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Identification, cloning and tissue localization of a rainbow trout (*Oncorhynchus mykiss*) intelectin-like protein that binds bacteria and chitin

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Abstract Intelectins are a recently identified group of animal lectins involved in innate immune surveillance. This paper describes the primary structure, expression and immunohistochemical localization of a rainbow trout plasma intelectin (RTInt). RTInt exhibited calcium-dependent binding to *N*-acetylglucosamine (GlcNAc) and mannose conjugated Toyopearl Amino 650M matrices. When GlcNAc eluates from chromatography matrices were analyzed by reducing 1D PAGE and Western blots, the lectin appeared as ~37 kDa and ~7 2kDa bands. Similar analysis of plasma revealed a single 72 kDa band under reducing conditions. MALDI-TOF MS demonstrated five, ~37 kDa isoforms (p*I* 5.3–6.1) separated by 2D-PAGE. A 975 bp cDNA sequence obtained by RT-PCR from liver and spleen tissue encoded a 325 amino acid secretory protein with homology to human and murine intelectins, which bind bacterial components and are induced during parasitic infections. Gene expression and immunohistochemistry detected RTInt in gill, spleen, hepatic sinusoid, renal interstitium, intestine, skin, swim bladder and within leukocytes. Direct binding assays demonstrated the ability of RTInt to bind relevant bacterial and chitinous targets. These findings suggest that RTInt plays a role in innate immune defense against bacterial and chitinous microbial organisms.

Introduction

Animal lectins are a diverse group of non-immunoglobulin, non-enzymatic carbohydrate binding proteins that are involved in a variety of biological processes including innate defense recognition of molecular patterns found on

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infectious agents [1]. Lectins are classified based upon their carbohydrate ligand specificity, biological function, cellular localization and dependence on cations for binding [1]. X-lectins or intelectins are a recently identified group of animal lectins which possess a fibrinogen-like motif similar to that of members of the ficolin/opsonin/p35 lectin family [2,3] and display calcium-dependant carbohydrate binding in the absence of a C-type carbohydrate recognition domain (CRD) [4]. Membrane bound, intracellular and soluble intelectin-like cDNA sequences and proteins with high predicted amino acid sequence homology have been reported from various species including humans [5,6], mice [7], frogs [8,9], channel and blue catfish [10,11], grass carp [12], trout [13,14], lamprey (BAB32787) and ascidians [15]. Although a limited number of these studies identify the proteins biological function and saccharide binding specificity, there is evidence implicating the involvement of intelectins in innate immune defense against microorganisms. For example, human intelectin 1 (hIntL) [5] bound D-galactofuranosyl residues of arabinogalactan cell wall components from the bacteria *Nocardia rubra* [16] and possessed 100% amino acid identity with human lactoferrin receptor; a protein with known biological functions including the facilitation of iron uptake and immune modulation of circulating leukocytes [6,17,18]. Similarly, the murine intelectin that was localized to Paneth cells of intestinal crypts by in situ hybridization [7], was induced by infection with the nematode *Trichinella spiralis* and believed to be involved in pathogen surveillance [19—21]. The galactose-specific lectin from the plasma of the ascidian invertebrate *Halocynthia roretzi*, which showed moderately high amino acid sequence homology to both human [5] and *Xenopus* [8] intelectin proteins, stimulated superoxide ion production in mammalian polymorphic leukocytes [22] and enhanced phagocytosis of sheep red blood cells by ascidian hemocytes [15].

In fish, the potential role of intelectin-like proteins as innate immune molecules is fast being realized. In rainbow trout, intelectin-like cDNA sequences and other putative acute phase genes were up-regulated in liver tissue following the injection of an emulsified, killed, Gram negative bacteria (*Listonella* (formerly *Vibrio*) *anguillarum*) [13,14]. Similarly, high-density in situ oligonucleotide microarrays were used to display the substantial up-regulation (>50 fold) of hepatic-induced genes (intelectin, haptoglobin, ferritin, and transferrin) in channel catfish (*Ictalurus punctatus*) following infection with the bacterial pathogen *Edwardsiella ictaluri* [10]. Recent investigations have identified two intelectin genes from channel catfish (*I. punctatus*) and blue catfish (*Ictalurus furcatus*) [11] with catfish intelectin 2 being strongly induced following intraperitoneal injection with *E. ictaluri*. In grass carp (*Ctenopharyngodon idella*) cDNA sequences encoding an intelectin (gcIntlL) protein was induced in multiple tissues following whole fish LPS stimulation [12]. cDNA sequences of intelectin-like proteins from several species of fish are available on the NCBI GenBank database including lamprey, *Lethenteron japonicum* (AB055981) and the zebra danio, *Danio rerio* (XM684148), however none have been characterized.

The goals of the present study were to identify and characterize the rainbow trout intelectin gene and to demonstrate the location of gene expression and protein distribution. In addition, the ability of this plasma lectin to bind to various Gram negative bacteria and chitin was determined.

Materials and methods

Chemicals and reagents

Monosaccharides and chitin used for affinity chromatography were obtained from Sigma (Oakville, ON, Canada). *Artemia* cysts were obtained from M & M Suppliers (Bothwell, WA, USA). Toyopearl AF-Epoxy 650M was obtained from TosoHass (Montgomery, PA, USA). Rainbow trout intelectin (RTInt) amino acid peptide conjugated with keyhole limpet hemocyanin (KLH) was obtained from Pacific Immunology (Ramona, CA, USA). Goat anti-rabbit polyclonal horseradish peroxidase (HRP) was obtained from Dako (Santa Barbra, CA, USA). Polyacrylamide gels were stained with SYPRO Ruby from Molecular Probes (Eugene, OR, USA) or Coomassie R-250 from Fischer Scientific (Ottawa, ON, Canada). Molecular weight protein standards were obtained from either GE Healthcare (Piscataway, NJ, USA) or New England Biolabs (NEB) (Ipswich, MA, USA). Bovine serum albumin standards used in the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) were from Fisher Scientific (Ottawa, ON, Canada). Trypticase soy broth was obtained from Difco Laboratories (Detroit, MI, USA). Total RNA isolation was performed using the RNA easy mini kit (Qiagen, Mississauga, ON, Canada). Platinum High Fidelity Taq DNA polymerase, Superscript III, TOPO TA Cloning© and the AEC Histostain SP immunohistochemistry kit were obtained from Invitrogen (Burlington, ON, Canada). Western blot was performed using ECT Plus blotting reagents from GE Healthcare. Ammonium bicarbonate, acetonitrile, dithiothreitol (DDT) and iodoacetamide used for in-gel protein extraction was obtained from Sigma. Modified porcine trypsin used for in-gel protein digestion was obtained from Promega (Madison, WI, USA). All other chemicals were obtained from Fisher Scientific.

Affinity chromotography and partial purification of RTInt

Plasma used for ligand binding assays with various chromatography matrices were dialyzed overnight in 50 mM Tris, 150 mM NaCl and 5 mM $CaCl_2$ at pH 7.8 (TBS-Ca^{2+}) and filtered (0.45 μm filter; Fischer Scientific). Re-hydrated Toyopearl AF-Epoxy 650M was conjugated to *N*-acetylglucosamine (GlcNAc) according to the manufacturer's protocol. Briefly, 5 g of each matrix were conjugated with GlcNAc (300 mM) in 0.1 M sodium hydroxide (NaOH; pH 12.0) at 37 °C with shaking for 16 h. Non-bound saccharides were washed from coupled matrices with 500 ml of 0.1 M NaOH (pH 12.0). Non-reacted epoxide groups were blocked using 1 M ethanolamine (pH 8.0) at 37 °C for 16 h with shaking. Ligand-coupled matrices were washed with 500 ml of milliQ (deionized and 0.22 μm filtered) water and equilibrated in TBS-Ca^{2+}.

All ligand-coupled chromatography was performed at 4 °C. Twenty-five milliliters of dialyzed plasma and 15 ml of TBS-Ca^{2+} was added to conjugated matrices and incubated overnight at 4 °C with gentle agitation. Matrices were