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Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): Inactivation of virus particles and induction of a type I interferon-related response

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Abstract

It is well known that human alpha-defensin-1, also designated as human neutrophil peptide 1 (HNP1), is a potent inhibitor towards several enveloped virus infecting mammals. In this report, we analyzed the mechanism of the antiviral action of this antimicrobial peptide (AMP) on viral haemorrhagic septicaemia virus (VHSV), a salmonid rhabdovirus. Against VHSV, synthetic HNP1 possesses two antiviral activities. The inactivation of VHSV particles probably through interfering with VHSV-G protein-dependent fusion and the inhibition of VHSV replication in target cells by up-regulating genes related to the type I interferon (IFN) response, such as Mx. Neither induction of IFN-stimulated genes (ISGs) by HNP1 nor their antiviral activity against fish rhabovirus has been previously reported. Therefore, we can conclude that besides to acting as direct effector, HNP1 acts across species and can elicit one of the strongest antiviral responses mediated by innate immune system. Since the application of vaccine-based immunization strategies is very limited, the used of chemicals is restricted because of their potential harmful impact on the environment and no antimicrobial peptides from fish that exhibit both antiviral and immunoenhancing capabilities have been described so far, HNP1 could be a model molecule for the development of antiviral agents for fish. In addition, these results further confirm that molecules that mediate the innate resistance of animals to virus may prove useful as templates for new antivirals in both human and animal health.

Keywords: HNP1; VHSV; IFN; Mx; AMP; Rhabdovirus; Alpha-defensin

1. Introduction

An increasing number of antiviral agents are presently in various stages of development and testing, and an increasing number have recently been licensed for use in humans and animals. However, most of the available antiviral drugs often lead to the development of viral resistance coupled with the problem of side effects, recurrence and viral latency. In this regard, antiviral drug development focusing on the regulation of innate defense system is an attractive approach.

The innate response is the first line of defense against infectious agents. Upon a first encounter with a virus, the innate immunity is triggered immediately in the organism. This early immune response is characterized by the production of different cytokines, as well as other immune intermediators and antiviral factors, such as antimicrobial peptides (AMPs) that control viral replication and provide time for the generation of a more-effective host adaptive immunity response. Defensins (Ganz et al., 1985; Selsted et al., 1985), a family of cysteine-rich cationic antimicrobial peptides, are probably the AMPs that show the broadest range of antiviral activity being active against both enveloped and non-enveloped virus (reviewed in reference Klotman and Chang, 2006). In addition to their direct antiviral effects, defensins also modulate the host immune response and provide a link between the innate (early) and the adaptive (late) mammal immune responses (Lillard et al., 1999; Selsted and Ouellette, 2005; Tani et al., 2000; Yang et al., 2002). This dual role of defensins as direct effectors and inducers of immune responses qualify defensins as potential antiviral drugs.

Defensins are polypeptides of fewer than 100 amino acids (Ganz, 2003) with β-pleated sheet structures stabilized by intramolecular disulphide bonds (Ganz, 2003; Lehrer and Ganz,

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2002). They are classified into α-, β- and θ-defensins, which differ in the distribution of the disulphide bonds between their six conserved cysteine residues (reviewed in references Klotman and Chang, 2006; Selsted and Ouellette, 2005; Yang et al., 2002). While disulphide bonds are not required for some of their functions such as the antibacterial activities of human α-defensin 1 (HNP1), human β-defensin-3 (HBD3) or the mouse Paneth-cell-derived α-defensin, cryptdin-4 (Klotman and Chang, 2006; Maemoto et al., 2004; Mandal and Nagaraj, 2002; Wu et al., 2003), they might be required for some others such as binding to HSV-1 and HIV virions (Daher et al., 1986; W. Wang et al., 2004).

Defensins and/or defensin-like peptides have been found in mammals (Ganz, 1999, 2003; Ganz et al., 1985; Klotman and Chang, 2006; Selsted and Ouellette, 2005), insects (Bulet and Stocklin, 2005; Lehrer and Ganz, 2002; Otvos, 2000), plants (Castro and Fontes, 2005; Garcia-Olmedo et al., 1998; Lay and Anderson, 2005; Lehrer and Ganz, 2002; Selsted and Ouellette, 2005) and birds (Martin et al., 1995; Sugiarto and Yu, 2004). Unexpectedly, no defensin-like peptides have been isolated from fish so far but genomic sequences show that β-defensins are most likely expressed. Thus, homologue sequences to β-defensins from higher vertebrates and one to human β-defensin-26 have been recently identified in non-salmonid fish (Zou et al., 2007) and rainbow trout (Falco et al., unpublished results), respectively, by bioinformatic analysis of EST and genome databases, demonstrating that the β-defensin family of AMPs is present in the fish genomes. Several other AMPs families have been reported in fish, including misgurin, pleurocidin, paradaxins, parasin I, hepcidin, piscidins/moronecidin, oncorhyncins, LEAP-2, perforin and cathelicidin (Cole et al., 1997; Fernandes et al., 2004; Lauth et al., 2002; Noga and Silphaduang, 2003; Oren and Shai, 1996; Park et al., 1997, 1998; Shike et al., 2002; Zhang et al., 2004; Hwang et al., 2004; C.I. Chang et al., 2005). To date, antiviral activity against fish virus has only been studied for piscidins (Chinchar et al., 2004) and the activity of non-piscine AMPs against fish viruses has been only reported for cecropins (Chiou et al., 2002). In addition, no AMPs from fish with both antiviral and immunoenhancing capabilities have been described.

Because of the economic and social impact of viral infections in aquaculture and since no efficient therapeutic agents against fish viral infections have yet been developed, antiviral agents, such as defensins, could be of great interest. Therefore, we have evaluated the ability of synthetic HNP1 to inhibit the infectivity of viral haemorrhagic septicaemia rhabdovirus (VHSV), one of the most devastating viruses for worldwide aquaculture (Lorenzen and LaPatra, 2005; Olesen and Korsholm, 1997). HNP1 was chosen because it has been shown to be effective against both non-enveloped (Buck et al., 2006) and enveloped viruses (reviewed in reference Klotman and Chang, 2006) including the rhabdovirus causing vesicular stomatitis in mammals (Daher et al., 1986), and the mechanism underlying the antiviral activity of HNPs against certain enveloped virus is partially known (Chang et al., 2003; T.L. Chang et al., 2005; Mackewicz et al., 2003; Sinha et al., 2003).

The results obtained in this work showed that HNP1 causes a dose-dependent inhibition of VHSV infectivity *in vitro* in the absence of cellular toxicity. Preliminary characterization of its mechanism of inhibition indicated that HNP1 exhibited anti-VHSV activity at least on two levels: directly by inactivating virus particles and indirectly by inducing cellular antiviral responses in the host fish cells. In addition, HNP1 showed immunomodulatory activity since *ex vivo* treatment of trout head kidney leucocytes with HNP1 increased the transcriptional expression level of IFN-stimulated genes (ISGs) and other immune related transcripts. Work is in progress to evaluate the possible *in vivo* activity of HNP1 by using protein- or gene-transfer based methodologies to explore the potential use of these AMPs for the development of novel fish therapeutic agents and/or vaccines.

2. Materials and methods

2.1. Fish cell lines and viral haemorrhagic septicaemia virus (VHSV)

The fish cell lines epithelioma papulosum cyprini (EPC) (Fijan et al., 1983), purchased from the European collection of cell cultures (ECACC No. 93120820), and RTG-2 (rainbow trout gonad) (Wolf and Quimby, 1962), purchased from the American Type Culture Collection (ATCC CCL 55), were used.

EPC cells were maintained at 28 °C in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen Corporation, UK) cell culture medium containing 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO, USA), 1 mM pyruvate (Gibco, Invitrogen Corporation, UK), 2 mM L-glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone. Likewise, RTG-2 were maintained at 20 °C in a 5% CO_2 atmosphere with MEM (with Earle's salts) cell culture medium (Gibco) containing 10% fetal calf serum (Sigma), 2 mM glutamine (Gibco) and 50 μg/ml neomycin sulphonate (Sigma).

Viral haemorrhagic septicaemia virus (VHSV 07.71) isolated in France from rainbow trout, *Oncorhynchus mykiss* (LeBerre et al., 1977) was propagated in EPC cells at 14 °C as previously reported (Basurco and Coll, 1989). Supernatants from VHSV-07.71 infected EPC cell monolayers were clarified by centrifugation at 1000 × *g* for 20 min and stored in aliquots at −70 °C. Viruses from clarified supernatants were concentrated to 10^{11} foci forming units (f.f.u.) per ml by ultracentrifugation at 100,000 × *g* for 45 min (Basurco and Coll, 1989).

2.2. Synthetic HNP1

Synthetic human alpha-defensin-1 (ACYCRIPACIAGERRYGTCIYQGRLWAFCC-NH_2) was purchased from Peptides International (Louisville, KY, USA). The purity of peptide was >98%. HNP1 was reconstituted to a final concentration of 1 μg/μl in sterile distilled water and stored until used in suitable aliquots at −20 °C. Several batches have to be tested for optimal activity, most probably due to differences in their disulphide bound patterns (native, fully active HNP1 has disulphide bonds between cysteins 2–30, 4–19 and 9–29).

2.3. Viral infectivity assays

To assay for VHSV infectivity, a previously developed immunostaining focus assay (focus forming units, f.f.u.) was used (Lorenzo et al., 1996; Mas et al., 2002, 2006; Perez et al., 2002; Micol et al., 2005). To test the influence of pre-incubation of VHSV with HNP1, different concentrations of HNP1 (up to 20 μg/ml) was incubated with 10^3 ffu from replication-competent stocks of concentrated VHSV (10^{10} ffu/ml) for 12 h at 14 °C in 25 μl serum-free cell culture medium supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin. After incubation, VHSV–HNP1 mixtures were added to the RTG-2 or EPC cell monolayers, grown in 96-well plates, in a final volume of 100 μl per well. The infected cell monolayers were then either not washed, or washed when indicated, and further incubated for 24 h at 14 °C. Alternatively, EPC and/or RTG-2 cell monolayers were either infected with VHSV (m.o.i. of 10^{-3}) in the presence of different concentrations of HNP1 or pre-incubated with HNP1 for 12 h at 28 and 20 °C, respectively, washed extensively and then infected with VHSV (m.o.i. of 10^{-3}). In both cases, infected cells were incubated for 24 h at 14 °C. The cell monolayers were then fixed for 10 min in cold methanol and air-dried. Monoclonal antibody (MAb) 2C9 directed towards the N protein of VHSV diluted 1000-fold in dilution buffer (0.24 mM merthiolate, 5 g/l Tween 20, 50/l mg of phenol red in PBS, pH 6.8) were added to the wells (100 μl/well) and incubated for 1 h at room temperature. After washing with distilled water, 100 μl of peroxidase-labelled rabbit anti-IgG mouse antibody (Ab) (Nordic, Tilburg, The Netherlands) were added per well, and incubation was continued for 30 min. After three washes by immersion in distilled water, 50 μl of 1 mg/ml per well of diaminobenzidine (DAB) (Sigma) in PBS containing H_2O_2 were added (Lorenzo et al., 1996; Sanz and Coll, 1992) and the reaction allowed to proceed until brown foci were detected with an inverted microscope (Nikon Eclipse TE2000-U, Nikon instruments Inc., NY, USA). Once washed with water and air dried, brown foci of DAB stained cells (VHSV-infected cell foci) were counted with an inverted microscope with a 10× ocular eye grid (Lorenzo et al., 1996). The results were expressed as the percentage of infectivity and calculated by the formula: (number of VHSV-infected cell foci in the presence of HNP1/total number of VHSV-infected cell foci in the absence of HNP1) × 100.

The mRNA corresponding to the N and G proteins of VHSV in EPC cells either infected with VHSV (m.o.i. of 10^{-3}) in the presence of 20 μg/ml of HNP1 or pre-incubated with 20 μg/ml of HNP1 for 12 h at 28 °C, washed and then infected with VHSV (m.o.i. of 10^{-3}) were also quantified 9 h post-infection by quantitative RT-PCR using specific primers for the VHSV-N and G genes (Chico et al., 2006).

2.4. Determination of VHSV binding to solid-phase HNP1 by enzyme-linked immunosorbent assay (ELISA)

DTT-treated HNP1 was obtained by incubating HNP1 (20 μg/ml) with 50 mM DTT for 30 min at 37 °C. Polystyrene plates (Dynatech, Plochingen, Germany) were coated with 0, 0.5, 1 or 2 μg of untreated HNP1 or with 2 μg of dithiothreitol (DTT)-treated HNP1 per well in 100 μl of distilled water, incubated overnight at 37 °C to dryness, and kept sealed with blue silica gel at 4 °C until used. After blocking with 3% dry milk in dilution buffer (0.24 mM merthiolate, 0.1% Tween 20, 0.005% phenol red in PBS) for 1 h at room temperature and washing, the plates were incubated for 3 h at 14 °C with concentrated VHSV (10^7 ffu/well) in 100 μl of distilled water or 100 μl/well of PBS. The unbound viral particles were then removed by washing the plates four times by immersion in distilled water. Polyclonal antibody (PoAb) anti-HNP1 (Alpha Diagnostic, San Antonio, USA) and MAb I10 (Fernandez-Alonso et al., 1998) reactive against VHSV-G protein diluted 500- and 200-fold, respectively, in dilution buffer (100 μl/well) was added to the wells and incubated for 90 min at room temperature. After washing with distilled water, 100 μl/well of a peroxidase-labeled goat anti-mouse IgG Ab (Sigma) diluted 300-fold in dilution buffer were added per well and the incubation was continued for 45 min. For color development, the plates were washed three times with distilled water and 100 μl of substrate buffer (150 mM sodium citrate, 3 mM H_2O_2 and 1 mg/l *o*-phenylenediamine, pH 4.8) were pipetted per well. The reaction was stopped after 30 min with 100 μl per well of 4N H_2SO_4. Absorbance readings at 492 nm to estimate enzymatic activity and 620 nm for estimation of each individual well background readings were measured using an ELISA plate reader (Anthos, LabTec Instruments).

2.5. Generation of a permanently transformed EPC cell line expressing green fluorescent protein (EPC-eGFP)

An EPC-eGFP cell line was obtained as previously described (Brocal et al., 2006) with minor modifications. Briefly, EPC cell monolayers in six-well plates were co-transfected with 1.5 μg of pMCV1.4-eGFP plus 0.5 μg of pAE6-*pac* (puromycin resistance gene) plasmid constructions (Brocal et al., 2006). The pMCV 1.4-eGFP plasmid was obtained by subcloning the eGFP cDNA sequence from the pGFP plasmid (Clontech, CA, USA) into pMCV 1.4 vector (Ready-Vector, Madrid, Spain) (Rocha et al., 2004) following standard procedures. After transfection, puromycin resistant cells were selected by adding 20 μg/ml of puromycin (Sigma) to the cell culture media at 6 days. Resulting puromycin-resistant cells were seeded in 96-well plates at a density from 1 to 50 cells/well (limiting dilution) and grown in cell culture medium conditioned by the growth of non-transfected EPC cells to favor growth of isolated cells. Twenty-four hours later, the wells were screened for the presence of single cells. Two weeks later, single colonies were transferred to wells of 48-well plates and grown in conditioned medium. Cell lines expressing eGFP were selected among the puromycin-resistant clones using an inverted fluorescence microscope (Nikon). Three EPC-eGFP cell lines were obtained and one of them selected for further work. The selected EPC-eGFP cell line was grown in 96-well plates and gradually transferred into cell culture flasks. The EPC-eGFP cell line has been maintained by continuous culture (about one subculture per week) in the absence of puromycin as described above for non-transformed EPC cell monolayers during more than 3 years.

2.6. Viral binding assays

To determine if HNP1 inhibited the binding of VHSV to cells, EPC-eGFP cell monolayers, grown on six-well plates, were incubated with VHSV (m.o.i. 0.3) in the presence or absence of 20 μg/ml of HNP1 or with VHSV pre-treated with HNP1 (12 h at 14 °C in serum-free cell culture medium) for 2 h at 4 °C. Cells were washed three times with PBS to remove unbound virus, and cell-bound virus was then detected by probing Western blots of cell lysates with the anti-VHSV N protein MAb 2C9 and an anti-GFP MAb (Santa Cruz Biotechnology, CA, USA) to control for protein loading. Briefly, infected cells were frozen and thawed and supernatants from cell lysates clarified by centrifugation (1000 × *g* for 10 min). SDS-polyacrylamide gels at 12% were loaded with 20 μl of samples in buffer containing β-mercaptoethanol. The proteins in the gel were transferred during 3 h at 125 mM in 2.5 mM Tris, 9 mM glycine, 20% methanol to nitro-cellulose membranes (BioRad, Richmond, VI, USA). The membranes were blocked with 2% dry milk, 0.05% Tween-20 and 0.3% rabbit serum in PBS and incubated for 2 h at room temperature with the above mentioned antibodies. Blots were then incubated with peroxidase-conjugated rabbit anti-IgG mouse antibody (SIGMA) diluted 1/500 in 2% milk-containing PBS for 45 min. Finally, the peroxidase activity was detected using the ECL chemiluminescence reagents (Amersham Biosciences, UK) and revealed by exposure to X-ray films (Amersham).

2.7. VHSV G protein-mediated syncytia formation in VHSV-infected cells in the presence of HNP1

EPC-eGFP cell monolayers, grown on 96-well plates, were infected with VHSV at a m.o.i. of 10^{-3} to 10^{-2}. Twenty-four hours post-infection, cells were washed and treated with HNP1 (20 μg/ml) in serum-free medium for 45 min at 14 °C or untreated. After cell washing, fusion was triggered by incubating cells with fusion medium (Mas et al., 2002) at pH 6 for 30 min at 14 °C. Monolayers were then washed and incubated with fusion medium at pH 7.5 for 2 h at room temperature. The cell monolayers were fixed with 4% paraformaldehyde in PBS (15 min at room temperature) and then incubated with the MAb anti-VHSV G protein I10 diluted 200-fold in PBS for 2 h at room temperature. The indirect staining was carried out by using rabbit anti-mouse antibody conjugate to rhodamine (TRITC, Sigma). To visualize nuclei, cells were incubated with 0.1 mg/ml of the DNA stain Hoechst (Sigma) for 10 min. Cells were viewed and photographed with an inverted fluorescence microscope (Nikon) provided with a digital camera (Nikon DS-1QM). To analyze the fusion in RTG-2 cells, cells were fixed with cold methanol, dried and stained with Giemsa (5 mg/ml in PBS). To measure the extent of fusion, the number of nuclei in syncytia of three or more nuclei per syncytia was counted among 10,000 nuclei per well as previously described (Estepa and Coll, 1997; Estepa et al., 2001).

2.8. Treatment of RTG-2 and head kidney leucocytes with HNP1

Confluent monolayers of RTG-2 cells in 24-well tissue culture plates were treated with PBS, 20 μg/ml of HNP1, 20 μg/ml of synthetic Pleurocidin (Ple) from winter flounder (Cole et al., 1997; Brocal et al., 2006) or 30 μg/ml of Polyribocytidylic acid (poly I:C) (Pharmacia, Piscataway, NJ, USA). At 24 h post-induction, cells in control and induced wells were harvested. In all cases, after incubation period, the medium was removed, cells desattached with Ca^{2+} and Mg^{2+}-free PBS and total RNA extracted.

Head kidney leukocytes were isolated following the method previously described (Graham et al., 1988). Briefly, fish were sacrificed by overexposure to MS-222 and the anterior kidney removed aseptically and passed through a 100 μm nylon mesh using RPMI-1640 Dutch modified culture medium supplemented with 10% fetal calf serum (Sigma), 1 mM pyruvate (Gibco), 2 mM glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone. The viable cell concentration was determined by Trypan blue exclusion. Cells were resuspended in RPMI-1640 with 10% FCS and 2% trout serum, dispensed into 24-well plates at a concentration of 1×10^6 cells/ml, and then incubated with PBS, 20 μg/ml of HNP1 or 30 μg/ml of poly I:C. After 24 h, total RNA was extracted from the cells as described below.

2.9. RNA isolation and cDNA synthesis

The "Total RNA Isolation System" (Promega) was used for cellular RNA extraction following manufacturer's instructions. Isolated RNAs were treated with DNase (RQ1 RNAase-Free Dnase, Promega), resuspended diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C until used. Two micrograms of RNA were used to obtain cDNA using the Moloney murine leukaemia virus reverse transcriptase (M-MLV) (Invitrogen). Briefly, RNA was incubated with 1 μl of random hexamers (50 μM) (Roche) and 1 μl 10 mM deoxynucleotide triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of M-MLV reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min and the resulting cDNA stored at −20 °C.

2.10. Detection of Mx1, Mx2, Mx3, Vig-1, TLR3, IL1β and iNOS cDNA by PCR

All amplification reactions contained 200 μM of each deoxynucleotide triphosphate, 1 unit of Taq polymerase (Roche), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 1 μM of each primer and 1 μl of cDNA in a final volume of 25 μl. PCR amplifications with primers for glyceraldehyde 3-phosphate dehydrogenase (GADPH) were performed with all samples as internal reference amplification for RT-PCR, since GADPH is constitutively expressed in all organs. Primers used

Table 1
Genes, sequence of primers and size of amplicons used in this study

Gene	Name	Sequence 5′–3′	Size (bp)	Reference
Mx1 protein	Mx1	**F**: ATGCCACCCTACAGGAGATGAT **R**: TAACTTCTATTACATTTACTATGCAA	421	Tafalla et al. (2007)
Mx2 protein	Mx2	**F**: ATGCCACCCTACAGGAGATGAT **R**: GGAAGCATAGTAACTTTATTATAAC	400	Tafalla et al. (2007)
Mx3 protein	Mx3	**F**: ATGCCACCCTACAGGAGATGAT **R**: CCACAGTGTACATTTAGTTG	381	McLauchlan et al. (2003)
VHSV-induced gene 1	Vig-1	**F**: CAGTTCAGTGGCTTTGACGA **R**: ACAAACGCCTCAAGGTATGG	232	Boudinot et al. (1999)
Toll-like receptor 3	TLR3	**F**: TGACAGAGCTTAACCTGGCT **R**: AAGAACTTCCAGCATGGACA	538	Rodriguez et al. (2005)
Interleukin 1 beta	IL1β	**F**: AGGGAGGCAGCAGCTACCACAA **R**: GGGGGCTGCCTTCTGACACAT	353	Wang et al. (2002)
Inducible nitric oxide synthase	iNOS	**F**: CATACGCCCCCAACAAACCAGTGC **R**: CCTCGCCTTCTCATCTCCAGTGTC	746	Lindenstrom et al. (2004)
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	**F**: ATGTCAGACCTCTGTGTTGG **R**: TCCTCGATGCCGAAGTTGTCG	514	T. Wang et al. (2004)

F, Forward primer; R, reverse primer.

for mRNA amplification and the sizes of the different PCR products are shown in Table 1. All PCRs were carried out in a Perkin-Elmer 2400 cycler and all samples were amplified twice to verify the results. Amplification conditions consisted of a denaturing step (5 min at 94 °C) followed by different specific cycling conditions and a final extension of 7 min at 72 °C. These conditions were established for each gene following protocols described previously by the references shown in Table 1. The PCR products in 8 μl were visualised on a 1.6% agarose gel stained with ethidium bromide. Samples that were to be compared were run in the same agarose gel. A 100 bp ladder was used as a size marker. The optical density (OD) of the amplification bands was estimated using the Scion image software. The mRNA expression for each gene was determined relative to the expression of the GADPH gene in the same sample using the formula: OD of mRNA band/OD of corresponding GADPH band.

2.11. Detection of the mRNA of the N protein of VHSV by quantitative RT-PCR

Primers and the FAM-labeled (TaqMan®) probe for the N gene of VHSV were described in a previous report (Chico et al., 2006). Quantitative PCR assays were performed using an ABI PRISM® 7300 Sequence Detector System. Reactions were carried out in a final volume of 25 μl, containing 300 nM of each primer, 100 nM of the probe, 2 μl of cDNA and 1× Absolute Q-PCR ROX Mix (ABGene). The polymerase chain reaction conditions consisted of one cycle of 2 min at 50 °C and 15 min at 95 °C followed by 40 cycles of 15 s

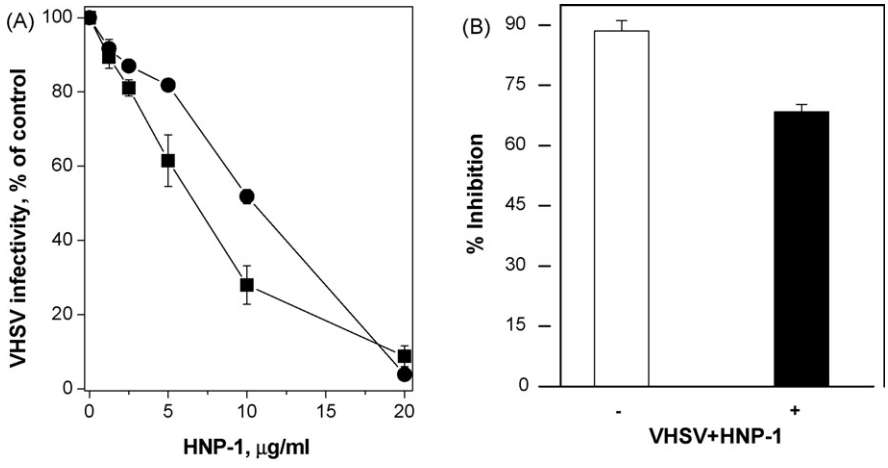

Fig. 1. Pre-incubation of VHSV with HNP1 in the absence of serum inhibits VHSV infectivity. 10^3 ffu of VHSV were mixed with increasing concentrations of HNP1 in 25 μl of serum-free cell culture medium and incubated at 14 °C for 12 h. The VHSV–HNP1 mixtures were then diluted five-fold to a final volume of 100 μl by adding 75 μl of cell culture medium containing 2% FCS. Then the mixtures were added to EPC (■) or RTG-2 (●) cell monolayers, grown in 96-well plates. Two hours later, the infected cell monolayers were either not washed (A and inset B−, white bar) or washed (inset B+, black bar) and further incubated at 14 °C during 24 h. The VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. Data are mean ± standard deviations (S.D.) from three different experiments, each experiment performed in triplicate.

at 90 °C and 1 min at 60 °C. Endogenous control included for quantitation was the 18S ribosomal RNA gene as determined with the TaqMan® Ribosomal RNA Control Reagents Kit (Applied Biosystems) following the manufacturer's guidelines.

3. Results

3.1. Pre-incubation of VHSV with HNP1 in the absence of serum inhibits VHSV infectivity

The ability of HNP1 to inactivate VHSV was studied by preincubating increasing concentrations of HNP1 with 10^3 ffu of cell-free VHSV in 25 μl of serum-free cell culture medium. The VHSV–HNP1 mixtures were then diluted with 75 μl of cell culture medium containing 2% of FCS (five-fold dilution) and added to and incubated with the fish cell monolayers until analysis of VHSV infectivity (24 h post-infection). Serum-free medium was used for those incubations because the inhibitory effect of HNP1 on other virions has been reported to be reduced by serum proteins (T.L. Chang et al., 2005; Daher et al., 1986; Mackewicz et al., 2003). Fig. 1A shows that HNP1 inhibited VHSV-infected cell foci formation in a dose-dependent manner in both EPC and RTG-2 cell lines, two fish cell lines of different origin, indicating that VHSV inhibition by HNP1 was independent on the cell line. Maxima inhibition (>90% of the ffu in both cell lines) was observed when 20 μg/ml of HNP1 were preincubated with VHSV.

On the other hand, no cytotoxicity was observed when the EPC cell monolayers were treated with HNP1 at different concentrations up to 20 μg/ml (data not shown), indicating that the effects of HNP1 on VHSV infectivity were not due to non-specific cytotoxicity.

3.2. Infection of EPC cell monolayers with VHSV in the presence of serum and HNP1 also inhibits VHSV infectivity without altering the VHSV binding to EPC cell monolayers

The previously commented inhibitory assays were performed by pre-incubating VHSV with HNP1, adding the VHSV–HNP1 mixture to the EPC cell monolayers and maintaining their presence throughout all the time of incubation until analysis. However, when the excess of HNP1 and unbound VHSV were removed from the cell culture medium 2 h post-infection and then incubation proceeded with fresh medium devoid of HNP1, the antiviral effect of HNP1 was ~30% reduced (Fig. 1B). To investigate any potential antiviral effects caused by the presence of HNP1 during the infection time, EPC cells were infected in the presence of HNP1 with non-treated VHSV in cell cultured medium containing 2% of serum. The results showed that HNP1 when added from the beginning of the infection ($t = 0$) also inhibited VHSV infectivity in EPC cells (Fig. 2A) with a very similar inhibitory profile to that shown when VHSV and HNP1 were pre-incubated in the absence of serum before infection (Fig. 1A). Thus, VHSV infectivity was reduced to 86, 80, 57 and 19% by the presence of 1.25, 5, 10 and 20 μg/ml of HNP1, respectively (Fig. 2A). This result suggested that in the presence of serum some HNP1 antiviral mechanism other than that exerted directly on VHSV particles could be operating.

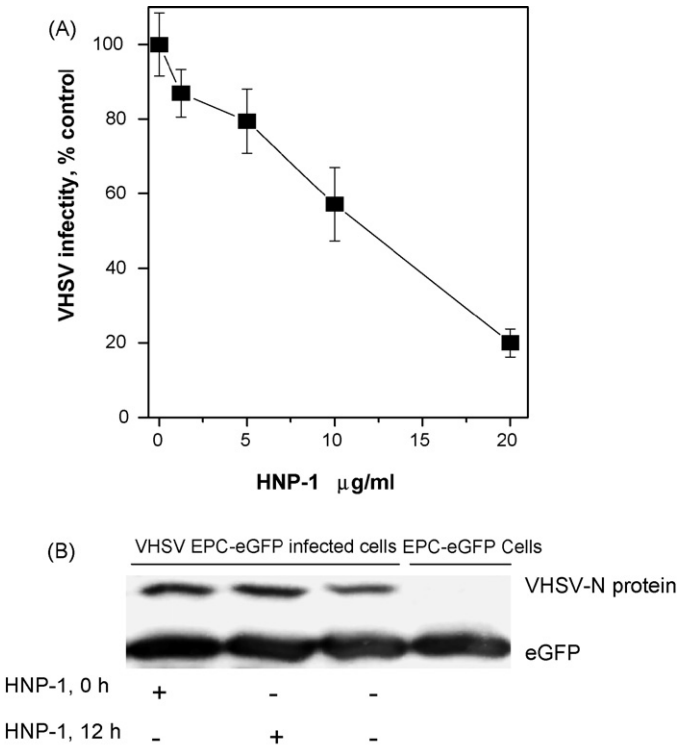

Fig. 2. Inhibition of VHSV infectivity by HNP1 (A) and effect of HNP1 on binding of VHSV to EPC cell monolayers (B). (A) EPC cell monolayers grown in 96-well plates, were infected with VHSV in the presence of different concentrations of HNP1 in cell culture media containing 2% of FCS. Twenty-four hours later, VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. Data are mean ± S.D. from three different experiments, each performed in triplicate. (B) EPC-eGFP cell monolayers (permanently expressing eGFP), grown in six-well plates, were incubated in 2% FCS containing cell culture medium during 2 h at 4 °C with VHSV in the absence of HNP1 (HNP1, 0 h, −), in the presence of 20 μg/ml of HNP1 (HNP1, 0 h, +) or with pre-incubated VHSV + HNP1 as in Fig. 1 (HNP1, 12 h, +). After washing unbound virus, cell lysates were prepared and separated by SDS-PAGE, and cell-bound virus was visualized by Western blotting with anti-N 2C9 or anti-eGFP MAbs. The inmunostained gel is representative of three independent experiments.

The inhibition of VHSV infectivity by the presence of HNP1 at the infection time could not be explained by changes in the binding of VHSV to the EPC cell monolayers because incubation of VHSV with EPC-eGFP cells at 4 °C with or without 20 μg/ml of HNP1 resulted in similar amounts of EPC cell-associated VHSV N protein (VHSV binding assay) (Fig. 2B). Similar results were found for VHSV pre-incubated with 20 μg/ml of HNP1 (Fig. 2B).

3.3. Pre-incubation of EPC cell monolayers with HNP1 in the presence of serum prior infection also inhibits VHSV infection

Cell-mediated antiviral effects induced by pre-incubation of cells with HNP1 before or during viral infection have been previously reported (Klotman and Chang, 2006). To investigate

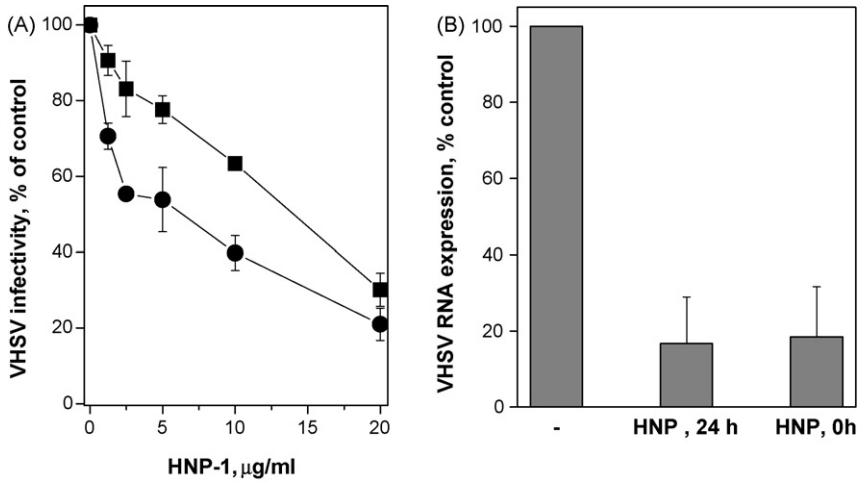

Fig. 3. Inhibition of VHSV infectivity (A) and VHSV RNA synthesis (B) by pre-incubation of cell monolayers with HNP1. EPC (■) and RTG-2 (●) cell monolayers were pre-incubated with HNP1 at the indicated concentrations in cell culture medium containing 10% FCS for 24 h. Cell monolayers were then washed, infected with VHSV in cell culture media containing 2% of FCS and incubated during 24 h at 14 °C. VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. Data are mean ± S.D. from three different experiments, each performed in triplicate. (B) EPC cell monolayers were incubated with 20 μg/ml HNP1 in cell culture media containing FCS during 24 h (24 h) before infection with VHSV or only during the time of VHSV addition (0 h). Viral RNA levels were determined at 9 h post-infection by TaqMan® quantitative RT-PCR. The average value of the non-treated VHSV-infected samples was 100%. The values are given as mean ± S.D. of duplicates.

whether cellular anti-VHSV defense mechanisms are induced by HNP1 in fish cells, monolayers of EPC and RTG-2 cells were treated with HNP1 for 24 h in the presence of 10% FCS followed by washing off HNP1 before infection with VHSV. Fig. 3A shows that also in this case, VHSV infectivity was similarly reduced in a dose-dependent manner.

The maximal inhibition of VHSV infectivity of 70–80% was observed in both EPC and RTG-2 cell monolayers incubated (Fig. 2A) or pre-incubated (Fig. 3A) with 20 μg/ml of HNP1. Similar results of 80–90% reduction were obtained by estimating VHSV infectivity by the mRNA levels of their proteins N (Fig. 3B) and G (data not shown).

Taken together, all these results indicate that in the presence of serum, HNP1 acts on fish VHSV target cells by inducing protection against the VHSV infection and that this effect is independent on the fish cell line and persisted after washing out the HNP1.

3.4. Interaction between HNP1 and VHSV in the absence of serum

If HNP1 inhibits VHSV infectivity in the absence of serum by inactivating VHSV virions, interactions of HNP1 with VHSV particles should be detectable. To investigate this possibility, VHSV binding to solid phase HNP1 was estimated by using specific MAbs to the G protein of VHSV. First, HNP1 was shown to be present in the solid phase since it could be detected by using a polyclonal antibody (PoAb) anti-HNP1 (Fig. 4A). The binding of DTT-treated HNP1 to solid phase was similar to that of the untreated HNP1 (not shown). Fig. 4A shows an HNP1 concentration- and disulphide-dependent recognition of VHSV bound to solid-phase HNP1 by a non-conformational anti VHSV-G protein MAb, indicating that a conformation-dependent HNP1 interacts with VHSV. The elimination of the HNP1 recognition of VHSV by reduction with DTT (Fig. 4A) suggests that the disulphide bonds are important for the recognition of VHSV by HNP1.

3.5. HNP1 inhibits VHSV G protein-mediated syncytia formation in VHSV-infected cell monolayers

The interaction of HNP1 with VHSV could be due to binding to the G protein or to the phospholipids of the VHSV membrane, the only types of molecules exposed on the VHSV surface. Because in the absence of serum, HNP1 can inactivate enveloped mammal virus particles by interacting with their surface glycoproteins (Klotman and Chang, 2006; Sinha et al., 2003; W. Wang et al., 2004; Yasin et al., 2004), we further studied the possible interaction of HNP1 with the VHSV-G protein by using a VHSV G protein-dependent fusion assay. VHSV-infected fish cells express VHSV G protein at the cell membrane of infected fish cells and those infected cells can be induced to fuse by lowering the cell culture media to pH 5–6. Cell-to-cell fusion of infected cells results in the formation of syncytia (multinucleated cells). Therefore, to examine whether HNP1 modifies the VHSV-G protein fusion properties, we assayed the possible interference of HNP1 in a syncytium-forming assay. This assay has been described before (Estepa et al., 2001; Mas et al., 2004), except in this case, we used the permanently transformed EPC cell line EPC-eGFP, to best detect the nuclei in syncytia. No syncytia of more than two to three nuclei per syncytia were detected in VHSV-infected EPC-eGFP cell monolayers incubated with HNP1 (Fig. 4D) despite the presence of protein G in the membrane of infected cells (Fig. 4D2). In contrast, syncytia were abundant in VHSV infected and non-treated cells (∼19 and 35% of nuclei in syncytia at m.o.i. 0.001 and 0.01, respectively) (Fig. 4B and C). On the other hand, the incubation of both uninfected EPC-eGFP cell monolayers (data not shown)

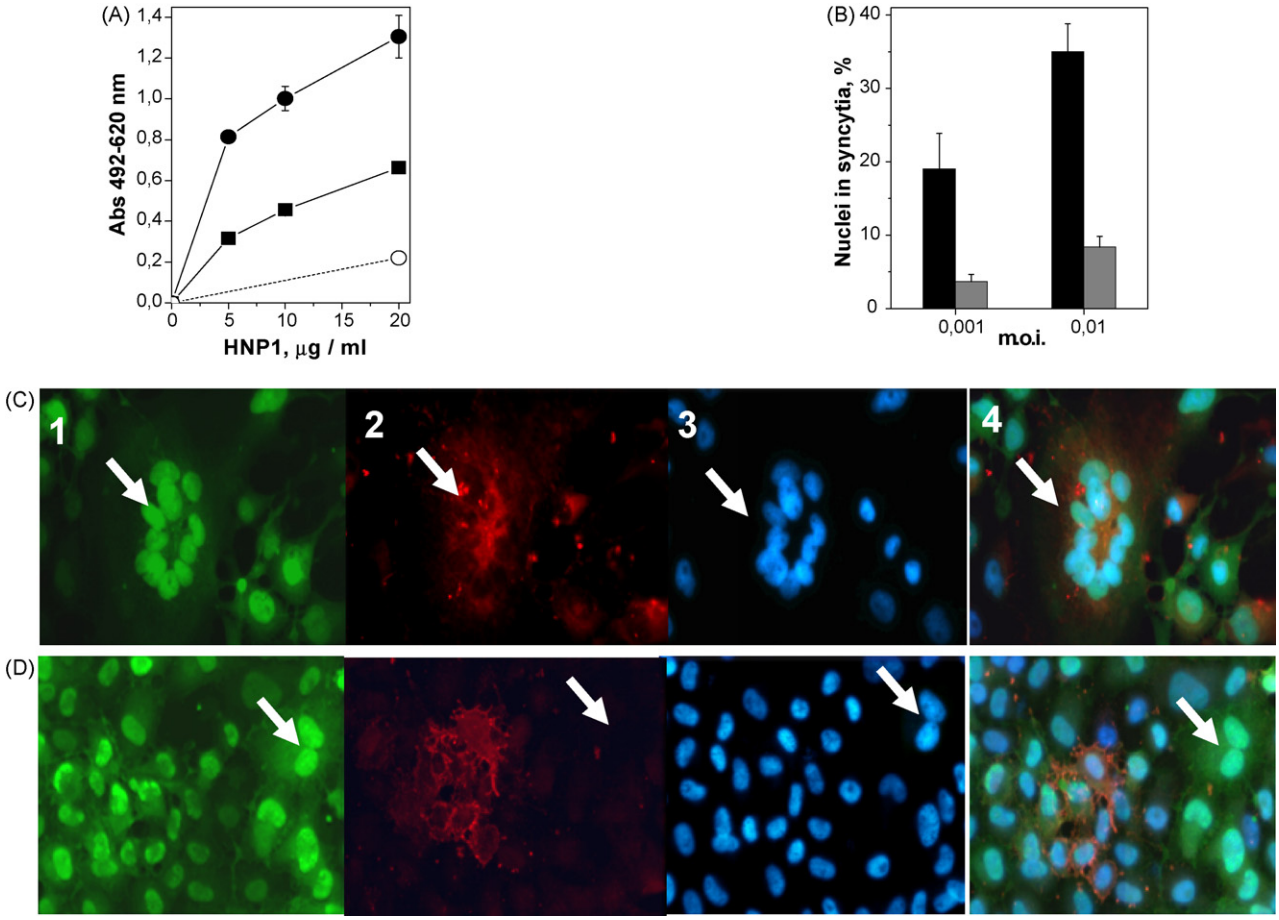

Fig. 4. Binding of VHSV to solid-phase HNP1 (A) and inhibition of syncytia formation in VHSV-infected EPC cell monolayers by HNP1 (B–D). (A) Different amounts of HNP1 or DTT-treated HNP1 were dried onto solid-phase 96-well plates and HNP1 was then detected by using a PoAb anti HNP1 (■). Concentrated VHSV was then added to each well and incubated during 3 h at 14 °C. After washing MAb I10 against VHSV-G protein was used to detect DTT-HNP1- (◯) and HNP1-bound VHSV (●). Data are means ± S.D. from two experiments, each performed in triplicates. (B) EPC-eGFP cell monolayers infected with VHSV during 24 h were washed and then incubated or not with HNP1 during 45 min. After washing the cell monolayers, cell-to-cell fusion was triggered by incubating with fusion medium at pH 6 during 30 min. Cell monolayers were then fixed, fluorescent microphotographies taken and number of nuclei in syncitya counted. Results are expressed as the percentage of nuclei in syncytia by the formula: number of nuclei in syncytia/total number of nuclei × 100. Data are the means ± S.D. from two different experiments, each performed in triplicates. Black bars, not incubated with HNP1. White bars, incubated with HNP1. (C and D) Fluorescent microphotographies of the VHSV infected EPC-eGFP cell monolayers at m.o.i. 0.001. (C) Not incubated with HNP1. (D) Incubated with HNP1. (1) GFP fluorescence (green fluorescence), (2) stained with MAb I10 anti-G protein of VHSV and anti Igs-TRITC (red fluorescence), (3) stained with the Hoechst DNA stain (blue fluorescence), (4) merged fluorescence of fields 1, 2 and 3. Arrows, syncytia.

and VHSV-infected EPC-eGFP cell monolayers with HNP1 (Fig. 4D) induced the unspecific formation of small syncytia (3–4%) of two to three nuclei per syncytia (Fig. 4B).

3.6. HNP1 induces mRNA expression of the Mx3 gene in RTG-2 cell monolayers

The inhibition of VHSV infectivity when using cell monolayers pre-incubated with HNP1 before the VHSV infection suggested that some cellular antiviral defense mechanisms might have been induced by HNP1 in fish cells. To investigate whether a type I IFN response could be induced by HNP1, RTG-2 cell monolayers were incubated during 24 h with HNP1 or poly I:C, a well-known type I IFN-inducer. As negative control, cell monolayers were not incubated or incubated with Pleurocidin (Ple), an AMP from flat fish that does not affect the expression levels of genes associated with the IFN response (Chiou et al., 2006). The TLR3 and the IFN-inducible Mx3 genes were selected to represent the IFN response genes. The Mx3 gene was chosen as marker for IFN responses among the three different rainbow trout Mx genes, because Mx3 was the Mx isoform predominantly expressed in RTG-2 cells in response to different IFN inducers (Tafalla et al., 2007). Both TLR3 and Mx3 genes were up-regulated in RTG-2 cell monolayers treated with HNP1 or poly I:C although induction was always higher in response to poly I:C (Fig. 5A and B). No changes in either TLR3 nor Mx3 gene expression levels were observed in response to Ple (data not shown).

3.7. HNP1 induces mRNA expression of immune response-related genes in trout head kidney leucocytes

To investigate the possible effect of HNP1 on fish immune responses *in vitro*, the mRNA expression profile of a set of repre-

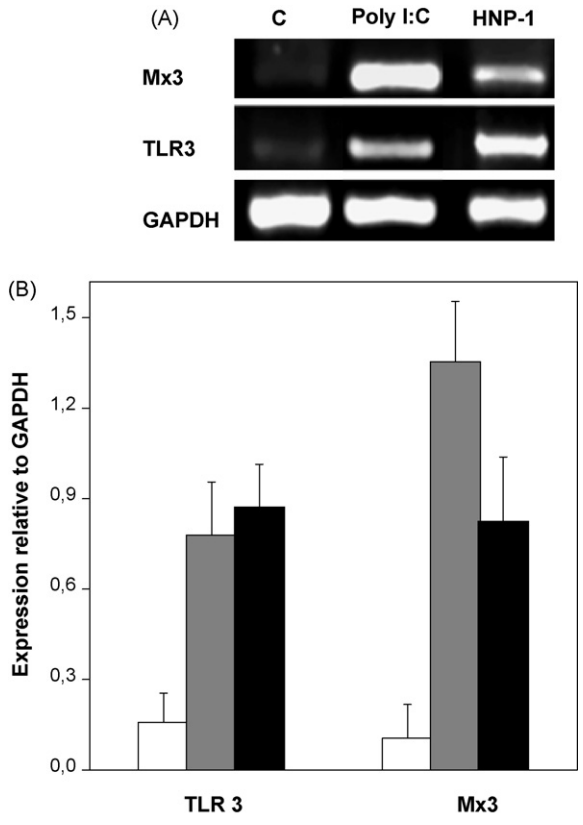

Fig. 5. Expression of transcripts from TLR3 and Mx3 genes in RTG-2 cells in response to HNP1 and poly I:C. Monolayers of RTG-2 cells were incubated with HNP1 or poly I:C. After 24 h of incubation at 20 °C, total RNA was extracted and the expression of transcripts from TLR3 and IFN-inducible Mx3 genes were then estimated by RT-PCR. The mRNA expression relative to GADPH was calculated by the formula: OD of mRNA band/OD of the corresponding GADPH band. (A) Photography of an agarose gel of Mx3, TLR3 and GAPDH mRNA amplified by RT-PCR. The gel is representative of three experiments. (B) Data are mean ± S.D. from two experiments, each performed in duplicate. White bars, untreated RTG-2 cells. Gray bars, HNP1-treated RTG-2 cells. Black bars, poly I:C-treated RTG-2 cells.

sentative immune response-related genes was analyzed in trout head kidney leucocytes. Fig. 6 shows, that the levels of Mx1, Mx2, Mx3, Vig-1, TLR3, IL1β and iNOS transcripts, as assayed by RT-PCR, were increased in head kidney leucocytes incubated with HNP1. A similar effect was shown for samples incubated with poly I:C with the exception of the iNOS gene transcripts.

4. Discussion

This work shows that synthetic human HNP1 is active in fish since it inhibits VHSV replication in fish cells and has immunoregulatory activity on fish leucocytes.

It has been previously described that the mechanisms underlying the antiviral actions of mammal defensins are multiple and complex and include direct effects on the virion as well as effects on the target cell and on innate and adaptive immunity (Klotman and Chang, 2006). In agreement with those findings HNP1 showed at least two mechanisms of anti-VHSV activity. Thus, HNP1 inhibits VHSV replication directly by interacting with the VHSV particles (Figs. 1 and 4) and indirectly by affecting the target cells (Figs. 2 and 3).

In the absence of serum, HNP1 inactivated VHSV before cell infection as it had been shown for type 1 and type 2 herpes simplex viruses (HSV-1 and -2, respectively) (Daher et al., 1986) and HIV-1 (Chang et al., 2003; T.L. Chang et al., 2005) as well as for vesicular stomatitis virus (VSV) (Daher et al., 1986), the prototype virus of rhabdoviridae family. In the presence of serum and at non-cytotoxic concentrations, as shown by the HNP1 solid phase binding assay (Fig. 4A), there was binding between VHSV and HNP1 and this binding is abrogated by treatment of HNP1 with DTT (Fig. 4A), suggesting that disulphide bonds were required for the interaction of this HNP1 with the surface of VHSV particles. Similarly, the direct effect of the HNP1 or θ-defensins on HSV-1 and HIV virions was abolished when their disulphide bonds were disrupted by treatment with the reducing agents DTT and iodoacetamide (Daher et al., 1986; W. Wang et al., 2004). HNP1 inhibited cell-to-cell fusion mediated by the low pH conformation of VHSV-G protein expressed at the surface of VHSV-infected EPC cells (Fig. 4). Consequently, inactivation of VHSV particles by HNP1 may involve interactions with VHSV-G protein rather than with the VHSV envelope. Recent studies have demonstrated that defensins, included HNP1, can inactivate enveloped virus by interacting with *N*-linked or *O*-linked glycans of viral surface glycoproteins in a lectin-dependant manner (Gallo et al., 2006; Hazrati et al., 2006; Klotman and Chang, 2006; Leikina et al., 2005; W. Wang et al., 2004; Yasin et al., 2004). This kind of binding alters the ability of these glycoproteins to bind to their receptors at the target cells or to fuse with the host cellular membranes. Whether the interaction of HNP1 with the G glycoprotein of VHSV occurs by a similar mechanism or by a different one remains to be determined. The relevant interest will be to determine whether HNP1 interacts with the VHSV-G glycoprotein regions implicated in the membrane fusion process since defensins also inhibit HIV-1 by preventing 6-helix bundle formation (Gallo et al., 2006) and a similar structure has been recently reported in the VSV G glycoprotein (Roche et al., 2006). In addition, HNP1 also promoted some cell-to-cell fusion among uninfected EPC cell monolayers as shown by induction of small syncytia of two to three nuclei per syncytia in those monolayers (Fig. 4D, arrows), showing that HNP1 also interacts with the cell membranes of fish cells.

Our data also suggest that HNP1 acted also intracellulary since the inhibition of VHSV infectivity was also obtained when VHSV was not treated with HNP1 but the fish cell monolayers were either infected in the presence of HNP1 (Fig. 2A) or pretreated with HNP1 before the VHSV infection (Fig. 3A and B). Regarding the mechanisms underlying the intracellular activity of HNP1, It has been shown that HNP1 in the presence of serum inhibited HIV-1 infectivity after HIV-1 entry into cells by interfering with cell-signalling pathways required for HIV-1 replication (T.L. Chang et al., 2005). Although a similar signalling pathway-related mechanism might also be implicated in the inhibition of VHSV infectivity by HNP1, other IFN-related mechanisms were operating in fish cells as shown by the up-regulation of Mx3, a well known marker of IFN-induction in

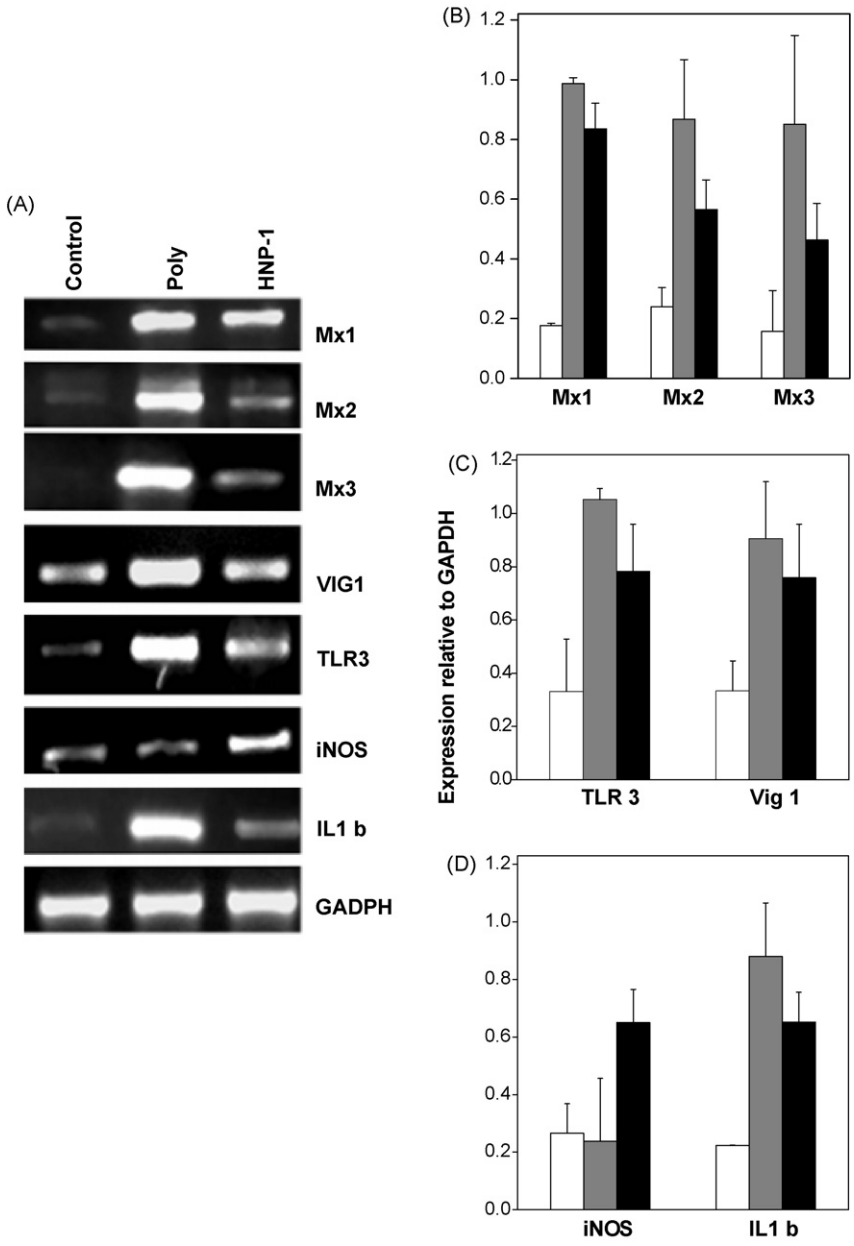

Fig. 6. Expression of transcripts from type I IFN-related genes Mx1, Mx2, Mx3 (A and D), Vig-1, TLR3 (A and C) and IL1β and iNOS (A and D) genes in head kidney leucocytes in response to HNP1. After incubating head kidney leucocytes with HNP1 during 24 h, total RNA was extracted and Mx1, Mx2, Mx3, TLR3, Vig-1, IL1β and iNOS mRNA were analyzed by RT-PCR and agarose gel electrophoresis. The mRNA expression relative to GADPH was calculated by the formula: OD of mRNA band/OD of the corresponding GADPH band. Data are means ± S.D. from three experiments, each in duplicate. White bars, untreated head kidney leucocytes. Gray bars, HNP1-treated head kidney leucocytes. Black bars, poly I:C-treated head kidney leucocytes. (A) Photography of a representative agarose gel ($n = 3$) of mRNAs amplified by RT-PCR. (B–D) Quantification of A by densitometry.

fish, in RTG-2 cells incubated with HNP1. In addition, similar up-regulated levels of TLR3 to those induced by poly I:C (a ligand of TLR3), were observed in response to HNP1, raising the question: How HNP1 activates TLR3? To date, the only evidence that HNPs are TLR ligands is the fact that murine β-defensin-2 interacts with TLR4 in dendritic cells (Froy, 2005; Yang et al., 1999). It could be possible however that for fish cells, the interaction is between HNP1 and TLR3. Learning how HNP1 induces Mx3 and TLR3 gene expression requires further study. Possibilities include the binding of HNP1 to a cellular receptor/s, receptor-mediated or independent endocytosis, transport across channels, binding to and endocytosis together with serum glycoproteins, such as transferrins (Hazrati et al., 2006), etc. In fact, accumulation of HNP1 within the cytoplasm of cells that do not synthesize HNP1, including CD4-T cells (Mackewicz et al., 2003; Zhang et al., 2002), smooth muscle cells (Nassar et al., 2002), epithelial cells and human cervical cells (CaSki cell line) (Hazrati et al., 2006) it has been previously reported although their uptake remains still unclear.

Because different effects of HNP1 have been described depending on the cells used (primary cells or transformed cell lines) (Chang et al., 2003; T.L. Chang et al., 2005; Klotman

and Chang, 2006), trout head kidney leucocytes were also used to define the HNP1 ability to modulate fish immune response. Four IFN-related genes (Mx1, Mx2, Mx3 and vig-1) were significantly modulated in head kidney leucocytes incubated with HNP1, thus confirming that HNP1 might trigger an antiviral response dependent of IFN induction. Moreover, IL1β and iNOs genes were also up-regulated indicating the immunomodulatory role of HNP1 on the leucocytes-regulated immune response in fish. In mammals the induction of proinflammatory cytokines (Froy, 2005; Lehrer and Ganz, 2002; Lillard et al., 1999; Yang et al., 2002) and IFN-γ but not IFN-α/β by HNPs has been previously reported (T.L. Chang et al., 2005; Klotman and Chang, 2006; Selsted and Ouellette, 2005).

Therefore, further studies of the antiviral activity of the heterologous HNP1 in fish and/or of HNP1-like homologous fish peptides could help the development of new orally- (medicated food) or bath immersion-administrated therapeutants for VHSV prevention as well as advance our understanding of how AMPs work to block virus replication. In addition, the inactivation of VHSV particles by HNP1 further confirms the previously proposed common mechanism that might account for a broad range of activity of the innate immune response against viruses that use a common pathway of membrane fusion for entering host cells (Klotman and Chang, 2006). Taken together, all these results suggest that HNP1, HNP1-like peptides and other innate immune system-related molecules may prove useful as templates for novel antivirals of broad range of activity in both human and animal health.

Acknowledgements

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