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The zebrafish galectins Drgal1-L2 and Drgal3-L1 bind *in vitro* to the infectious hematopoietic necrosis virus (IHNV) glycoprotein and reduce viral adhesion to fish epithelial cells*

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Abstract

The infectious hematopoietic necrosis virus (IHNV; *Rhabdoviridae*, *Novirhabdovirus*) infects teleost fish, such as salmon and trout, and is responsible for significant losses in the aquaculture industry and in wild fish populations. Although IHNV enters the host through the skin at the base of the fins, the viral adhesion and entry mechanisms are not fully understood. In recent years, evidence has accumulated in support of the key roles played by protein-carbohydrate interactions between host lectins secreted to the extracellular space and virion envelope glycoproteins in modulating viral adhesion and infectivity. In this study, we assessed *in vitro* the potential role(s) of zebrafish (*Danio rerio*) proto type galectin-1 (Drgal1-L2) and a chimera galectin-3 (Drgal3-L1) in IHNV adhesion to epithelial cells. Our results suggest that the extracellular Drgal1-L2 and Drgal3-

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L1 interact directly and in a carbohydrate-dependent manner with the IHNV glycosylated envelope and glycans on the epithelial cell surface, significantly reducing viral adhesion.

Keywords

infectious hematopoietic necrosis virus (IHNV); zebrafish; epithelial cells; galectin; viral adhesion; IHNV envelope glycoprotein

Introduction

Viral infections cause severe diseases in farmed and wild fish populations and have a detrimental impact in the aquaculture industry and the environment. Both rhabdoviruses, such as the infectious hematopoietic necrosis virus (IHNV), snakehead rhabdovirus (SHRV) and viral hemorrhagic septicemia virus (VHSV), and birnaviruses such as the infectious pancreatic necrosis virus (IPNV) are responsible for massive losses of fish in both wild and farmed salmon and trout species by highly pathogenic local and systemic infections that lead to significant morbidity and mortality (Wolf 1988; Crane and Hyatt 2011; Purcell et al. 2012).

Rhabdoviruses are characterized by a single-stranded, negative-sense RNA genome, and an envelope consisting of trimers of a glycoprotein that displays multiple *N*-linked oligosaccharides and plays an integral role in the pathogenesis of viral infection. IHNV (family *Rhabdoviridae*, genus *Novirhabdovirus* type species) infects teleost fish, such as salmon and trout, and is responsible for catastrophic losses in the aquaculture industry. The genome of IHNV encodes for six gene products, the nucleocapsid (N), phosphoprotein (P), matrix protein (M), envelope glycoprotein (G), non-virion protein (NV) and polymerase or large protein (L), and is organized in the following order: 3'-leader-N-P-M-G-NV-L-trailer-5'. Three major genetic groups of North American IHNV isolates have been defined based on their envelope protein sequence similarity, designated as the U, M, and L genogroups. The M genogroup is endemic in the rainbow trout farming region in Idaho, where phylogenetically distinct sub-groups, designated MA-MD have been reported. The MB, MC, and MD sub-groups are the three most prevalent and widely distributed types of IHNV in the virus-endemic region, and they have been shown to co-circulate in the field for over 20 years (Troyer and Kurath 2003).

IHNV infections spread throughout fish populations by waterborne transmission (Batts et al. 1991), as the virus shed by the infected animals into the water enters the new host, and soon infections can be detected in gills, esophagus and cardiac stomach (Chilmonczyk and Monge 1980; Helmick et al. 1995). The primary infection site(s) of IHNV have not been rigorously identified but it has been reported that the virion enters the host through the skin at the base of the fins (Harmache et al. 2006). Skin has also been identified as a main entry site for the koi herpesvirus in *Cyprinus carpio* (Costes et al. 2009). The viral adhesion and entry mechanisms, however, are not fully understood but a unique fibronectin has been identified as an IHNV attachment factor present at the cell surface (Bearzotti et al. 1999; Liu, X. and Collodi 2002).

In recent years, evidence has accumulated in support of the key roles played by protein-carbohydrate interactions between host lectins and virion envelope glycoproteins in modulating viral adhesion and infectivity (Ouellet et al. 2005; St-Pierre et al. 2011; Yang et al. 2011; Toledo et al. 2014). Glycans on the viral surface can be recognized by host lectins that function as pattern recognition receptors (PRRs) that signal to activate and regulate the appropriate innate and adaptive immune responses (Barrionuevo et al. 2007; Jeon et al. 2010). However, multiple examples reveal that co-evolution of host-pathogen consortia has led to subversion of the immune recognition roles of lectins to facilitate adhesion and entry of the pathogens into the host cells (Kamhawi et al. 2004; Ouellet et al. 2005; Okumura et al. 2008; Yang et al. 2011). Galectins are good examples of these opposing recognition functions of lectins that may be either beneficial for the host in their roles as PRRs, but also detrimental by functioning as facilitators of viral, bacterial, and parasitic infection (Tasumi and Vasta 2007; Nieminen et al. 2008; Stowell et al. 2008; Vasta 2009; St-Pierre et al. 2011; Yang et al. 2011; Vasta 2012).

Galectins comprise an evolutionary conserved family of β -galactoside binding proteins, ubiquitous in mammals and other vertebrate taxa, invertebrates, and fungi. Galectins are defined by a unique sequence motif in their carbohydrate-recognition domains (CRDs), and are classified into three major structural types: (i) proto-type galectins, which contain one CRD and form homodimers; (ii) chimera-type galectins, which have a single CRD and can oligomerize forming trimers and pentamers; (iii) tandem-repeat-type galectins, which are characterized by two CRDs joined by a linker peptide (Vasta and Ahmed 2008). Since their discovery in the 1970s, over a dozen galectin subtypes (galectins 1-14, numbered in the order of their discovery) have been identified in mammals. Our understanding of their biological roles, initially limited to the recognition of cell surface glycans in embryogenesis and early development (Camby et al. 2006), has expanded in recent years by the discovery of their immunoregulatory activities (Rabinovich and Toscano 2009). In this regard, galectin 1 largely displays anti-inflammatory activity, whereas galectin 3 is endowed with pro-inflammatory properties (Di Lella et al. 2011). A gradual paradigm shift has taken place in the past few years through the realization that galectins also bind glycans on the surface of potentially pathogenic microbes, and function as recognition and effector factors in innate immunity (Stowell et al. 2008). Galectins can function as PRRs, recognizing various pathogen-associated molecular patterns (PAMPs) such as glycans, like lipopolysaccharides and peptidoglycan on the surface of pathogenic microbes, parasites, and fungi (Vasta 2012). Most galectins are either bivalent or multivalent with regard to their carbohydrate-binding activities, which enable the simultaneous recognition of multiple binding partners ("self" or "non self"). This suggests that galectins can act as bridging molecules between immune cells such as phagocytes and their targets, acting as opsonins (Karlsson et al. 2009) or between microbes and other host cells modulating the immune response (Rabinovich and Toscano 2009; Vasta 2009; Davicino et al. 2011). Galectins of all three structural types have been identified and characterized in various tissues, plasma and mucus of teleost fish, and their roles in recognition of bacterial pathogens has been reported for multiple fish species (Vasta et al. 2011). Among these, the zebrafish offers multiple advantages over mammalian models for the elucidation of developmental and immune processes (Patton and Zon 2001; Jesuthasan 2002). The value of the zebrafish as a model system is buttressed by the

observation that many orthologous genes, including galectins (Ahmed et al. 2004; Ahmed et al. 2009), are shared with mouse and man. Thus, the use of zebrafish for addressing basic questions about host-pathogen interactions and infectious disease has expanded dramatically in recent years (Yoder et al. 2002). All three galectin types (proto, chimera and tandem repeat) are present in zebrafish, and although its galectin repertoire is less complex than that of mammals, subtype isoforms are expressed, most likely resulting from genome duplications in this species (Ahmed et al. 2004). Thus, for our initial studies aimed at assessing *in vitro* the potential roles of zebrafish galectins in viral adhesion to epithelial cells, we selected members of the “proto” and “chimera” galectin types (galectin 1, and galectin 3, respectively) that in mammals: (a) are secreted to the extracellular space and bind to viral, bacterial, and fungal pathogens and parasites (Vasta 2009), and (b) have opposite roles in the regulation of immune responses (Di Lella et al. 2011). Further, to facilitate this initial study, the subtypes/isoforms selected (DrGal1-L2 and DrGal3-L1) were those that are most abundantly expressed in zebrafish. Our results suggest that the extracellular Drgal1-L2 and Drgal3-L1 interact directly with the IHN virus glycosylated envelope in a carbohydrate-dependent manner, as well as with galactosyl moieties on the epithelial cell surface, significantly reducing viral adhesion. A preliminary glycosylation study of the IHN virus envelope glycoprotein identified oligosaccharides that may potentially constitute the galectin ligands responsible for the observed inhibition of viral adhesion to the host.

Materials and Methods

Cell cultures

The epithelial EPC (epithelioma *papulosum cyprini*) cell line (CRL-2872, ATCC) from the fathead minnow (*Pimephales promelas*) was grown in minimal essential medium (MEM; Cellgro) supplemented with 10% fetal bovine serum (FBS; Quality Biological), 2 mM L-glutamine and penicillin-streptomycin at 26 °C. The ZFL (zebrafish liver) cell line was grown in Leibovitz's L-15 medium supplemented with penicillin-streptomycin at 28 °C. All cell lines were grown in an atmosphere of 100% air with humidification.

Virus purification and labeling

The IHN virus strain 220-90 was propagated by multiple passages on confluent layers of EPC cells, as described (Ammayappan et al. 2010). In brief, confluent EPC cells grown in 75 cm² flasks at 26 °C were infected at a multiplicity of infection (MOI) of 0.1 in the presence of MEM and 2% FBS. The infection was allowed to proceed at 16 °C until extensive cytopathic effect was observed. The supernatant was harvested 3-4 days post-infection (dpi), clarified by low-speed centrifugation, then separated on a discontinuous cesium chloride gradient by ultracentrifugation at 28,000×g. The banded virus was resuspended in TEN buffer (1 mM EDTA, 10 mM Tris-HCl, 1 M NaCl, pH 7.4) and stored at -80°C. Virus titers were measured by endpoint dilution on EPC cells (Burke and Mulcahey 1980). For biotinylation, the 1× TEN buffer was exchanged with phosphate-buffered saline (PBS) by overnight dialysis at 4-8 °C prior to the purified recombinant virus being reacted with 1 mg/mL EZ-Link Sulfo-NHS-Biotin (Pierce) in accordance with the manufacturer's recommendations. Briefly, virus (5 mg/ml) were incubated with 10 mM of biotin reagent for 2 hours on ice, followed by buffer exchange to remove the non-reacted reagent.

Expression and purification of recombinant zebrafish galectins

The cloning of the zebrafish galectins has been described elsewhere (Ahmed et al. 2004). Histidine-tagged recombinant Drgal1-L2 (Genbank accession no. AY421704) and Drgal3-L1 (Genbank accession no. AY421705) were separately expressed from the pET-28b (+) vectors in transformed Rosetta (DE3)pLysS competent cells (Novagen). Plasmid-carrying cells were grown in Luria-Bertani broth with antibiotics (chloramphenicol, 34 μ g/ml; kanamycin, 15 μ g/ml) to an OD₆₀₀ of 0.600 at which time recombinant protein expression was induced with the addition of 0.1 mM isopropyl D-thiogalactoside for 16 h at 23 °C. After pelleting, the soluble protein fraction was extracted with BugBuster Protein Extraction Reagent (Novagen) containing 1 mM phenylmethylsulfonyl fluoride, 1× Protease Inhibitor Cocktail Set I (Calbiochem), 20 μ g/ml lysozyme, 25 U/ml Benzonase Nuclease (Novagen) and 0.07% β -mercaptoethanol (BME). The clarified soluble fraction was loaded onto a pre-equilibrated chromatography column packed with 4 ml of divinyl sulfone-conjugated lactosyl-Sepharose slurry. After rinsing the column thoroughly with wash buffer (1:10 PBS, 0.07% BME), the galectins were eluted with elution buffer (1:10 PBS, 0.07% BME, 100 mM lactose). Glycerol was added to a final concentration of 50% (v/v); stocks were made and stored at -20 °C.

Production of anti-galectin (zebrafish) and anti-IHNV antibodies

Anti-Drgal1-L2 and Drgal3-L1 antisera were prepared in New Zealand White rabbits as previously described (Ahmed et al. 2004). Immunoglobulins were purified on Protein A-Sepharose, and tested for specificity in Western blot. Preparation of the rabbit anti-IHNV (whole virions) antiserum was prepared in rabbits (Proteintech Group, Inc.) using IHNV (5 mg total inoculum) purified from cell cultures as described above. Purification of immunoglobulins was carried out Protein A-Sepharose, and a specificity test by Western blot revealed that all the structural proteins (N, M, P, and G) of IHNV were strongly recognized.

Binding of zebrafish galectins to IHNV

Binding of recombinant zebrafish galectins (Drgal1-L2 and Drgal3-L1) to IHNV was detected by ELISA. A 96-well microtiter plate was coated with 1 μ g/well of recombinant Drgal1-L2 or Drgal3-L1 in coating buffer (15 mM sodium carbonate, 85 mM sodium bicarbonate, pH 9.6), or buffer only (background control) overnight at 4-8 °C overnight in the presence of 100 mM lactose to stabilize the CRD binding pocket and 10 mM BME to prevent oxidation. As a negative control, the remaining wells were coated with 1 μ g of BSA in PBS. The plate was washed with cold PBS/lactose/BME and incubated with blocking buffer diluted in PBS/lactose/BME for 8 h at 4-8 °C. The plate was then washed 6 times with PBS/10 mM BME (PBS/BME) and incubated with serial dilution of the purified viral preparation overnight at 4-8 °C. After three washes with PBS/BME, the plate was fixed with 2% paraformaldehyde for 1.5 h at room temperature. The wells were washed extensively with PBS to remove formaldehyde. Binding of the virus to galectins was detected by incubation with a 1:1000 dilution of anti-IHNV rabbit antiserum followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG. The plate was developed using TMB substrate (KPL) for HRP and the reaction was stopped by adding 1 M hydrochloric acid (HCl).

Absorbance measurements were read on a SpectraMax plate analyzer at a 450 nm wavelength. Specificity of binding of the recombinant zebrafish galectins to IHNV was assessed by binding-inhibition by free monosaccharides and disaccharides (lactose, sucrose, glucose or N-acetyllactosamine). Sugars (at increasing concentrations) or buffer alone (0% inhibition control) were added to the galectin-coated wells and incubated for 30 min, followed by the IHNV suspension or buffer alone ("no virus" control) added to the wells. After a 30 min incubation, the plates were gently rinsed three times to remove unbound virus, fixed with paraformaldehyde, and the galectin-bound virus assessed by an anti-IHNV antibody followed by an HRP-conjugated anti-rabbit IgG, as indicated above. The % inhibition of binding was calculated for each sugar relative to the "no sugar added" control.

Assessment of IHNV infection on galectin levels and secretion in fish cell lines

Confluent monolayers of EPC cells grown in L-15 medium were infected with purified IHNV at MOI of 10 in the presence of 10% FBS. Infections were allowed to proceed for 48 h at 16 °C. At designated time points, the inoculums were removed and RNA was harvested with TRI Reagent (Sigma-Aldrich) and further purified following the directions to the RNeasy Mini Kit (Qiagen). Purified RNA was stored at -80°C.

Relative quantification of gene expression by real-time PCR

Complementary DNA (cDNA) was synthesized from 1 µg of total cellular RNA by reverse transcription using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) following the manufacturer's instructions. For quantitative PCR, 0.1-100 ng of cDNA was used in a reaction containing 1× RT² SYBR Green ROX qPCR Mastermix (Qiagen) and 500 nM of the following oligonucleotide primer sets (Sigma-Aldrich) designed to match both the zebrafish and fathead minnow galectin gene sequences previously obtained in our laboratory (Ahmed et al. 2004); Jackson and Vasta, unpublished): Gal1-L2, forward, 5'-CCAGTGCCTATAGTGTGCAATTC-3'; reverse, 5'-TCATTGGTGAATGTGATTTTATCTG-3'; Gal3-L1, forward, 5'-GAGGCAATGACATTGCATTTTC-3'; reverse, 5'-GATCTTCATCTCAAAAGGCTTTCC-3'; 18S ribosomal RNA, forward, 5'-AATGTCTGCCCTATCAACTTTC-3'; reverse, 5'-TGGATGTGGTAGCCGTTTC-3'. Amplification was carried out on a Fast 7500 Real-Time PCR System (Applied Biosystems). The thermal cycling conditions were as follows: 95 °C for 10 min; 40 cycles of 95°C for 15 s, 57°C for 30 s, 72°C for 30s. The identity of the resulting amplicons was confirmed by sequencing. For this, amplicons were purified with High Pure PCR Product Purification Kit (Roche), ligated into pGEM-T vector system (Promega), and transformed into JM109 competent cells following the manufacturer's recommendations. Plasmids with PCR insert were selected, amplified, and extracted with QIAprep Spin Miniprep Kit (Qiagen), and subjected to sequence analysis (BioAnalytical Services Laboratory, IMET, MD). Obtained sequences were aligned with either Drgal1-L2 or Drgal3-L1 using Blastn (NCBI, NIH).

Western blot

Cell lysates and supernatant precipitates were fractionated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature, then incubated with a 1:1000 dilution of purified polyclonal

rabbit immunoglobulins specific for zebrafish galectins Drgal1-L2 and Drgal3-L1 (Open Biosystems) overnight at 4-8 °C. Indirect detection was carried out with a 1:1000 dilution of HRP-conjugated anti-rabbit IgG. All membranes were developed using Western Lightning Plus-ECL reagent (PerkinElmer) followed by exposure to autoradiography film.

Detection and identification of viral proteins by Western blot

Whole purified IHN virus was fractionated on a 10% SDS-PAGE gel and transferred to PVDF membranes as described above. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature, then incubated with a 1:1000 dilution of polyclonal rabbit antiserum specific for IHN virus strain 220-90 overnight at 4-8 °C. Indirect detection and development were carried out as described above.

Glycotyping of viral glycoprotein(s) by a lectin overlay binding assay

For the characterization of the glycosylation of viral components, whole purified IHN virus was separated on a 10% SDS-PAGE gel and transferred to PVDF membranes as described above. Glycosylated viral components were detected with the Glycoprotein Staining Kit (Thermo Scientific). The detection and preliminary characterization of specific carbohydrates on the IHN virus envelope were carried out using digoxigenin (DIG)-labeled lectins from the DIG Glycan Differentiation Kit (Roche).

Fractionation and isolation of the viral glycoprotein

The IHN virus envelope glycoprotein was isolated by affinity chromatography on a column consisting of *Datura stramonium* agglutinin (DSA) conjugated to agarose (EY Laboratories), subsequently subjected to immunoprecipitation using the Classic IP Kit (Pierce) in conjunction with polyclonal anti-IHN virus antibody, and finally analyzed by SDS-PAGE and Western blot.

Galectin binding to the isolated viral glycoprotein

The binding of the zebrafish galectins Drgal1-L2 and Drgal3-L1 to the isolated viral glycoprotein fraction was assessed by the lectin overlay binding assay as described above, with the PVDF membrane strips displaying the isolated viral glycoprotein probed with recombinant Drgal1-L2 and Drgal3-L1, followed by anti-Drgal1-L2 and anti-Drgal3-L1 antibodies. Binding of DSA was assessed similarly using a DSA-HRP conjugate (EY Laboratories).

N-glycosidase F and endoglycosidase H treatment of the viral glycoprotein

In order to release the N-linked glycans from the sample, whole purified IHN virus was digested with 100 U of either PNGase F or EndoH (New England Biolabs) for 1 h at 37 °C and further analyzed for lectin binding after separation on a 10% SDS-PAGE gel and transfer to a PVDF membrane. Control samples were incubated without enzymes.

Glycomics analysis of the EPC cell line

This was carried out as described elsewhere (Jang-Lee et al. 2006). Briefly, cells were homogenized in an extraction buffer of 1% CHAPS (v/v) in 25 mM Tris, 150 mM NaCl, 5

mM EDTA in water, pH 7.4 and subsequently dialyzed against a 50 mM ammonium bicarbonate buffer. Glycoproteins were reduced, carboxymethylated by dithiothreitol (Roche Applied Science) and iodoacetic acid (Sigma-Aldrich), and were tryptic-digested by trypsin (Sigma) prior to the release of protein linked N-glycans by PNGase F (Roche Applied Science) digestion. Released N-glycans were permethylated and purified by Sep-Pak C18 (Waters) prior to MS analysis.

Glycoproteomic analysis

The IHNV glycoproteins were first isolated by DSA-agarose affinity followed by a second round of purification by immunoprecipitation with antibody against IHNV as described above. The viral glycoprotein interacting with zebrafish galectins was cut out for glycopeptide MS identification carried out as described elsewhere (Harrison et al. 2012). Briefly, gel bands were excised and destained using 50 mM ammonium hydrogen carbonate (Sigma-Aldrich) mixed with an equal amount of acetonitrile (Romil). The samples in the gel pieces were reduced and carboxymethylated by dithiothreitol (Roche Applied Science) and iodoacetic acid (Sigma-Aldrich). They were then digested by sequencing grade modified trypsin (Promega) and the supernatant, which contained (glyco) peptides, was transferred to a new tube. The (glyco) peptides remaining in the gel were extracted using 0.1% (v/v) trifluoroacetic acid (Romil), followed by acetonitrile. The supernatants were combined and concentrated for mass spectrometric analysis. Online nanoLC-MS was run on an API QSTAR Pulsar Hybrid LC/MS/MS system (Applied Biosystems/MDS Sciex). The data were analyzed using Analyst QS Software (Applied Biosystems).

Binding of zebrafish galectins to EPC cells

Each well of a 96-well microtiter plates was seeded with 5×10^4 EPC cells and incubated overnight, then incubated with various concentrations (0 to 30 $\mu\text{g/ml}$) of recombinant Drgal1-L2 or Drgal3-L1 for 1 h at room temperature. After blocking with a 1% BSA solution for 1 h at room temperature, galectin binding to EPC cells was indirectly detected by incubation with polyclonal rabbit anti-Drgal1-L2 (1: 500) or Drgal3-L1 (1:500) antiserum followed by HRP-conjugated anti-rabbit IgG. The plate was developed using TMB substrate, and the reaction was stopped by adding 1 M HCl. Absorbance values were read at 450 nm.

IHNV binding to EPC cells in the presence or absence of zebrafish galectins

EPC cells were grown in a 96-well microtiter plate to 100% confluence, and incubated with biotinylated IHNV (MOI of 3 or 6) previously incubated with 30 $\mu\text{g/ml}$ of recombinant Drgal1-L2 or Drgal3-L1 for 30 min, followed by three washes to remove the unbound virus. The 30 min time point was selected based on the reported period at which viral adhesion is optimal, without viral replication taking place yet (Liu, H. et al. 2011). The IHNV binding was detected with HRP-conjugated streptavidin (Thermo Scientific). The plate was developed using TMB substrate and the reaction was stopped by adding 1 M HCl. Absorbance values were read at 450 nm.

Statistical analysis

The mRNA and protein expression levels were quantified using Image J software to estimate the relative band intensities. Comparison of two groups was performed by Student's T-test for the comparison of non-paired samples. All results with $p < 0.05$ were considered statistically significant.

Results

Our prior studies have shown that the zebrafish represents a suitable model system to elucidate the functional aspects of galectins in both development and immunity (Vasta et al. 2004; Vasta and Ahmed 2008; Ahmed et al. 2009; Feng et al. 2015). As studies in mammals have shown that galectin-1 and galectin-3 are involved in interactions with potential bacterial and viral pathogens, we selected the galectin-1 and galectin-3 homologs in zebrafish, Drgal1-L2 and Drgal3-L1, to examine their role in pathogen recognition and entry. Thus, to gain insight into the possible participation of galectins as PRRs or innate immune effectors in IHNV infection in teleost fish, we examined the *in vitro* expression and secretion of selected proto and chimera type galectins in cultured fish cell lines, their binding to both the epithelial cell surface and IHNV, identified their ligands on the virion envelope, and experimentally tested their effects on viral infectivity.

The ZFL and EPC cell lines express and secrete homologous galectin-like proteins

As the major entry portal of IHNV has been identified as the epithelium at the base of the fins, and given the lack of established zebrafish epithelial cell lines that can be productively infected with IHNV, we selected the EPC epithelial cell line for further study. Interestingly, IHNV is rapidly propagated *in vitro* in EPC cells but, although IHNV can infect adult and larval zebrafish, *in vitro* propagation of IHNV in ZFL (hepatic cells) has been mostly unsuccessful (data not shown). To confirm that the epithelial EPC cell line would be a suitable model for the zebrafish galectin functions, however, we compared the expression and secretion of the zebrafish Drgal1-L2 and Drgal3-L1 in the ZFL with those from the EPC epithelial cell line (Fig 1). Our study by qPCR on total RNA, and by Western blot on cell lysates and the culture supernatants not only confirmed that the EPC cell line expresses galectins of similar sequence with either the zebrafish Drgal1-L2 or Drgal3-L1 and cross-reactive with their antibodies (Fig. 1A, B), but also that these galectins are similarly secreted to the extracellular environment at comparable levels by the two cell lines (Fig. 1C). To confirm the identity of the PCR amplicons from EPC samples in Figure 1A, we performed sequence analysis. Results show that amplicons from EPC share 86% (103/120) and 91% (143/158) identity with Drgal1-L2 (Fig. 1E) and Drgal3-L1 (Fig. 1F), respectively. The corresponding amino acid sequences are 77% (30/39) and 90% (47/52) identical. Most importantly, the key residues of the CRD that recognize the carbohydrate ligand are fully conserved in both galectins.

Exposure of fish epithelial cells (EPC) to IHNV modulates of galectin expression at later stages of infection

Based on our observations that galectins of similar sequence are synthesized and secreted by the ZFL and EPC cells, we examined the potential effects of IHNV exposure on their

transcription. Exposure of EPC cells to IHNV revealed only slight to moderate increases in Drgal1-L2 expression during the first 12h, followed by a dramatic downregulation of expression of both Drgal1-L2 or Drgal3-L1 at 48h (10-15% of the unexposed controls) (Fig. 2), a time point in the experimental exposure that coincides with the full-fledged infection. However, no significant changes in galectin expression were observed during the early stages of infection, particularly during viral adhesion, which takes place from the first few minutes to reach optimal levels from 30 min to 2h after exposure.

Recombinant zebrafish galectins Drgal1-L2 and Drgal3-L1 bind to IHNV envelope in a dose-dependent and carbohydrate-specific manner

Given that the epithelial cells express and secrete proto- and chimera-type galectins to the extracellular space, we investigated whether these galectins could bind to the IHNV glycosylated envelope. For this, we tested the binding of the recombinant Drgal1-L2 and Drgal3-L1 to intact IHNV virions in a “capture” format ELISA. Our results revealed that both Drgal1-L2 and Drgal3-L1 recognized the IHNV virus in a dose-response manner, although the latter showed a significantly stronger binding at the three concentrations tested, and greatest difference in IHNV binding with Drgal1-L2 at 0.25 μ g protein/well (Fig. 3A). Based on these results we tested the carbohydrate specificity of the galectin-virion interactions by a binding-inhibition capture ELISA, in which the potential inhibitory effects of the disaccharides *N*-acetyllactosamine (LacNAc), lactose, and sucrose were examined (Fig. 3B-D) over the “no sugar added” control. The most effective inhibitor for the interactions of both Drgal1-L2 and Drgal3-L1 with IHNV was LacNAc, although it was significantly more effective for Drgal1-L2 than for Drgal3-L1: while only 0.5 mM was required for 50% inhibition for Drgal1-L2 binding to IHNV, a 3-fold higher concentration (1.8 mM) was required for Drgal3-L1 (Fig. 3B). In contrast, lactose concentrations of 25 mM and 55 mM were required for 50% inhibition of Drgal3-L1 and Drgal1-L2, respectively (Fig. 3C), while sucrose had a negligible inhibitory effect on both galectins (Fig. 3D). These results confirmed that the binding of the zebrafish galectins Drgal1-L2 and Drgal3-L1 to IHNV is carbohydrate-dependent and can be specifically blocked in a concentration-dependent manner by disaccharides that we have previously shown as high (LacNAc) or moderate (lactose) affinity ligands for these galectins (Ahmed et al. 2004).

Identification of galectin ligands on the IHNV envelope

To elucidate the structural basis of the interactions between Drgal1-L2 and Drgal3-L1 with IHNV, we first characterized the carbohydrates present on IHNV proteins via the binding of a set of DIG-labeled plant lectins of well-established carbohydrate specificity. These included GNA, specific for terminally linked mannose, MAA, that binds sialic acid terminally linked in α 2-3 to galactose, PNA, that binds galactose in galactose- β (1-3)-*N*-acetylgalactosamine [Gal β (1-3)-GalNAc], SNA, that is specific for sialic acid terminally linked in α 2-6 to galactose or *N*-acetylgalactosamine, and galactose- β (1-4)-*N*-acetylglucosamine [Gal β (1-4)-GlcNAc], and DSA, that binds *N*-acetylglucosamine (GlcNAc) oligosaccharides and Gal β (1-4)-GlcNAc. The results revealed that, except for PNA, all lectins tested recognized the envelope glycoprotein (data not shown), indicating that it displays complex or hybrid type *N*-linked oligosaccharides that are sialylated via both 2-3 or 2-6 linkages to the non-reducing terminal sugar moiety, most likely galactose.

To confirm our findings from the IHNV glycotyping, we treated the virions with the glycosidases PNGase F or Endo H, and assessed DSA binding and potential shifts in the mobility of the envelope glycoprotein. The IHNV glycoprotein (G) and additional IHNV structural proteins were identified following Schütze *et al* (Schutze et al. 1995), together with information reported in Lee *et al* (Lee et al. 1996) and Biacchesi *et al* (Biacchesi et al. 2002). The identification of the IHNV glycoprotein bands corresponding to the G monomer, dimer, and trimer was based on Anderson *et al* (Anderson et al. 2008). While PNGase F cleaves the bonds between the basal GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins, Endo H cleaves glycosidic linkages within the chitobiose core of high mannose and hybrid oligosaccharides. The PNGase F treatment abolished the recognition of the IHNV glycoprotein by DSA (Fig. 4A, lane “F”), and increased the electrophoretic mobility of about 50% of the IHNV glycoprotein, most visibly in the band of approximately 67 kDa that corresponds to the glycoprotein monomer [Fig. 4B, lane “F”, G(m)]. This increase in mobility of the IHNV glycoprotein by PNGase F deglycosylation was consistent with that reported in Huang *et al* (Huang C et al. 1994). The increase in mobility of the IHNV glycoprotein monomer was less evident for the dimer [G(d)] and trimer [G(t)], possibly due to the deglycosylation of only part of the aggregated subunits, or a reduced susceptibility of G(d) and G(t) to the enzyme due to steric hindrance in these glycoprotein oligomers. In contrast, Endo H treatment of the virions did not affect the interactions between DSA and the viral glycoprotein (Fig. 4A, lane “H”), and only marginally increased the electrophoretic mobility of the bands corresponding to the glycoprotein monomer, dimer or trimer (Fig. 4B, lane “H”). These results suggest that mostly complex *N*-glycans carrying galactose- β (1-4)-*N*-acetylglucosamine moieties are present on the viral glycoprotein and are involved in the IHNV-lectin interactions.

Finally, we examined by Western blot analysis the recognition of the DSA-affinity/antibody-immunoprecipitation-purified IHNV glycoprotein by the zebrafish galectins Drgal1-L2 and Drgal3-L1. Indeed, the IHNV glycoprotein was not only strongly recognized by the anti-IHNV antibodies and DSA, but also by Drgal1-L2 and Drgal3-L1 (Fig. 4C), indicating that the aforementioned terminal galactose- β (1-4)-*N*-acetylglucosamine moieties in complex *N*-linked oligosaccharides decorating the IHNV envelope are the most likely Drgal1-L2 and Drgal3-L1 ligands responsible for the galectin-IHNV specific interactions previously identified (Fig. 3).

Glycosylation analysis of the EPC cell line and the IHNV envelope glycoprotein revealed potential galectin ligands

To obtain information on the types of oligosaccharide that are expressed on the IHNV envelope, we carried out glycomic profiling of the EPC cell line together with a glycoproteomics analysis of an IHNV glycoprotein sample. Although the dominant glycans expressed by the EPC cells were found to be high mannose in our glycomics experiment, a significant minority of the glycans are complex-type and some of the latter have LacNAc antennae that are potential galectin ligands (Fig. 5A). Importantly, our glycoproteomics analysis of the IHNV glycoprotein sample, revealed a glycopeptide that was solely glycosylated with complex-type glycans (Fig. 5B). Seven glycoforms of this peptide were observed as doubly charged molecular ions at *m/z* 1211, 1292, 1313, 1373, 1394, 1446 and

1475. With the exception of m/z 1211 and 1313, which have non-galactosylated antennae (see annotations on Fig. 5B), all the glycans have compositions consistent with biantennary structures carrying one or two potential galectin ligands--LacNAc.

Recombinant galectins bind to the epithelial cell surface in a dose-dependent manner

As our results revealed the specific binding of Drgal1-L2 and Drgal3-L1 to the IHNV envelope glycoprotein, and tentatively identified the galectin ligands as complex biantennary *N*-glycan structures containing LacNAc unit, it became of interest to investigate if these galectins could also bind to the surface glycans on the epithelial cells. For this, we first tested the binding of recombinant Drgal1-L2 to EPC cells grown in ELISA plates, using asialofetuin-coated wells as a positive control for galectin binding. The results showed that like the mammalian proto-type galectins, DrGal1-L2 binds to asialo-fetuin and to the EPC (most likely to polylactosamine chains from cell surface glycans) with similar binding profiles (Fig. 6A). Like its mammalian counterpart, DrGal3-L1 also has a preference for polylactosamines present on the cell surface, ECM glycans, and other glycoproteins, but binds weakly to asialofetuin (data not shown). A comparative analysis of the binding of Drgal1-L2 and Drgal3-L1 at two concentrations (15 μ g/ml and 30 μ g/ml) confirmed that both recombinant Drgal1-L2 and Drgal3-L1 bound in a dose-response manner to the surface of the epithelial cells (Fig. 6B). A preliminary study to examine whether endogenous galectins secreted by the EPC cells into the culture medium could bind to the cell surface was carried out by exposing the cells to culture medium containing 100 mM lactose to displace any cell surface-bound endogenous galectins prior to adding the exogenous recombinant Drgal1-L2 to the culture. The results showed a 10-20% increase in binding of the exogenous Drgal1-L2 (data not shown), suggesting that as the cells grow in culture and secrete galectins to the extracellular space, these may remain bound to the cell surface.

Pre-incubation of recombinant Drgal1-L2 or Drgal3-L1 with IHNV inhibits viral adhesion to the host cell surface

Finally, the observations that both Drgal1-L2 and Drgal3-L1 recognized the IHNV envelope glycoprotein, and strongly bound to the epithelial cell surface led us to inquire whether the zebrafish galectins either facilitate IHNV infection by cross-linking the virus to the cell surface or prevent infection by hindering viral adhesion. Thus, we investigated the effect of binding of zebrafish galectins to the IHNV on adhesion of the virions to the surface of epithelial cells by pre-incubating the biotinylated IHNV with either Drgal1-L2 or Drgal3-L1 for 1 hour, before adding the virus to the EPC culture. The cells were incubated with virus for 30 min, a time point selected from the kinetics of IHNV adhesion to the host cells reported elsewhere (Liu, H. et al. 2011) as the time for optimal adhesion to the cell surface. Our results indicate that pre-exposure of IHNV to recombinant Drgal1-L2 or Drgal3-L1 significantly decreased the viral adhesion to the EPC cells (Fig. 7). Pre-incubation of IHNV with 30 μ g/ml recombinant Drgal1-L2 decreased IHNV binding to EPC cells by over 40% relatively to the unexposed controls, whereas Drgal3-L1 had a more dramatic effect, decreasing IHNV binding to EPC cells by approximately 65% (Fig. 7).

Discussion

Although some galectin family members have been shown to participate in early embryogenesis and developmental processes (Camby et al. 2006; Di Lella et al. 2011), limited information is currently available concerning their potential roles in infectious disease (Vasta 2009; Vasta et al. 2012). In recent years, however, evidence has accumulated not only in support of the role(s) of some galectins as PRRs (Vasta 2009; Vasta 2012), but also as effectors displaying bacteriocidal activity (Stowell et al. 2010). Furthermore, some pathogens have co-evolved with their hosts to take advantage of the recognition functions of their galectins to facilitate entry and infection (Pelletier and Sato 2002; Pelletier et al. 2003; Tasumi and Vasta 2007; Okumura et al. 2008; Feng et al. 2013). Fragmentary evidence suggests that in mammals, selected galectin types can mediate very different mechanisms to elicit protective functions during microbial infections (Farnworth et al. 2008; Yang et al. 2011). For example, galectin-3 mediates neutrophil recruitment into the lung in an integrin-independent manner upon pneumococcal infection (Sato et al. 2002; Nieminen et al. 2008). In contrast, galectin-1 directly inhibits hemagglutination activity and infectivity of influenza A virus by binding its envelope glycoproteins and suppresses virus-induced cell death (Yang et al. 2011).

In this study we investigated the potential role(s) of galectins as PRRs in IHN virus infection using the zebrafish as a model system. The initial studies for the identification and characterization of galectins in zebrafish (Ahmed et al. 2004) had revealed that this teleost species expresses a diverse galectin repertoire that consists of the same proto, chimera and tandem repeat structural types that have been defined in the mammals (Cooper 2002). In this study we tentatively selected their zebrafish homologues, Drgal1-L2 and Drgal3-L1, to examine their potential role(s) in pathogen recognition and entry. The expression and secretion of zebrafish Drgal1-L2 and Drgal3-L1 galectin homologues by the epithelial cell line EPC and the zebrafish also confirmed its suitability for *in vitro* studies aimed at investigating the potential effect of fish galectins secreted by epithelial cells on IHN virus infectivity. The skin of teleost fish is covered by mucus secreted by specialized mucous glands, and galectins secreted by these glands as well as epithelial cells have been identified and their binding activity demonstrated in several studies (Suzuki et al. 2003; Tasumi et al. 2004; Takayama et al. 2009). Our observation that exposure of the fish epithelial cells to IHN virus has negligible effects on expression of Drgal1-L2 and Drgal3-L1 during the viral adhesion stage, but strongly downregulates galectin expression at later infection stages. Recently, human galectins have been shown to be induced *in vivo* in response to several different viral infections, including influenza (Katoh et al. 2014), Nipah (Garner et al. 2015), and human immunodeficiency virus (Tandon et al. 2014). It is tempting to speculate that the downregulation of galectin expression observed at 48h may reflect a viral-mediated mechanism for subversion of galectin function at the later stages of viral infection, and current studies in our laboratory are addressing this intriguing observation.

The finding that both Drgal1-L2 and Drgal3-L1 recognized and bound the IHN virus virion glycosylated envelope was strongly suggestive of a role in the teleost antiviral defensive mechanisms, and we focused subsequent studies on the structural basis of the galectin-ligand recognition. The results confirmed that the binding of the zebrafish galectins Drgal1-L2 and

Drgal3-L1 to IHNV is carbohydrate-dependent and can be specifically blocked in a concentration-dependent manner by the disaccharides LacNAc or lactose, but not by sucrose. In a previous study we had shown that LacNAc behaves as a high affinity ligands for zebrafish galectins, whereas lactose is less effective (Ahmed et al. 2004).

Glycotyping with plant lectins and enzymatic treatment with glycosidases revealed that the IHNV envelope glycoprotein displays complex or hybrid type *N*-linked oligosaccharides that are sialylated *via* both 2-3 or 2-6 linkages to the non-reducing terminal sugar moiety, most likely Gal. The lack of binding by PNA, which preferentially recognizes Gal β (1-3)GalNAc, together with strong binding by DSA, which in addition to chitobiose binds to Gal β (1-4)GlcNAc provided the structural basis for the observation that the envelope glycoprotein is effectively recognized by both Drgal1-L2 and Drgal3-L1, and that these interactions can be most effectively inhibited by LacNAc. The preliminary glycosylation analysis of the purified IHNV envelope glycoprotein by mass spectrometry revealed the presence of complex biantennary *N*-glycan structures containing terminal LacNAc. These terminal LacNAc units were also present in the glycome of the uninfected fish epithelial cells, suggesting that like other viruses (St-Pierre et al. 2011; Yang et al. 2011), IHNV takes advantage of the glycosylation systems of the host cells in which it replicates.

Upon the observations that both Drgal1-L2 and Drgal3-L1 bind to the IHNV envelope glycoprotein, and also effectively bind to the epithelial cell surface, it became of interest to investigate if these galectins could either prevent infection by hindering viral adhesion as shown for the influenza virus (Yang et al. 2011) or facilitate viral adhesion by cross-linking the virus to the cell surface as proposed for HIV (Sato and Nieminen 2004). At the concentrations tested, both Drgal1-L2 and Drgal3-L1 inhibited IHNV infection of the epithelial cells, suggesting that both may function by coating the virus envelope and interfering with the viral adhesion mechanisms, such as that mediated by the truncated fibronectin identified as the IHNV co-receptor on the zebrafish cell surface (Liu, X. and Collodi 2002). However, it is also possible that by partially coating the host cell surface, the galectins also contribute to hinder, sterically or specifically, the cell surface sites to which the IHNV virions adhere. The relatively higher degree of inhibition of viral adhesion observed for Drgal3-L1 as compared to Drgal1-L2, was consistent with Drgal3-L1's more effective binding to the epithelial cell surface. Further, fibronectin is a glycoprotein that is recognized as an effective galectin ligand (Ozeki et al. 1995). Thus, our results suggest the possibility that both galectins may inhibit IHNV adhesion by coating the virions surface glycoproteins, as well as the truncated fibronectin co-receptor. In some teleost fish species, galectins have been shown to participate in innate immune mechanisms against eukaryotic parasites, bacteria, and viruses. In the conger eel (*Conger myriaster*) both isotypes of congerin, a proto-type galectin, participate in cellular encapsulation of the parasitic nematode (*Cucullanus* spp.) via the induction of cellular adhesion to the parasites through galectin-glycan recognition (Nakamura et al. 2012; Watanabe et al. 2012). Other examples have revealed direct and specific interactions of fish galectins with the pathogens (Tasumi et al. 2002; Tanguy et al. 2004; Dutta et al. 2005) and by controlling virulence and viral replication rate by modulating the viral adhesion to the host (Poisa-Beiro et al. 2009; Liu, S. et al. 2013). Current studies in our laboratory are aimed at elucidating the mechanisms

involved in the modulation of viral adhesion by Drgal1-L2 and Drgal3-L1, and other galectins from the zebrafish repertoire.

Acknowledgments

Note: This article is dedicated to the memory of Barbara Giomarelli, Ph.D

Abbreviations

IHNV	infectious hematopoietic necrosis virus
Drgal1-L2	zebrafish galectin-1
Drgal3-L1	zebrafish galectin-3
PRR	pattern recognition receptors
CRD	carbohydrate-recognition domain
FBS	fetal bovine serum
MOI	multiplicity of infection
PBS	phosphate-buffered saline
BME	β-mercaptoethanol
HRP	horseradish peroxidase
LacNAc	<i>N</i> -acetyllactosamine
GlcNAc	<i>N</i> -acetylglucosamine
DSA	<i>Datura stramonium</i> agglutinin
Galβ(1-3)GalNAc	galactose-β(1-3)- <i>N</i> -acetylgalactosamine
Galβ(1-4)GlcNAc	galactose-β(1-4)- <i>N</i> -acetylglucosamine

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Highlights

- Exposure to IHNV upregulates galectin expression in fish epithelial cells
- Extracellular galectins interact directly with the IHNV glycosylated envelope
- Identified galectin oligosaccharide ligands on IHNV responsible for galectin binding
- Extracellular galectins significantly reduce viral adhesion to epithelial cells

Fig 1. EPC cells express and secrete galectin homologous to Drgal-1 and Drgal-3

Total RNA and cell lysates of *epithelioma papulosum cyprini* cells (EPC) or zebrafish liver cells (ZFL) and their spent culture media were collected to analyze relative levels of Drgal1-L2 and Drgal3-L1 transcripts (A), expressed protein (B), and proteins secreted to the extracellular space (C). Recombinant Drgal1-L2 and Drgal3-L1 were subjected to Western blot to verify the antibody specificity for Drgal1 and Drgal3 (D). Purified DNA fragments from RT-PCR from EPC cells were subjected for sequence analysis, and obtained sequences and the deduced amino acid were aligned with either Drgal1-L2 (E) or Drgal3-L1 (F) using Blastn and Blastp (NCBI, NIH). The key residues of the CRD were boxed.

Fig. 2. Exposure of fish epithelial cells (EPC) to IHNV modulates galectin expression

Relative levels of Drgal1-L2 and Drgal3-L1 mRNA transcript were measured at various timepoints, indicated as hours post-infection (hpi) with IHNV. All threshold cycle (C_T) values were normalized to 18S rRNA endogenous controls. Relative fold changes in gene expression were calculated using the $-C_T$ method (Livak and Schmittgen 2001). The $\log(10)$ -transformed fold changes were evaluated in a two sided t test. The derived p values less than 0.05 were considered to be significantly different. * $p < 0.05$, ** $p < 0.005$ vs 0 hpi.

Fig. 3. Recombinant zebrafish galectins Drgal1-L2 and Drgal3-L1 bind to the IHN virus envelope in a carbohydrate-specific manner

A capture ELISA was performed to detect and analyze the binding properties of exogenous zebrafish galectins to IHN virus envelope. Recombinant Drgal1-L2 or Drgal3-L1 was immobilized on a microtiter plate (1 µg/well) and incubated with increasing concentrations (from 0.5 to 0.125 µg/well; 200 µl/well) of purified IHN virus (A); or incubated with IHN virus (0.5 µg/well) in the presence of different concentration of LacNAc (B), lactose (C) or sucrose (D). Binding was detected with antibodies specific for IHN virus. Nonspecific background absorbance (no virus) was subtracted from the absorbance values. Percent inhibition was

calculated as $\left(1 - \frac{\text{Absorbance with sugar}}{\text{Absorbance without sugar}}\right) \times 100\%$ (B- D). Average of triplicate data (+/-) standard deviation is represented. *p<0.05 vs the group with 0.125 µg/well IHN virus

Fig 4. Identification of galectin ligands on the IHNV envelope

A. Viral samples were treated with enzymes Endo H (H) or PNGase F (F). Enzyme-treated and untreated IHNV (C) were loaded onto a 10% SDS-PAGE and the protein and glycoprotein components detected either by HRP-conjugated *Datura stramonium* agglutinin (DSA) (A) or by anti-IHNV antibody (IHNV) followed by an HRP-conjugated anti-rabbit antibody (B). The (m) enriched fraction was characterized in WB with anti-IHNV, HRP-conjugated DSA, or biotinylated zebrafish galectins Drgal1-L2 and Drgal3-L1 (C). N, nucleocapsid; P, phosphoprotein; M, matrix protein; G, surface glycoprotein (m: monomer; d: dimer; t: trimer, based on Anderson *et al* (Anderson et al. 2008).

Fig 5. Glycosylation analysis of the EPC cell line and the IHNV envelope glycoproteins

(A) MALDI-TOF mass spectrum of permethylated N-glycans of EPC cell line. Annotated structures are according to the Consortium for Functional Glycomics guidelines. All molecular ions are $[M+Na]^+$. Putative structures are based on m/z values, monosaccharide composition, and knowledge of N-glycosylation pathways.

(B) Electrospray mass spectrum of a glycopeptide from IHNV envelope glycoproteins. EPC viral glycoproteins were purified on a DSA-agarose column followed by immunoprecipitation with anti-IHNV antibodies. Purified viral glycoproteins were separated by SDS-PAGE and the glycoproteins that were recognized by Drgal1-L2 and Drgal3-L1 in a Western blot were excised from the gel for glycoproteomics analysis. Glycopeptide molecular ions are annotated with sugar sequences that are inferred from their compositions and biosynthetic knowledge. Signals marked with a cross are not glycopeptides.

Fig 6. Recombinant zebrafish galectins Drgal1-L2 and Drgal3-L1 bind to the surface of EPC cells

The binding of Drgal1-L2 to the EPC cell surface was quantified by ELISA, with Drgal1-L2 binding to asialofetuin as a reference. EPC cultured in ELISA plates (EPC) or asialofetuin coated plates (ASF) were incubated with Drgal1-L2 (0-30 mg/ml) (A); or EPC cultured in ELISA plates were incubated with 15 or 30 μg/ml of recombinant Drgal1-L2 and Drgal3-L1 (B). The binding of by Drgal1-L2 and Drgal3-L1 was detected with rabbit antibodies against specific galectins followed by HRP-conjugated anti-rabbit antibody. Nonspecific background absorbance (cells only) was subtracted from the absorbance values. The fold change in binding of recombinant Drgal1-L2 and Drgal3-L1 to EPC is shown relative to the cells incubated with buffer only (“no-galectin” negative control) (B). Data shown and the bar graphs are representative data from three independent experiments. *p < 0.05; **p < 0.005; ***p < 0.001 vs control.

Fig 7. Pre-incubation of IHN with recombinant Drgal1-L2 and Drgal3-L1 inhibits viral adhesion to the host cell surface

Biotinylated IHN virus was pre-incubated with Drgal1-L2 or Drgal3-L1 (30 mg/ml) for 1 hour, or without galectin incubation (IHN), and applied to EPC cells cultured in ELISA plates (MOI 1). IHN binding to the cell surface was detected by HRP-conjugated streptavidin. Nonspecific background (EPC cells only) was subtracted. Bar graph shows the fold change in adhesion to EPC cells of IHN that had been pre-incubated with recombinant Drgal1-L2 or Drgal3-L1 relative to the unexposed IHN. Representative data from three independent experiments are shown. * $p < 0.05$, ** $p < 0.005$ vs control.