition of protein synthesis in CHSE cells through phosphorylation of eIF2α with minimal

involvement of apoptosis. The anticipation is that protein inhibition is used by the virus to evade the host innate antiviral responses.

Keywords: Birnavirus; infectious pancreatic necrosis virus; eIF2α; inhibition of protein synthesis; Mx; apoptosis

1. Introduction

Cells employ inhibition of protein synthesis as a defensive mechanism in response to virus invasion, the aim being to limit the production of virus progeny and consequently arrest the spread within the organism. In higher vertebrates, there are mainly three well-known mechanisms by which inhibition of protein synthesis is induced during virus infections: (1) through the activation of the interferon inducible, dsRNA-activated protein kinase R (PKR); (2) through the activation of the PKR-like endoplasmic reticulum (ER) kinase (PERK); and 3) through factors associated with the induction of apoptosis. PKR is activated and auto-phosphorylated upon recognition and binding of dsRNA to its binding motif in the N terminus [1]. Phosphorylated PKR in turn phosphorylates the eukaryotic initiation factor 2-alpha (eIF2α). eIF2α phosphorylation blocks eIF2B-mediated GDP−GTP exchange preventing the formation of GTP–eIF2–tRNAi^{Met} ternary complex, which is crucial for translation initiation, and results in translation inhibition [2]. eIF2α phosphorylation can also be induced through PERK which is activated due to the accumulation of unfolded protein in the ER lumen, as a result of high virus replication, leading to the initiation of what is known as the unfolded protein response (UPR) [3]. Apoptosis on the other hand is induced by many innate and adaptive antiviral mechanisms [4], thereby leading to the activation of intracellular caspases during the early stages of apoptosis. Some of the caspases executing apoptosis can target components of the translation machinery for proteolysis [5].

Viruses, as obligatory pathogens, depend on the host machinery to produce their own proteins. They have evolved different strategies of taking advantage of the host machinery of protein synthesis that may or may not be associated with inhibition of protein synthesis. Picorna- and rotaviruses break translational control by targeting and disrupting different components of the translational apparatus [6–8]. As a result, the cells lose control of the translation machinery leading to the inhibition of host protein synthesis [9,10]. Other viruses such as adeno- and herpesviruses disrupt the regulatory pathway that inhibits protein synthesis in response to virus infection through PKR activation [11,12]. For birnavirus infections, there are no studies that clearly demonstrate how inhibition of protein synthesis occurs and to whose benefit.

IPNV is a type species of the genus Aquabirnavirus. It is an icosahedral, non-enveloped, double-stranded RNA virus consisting of two segments [13]. The first segment (A) is 3,092 bp long and contains two overlapping open reading frames (ORFs): a short one encoding a non-structural protein VP5 [14,15] and a long ORF encoding structural proteins VP2 and VP3 as well as a non-structural protein VP4 [16,17]. Segment B is 2,784 bp long and contains only one ORF encoding the viral polymerase VP1 [18]. Early studies using a piscine birnavirus infectious pancreatic necrosis virus (IPNV) showed that the virus was not associated with inhibition of protein synthesis although it suppressed DNA synthesis in permissive host cells [19,20]. In another study characterizing eIF2α and its response to endoplasmic reticulum stress, IPNV phosphorylated eIF2α [21], suggesting that inhibition of protein synthesis is in fact induced

although this was not the focus of the study. In a recent study, Chen *et al.* showed that a low virulent recombinant strain of IPNV is able to induce inhibition of protein synthesis in RTG-2 cells [22]. In the present study, we demonstrate not only that this virus induces inhibition of protein synthesis in CHSE-214 cells but also that the virus probably uses this mechanism to its advantage.

2. Materials and Methods

2.1. Cell Lines

Chinook salmon embryonic (CHSE-214) [23] and Asian Grouper strain K (AGK) [24] cells were maintained in L-15 media with Glutamax® (Gibco, Carlsbad, CA, USA) supplemented with 5% FBS (Sigma Aldrich, St. Louis, MO, USA). For maintenance, CHSE-214 cells were grown at 20 °C while AGK cells were kept at 28 °C.

2.2. Virus Propagation

A recombinant IPN virus (rNVI-15R^b) previously produced by reverse genetics [25] was used. In order to obtain adequate amounts of virus for use in the inhibition of protein synthesis experiment, the virus was first inoculated into 70%–80% confluent AGK cells followed by incubation at 15 °C until full CPE. The supernatant containing the virus was then harvested and clarified by centrifugation at 2500 rpm for 10 min. The concentration of the virus was estimated by titration in 96-well plates (Falcon, Bedford, MA, USA) containing 80%–90% confluent CHSE cells.

2.3. Virus Infection and Metabolic Labeling

Six-well plates containing approximately 90% confluent CHSE cells were used. Inoculation of cells in wells was done sequentially, one well for each time point to yield cells infected either for 3, 12, 24 or 48 h by the time of sampling. The cells were infected at a multiplicity of infection (MOI) of 20 with the purpose of obtaining one-cycle infection kinetics, and the experiment was repeated four times.

Level of protein synthesis was evaluated by monitoring S^{35} methionine incorporation into proteins. Cells were washed 3× with PBS followed by incubation with Methionine, Lysine and L-glutamine free Dulbecco's modified Eagle's medium (sigma Aldrich) containing 20μCi/mL S^{35} Methionine (Hartmann analytic, Braunschweig, Germany), 1% L-glutamine (Sigma Aldrich) and 2% FBS for 30 min. Thereafter, the cells were washed once with PBS, lysed using 250 μL Cell M lysing reagent (Sigma Aldrich) and then slowly agitated for 15 min. Cells were then scraped from the wells and transferred to 1.5 mL Eppendorf tubes together with the supernatants. Lysates were centrifuged at 13,000 rpm for 5 min to remove the cell debris and nucleus. Finally, the supernatants were transferred to new Eppendorf tubes and kept at −80 °C until required.

To evaluate the level of protein synthesis, cell lysates were subjected to gel electrophoresis. 10 μg of total cell proteins from each sample was applied to a Nupage mini gel (Invitrogen, Carlsbad, CA, USA) and subjected to electrophoresis at 200V. The separated proteins were transferred from the gel to a PVDF membrane using a semi-dry blotter (Biorad, Hercules, CA, USA). The membrane was incubated in a storage phosphor cassette overnight. Finally, radioactivity was detected using the Typhoon (GE Healthcare, Piscataway, NJ, USA).

2.4. Western Blot Analysis

Following radioactivity detection, the membranes were rehydrated in Tris buffer saline with 0.5% Tween 20 (0.5% TBST). Of the non-fat dry milk (Biorad), 5% was prepared in 0.1% TBST and was used to block non-specific reactions. The membrane was then probed by using antibodies against actin (Sigma Aldrich), eIF2α and phosphorylated eIF2α, ser51 (peIF2α) (Cell Signaling, Beverly, MA, USA) while aliquots from the same sample were used in another blot to detect virus protein synthesis using K95 polyclonal rabbit anti-IPNV [26]. This was followed by incubation with HRP-labeled secondary antibodies. The signal was developed using SuperSignal West Dura Extended Duration Substrate (Pierce) and detected using the Typhoon (GE healthcare). After the first detection, the membrane was washed twice with 0.1% TBST, stripped using Restore™ Plus buffer (Pierce, Rockford, IL, USA), washed twice as above, and re-probed with a new primary antibody and developed as already described.

2.5. Cell Viability Assay

Cell viability at different times post IPNV infection was determined by using CellTiter 96® AQueous One Solution Assay (Promega, Madison, WI, USA). The assay measures the reduction of a tetrazolium compound by the cells into a colored formazan product, which is directly correlated to the cell number. Confluent CHSE-214 cells seeded in 96-well plates (Corning, Life Science, Lowell, CA, USA) were infected with IPNV as described above or left uninfected. Cells were kept at 15 °C during the infection period. At 3, 12, 24 and 48 hpi, 20 μL of the CellTiter 96® AQueous One Solution reagent was added to the cells. Cells were incubated at 20 °C for 6 h and the absorbance at 490 nm was measured using a GENios microplate reader (Tecan, Männedorf, Switzerland). The viability was calculated and expressed as a percentage of the OD_{490} values obtained from the corresponding controls.

2.6. Assessment of Apoptosis by DNA Fragmentation

The fragmentation of DNA was assessed by using Apoptotic DNA Ladder Kit (Roche, Basel, Switzerland). Six-well plates containing confluent CHSE cells were used for this purpose. Cells were infected in quadruplicates with IPNV as described above while the remaining two wells were left uninfected as negative controls. Infected cells were sampled at 3, 12, 24 and 48 h post infection, while untreated cells were sampled at 48 h post infection. Cells were harvested by lysis using 400 μL binding/lysis buffer diluted v/v in PBS. Following incubation for 10 min, 100 μL isopropanol was added and mixed with the sample. The samples were then loaded in polypropylene tubes containing two layers of glass fiber fleece, washed using washing buffer, and finally eluted in 200 μL elution buffer. Samples were treated with 2 μg/mL DNase free RNase (Roche) before being analyzed by gel electrophoresis.

2.7. Caspase 3 Assay

The activity of caspase 3 in the cell lysates was assessed by using the Caspase-Glo® 3/7 assay kit (Promega) following the protocol as described by the manufacturer. Briefly, CHSE-214 cells were seeded in white-walled 96-well plates (Corning) to a final density of 10^4 cells/well and incubated for about 14 h. Cells were then infected with IPNV in triplicates sequentially as already described above. Duplicates of uninfected cells or cells treated with 2.5 μM staurosporine for 3, 12, 24 and 48 h were

included as negative and positive controls, respectively. At the time of sampling, the volume of media in the wells was adjusted to 50 µL and equal volumes of Caspase-Glo® 3/7 were added to each well. Cells were incubated at room temperature for 1 hour prior to bioluminescence reading (490 nm) using a GENios microplate reader (Tecan). The results were calculated by subtracting the mean values obtained from the control samples from the readings obtained from each well. The whole experiment was repeated twice.

2.8. Assessment of Apoptosis by Flow Cytometry

Six-well plates containing confluent CHSE cells were used in a similar setup as the one described in the section above. In addition, three parallel wells in a separate plate were treated with 2.5 µM staurosporine for 12, 24 or 48 h as positive controls of apoptosis. During each sampling, the supernatant was transferred to a 5 mL polystyrene round-bottomed tube (BD Biosciences, San Jose, CA, USA) while adherent cells were washed twice with PBS prior to trypsinization. Trypsinization was done for 5 min following which the reaction was stopped by adding fresh media. The trypsinized cells were then pooled with the original supernatant in the 5 mL polystyrene tubes. The cells were pelleted by centrifugation at $300\times g$ for 10 min. The supernatant was then removed and cells were re-suspended in 100 µL Hepes buffer containing 2 µL fluorescein conjugated annexin-V staining reagent (Annexin-V-FLUOS Staining Kit, Roche). After incubating for 30 min, the volume was adjusted to 300 µL. To differentiate between apoptotic and necrotic cells, membrane permeability was assessed by adding propidium iodide (PI) (Sigma Aldrich), just before the analysis to a final concentration of 8 µg/mL. Flow cytometry was performed for 30,000 events using a BD FACSARIA™ cell sorter (BD) while data analysis was performed using BD FACS DiVa Software, version 5.0.2 (BD).

The following parameters were measured to identify the apoptotic cells: (1) the area pulse of forward light scatter (FSC-A) *versus* side scatter (SSC-A), and (2) fluorescent intensities of FITC (filter 530/30) and PI (filter 630/30) upon excitation with 20 mW 488 nm laser. Cell aggregates were identified and excluded by using the width pulse of FSC-A *versus* area width of SSC-A. The whole experiment was repeated four times.

2.9. Quantitative Real-Time PCR Analysis

To assess the level of replication of IPNV as well as the expression of IFNα, Mx1 and PKR, real-time RT-qPCR was used. Cells were infected with IPNV as described above with the exception that this time three parallels were used per treatment. In addition, one parallel of cells was treated with IFNα for 4 days, as described previously, [27] as a control.

Total RNA was isolated by using the RNeasy Plus minikit (Qiagen, Hilden, Germany) according the manufacturer's instructions and the concentration of RNA was determined by using the Nanodrop ND1000 (NanoDrop technologies, Wilmington, DE, USA). Of total RNA, 400 ng from each sample were used to synthesize cDNA using a Transcriptor first-strand cDNA synthesis kit (Roche) according to the manufacturer's instructions. The cDNA was diluted five times and stored at −20 °C until required.

Quantitative PCR was performed in 96-well plates using the LightCycler 480 system (Roche). For each reaction, 2 µL cDNA was mixed with 10 pmol gene-specific primers and 10 µL LightCycler 480

SYBR green I master mix (Roche). The final concentration was adjusted to 20 μL using RNase-free water. The sequences of primers used in the reactions are provided in Table 1.

The cycling conditions for the PCR reactions were as follows: denaturation 94 °C for 10 s; annealing 60 °C for 10 s; elongation 72 °C for 10 s. The results were analyzed by the $\Delta\Delta CT$ relative quantification approach [28] using β-actin as reference gene. Graphs were drawn with the help of GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Table 1. Primer sequences used for real-time PCR

Name	Primer sequence (5'-3')	Genbank accession no.
Mx-Forward	TGCAACCACAGAGGCTTTGAA	U66475
Mx-Reverse	GGCTTGGTCAGGATGCCTAAT	
B actin Forward	CCAGTCCTGCTCACTGAGGC	AF012125
B actin Reverse	GGTCTCAAACATGATCTGGGTCA	
PKR Forward	TGAACACAGCCAGAAGAACAA	EF523422.1
PKR Reverse	GACTACCGCCACATAACTCCA	
IPNv Forward	CAACAGGGTTCGACAAACCATAC	
IPNv Reverse	TTGACGATGTCGGCGTTTC	
IFNα Forward	TGGGAGGAGATATCACAAAGC	NM_001123570.1
IFNα Reverse	TCCCAGGTGACAGATTTCAT	

2.10. Statistical Analysis

For the caspase assay, two-way analysis of variance was used to test for differences between staurosporine-treated and infected cells followed by the Bonferroni test to compare the mean of each treatment to the mean of the RLU values obtained at 3 h. An Anova test was performed to assess reduction in protein synthesis and viability with a Dunnett *post hoc* test using GraphPad Prism 5.0 (GraphPad Software Inc.).

3. Results

3.1. Virus Replication in CHSE-214 Cells

Initially, we wanted to establish a system by which virus replication and its effect on cellular responses could be monitored over a short period of time thereby allowing, us to assess the effect of virus infection from the early replication stages until the endpoint in cell culture. We found that infection with 20 pfu/cell results in appearance of evident CPE by 48 h from the time of infection (Figure 1).
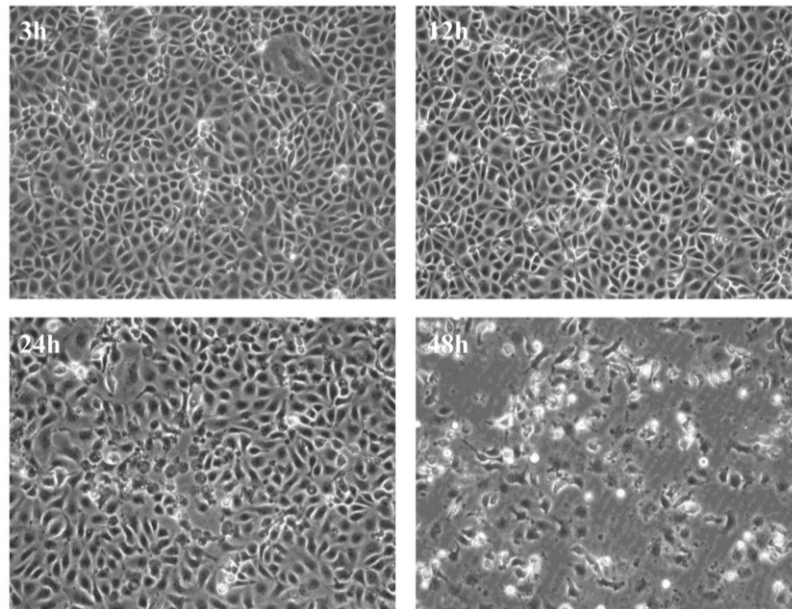
Figure 1. Cytopathic effects in CHSE cells**.** Development of cytopathic effect in CHSE-214 cells infected with recombinant strain rNVI-15R[b] of infectious pancreatic necrosis virus.

3.2. Virus Replication Results in Inhibition of Protein Synthesis

After establishing our infection model we examined protein synthesis in CHSE cells infected with IPNV at different times post infection (p.i.). At three hours p.i. (hpi), no difference in protein synthesis was observed between infected cells and uninfected controls (Figure 2a). At 12 and 24 hpi, however, a progressive reduction in protein synthesis was observed in infected cells, initially with a moderate reduction at 12 h (80% protein synthesis compared to uninfected controls; $p < 0.01$) followed by a marked reduction at 24 hpi, 65% reduction compared to uninfected controls (Figure 2b, $p < 0.001$). The loss of cell viability was 6% ($p < 0.05$) and 9% ($p < 0.01$), respectively, at these time points (Figure 2c), showing a pronounced reduction in protein synthesis with marginal reduction in viability.

3.3. Virus Replication Increases despite Inhibition of Host Protein Synthesis at Early Time Post Infection

Next we wanted to investigate whether inhibition of protein synthesis has any effect on virus replication. We first examined IPNV replication at different stages of infection by real-time RT-qPCR using primers specific for the virus VP2 protein. The results show that the viral mRNA levels increased rapidly in CHSE cells, peaking at 24 h (Figure 3a). Since production of new viruses can be inhibited at the translation level, we used Western blot as an additional tool to monitor the production of virus proteins. Our results showed that inhibition of protein synthesis had no effect on production of virus proteins (Figure 3b).
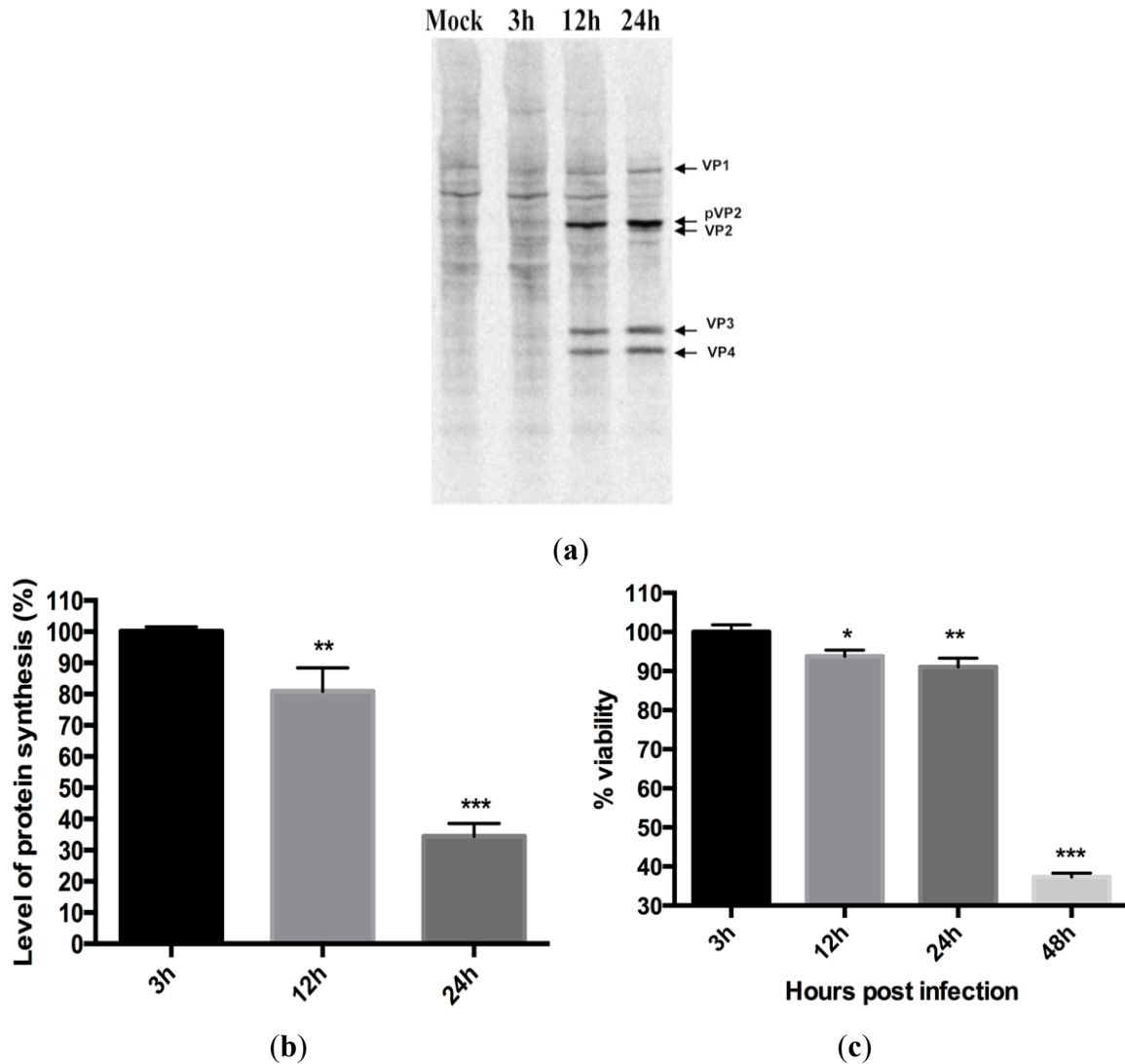
(a)

(b)

(c)

Figure 2. Infectious pancreatic necrosis virus induced inhibition of protein synthesis in CHSE 214 cells. Cells were first infected and, at indicated times, proteins synthesized were labeled using S^{35} Methionine and then harvested. (**a**) Prepared lysates were subjected to SDS-PAGE, blotted onto a PVDF membrane and autoradiographed in storage phosphor cassettes before analysis using the Typhoon. The numbers represent time in hours post infection; (**b**) Levels of protein synthesis expressed as percentages of the mock-infected cells after measurement of mean density protein amounts of three different bands using ImageQuant software (GE Healthcare). The results are representative of four independent experiments. *$p < 0.05$; **$p < 0.01$; (**c**) Cell viability post IPNV infection. Cells were infected with IPNV and cell viability was assayed at the indicated time post infection using CellTiter 96® AQueous One Solution assay (promega). The percent viability was calculated as described in the methodology section. Bars represent the average of 8 measurements taken from two independent infections ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

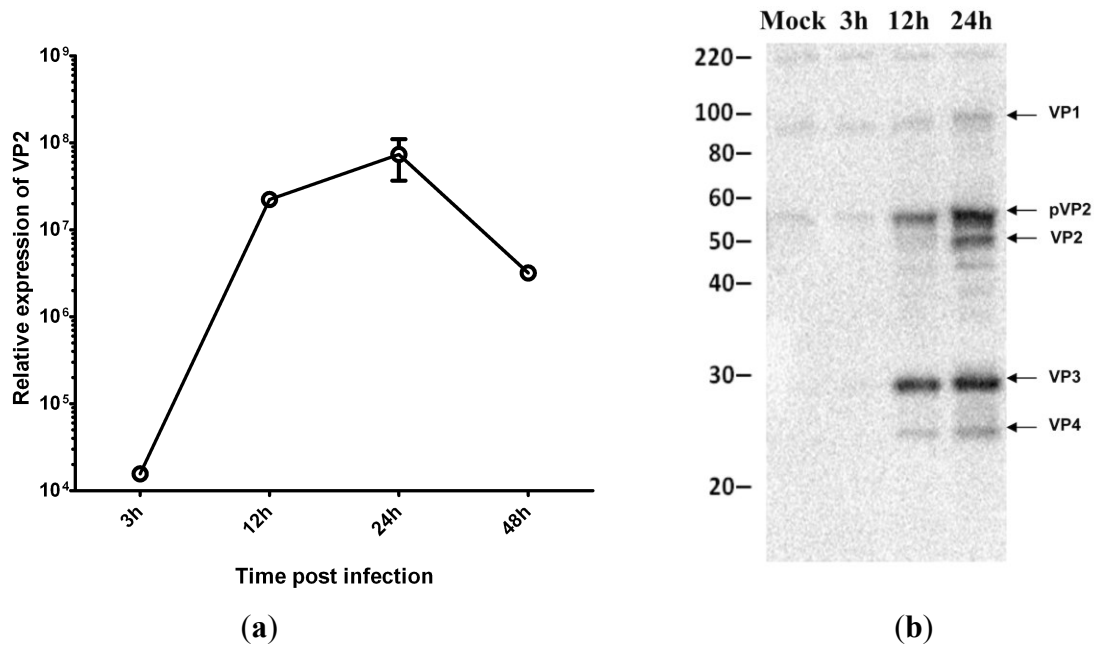**Figure 3.** IPNV replication in virus-infected CHSE cells. (**a**) Relative expression of infectious pancreatic necrosis virus mRNA at different time post infection (n=3); (**b**) Western blot analysis for IPNV proteins synthesis at different times post infection.

3.4. IPNV Infection of CHSE Cells Results in eIF2α Phosphorylation

Phosphorylation of eIF2α by different kinases is an important mechanism of protein synthesis regulation. In the present study, the onset of eIF2α phosphorylation was 12 hpi (Figure 4) and coincided with that of protein synthesis inhibition (Figure 2). At 24 h, the intensity of eIF2α phosphorylation increased (Figure 4) while there were not enough proteins left from the infected cells at 48 hpi to allow assessment.
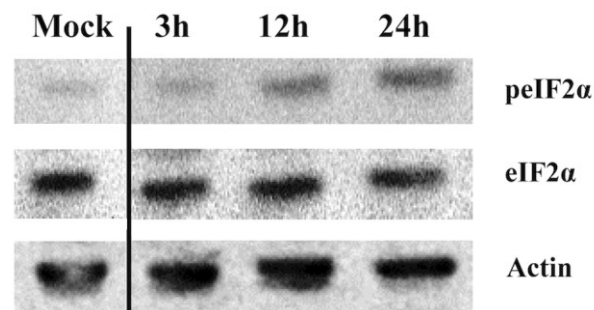

Figure 4. eIF2α phosphorylation in IPNV-infected CHSE-214 cells. CHSE-214 cells were infected with IPNV, cells were lysed at the indicated time points, and lysates were subjected to Western blot analysis using rabbit polyclonal antibodies against the phosphorylated form of eIF2α (Invitrogen). The blot is representative of a minimum of three independent observations. The numbers represent time in hours post infection.

3.5. The Effect of Protein Synthesis Inhibition on Type I IFN Response

Inhibition of protein synthesis is a global effect at cellular level and thus affects IFNα expression *per se* and also downstream effector responses. Antibodies to salmon IFNα are not available to us and therefore we examined this effect by measuring IFNα mRNA levels. To estimate the production of IFNα at the protein level, an indirect approach using Mx1 and PKR mRNA expression, which comes down stream of IFNα signaling, was used. The results show that while IFNα mRNA gradually increased following infection (Figure 5a) and peaking at 24 hpi, Mx1 or PKR expression was not induced at any of the time points examined (3−48 hpi, Figure 5b,c). Our interpretation is that IPNV signals off a response in the cell that interferes with IFNα responses, either through a direct impact on IFNα levels or indirectly through downstream effects of IFNα.
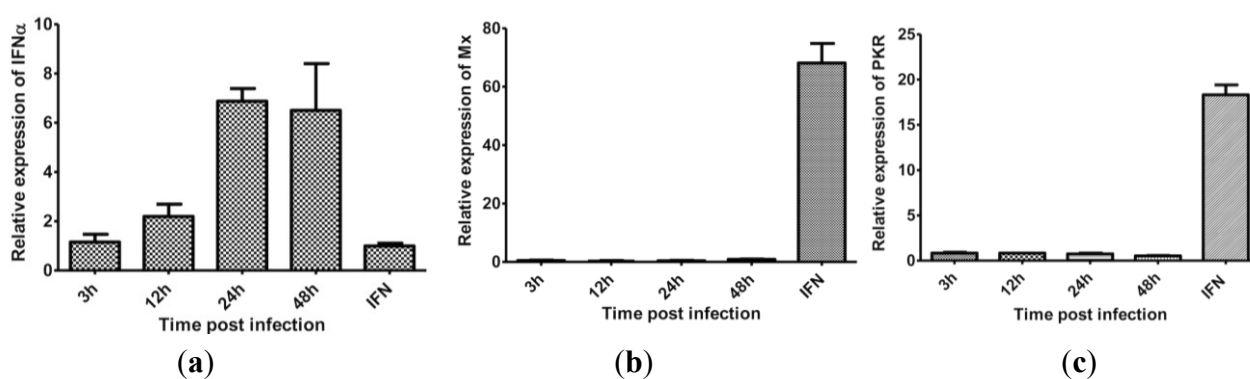

Figure 5. IFNα and Mx1 mRNA expression in IPNV-infected CHSE-214 cells. CHSE-214 cells were infected with IPNV, harvested at indicated time points, and IFNα (**a**), Mx1 (**b**) and PKR (**c**) mRNA expression were measured by real-time PCR. IFN is cells treated with recombinant IFNα for 4 days and used as positive control (n = 3).

3.6. IPNV Induces More Necrosis than Apoptosis at Early Time Post Infection

Previous studies have shown that IPNV strains of serotype Ab induce apoptosis at early time post infection [29]. Since apoptotic cells exhibit decreased protein synthesis, this could serve as an explanation to the reduced protein synthesis referred above and therefore we needed to understand what role apoptosis played with regard to reduced protein synthesis. First, a DNA laddering assay was used. At 3 and 12 hpi, no DNA fragmentation was observed; however, at 24 and 48 hpi, fragmentation of DNA was visible (Figure 6), thus suggesting involvement of apoptosis. Since apoptosis-like DNA laddering is known to occur also as a result of necrosis [30,31], DNA laddering alone cannot fully distinguish between apoptosis and necrosis. The caspase 3 assay and flow cytometry were therefore used to validate these findings. In IPNV-infected cells, a significant increase in caspase 3 activity was observed both at 24 ($p < 0.05$) and 48 hpi ($p < 0.001$) (Figure 7), in agreement with the DNA laddering (Figure 6). Caspase 3 activity in staurosporine-treated cells (positive controls) was observed from 12 h post treatment and onwards (Figure 7). For flow cytometry, we used double-staining for annexin V (AV) and Propidium Iodide (PI) to represent apoptosis and necrosis, respectively. Cells that are AV$^+$/PI$^-$ are apoptotic while AV$^+$/PI$^+$ or AV$^-$/PI$^+$ are necrotic [32]. IPNV-infected cells showed a marked increase for AV$^+$/PI$^+$ staining from 12 h onwards (Figure 8; from 10 to 59% double-positive cells), demonstrating

that the cells' membrane integrity was compromised. On the other hand, AV^+/PI^- stained cells remained low, and increased from 1 to 2% by 24 h and 1.7% by 48 h.
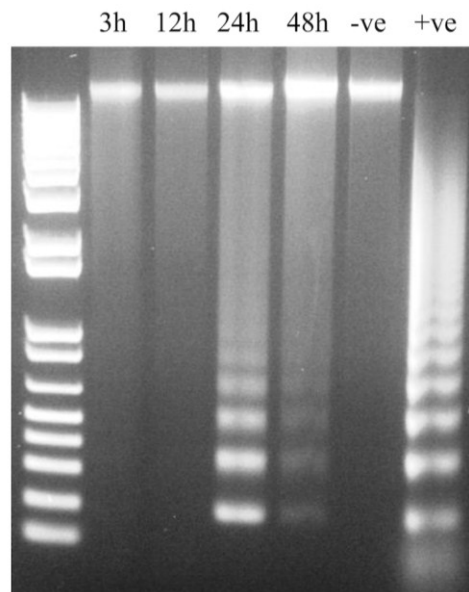

Figure 6. DNA laddering in IPNV-infected CHSE-214 cell lines. Cells were infected and harvested at the indicated time points. DNA was extracted using the Apoptotic DNA Ladder Kit (Roche) before being analyzed by gel electrophoresis. The result is representative of three independent experiments. −ve: uninfected cells; +ve: positive control.
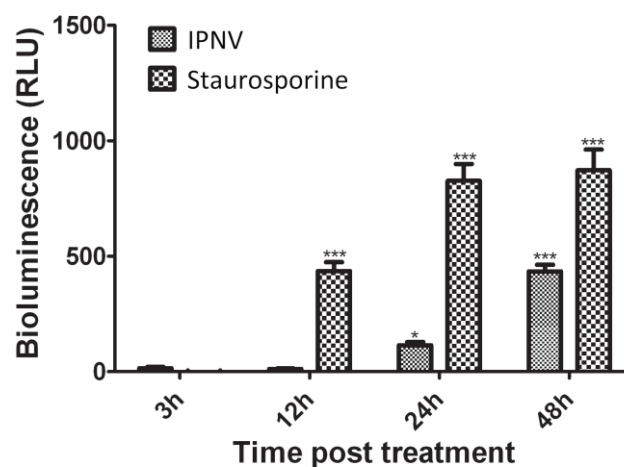

Figure 7. Caspase 3 activity in cells infected with infectious pancreatic necrosis virus. Cells were infected with IPNV or treated with 2.5 µM staurosporine (positive control) for the indicated periods. Uninfected and untreated cells were used as negative controls. Caspase 3 activity was assessed by measuring the bioluminescence resulting from cleavage of a luminogenic substrate using Caspase-Glo® 3/7 assay kit (promega). The bars represent the relative luminescence units after subtracting the mean values obtained from the uninfected/untreated cells. Bars represent means of data obtained from two independent experiments each conducted using three parallels ± S.E.M. Statistical significance compared to cells treated at 3 h is indicated by asterisks; * = $p < 0.05$; *** = $p < 0.001$.
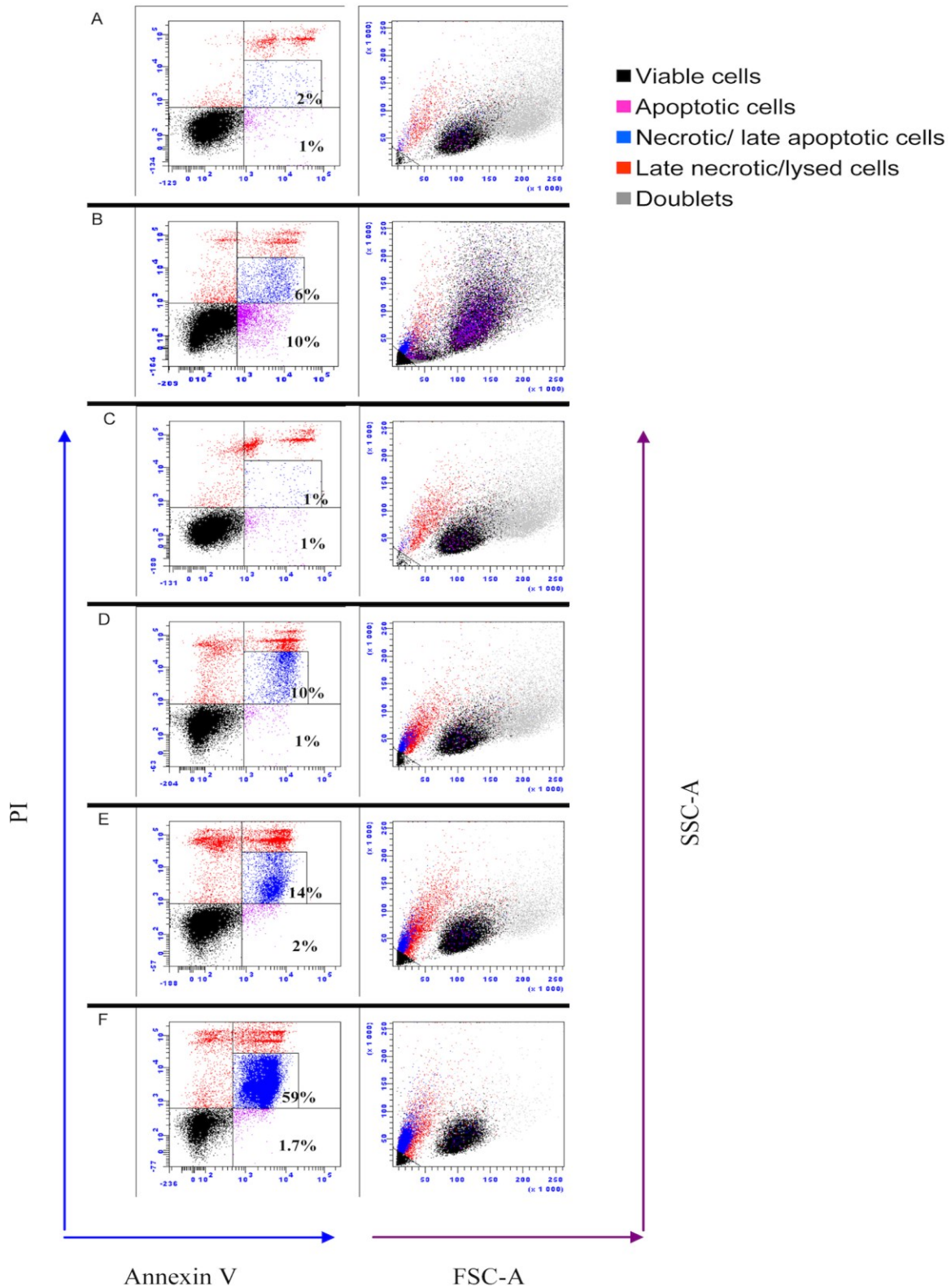
Figure 8. Flow cytometric analysis of infectious pancreatic necrosis virus-infected CHSE-214 cells. Cells were stained with annexin V and propidium iodide (PI) prior to analysis at prescribed time points. **A** = non infected cells; **B** = cells treated with staurosporine for 48 h to induce apoptosis; **C-F** = IPNV-infected cells for 3, 12, 24 and 48 h, respectively. Necrotic cells (blue) have low forward (FSC-A) and side scatter (SSC-A) values. Results provided are representative of at least four independent observations.

4. Discussion

Here we show for the first time that infectious pancreatic necrosis virus induces inhibition of protein synthesis in the permissive cell line, CHSE. The inhibition involves phosphorylation of eIF2α (from 12 hpi and onwards). While apoptosis plays less of a role in protein synthesis inhibition, and DNA laddering is seen by 24 hpi, the highest percentage of apoptotic by 24 hpi is 2% by flow cytometry. Despite cellular protein shutdown, virus replication peaks at 24 hpi and a drop in cell viability is seen earliest at 48 hpi, coinciding with the loss of membrane integrity (by flow cytometry). Because inhibition of protein synthesis did not hinder virus production, we propose this is a strategy of the virus to circumvent IFNα-induced cellular, antiviral mechanisms and to induce cell lysis in order to facilitate its release from infected cells.

Phosphorylation of eIF2α and the subsequent inhibition of protein synthesis are important host defensive mechanisms to limit the replication of RNA viruses [33]. eIF2α phosphorylation following IPNV infection (Figure 4) is consistent with a previous report [21] and results in inhibition of protein synthesis in CHSE-214 cells. Two kinases have been shown to play roles in eIF2α phosphorylation during virus infections in higher vertebrates, namely the PKR and PERK [1,3]. Fish possess an additional kinase named Z-DNA binding protein kinase (PKZ) and Atlantic salmon PKZ has been shown to phosphorylate eIF2α in response to Z-DNA, but its activation by Z-dsRNA has not yet been demonstrated [34]. Antibodies against PKR, PERK, or their phosphorylated forms are not available for salmonids at the moment. It was therefore difficult for us to further study and demonstrate the mechanisms by which eIF2α is induced. Both PKR and PERK have been suggested to be involved in inducing cell death following IPNV infection [35]. Indeed, high virus replication and protein synthesis as observed in this study and accumulation of folded proteins may have led to PERK activation as a consequence of ER stress followed by eIF2α phosphorylation. However, ER-stress response has only been shown to occur during infection with enveloped viruses [36]. PKR, on the other hand, is found in the cytoplasm where IPNV replication occurs. In fact, IPN viral RNA is exposed in the cytoplasm and can be detected in infected cells during replication through various receptors [37]. Despite PKR not being found as upregulated, basal levels of PKR could be responsible for eIF2α phosphorylation during IPNV infection. However, additional studies should be performed to elucidate this in detail.

Contradicting reports have been published regarding the occurrence of apoptosis following IPNV infections [29,38–41]. Since eIF2α has previously been implicated in decreased protein synthesis of apoptotic cells [42], we needed to understand to what extent apoptosis *per se* contributed to protein shutdown. All apoptosis assays used in the present study, namely DNA laddering, caspase 3 and flow cytometry, showed that apoptosis occurred from 24 h onwards (Figures 6, 7 and 8), but the fraction of apoptotic cells is very small, based on flow cytometry (Figure 8), which is consistent with our earlier results [41]. Intriguingly, loss of membrane integrity as detected by PI staining (Figure 8) appears earlier than apoptosis, similar to the findings of some [38] but at variance with results of others [29,39,41]. The differences in apoptosis/necrosis profiles between our findings and other researchers [29,38–41] demonstrate that different IPNV isolates are associated with variable apoptotic/necrotic characteristics. Care should therefore be taken not to generalize results beyond isolates or geno-groups.

Perforation of cell membranes and the subsequent changes in membrane permeability in virus-infected cells may occur at early stages due to virus entry, during virus maturation or late stages

of virus replication resulting in cell lysis and virus release [43]. It has also been suggested that permeability changes may be essential for cells to switch from synthesis of cell proteins to virus proteins [43]. Forming of pores during the entry process is sensitive to the concentration of extracellular Ca^{2+} and can be inhibited by higher concentrations [43]. In the present study, changes in membrane permeability started at middle stages of virus replication (12 hpi) and reached the peak at late stages of virus replication with the onset of cell lysis. No change in membrane permeability was detected at early stages. This suggests that changes in membrane permeability during IPNV infection may be essential for virus replication and/or release. This is similar to what has been found for infectious bursal disease (IBDV), another member of the family *Birnaviridae* [44] where no virus was rescued after disrupting the pore forming mechanism by mutagenesis. Pore formation by IBDV could also be inhibited by increasing Ca^{2+} concentrations. The ability of IPNV to perforate cell membranes is yet to be demonstrated. However, high extracellular Ca^{2+} was shown to decrease plaque formation in CHSE cells while the opposite was observed when extracellular Ca^{2+} was decreased or with Ca^{2+} blockers [45].

To overcome the effect of protein shutdown, several species of RNA viruses have evolved a cap-independent (eIF2α-independent) mechanism of initiation of translation. For example, viruses belonging to the family *Picornaviridae* possess internal ribosome entry sites (IRES) that enable them to preferentially translate their proteins in a cap-independent manner [46]. Hantaviruses have a unique cap-independent mechanism by which they utilize their nucleocapsid protein to replace all or part of the cap-binding complex [47]. For the *Birnaviridae* family, there is limited information on the initiation of translation and replication and it is not known if they possess a ribosome entry site (IRES) of normal (*i.e.*, >300 nt) length or another mechanism that is needed to recruit host cell-encoded initiation factors [48]. We found that eIF2α phosphorylation was induced as early as 12 hpi and the phosphorylation levels continued to increase for the duration of the infection cycle accompanied by marked inhibition of cellular protein synthesis (Figures 2 and 7). On the other hand, virus protein synthesis increased during the time of host protein inhibition (Figure 3b). What mechanisms are used by IPNV to initiate translation under these circumstances remains unresolved and additional studies are needed to understand this in more detail.

Inhibition of protein synthesis is a double-edged sword; whereas it is an important defensive tool for the host cell [49], it nevertheless limits the antiviral effects downstream of interferon responses, and can thus be a survival strategy for the virus [50]. Previous studies show that IPNV infections do not suppress IFN production but interfere with downstream signaling [51]. Skjesol *et al.* [52] confirmed this and further demonstrated that IPNV infections are able to attenuate IFN induced responses even when the cells were pretreated with IFN for 4 h. Herein we show that inhibition of protein synthesis was induced while virus replication was at its highest (Figures 2 and 3) and with a parallel increase of IFNα mRNA expression (Figure 8a). Regardless, neither Mx1 nor PKR mRNA was induced and from this we interpret the results to imply that IPNV induced inhibition of protein synthesis may serve as a strategy for the virus to evade or attenuate interferon-induced anti-viral responses and/or to facilitate the release of viral progeny.

Inhibition of protein synthesis post IPNV infection found here is in contrast with previous reports where no protein inhibition was observed following infection of RTG cells although host DNA synthesis was inhibited [19,20,53]. The reasons for this are not clear but there are several differences between the present study and the previous ones. We used CHSE cells instead of RTG-2 cells but we consider it

unlikely that this would account for the particular differences observed since the isolate used has also the ability to inhibit protein synthesis in RTG-2 cells [22]. Moreover, the virus isolates used in previous studies are different from ours; they used the so-called Dry Mills strain [19,20], which is closely related to AF343571 and isolate VR-299 [53]. Both isolates belong to the North American West Buxton (WB) serotype (serotype A1); we used an Sp serotype (serotype A2) that is antigenically and pathogenically distant from the WB isolates [54] in the present study. In addition to this, there are differences in the multiplicity of infections used or the time of sampling. While Nicholson and Lothrop used very high MOI, 100TCID_{50}/Cell [19,20], Dobos used similar MOI to ours, 20 PFU/cell, but the last sample was obtained at 12 hpi which in our study was found to be the start of protein inhibition [53].

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Author Contributions

AAG contributed to the planning of the research experiment, carried out the work in the lab, analyzed the data and contributed to the writing of the manuscript; SM contributed to the planning of the research experiment, participated in analyzing the data, and contributed to the writing of the manuscript; ØE contributed to the planning of the research experiment, participated in interpreting the data, contributed to the writing of the manuscript, and provided the funding.

Conflict of Interest

The authors declare no conflict of interest.

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III

PKR ACTIVATION FAVORS INFECTIOUS PANCREATIC NECROSIS VIRUS REPLICATION IN INFECTED CELLS

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ABSTRACT

PKR is a Type I IFN stimulated gene that plays important biological and immunological functions. In viral infections in general PKR inhibits or promotes viral replication, but PKR-IPNV interaction has not been studied previously. We investigated the involvement of PKR during IPNV infection using a custom-made rabbit antiserum and the PKR inhibitor C16. Reactivity of the antiserum to PKR in CHSE-214 cells was confirmed after IFNα treatment giving an increased protein level. IPNV infection alone did not give increased PKR levels by western blot, while pre-treatment with PKR inhibitor before IPNV infection gave decreased eIF2α phosphorylation. This suggests that PKR, despite not being upregulated, is involved in eIF2α phosphorylation during IPNV infection. PKR inhibitor pre-treatment resulted in decreased virus titers, extra- and intracellularly, concomitant with reduction of cells with compromised membranes in IPNV-permissive cell lines. These findings suggest that IPNV uses PKR activation to promote virus replication in infected cells.

Key words: PKR, cloning, PKR inhibitor, IPNV, eIF2α phosphorylation, necrosis.

INTRODUCTION

Type I interferon (IFNα/β) responses are some of the crucial innate responses against virus infections. The induction of these responses begins with the stimulation of IFN production that can be induced through different pathways (Honda et al., 2005). The produced IFN binds its receptor on the cell surface and induces signaling cascades through the JAk/STAT pathway. As a result, an antiviral state is created characterized by increased transcription of several antiviral IFN stimulated genes (ISGs). Examples of these genes are those encoding the myxovirus resistance protein (MX), Protein kinase R (PKR) and ISG15 (Schoggins and Rice, 2011).

PKR is one of the best-studied ISGs and has been shown to play important roles during development, stress responses and virus infections. Genes encoding PKR have been characterized in different mammalian and fish species (Perelygin et al., 2006; Rothenburg et al., 2008). Analysis of amino acid sequences of PKR from different species revealed a common structure consisting of amino-terminal regulatory domain containing the dsRNA binding motif (dsRBM) and carboxy-terminal catalytic domain containing eleven kinase subdomains (Hu et al., 2013; Perelygin et al., 2006). Phylogenetic analysis and amino acid sequence alignment of the kinase domain from mammalian and fish PKR revealed low sequence conservation although some of the domains that are important for dimerization or substrate interaction were conserved (Rothenburg et al., 2008).

The classical mode of action of PKR is the induction of translation arrest in order to prevent production of new virus progeny thereby limiting virus spread. For this to happen, PKR must first be activated by binding of the dsRNA and other specific type of RNAs to its binding motif (Bevilacqua et al., 1998; Dauber et al., 2009; Manche et al., 1992). Once bound, a series of phosphorylation events are induced that ultimately lead to PKR activation. Activated PKR phosphorylates the eukaryotic initiation factor α (eIF2α), the only known substrate so far (Dey et

al., 2005). Phosphorylation of eIF2α prevents the recycling of GDP to GTP by the guanine nucleotide exchange factor eIF2B, which is important for the function of the translation initiator ternary complex Met.tRNA-eIF2-GTP, resulting in global inhibition of protein synthesis (Asano et al., 2000).

Recently, PKR was found to be involved in apoptosis, formation of stress granules (SGs) (Gil and Esteban, 2000; Lindcluist et al., 2011) and IFN-induced cellular necrosis (Thapa et al., 2013). Induction of apoptosis by PKR can be initiated via the death receptor, caspase 8 mediated or mitochondrial, caspase 9 mediated pathways; and involves both NFkB activation and eIF2α phosphorylation (Gil and Esteban, 2000; Gil et al., 2002). IFN-induced necrosis, on the other hand, requires IFN as well as interaction between PKR and RIP1 (receptor-interacting protein 1) to trigger necrosome formation and is licensed by FADD and caspases (Thapa et al., 2013). The detailed mechanism by which PKR participates in forming the SGs is not well described although it has been shown to involve eIF2α phosphorylation (Courtney et al., 2012).

Infectious pancreatic necrosis (IPN) is an important disease of farmed Atlantic salmon (*Salmo salar* L.). The causative agent, IPN virus (IPNV), is non-enveloped virus and is classified under the family *Birnaviridae*, genus *Aquabirnavirus*. The virus is ~60 nm in diameter and consists of a capsid that contains two genome segments (A and B) of double-stranded RNA (Dobos, 1995). The relatively small segment B encodes the virus polymerase, VP1 (Duncan et al., 1991) whereas segment A contains a large open reading frame (ORF) encoding a 107-kDa polyprotein (NH$_2$-pVP2-VP4-VP3-COOH). This polyprotein is post translationally cleaved to produce the major structural proteins VP2 and VP3 by the action of viral protease (VP4) (Duncan et al., 1987; Macdonald and Dobos, 1981; Petit et al., 2000). A second ORF overlapping the N-terminal region of VP2 encodes a small non-structural protein VP5 (Havarstein et al., 1990; Magyar and Dobos, 1994).

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At cellular level, IPNV-infected cells exhibit morphological and functional signs of necrosis (Evensen and Santi, 2008) but the events preceding the necrotic stage are less known. Some IPNV strains are reported to induce necrosis in infected cells in a post-apoptotic phenomenon (Hong et al., 1998) while others induce necrosis without being preceded by cellular apoptotic events or to a very limited extent (Espinoza et al., 2005; Gamil et al., 2015). In a previous study, we showed that IPNV infection of permissive cells resulted in eIF2α phosphorylation but PKR expression was not increased. Infected cells showed membrane leakage and reduced viability at 48h post infection, but this could not be linked to PKR involvement (Gamil et al., 2015). Here we have cloned Atlantic salmon PKR, developed custom made antibodies against it and investigated its role during IPNV infection with the aim to understand the involvement of PKR in virus replication and release of virus from infected cells. We did not see any increase in PKR protein expression post IPNV infection but despite so, inhibition of PKR using the small molecule inhibitor of PKR C16 resulted in reduced eIF2α phosphorylation. Moreover, release of virus to the supernatant was reduced by 2 $\log_{10}$ in two permissive cell lines. A similar reduction was observed for the intracellular viral load. IPNV infected and C16 treated cells exhibited lower number of cells with compromised membranes compare to untreated-IPNV infected cells. These findings are indicative of PKR playing a role in promoting IPNV replication in infected cells.

MATERIALS AND METHODS

Cell lines

Chinook salmon embryonic (CHSE-214) (Lannan et al., 1984), TO (Froystad et al., 1998) and Asian Grouper strain K (AGK) cells (Munang'andu et al., 2012) were maintained in L-15 media with Glutamax® (Gibco) supplemented with 5-10% FBS (sigma Aldrich). For maintenance, CHSE-214 and TO cells were grown at 20ºC while AGK cells were kept at 28 ºC. *Epithelioma papulosum cyprini* (EPC) cells were maintained at 20°C with L-15 medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS), L-glutamine, and gentamicin.

Virus propagation

A recombinant IPN virus (rNVI-15R[b]) previously produced by reverse genetics (Song et al., 2005) was used. For propagation, the virus was inoculated into 70-80% confluent AGK cells and incubated at 15ºC until full CPE. The supernatant containing the virus was then harvested and clarified by centrifugation at 2500 rpm, 4 °C for 10 min. The concentration of the virus was estimated by titration in 96 well plates (Falcon) containing 90-100% confluent CHSE-214 cells.

Cloning, prokaryotic expression of salmon PKR and production of rabbit antiserum

Total RNA from TO cells that had been treated with recombinant IFNα as previously described (Xu et al., 2010) was used as a template for cDNA synthesis. Transcriptor first-strand cDNA synthesis kit (Roche) was used to make cDNA according to the manufacturer's instructions. For initial cloning, one pair of primers, PKR-F1 and PKR-R1 were designed on the basis of Atlantic salmon PKR mRNA sequence (GenBank accession no. EF523422). A region from 73 bp upstream of the start codon of the open reading frame (ORF) to 412 bp downstream of the stop codon was amplified. The PCR products were purified by using the QIAquick gel extraction kit (Qiagen) and cloned into the pGEM-T Easy vector (Promega). The ORF of salmon PKR gene was subcloned from pGEM-T into the prokaryotic vector pET-32c (Novagen) by using

primer set pET32c-PKR-F and pET32c-PKR-R. The recombinant vector containing a 6×His-tag at the N-terminal of the protein was used to facilitate purification using a His-Bind column. The recombinant vector, named pET32c-PKR, was confirmed by DNA sequencing and transformed into the bacterial host BL21 (DE3) for expression driven by the T7 polymerase. Induction was carried out at 37°C for 2 h with 1mM Isopropyl-β-D-Thiogalactopyranoside (IPTG). The fusion protein was purified according to the protocol of the His-Bind purification kit (Novagen) and used to immunize a rabbit for production of polyclonal anti-PKR serum.

Recombinant IFNα treatment

To test the effect of IFNα treatment on the expression of salmonid PKR, CHSE-214 cells grown in 6 wells plates (Corning) were treated with 500 ng/ml IFNα as described previously (Xu et al., 2010) and harvested at 4, 8, 16, 24 and 48 hours post treatment. Parallel wells were left untreated and harvested together with cells treated for 48 hours. At the indicated times post treatment, cells were sampled for western blot and real-time PCR.

Effect of IPNV infection on PKR expression

Six well plates containing approximately 90% confluent CHSE-214 cells were infected sequentially in reverse order with 20 PFU/cell IPNV to produce cells infected for 3, 12, and 24 hours at the time of sampling. Negative and positive controls were uninfected cells and cells treated with recombinant IFNα (500 ng/ml of medium), respectively, harvested after 4 days.. The cells were sampled by washing once with PBS prior to lysis and western blot analysis.

Western blot

Following IPNV infection or recombinant IFNα treatment, CHSE-214 cells were lysed by using CelLytic M reagent and scraped from the plates. Lysates were separated in 12% NuPAGE Bis-Tris gels (Invitrogen) and transferred to PVDF membrane using Trans-Blot SD semi-dry transfer cell (BioRad). The membrane was blocked for 2 hours using 5% dry milk in TBST (0.02

M Tris-HCl, 0.9% NaCl, 0.05% Tween 20, pH 7.6) and then incubated overnight at 4 °C with polyclonal antibodies against PKR diluted in 5% dry milk in TBST. Horseradish peroxidase (HRP) conjugated anti-rabbit antibody (GE healthcare) diluted 1:2000 was added and incubated for 1 h. The signal was finally developed using SuperSignal West Dura Extended Duration Substrate (Pierce) or the ECL Plus™ Western Blotting detection reagents and detected using the Typhoon (GE healthcare). After detection, the membrane was washed twice with 0.1% TBST, stripped using Restore™ Plus buffer (Pierce), washed twice as above, and re-probed using antibodies against actin (Sigma Aldrich) as described above.

Quantitative real-time PCR analysis

To quantify the expression of PKR following IFNα treatment RT-qPCR was used. Cells were treated with IFNα, as described above, and total RNA was isolated by using the RNeasy Plus minikit (Qiagen) according the manufacturer's instructions. RNA concentrations were determined by using the Nanodrop ND1000 (Thermo Scientific). 1µg of total RNA was used to synthesize cDNA using a Transcriptor first strand cDNA synthesis kit (Roche) according to the manufacturer's protocol. The cDNA was diluted five times and stored at -20°C until required. Quantitative PCR was performed in 96 well plates using the LightCycler 480 system (Roche). For each reaction, 2µl cDNA was mixed with 10 pmol gene-specific primers and 10µl LightCycler 480 SYBR green I master mix (Roche). The final concentration was adjusted to 20µl using RNase free water. The sequences of primers used in the reactions are provided in Table 1. The cycling conditions for the PCR reactions were as follows: denaturation 94 ºC for 10 sec; Annealing 60 ºC for 20 sec; elongation 72 ºC for 8 sec. The results were analyzed by the ΔΔCT relative quantification approach (Livak and Schmittgen, 2001) using β-actin as reference gene.

Pre-treatment of cells with PKR inhibitor C16 on eIF2α phosphorylation

Both PKR inhibitor C16 and its inactive form (Merck Millipore) were dissolved in DMSO to 5mg/ml. CHSE-214 cells were then incubated with 2µM of either solution for 30-45 min at 15°C. Parallel wells of pretreated and untreated cells were subsequently infected with IPNV for 24 hours, as described above, in the presence or absence of C16. Uninfected cells, both from C16 treated and untreated cells, were also included as controls. At 24 hours post infection (hpi) cells were lysed and lysates were purified using ReadyPrep TM 2-D cleanup kit (Biorad). 30µg of purified lysates were subjected to 2D-western blot analysis to evaluate the levels of phosphorylated eIF2α (peIF2α) in infected and uninfected cells. Samples were first subjected to isoelectric focusing (IEF) using 11cm, nonlinear, pH 3-10 IPG strips (Biorad) followed by second dimension run using 8-16% Tris-HCl Criterion precast gels and finally blotting into PVDF membrane before being probed using monoclonal anti eIF2α antibodies. Anti-mouse IgG HRP labeled secondary antibodies (GE healthcare) were applied and the signal was developed using SuperSignal West Dura Extended Duration Substrate (Pierce) and finally detected using the Typhoon (GE healthcare).

Flow cytometry

CHSE-214 or TO cells grown in 24 wells plates were either pre-treated for 30min with 2µM C16 or left untreated. Three wells of each (C16 pretreated and untreated cells) were then infected with IPNV at 20 PFU/cell, and incubated for 24 hours in the presence or absence of the PKR inhibitor, respectively. Uninfected cells in triplicate were also included as negative controls. After 24 hours incubation, the media was removed and cells were washed once with PBS before trypsinisation. Trypsinized cells were re-suspended in FBS and centrifuged for 10 min at 300 x g. Cells were then stained using Annexin V/ Ethidium homodimer III (EtD-III) staining kit (Biotium) according to the manufacturer's protocol, in order to determine the fraction of cells

with apoptotic (Annexin V positive) or membrane permeability (EthD-III positive) changes. Flowcytometry was performed for 10,000 events with a Guava easyCyte™ Flow Cytometer (Merck Millipore) and the acquired data were analyzed using InCyte™ software version 0.2 (Merck Millipore). The following parameters were measured: 1) the area pulse of forward light scatter (FSC-A) versus side scatter (SSC-A), and 2) fluorescent intensities of $CF^{TM}488A$ (filter 525/30) and EthD-III (filter 690/50) upon excitation with 20 mW 488 nm laser. Cell aggregates and debris were identified and excluded by using the width pulse of FSC-A versus area width of SSC-A.

Effect of PKR inhibition on virus loads in the supernatant

To determine the impact of PKR inhibition on IPN replication, confluent CHSE-214 or TO cells seeded in 24 wells plates (Corning) were either pretreated for 30-45min with either an active or inactive form of the PKR inhibitor C16 (Merck Millipore) as described above, or left untreated. Cells were then infected with IPNV at 20 PFU/cell and after one hour adsorption, they were washed once with PBS. After washing, L-15 glutamax media (Invitrogen) supplemented with 1% FBS and 50µg/ml Gentamycin was added to the cells. For cells pre-treated with either forms of the PKR inhibitor C16, 2µM of the inhibitor was also included in the media. Supernatants were harvested at 10, 24 and 48hpi and the amounts of virus in the supernatants were determined by titration in CHSE-214 cells using Kärber's method (Kärber, 1931).

Effect of PKR inhibition on the intracellular virus loads

Intracellular virus loads were determined in cells infected with IPNV in the presence or absence of C16, as described above. At 10, 24 and 48hpi supernatants were removed from the cells. Attached cells were then washed once with PBS. 200µl of fresh cell culture media was subsequently added to the cell layers, which were then lysed by subjecting them to two rounds of

freeze-thawing in order to release virus to the supernatant. The amount of virus released from the cells was finally determined by titration in CHSE-214 cells as already described.

Effect of over-expressing a catalytic, inactive form of PKR

Eukaryotic expression plasmid encoding a mutated version of carp PKR (pcDNA-mutcarpPKR, Lys419Arg), which expresses a catalytically inactivated form, was a kind gift from professor Gui (Liu et al., 2011). For over-expression of carp PKR proteins, EPC were transfected by electroporation with 2 µg of pcDNA-mutcarpPKR or pcDNA3.1-myc-His per 106 cells using the Neon transfection system (Invitrogen, Carlsbad, CA) with one pulses of 1200V for 40 ms. After transfection, cells were kept at 20°C until further examination.

Statistics and graphics

Differences in virus titers between groups were determined by Wilcoxon Rank Sum test using JMP 11 statistical software (SAS institute Inc.). Two-way analysis of variance (ANOVA) followed by Bonferroni post test was performed to compare the differences in the percentage of necrotic cells between the different groups. The ANOVA analysis and all the graphs were done using GraphPad Prism 5.0 (GraphPad Software Inc.).

RESULTS

Production of rabbit antiserum against Atlantic salmon PKR

Polyclonal antiserum against PKR (anti-PKR) was made by immunizing a rabbit with recombinant proteins produced by expressing the ORF of PKR in *Escherichia coli* BL21 (DE3) cells using the pET prokaryotic expression system. Purification of proteins was performed using His-Bind columns under denaturing conditions because the protein yield in the cell soluble fraction was much lower than that of the inclusion bodies (data not shown). The expression and purification of recombinant proteins were identified by SDS-PAGE (Fig. 1). The predicted molecular weight in CHSE-214 cells is around 85 kD which corresponds to the size of trout PKR (Accession no. NM_001145891). The polyclonal antibody was used to detect PKR expression in CHSE-214 cells in response to recombinant IFNα treatment and IPNV infection.

PKR expression in CHSE-214 cells is induced following IFNα treatment

To confirm that the antibodies described in the section above bind to PKR in CHSE cells using western blot, the latter were pretreated with recombinant IFNα. Thereafter anti PKR antibodies were used to detect PKR expression. This approach was used since it is well-known that PKR expression is induced following IFNα pretreatment. The results show that while PKR is constitutively expressed at low levels in untreated cells, a strong induction was observed from 16 h post IFN-α treatment (Fig. 2a). These findings correlate with PKR mRNA expression by real time PCR (Fig. 2b).

PKR expression is not induced in IPNV infected cells

PKR has previously been suggested to be involved in mediating cell death during IPNV infection (Huang et al., 2011). To test this, the anti-PKR antibodies (above) were used. Our findings show that PKR is not induced in IPNV infected cells at any of the time points examined

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(3-24 hpi) while IFNα treated cells (positive controls) showed induction of PKR expression (Fig. 3).

Pretreatment of cells with PKR inhibitor alters eIF2α phosphorylation after IPNV infection

Constitutively expressed basal levels of PKR can contribute to eIF2α phosphorylation when activated. Given that eIF2α was previously found to be phosphorylated following IPNV infection (Gamil et al., 2015; Garner et al., 2003), we wanted to know whether PKR is involved in this phosphorylation. We therefore pretreated cells with the PKR inhibitor C16 followed by infection with IPNV and then observed eIF2α phosphorylation through the course of infection. Since salmonid antibodies against phosphorylated PKR are presently not available, PKR phosphorylation could not be measured directly, instead eIF2α phosphorylation was used as a downstream indicator. The results show that pretreatment of cells with C16 followed by IPNV infection inhibited eIF2α phosphorylation (Fig. 4b). Pretreatment of cells with inactive C16 also resulted in some decreased eIF2α phosphorylation (Fig. 4d). In contrast, IPNV infection alone resulted in increased eIF2α phosphorylation as indicated by a shift in the protein band corresponding to the size of eIF2α (36Kda) towards more acidic pH (Fig. 4f). It is also noteworthy that in all lysates, non-specific bands (>40 KDa) were detected in addition to specific ones but no effort was put into identifying them. These findings suggest that PKR contributes to eIF2α phosphorylation in IPNV infected CHSE-214 cells.

Inhibition of PKR reduces virus yield in the supernatant

To further investigate the role of PKR activation during IPNV infection we used two different cell lines to measure the effect of inhibitor on virus loads in the supernatants by titration. For CHSE-214 cells (Fig. 5a), treatment with PKR inhibitor resulted in a significant reduction of virus titer in the cell supernatant by 1.7 $\log_{10}$ at 10 hpi (p=0.0126), over 2.5 $\log_{10}$ by 24 (p=0.0033) and 48 hpi (p=0.0039). The same effect was seen in TO cells (Fig. 5b) where the

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virus titer in the supernatant was reduced 1.3 $\log_{10}$ at 10 hpi (p= 0.0039), 0.8 $\log_{10}$ at24 hpi (p= 0.037) and thereafter 2.4 $\log_{10}$ at 48 hpi (p=0.0036) in C16-treated cells.

To validate that the effect observed on virus titer is due to PKR activation we treated CHSE-214 cells with an active and inactive form of C16 and compared the virus loads on the supernatants to untreated cells at 24 and 48 hpi. We found that treatment with the inactive form of the inhibitor had minor impact on virus titer ($\pm$ 0.3 $\log_{10}$ change) while treatment with the active form of C16 resulted in a significant reduction (P<0.05) in virus titers by 1.5 $\log_{10}$ at 24hpi and 0.6 $\log_{10}$ at 48hpi (Fig 5c).

Since PKR knock-out cell lines of piscine origin are not available, we used an approach to transfect permissive cells (EPC cells) with a catalytic inactive form of PKR. EPC cells were transfected with pcDNA-mutcarpPKR, which will out-compete the function of cellular PKR (Liu et al., 2011). Transfected cells were infected with IPNV at 1 and 0.1 MOI, which resulted in significant reduction of endpoint titer at 1 MOI (not significant at 0.1 MOI; Supplementary Fig. 1), further corroborating the importance of functional PKR for IPNV replication in permissive cell lines.

Inhibition of PKR results in decrease IPNV induced membrane damage

To understand the mechanism behind the decreased virus titers we further tested whether treatment with the PKR inhibiter would affect the ability of the virus to compromise the cell membrane, a well-known trait of IPNV (Gamil et al., 2015). We compared membrane permeability and apoptotic changes following IPNV infection in untreated and C16 treated cells. In both CHSE and TO cells, IPNV infection resulted in an increase in the percentages of cells with compromised membranes (EthD-II positive) compared to uninfected cells (Fig. 6). No significant effect on the percentage of apoptotic cells (Annexin-V positive) was observed (Fig. 6).

Notably however, the percent of cells with compromised membrane was significantly lower in cells treated with C16.

Decrease membrane damages and virus replication results from inhibition of virus replication

At this point there are two explanation for the observed reduction in virus titer and the virus induced cell damage; either the PKR mediates the induced membrane damage which subsequently promote virus release, and therefore inhibition of PKR results blocking virus release or the effect is due to an effect on the virus replication. If the former is true then the virus will accumulate in the infected cells and it is expected to find a higher virus titer in the intracellular compartment. On the contrary, a lower virus titer is expected in C16 treated cells if there is a direct effect one IPNV replication. To clarify this point, the virus amounts intracellularly were assessed by titration of washed, freeze/thawed cells. In CHSE-214 cells (Gig 7a) there was a significant reduction in titer by 1.6 $\log_{10}$ at 24hpi (p=0.0038) and by 1.75 $\log_{10}$ at 48h (p=0.0038). The amount at 10hpi was undetectable. Similar to CHSE-214, virus titer intracellularly at 10hpi below detection limit in TO cells, whereas there was a 1.7 (p=0.0038) and 1 $\log_{10}$ (p=0.0435) reduction in virus titers at 24 and 48 hpi, respectively (Fig. 7b).

DISCUSSION

In this study, we show that inhibition of PKR results in decreased virus titer in two IPNV permissive cells lines, strongly indicating that a functional PKR favors IPNV replication. The use of PKR activation in favor of the virus has been shown previously for other virus species. For example, hepatitis C virus (HCV) replicates more efficiently when PKR is activated and inhibition of PKR activation decreases virus yield (Arnaud et al., 2010). This has been attributed to inhibition of IFNα production resulting from the global inhibition of protein synthesis induced through PKR activation. Our previous findings (Gamil et al., 2015) suggest that the same IPNV strain used in this study uses inhibition of protein synthesis as a strategy to evade IFN responses. In the present study, we additionally show that inhibition of PKR activation results in decreased virus titer intracellularly and in the supernatant (Fig. 5,7). The level at which the PKR inhibitor interfere with virus replication is not clear but the decrease in intracellular titers suggests that it occurs prior to the viral release stage, possibly at replication levels. Interference at multiple stages is also a possibility. The detailed mechanism underlying this interference remains unknown and requires further investigation. It is known that PKR activation results in transcriptional activation of genes and several transcription factors involved in different cellular processes some of which could be beneficial for virus replication. For example, it was previously shown that activation of the transcription factor ATF3 (activating transcription factor 3), whose transcription can be induced by PKR (Garcia et al., 2006), is beneficial for murine cytomegalovirus (MCMV) infection (Rosenberger et al., 2008). Similarly the NFkB (Nuclear factor kappaB) is an important transcription factor that is activated by PKR and it has been shown that activation is to the advantage of several virus species (Garcia et al., 2006; Hiscott et al., 2001). One possibility could therefore be that activation of some of the PKR-induced genes or

transcription factors promotes IPNV replication and inhibition of their activation consequently hampers virus replication but this remains to be decided.

Since PKR inhibition resulted in reduced phosphorylation of eIF2α, decreased virus titer upon PKR inhibition may also indicate that eIF2α phosphorylation is used as a means to enhance IPNV replication. It was previously shown that translation of some cellular mRNAs is enhanced when eIF2α is phosphorylated. The common features for these mRNAs are: 1) low translation and transcription under normal conditions; 2) possession of translated upstream ORFs (uORFS) at their 5' region; and 3) at least one of the uORFs is longer than 20 codons and is translated under normal conditions (Andreev et al., 2015). Ebola virus is able to translate its protein when eIF2α is phosphorylated and the translation of the virus polymerase as well as virus replication was influenced by the presence of uORF. Notably, under high levels of eIF2α phosphorylation, the presence of the uORF enhanced the translation of the L polymerase and removal of the uORF by mutating the start codon resulted in decreased virus replication (Shabman et al., 2013). IPNV possesses an uORF that encodes for VP5 and overlaps the main ORF in segment A (Weber et al., 2001). It is possible that presence of the uORF will result in enhanced translation under eIF2α phosphorylation. Previous study performed in our group, however, demonstrated that ablation of VP5 expression by mutating the start codon as well as truncation by inserting premature stop codon did not affect virus replication (Santi et al., 2005). However, the mutation was made in the second in-frame start codon that was suggested to be the site at which the translation of VP5 is initiated (Weber et al., 2001). It remains possible that the first in-frame start codon is the one responsible for the translation control of the main ORF especially that mutations in the region containing this codon did not yield viable virus (Weber et al., 2001). Further studies are therefore warranted to better understand the role of uORF encoding VP5 in the control of virus replication.

The decrease in virus titer was accompanied by decrease in number of necrotic cells (or cells with compromised membranes) (Fig. 6). One interpretation of these findings could be that the virulent Sp strain of IPNV uses PKR-mediated cellular necrosis to induce cellular lysis and subsequently facilitate virus release. In this case the anticipation would be that PKR interacts with RIP1 and induce cellular necrosis via RIP1/RIP3 pathway as previously described (Thapa et al., 2013). Due to the paucity of reagents and antibodies we could not further investigate this possibility. However, since the decrease in number of cells with compromised membrane coincided with decreased intracellular virus titers, we hypothesize that reduced number of cells with compromised membranes is a direct consequence of reduced virus replication (Fig. 7), although we cannot completely rule out the former possibility. This latter interpretation is supported by the fact that birnaviruses were shown to possess mechanisms that enables them to perforate cell membranes (Galloux et al., 2007).

Although the induction of eIF2a phosphorylation following IPNV infection has been reported previously (Garner et al., 2003), no efforts were directed at investigating the mechanisms involved. The eIF2a kinases that activated during IPNV infection are PKR and PERK (Huang et al., 2011; Wang et al., 2011). In this study we did not detect PKR upregulation post IPNV infection. However, treatment with a PKR inhibitor resulted in decreased eIF2α phosphorylation (Fig. 4) and through a competitive approach (transfection with mutated form of PKR) we also showed a significant reduction of IPNV titer in a permissive cell line (Suppl. Fig. 1). Upregulation of PKR is not necessary for the PKR function and basal levels of activated PKR are sufficient to induce eIF2α phosphorylation and inhibit translation in response to virus infection (Gainey et al., 2008). Our findings therefore suggest that PKR participates, at least partially, in eIF2α phosphorylation during IPNV infection. This is in agreement with what has been reported for another member of the family *Birnaviridae*, the infectious bursal disease virus

(IBDV), where the expression of VP2 in HeLa cells was associated with PKR activation and subsequently eIF2α phosphorylation (Busnadiego et al., 2012). It is therefore possible that VP2 of IPNV plays a similar role but further studies are needed to demonstrate this.

The ultimate goal for a virus infection is to use the cell's machinery to produce new virus progeny. PKR activation leads to attenuation of global protein synthesis through eIF2α phosphorylation and could therefore have severe impact on production of new virus progeny (Barry et al., 2009). Consequently, it is not surprising that viruses have developed different strategies to prevent PKR activation (Dauber and Wolff, 2009). Many viruses, on the other hand, are capable of producing their progenies through cap-independent mechanisms and are thus not affected by the translation inhibition induced by PKR/eIF2α (Schneider and Mohr, 2003).The replication of IPNV despite eIF2α phosphorylation (Fig. 4) is consistent with our previous findings (Gamil et al., 2015) and implies that translation of IPNV possesses cap independent mechanism to translate its own protein. Since IPNV possesses uORF in segment A as indicated above, another possibility is that this uORF inhibits the translation of IPNV proteins and eIF2α phosphorylation will mediate the bypass of this uORF and help to promote the translation of the second ORF encoding the107-kDa polyprotein. Further studies are required, however, to investigate these possibilities.

PKR was shown to be responsible for NFkB activation in herpes simplex virus infected cells. This activation participates in the control of virus replication as the virus yields in PKR or NFkB deficient cells were 10 fold higher than in PKR and NFkB sufficient cells (Taddeo et al., 2003). For other different viruses however, NFkB activation can be used in favor of the virus either as a means to inhibit apoptosis and prolong cell survival in order to gain time for replication or to directly enhance viral replication for viruses that possess NFkB binding sites in their promotor (Santoro et al., 2003). It was shown previously that NFkB is activated in IPNV

infected cells (Hong et al., 2008). Although, involvement of tyrosine kinase pathway was suggested, the mechanisms involved have not been dissected. It is known that PKR induced necrosis requires interaction with RIP1 and is negatively regulated by FADD and caspases (Thapa et al., 2013), both are components of the apoptotic signaling (Gupta et al., 2004; Thorburn et al., 2003). RIP1 activation can also lead to NFkB activation (Lin, 2014). However, NFkB signaling is generally considered as an anti-apoptotic response although in some cases it can also be linked to cell death (Lin, 2014). In the current study, we found an increase in the number of necrotic cells following IPNV infection while the apoptotic cell number remained unchanged (Fig. 6). The mechanism by which cell death is induced during IPNV infection remains obscure and further studies are require to investigate the possible involvement of PKR and NFkB signaling pathways.

The oxindole/imidazole derivative C16 is widely used as specific PKR inhibitor *in vitro*. Recently it was shown that systemic injection of rats with this inhibitor resulted in *in vivo* PKR inhibition suggesting that it can be used to reverse the undesirable effect of PKR activation in certain diseases such as the neurodegenerative disorder (Ingrand et al., 2007). The finding that treatment with this inhibitor reduced IPNV titers *in vitro* is interesting and demonstrates that C16 can be used to inhibit IPNV replication as a novel anti IPNV drug target. A similar *in vivo* effect in addition to safety must however, first be demonstrated and considered.
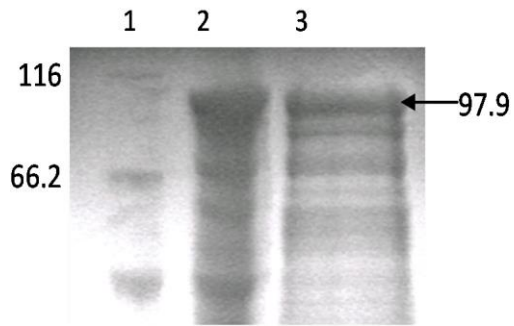
Figures

Figure 1. Expression of recombinant PKR protein in *E. coli* and its purification. 1. Protein marker; 2. pET32-PKR induced with IPTG; 3. Purified, recombinant PKR of a size of 97.9 kD.
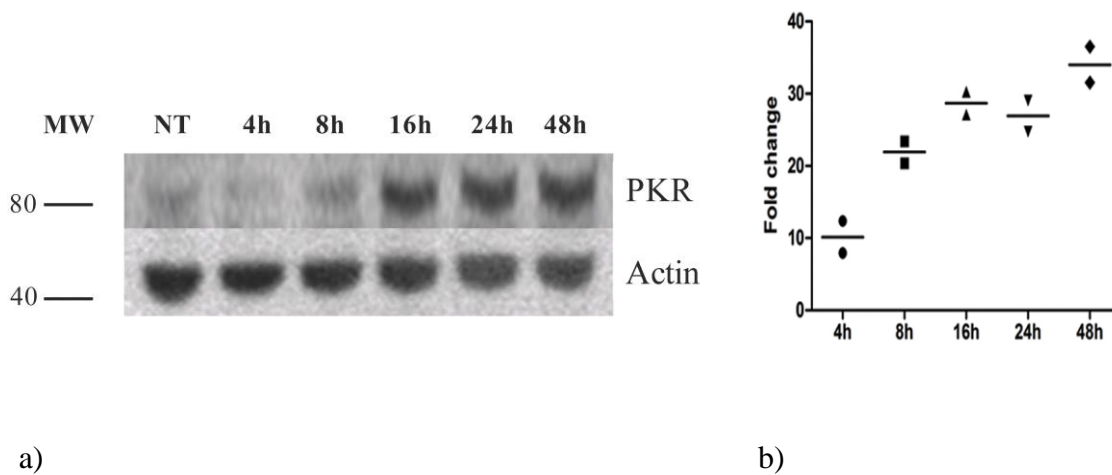

a)   b)

Figure 2. Time course analysis of PKR protein (a) and mRNA (b) expression in CHSE-214 after recombinant IFN-α treatment detected by Western blot (a) and real-time PCR (b). The real-time data are expressed as the mean fold changes in gene expression for IFN-α treated samples relative to the untreated control after normalization to β-actin (n=2). MW= molecular weight; NT − non-treated; different timepoints are post IFN treatment.
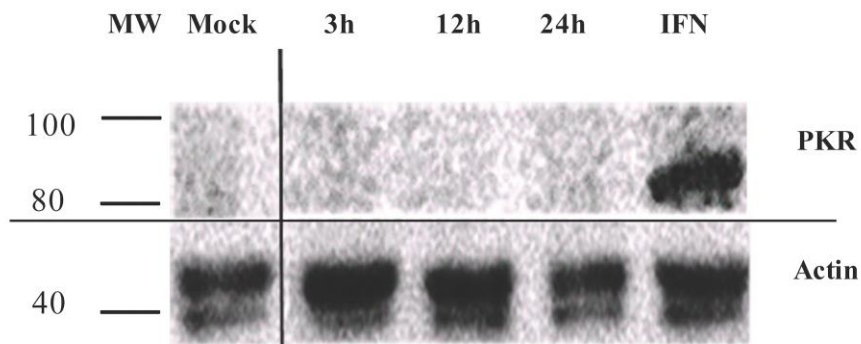
Figure 3. PKR expression in cells infected with infectious pancreatic necrosis virus assessed by Western blot analysis. The cells were harvested at indicated times (hours;h) following infection. IFN = cells were treated with interferon α for 4 days prior to sampling. MW=molecular weight.
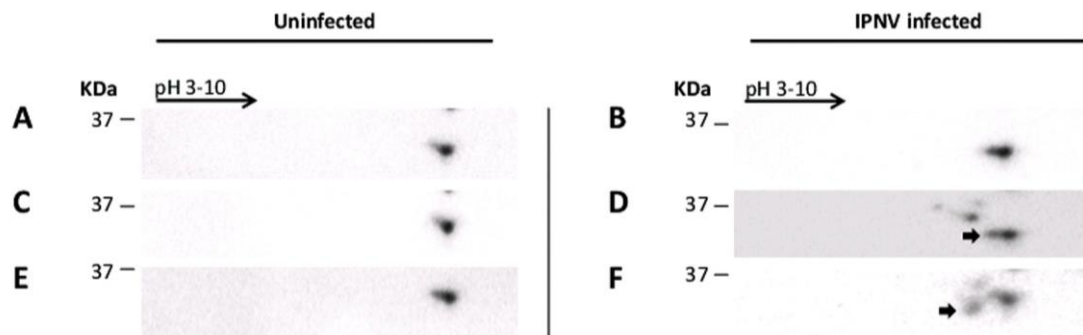

Figure 4. Effect of PKR inhibitor C16 on eIF2α phosphorylation in CHSE-214 cells. Parallel wells of C16-treated (A&B), C16 inactive form (C&D) or untreated cells (E&F) were infected with 20pfu/ml IPNV (B,D.F) or left uninfected (A,C,E). Non-infected cells pretreated with activated C16 (A), non-activated C16 (C) or left untreated (E) all exhibited an identical protein pattern. Cells infected with IPNV and pretreated with active C16 (B) showed inhibition of eIF2α phosphorylation. Pretreatment with inactive C16 (D) gave some shift of eIF2α phosphorylation towards more acidic pH (small tale; arrow in D), possibly because of spontaneous activation of C16. IPNV infection alone (F) resulted in increased eIF2α phosphorylation shown by a shift towards more acidic pH and a protein band corresponding to the size of eIF2α (36Kda). Total cell lysates were fractioned on 2D gels and subjected to western blot using anti-eIF2α monoclonal antibodies. Arrows indicate the shift of eIF2α to more acidic pH due to phosphorylation.
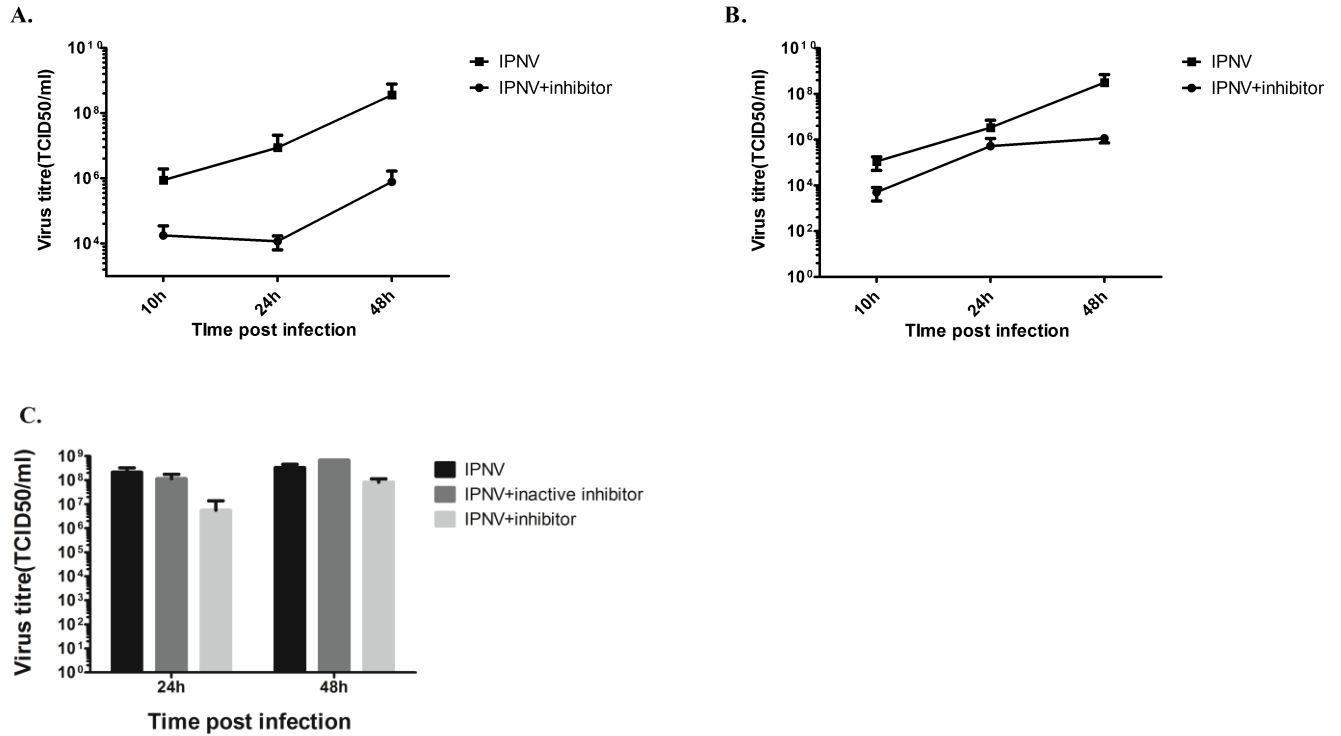
Figure 5. Effect of the PKR inhibitor C16 on IPNV replication. CHSE-214 (a) and TO (b) cells were either treated with 2μM of the PKR inhibitor C16 or left untreated and then infected with IPNV at 20 PFU/ml. and the amount of virus titers in the supernatants at different times post infection was determined by titration in CHSE-214 cells. Data represent an average of 6 parallels taken from 2 independent infections ± SD. (C) Virus titers in the supernatants in CHSE-214 cells following pretreatment with active and inactive form of C16 compared to untreated cells. Data represent an average of 3 parallels from a single infection ± SD.
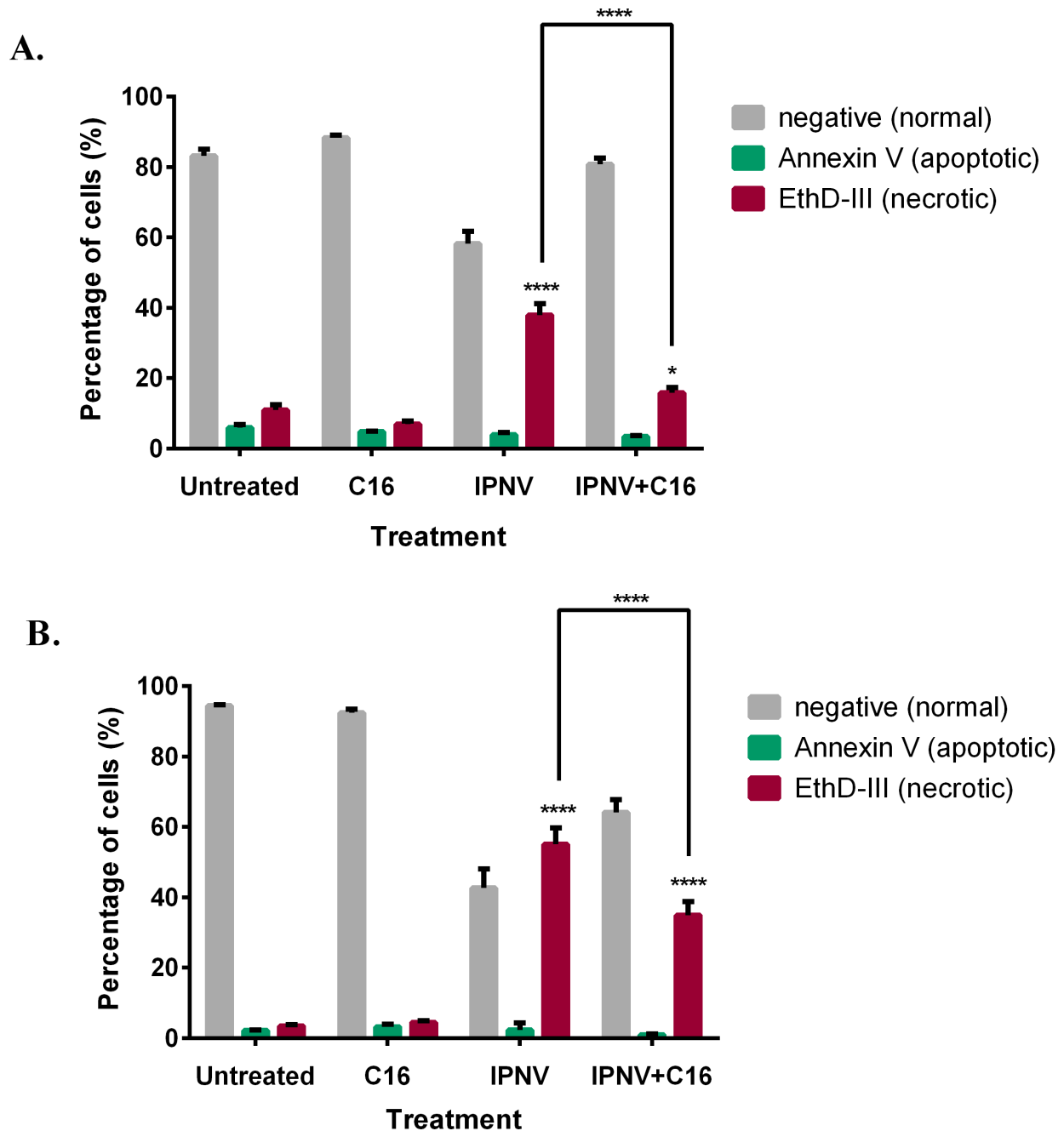
Figure 6. PKR induced membrane damage promotes virus release from infected cells. CHSE-214 (a) and TO (b) cells were infected with IPNV for 24 hours in the presence or absence of the PKR inhibitor C16 and apoptotic (Annexin-V) or membrane permeability (EthD-III) changes were assessed by flow cytometry as described in the methodology. The number of cells with compromised membranes (EthD-III) was significantly reduced by C16 treatment while the number of apoptotic cells (Annexin-V) remained unchanged. Bars represent the mean of three parallels ± SD. Asterisks indicate statistical significance; * P<0.05, **** P<0.0001. N=3.
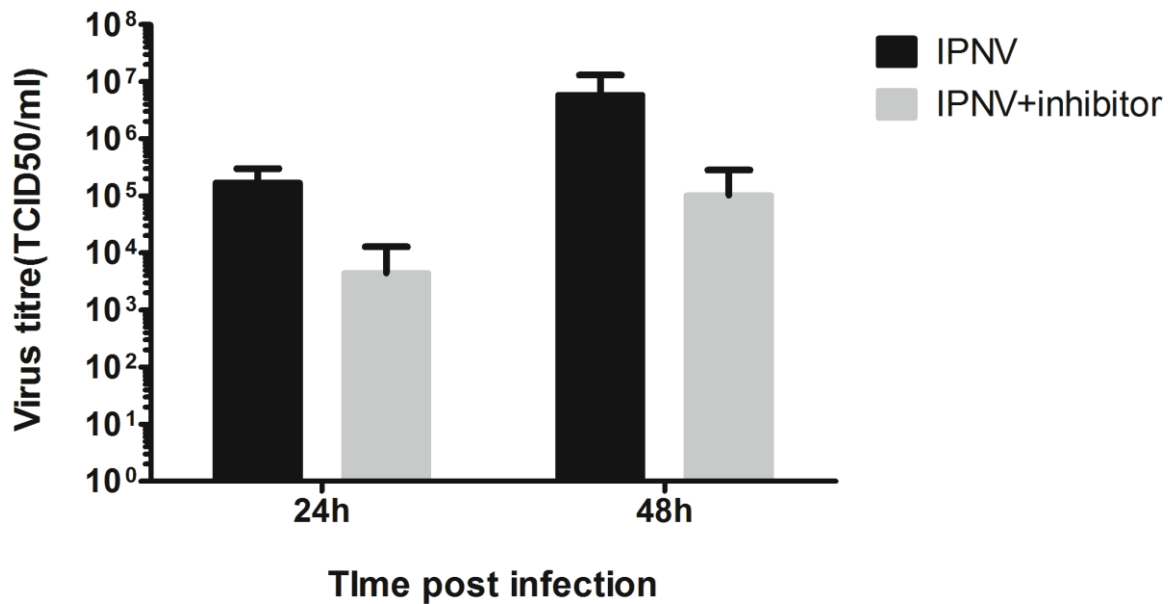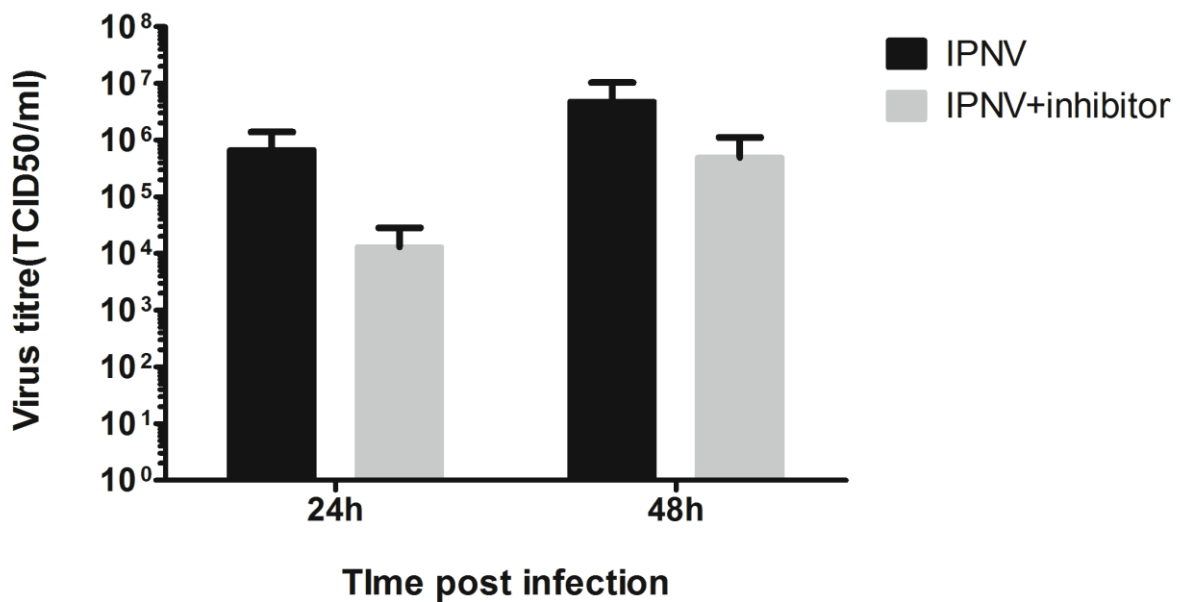
Figure 7. Effect of the PKR inhibitor C16 on the intracellular virus loads. CHSE-214 (a) and TO (b) cells were either treated with 2μM of the PKR inhibitor C16 or left untreated and then infected with IPNV at 20 PFU/ml. The virus was released from attached cells by 2 rounds of freeze-thaw at different time post infection followed by titration in CHSE-214. Data represent an average of 6 parallels taken from 2 independent infections ± SD.
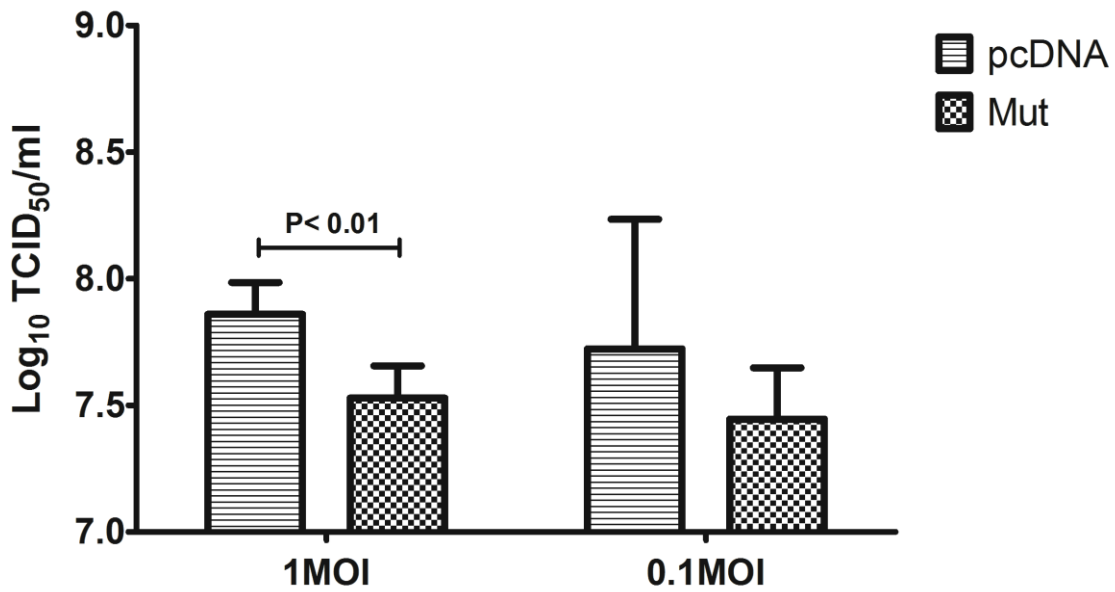
Supplementary Figure 1. mutcarpPKR effect on IPNV titer in EPC cells. EPC cells in each well of 6-well plates were transfected with a total amount of 2 µg of pcDNA3.1- mutPKR or pcDNA3.1-myc-His plasmids. At 2 hours post transfection, the cells were infected with 1 and 0.1MOI IPNV. Cell supernatants were collected at 72 hpi for virus titration (in CHSE cells; mean+SEM, n = 3).

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