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# A Novel Ladder-like Lectin Relates to Sites of Mucosal Immunity in Atlantic Halibut (*Hippoglossus hippoglossus* L.)

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The immune system of Atlantic halibut (*Hippoglossus hippoglossus* L.) has been studied in our group in previous years (Lange 2001, Lange, 2004a; Gudmundsdottir et al., 2003), with a particular emphasis on early ontogeny (Lange, 2004b; Lange 2006; Magnadottir et al., 2005; Magnadottir et al., 2018).

This study aimed at identifying putative novel lectins in halibut serum and sites of detection during early development.

Lectins are key components of the innate immune system and play important roles in plasma, skin mucus and the gastrointestinal tract of fish (Vasta et al., 2011, Ng et al., 2015). Lectins can also activate the complement system, which contributes to the first line of immune defences against invading pathogens and in the clearance of potentially damaging debris and necrotic or apoptotic cells (Dodds and Law, 1998; Sunyer and Lambris, 1998; Carrol and Sim, 2011). Furthermore, diverse roles for the complement system are also implicated in regeneration (Del-Rio-Tsonis et al., 1998; Haynes et al., 2013) and tissue remodelling during development (Lange et al., 2004b; 2004c; Lange et al., 2005; Lange et al., 2006). The lectin pathway of the complement system is activated via binding of lectins or ficolins to carbohydrates and is evolutionarily conserved in bony fish (Dodds, 2002; Endo et al., 2006; Nakao et al., 2006; Kania et al., 2010; Rajan et al., 2011). Lectins isolated from fish differ in molecular weight, sugar binding specificity, glycosylation and in the number of subunits, and have active antimicrobial, immunoregulatory, anti-tumour and developmental roles (Ng et al., 2015).

A novel 27 kDa ladder-like lectin protein was isolated from halibut serum by binding to N-acetyl glucosamine (GlcNAc), based on a method by Nakao et al. (2004). Blood was collected from a gill vessel of experimentally farmed adult halibut (weight 4.5 – 5.0 kg); the blood was allowed to clot overnight at 4 °C, and thereafter serum was collected by centrifugation at 2000 rpm for 20 min, yielding an overall pool of 150 ml serum from 10 fish. Large molecules were precipitated by adding $PEG_{3350}$ to 7 % by stirring gently on ice for 50 min, followed by centrifugation at 7000 rpm for 20 min at 4 °C. The supernatant was discarded; the precipitated protein was solubilised in 30 ml of sample buffer (50 mM Tris-HCl pH 7.8, 200 mM NaCl, 10 mM $CaCl_2$) and centrifuged again for 30 min at 12,000 rpm at 4 °C, discarding any insoluble material. The collected supernatant was then loaded onto a 2 ml N-acetyl glucosamine (GlcNAc; Sigma) column, which had been prepared by linking GlcNAc on Sepharose medium and loading it onto an empty PD10 column. The GlcNAc-sepharose column was thereafter washed with the sample buffer, bound protein was gradually eluted with a gradient of 5-50 mM methyl-α-mannopyranoside and the column washed again with 150 mM GlcNAc in sample buffer, followed by 10 mM EDTA in PBS. The collected fractions of the eluted

protein were analysed by SDS-PAGE (Fig. 1A) and polyclonal antibodies were produced against the halibut GlcNAc-binding protein in mouse ascitic fluid according to the method of Harlow and Lane (1988), as previously described for halibut C3 (Lange et al., 2004a and 2004b). In brief, the GlcNAc-binding protein was electrophoresed on SDS-PAGE and the gels were stained with 0.01 % Coomassie blue for 10 min, followed by washing in distilled water for 1 h. The bands, containing 100 µg purified protein, were excised from the gels and emulsified by passing the gel-sections repeatedly through a 1 ml syringe and a 21G needle before intraperitoneal injection into Balb/c mice. The injection was repeated on day 14, when the mice also received 500 µl i.p. of Pristane (Sigma, U.K.). On day 21 the protein injection was repeated and in the following weeks the mice were sacrificed, the ascitic fluid collected, aliquoted and stored at -80°C. Antibody specificity was tested by Western blotting on the purified protein fractions (Fig. 1B) and on halibut sera (Fig. 1C).

Comparing the GlcNAc-binding protein in halibut sera under reducing and non-reducing conditions, it showed a multimeric ladder-like structure under non-reducing conditions, compared to under reducing conditions where only one approximately 27 kDa band was observed, as assessed by Western blotting on halibut serum, using the anti-halibut GlcNAc-binding protein antibody (Fig. 1D).

The isolated protein fractions (see boxed areas in Fig 1) were analysed by mass spectrometric analysis and the partial sequences obtained for the approximately 27 kDa band of the GlNAc-binding halibut protein (Table 1) did not show any significant homology with other known proteins, or protein sequences, in UniProt.

**Table 1:** Amino acid sequences of the halibut GlNAc-binding protein obtained by mass spectrometry analysis revealed six sequences that did not show significant homology with known proteins.

| | ***Peptide amino acid sequence*** |
|---|---|
| msms 1 | SDNGLAQL(DA)VDRPTLK |
| msms 2 | NYGLFSLSTAAHQSQFVLYK |
| msms 3 | (HP) TPGNVFNADR |
| msms 4 | TPQTEFSSVTV (partial only) |
| msms 5 | ALDYVLSGK |
| msms 6 | PGNVFNADR |

Detection of the GlNAc-binding protein was further assessed during early halibut ontogeny using the mono-specific polyclonal anti-halibut GlcNAc-binding protein antibody (Fig. 1B and 1C) in immunohistochemistry. For this purpose, farmed Atlantic halibut larvae were used, which were obtained from the Icelandic fish farm Fiskey hf, during routine health checks by the Fish disease Laboratory, Institute for Experimental Pathology, University of Iceland. The rearing of the larvae has been described before (Lange et al., 2004b, Magnadottir et al., 2018). Three to five larvae were

collected for each developmental stage at 109, 206, 408, 495, 655, 860 and 1050 degree days (° d) post hatching, fixed in 4 % formalin in buffered PBS for 24 h and consecutively embedded in paraffin. Immunohistochemical staining of paraffin tissue sections (5 µm) was performed according to our previously published protocols (Lange et al., 2004b and 2004c), using the primary anti-halibut GlcNAc-binding protein antibody, secondary anti-Mouse IgG AP-conjugated antibody (DAKO), ABComplex/AP (DAKO), fast red solution (DAKO) and counterstaining with methylene green.

The halibut GlcNAc-binding protein was detected in immunogenic sites and neuronal tissue at the different developmental stages tested (Fig. 2). At 119 °d, a positive detection was seen in the mucosa of intestines (Fig. 2A), skin and gills, as well as in the liver, muscle and plexiform layer of eye, while a faint response was also seen in the brain. At 206 °d, mucosal layers of skin were strongly positive, particularly in sacchiform cells (Fig. 2B), and this was also observed at 408 °d, at which point a clear detection was observed in hepatocytes of the liver (Fig. 2C). At 495 °d, the medulla oblongata and optic lobe of the brain were strongly positive (Fig. 2D and E), mucosa of intestine remained strongly positive and a positive detection was also seen in hepatocytes of the liver, in spinal cord and plexiform layer of eyes. At 655 °d, goblet cells in the oesophagus were strongly positive (Fig. 2F) and a clear detection was seen in the mucosal layers of gills (Fig. 2G). At 860 °d, arteries of gills and gill mucosa were positive (Fig. 2H), myeloma of kidney showed strong positive, while tubuli were negative (Fig. 2I), and the eye showed strong positive in ganglion cells and the plexiform layers (Fig. 2J). Mucosal cells in the oesophagus were also strongly positive, as well as sacchiform cells in the skin and goblet cells in the gut. At 1050 °d, a strong response was still seen in the myeloma of kidney, while the response in brain and spinal cord was reduced. A prominent detection was seen in skin mucosa (Fig. 2K) and in mucosal cells of the oesophagus (Fig. 2L) at 1050 °d. Mouse ascitic fluid, used as a negative control, showed negligible staining (not shown).

The strong positive detection in the mucosal cells of the skin, gills and gut, and a notable predominant detection in mucosal surfaces at the later stages of development tested (1050 °d), indicate a role for this GlcNAc-binding protein in mucosal immune defences. Further detection was found in immune-related organs: liver and myeloma of kidney, which were positive at different developmental stages tested. A positive detection in brain was specifically strong around 495 °d in the medulla oblongata and the optic lobe, and a strong detection was also seen in the plexiform layer of eye. This may indicate putative roles in neuronal remodelling during development as lectins can be involved in the clearance of apoptotic cells via the complement pathway; a similar detection in neuronal and ocular tissue has previously been observed for complement component C3 during early halibut ontogeny (Lange et al., 2004b; Lange et al., 2006).

Under non-reducing conditions, the isolated GlcNAc-binding halibut protein showed a multimeric ladder-like structure, which is somewhat similar as previously seen for a "ladder-lectin" described in rainbow trout (*Oncorhynchus mykiss*) (Jensen et al., 1997), which has though a smaller subunit size at 16 kDa, compared to the halibut GlcNAc-binding protein which showed a 27 kDa subunit size. The halibut protein sequences analysed by mass spectrometry did however not cover the N-terminal amino acid sequence of the GlcNAc-binding protein and thus it was not possible to align it with the N-terminal amino acid sequence of the trout ladder-lectin, which interestingly did not show any significant homology with known proteins either (Jensen et al., 1997). The rainbow trout ladder-lectin has been identified as being a group of C-type lectins, with multiple isoforms that bind pathogen-associated molecular patterns, such as chitin and microbial surfaces (Russel et al., 2008), and these show similar detection sites as were found for the halibut GlcNAc-binding protein here, including both in mucosal surfaces as well as in liver and kidney, but have however not been reported in brain or eye (Russel et al., 2008).

The strong positive detection of the halibut GlcNAc-binding protein in gill mucosa, sacchiform cells of the epidermis and in mucosal goblet cells of the intestine; indicate similar sites of expression as various lectins described in other fish (Jung et al., 2002; Suzuki et al., 2003; Jensen et al., 1997; Tsutsui et al., 2005). In pufferfish (*Takifugu rubipres*), a similar mucosal-related pattern of detection, as observed here, was seen for mucosal lectins (Tsutsui et al., 2005) and has also been observed in an immunohistochemical study of six lectins in the flatfish *Paralicthys olivaceus* (Jung et al., 2002). Furthermore, galectins are also present in teleost fish, and have a wide range of functions in embryogenesis and innate immunity (Vasta et al., 2004; Zhou et al., 2016). Galectins have been described in mucosal immunity in various fish (Rajan et al., 2013, Nita-Lazar et al., 2016; Chen et al., 2013). For example, in Japanese conger eel (*Conger myriaster*), galectin-containing cells have been detected in the skin and in mucosal tissues by immunohistochemistry (Nakamura et al., 2001), and have been found to have agglutinating and opsonising activities (Nakamura et al., 2007) as well as to participate in cellular encapsulation of parasitic nematodes (Nakamura et al., 2012).

Here, a novel GlcNAC-binding ladder-like lectin from halibut is described for the first time and was detected at primary sites of innate immune defences. As the peptide sequences identified by mass-spec analysis did not show homology with any other proteins it still remains uncertain whether this GlcNAc-binding lectin forms part of the complement pathway, as a lectin or ficolin, or if it belongs to galectins. A strong detection in mucosal surfaces of skin, gills and gut, shows a similar pattern of expression as both mucosal lectins and galectins in other fish. Detection in neuronal tissue may indicate putative roles in tissue remodelling of the brain and in ongoing neurogenesis in the fish eye, and follows a pattern previously observed in halibut ontogeny for complement C3.

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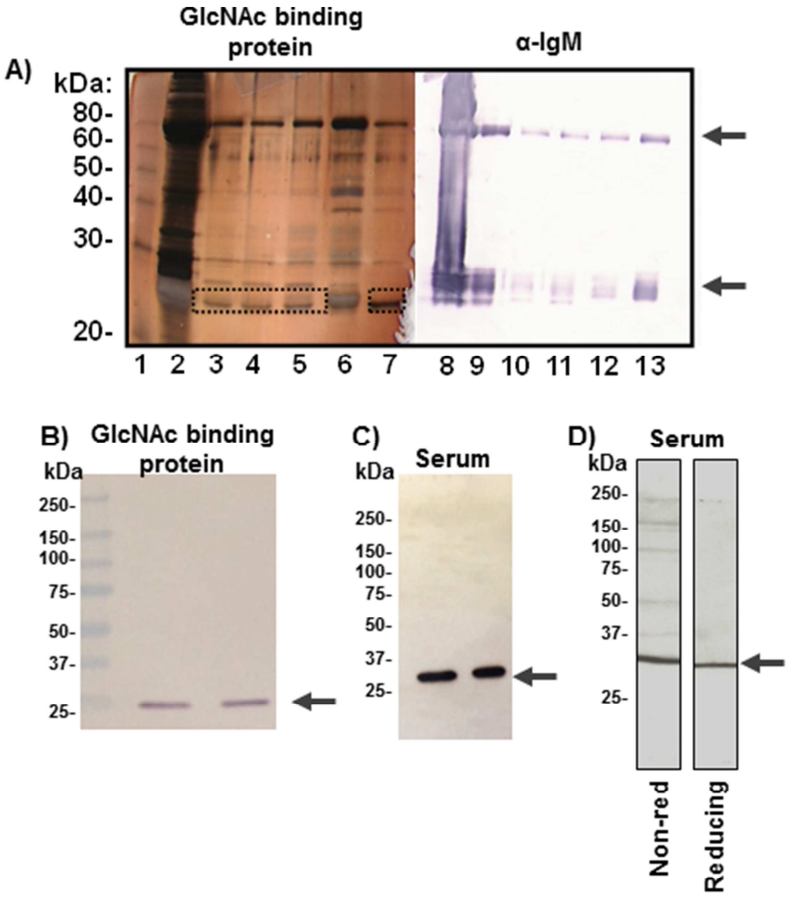
**Figure legends**

**Figure 1.** Halibut GlcNAc-binding protein isolated from halibut serum on an N-acetyl glucosamine column is shown by SDS-PAGE and by Western blotting. **A)** A silverstained SDS-PAGE gel showing halibut fractions eluted from the GlcNAc column: Lane 1 is a standard, lane 2 is the flowthrough, lanes 3-5 are eluted protein with 5, 50 and 300 methyl-α-mannopyranoside respectively, lane 6 is eluted protein with 150 mM GlcNac and lane 7 is eluted protein with 10 mM PBS. For detection of the presence of IgM in the fractions lanes 8 to 13 are corresponding to lanes 2-7, tested on WB using anti-halibut IgM antibody; the IgM heavy and light chains are indicated by arrows. The dashed boxes represent the bands excised and analysed by mass spectrometric analysis (see Table 1). B) Western blotting showing the specificity of the newly generated mouse polyclonal anti-halibut GlcNAc-binding protein antibody, reacting with the purified GlNAc-binding protein fractions and showing a strong positive band at approximately 27 kDa. **C)** Mono-specificity of the generated mouse polyclonal anti-halibut GlcNAc-binding protein antibody is shown on halibut serum (under reducing conditions), with one specific band detected at approximately 27 kDa. **D)** A ladder-like structure of the GlcNAc-binding protein is observed under non-reducing conditions as assessed by Western blotting on halibut serum using the polyclonal mouse antibody generated against the anti-halibut GlcNAc-binding protein (left panel). A single 27 kDa band (indicated by an arrow) is seen in halibut serum under reducing conditions (right panel). The molecular weight standard is indicated on the far left (25-250 kDa). Western blots in A and B were developed using alkaline phosphatase conjugated secondary mouse-IgG (Dako, Denmark) and 0.1 M ethanolamine/HCl buffer (pH 9.6) containing NBT (1 mg ml−1p-nitroblue tetrazolium), 0.1 M $MgCl_2$ and BCIP (4 mg ml−15-bromo-4chloro-indolyl phosphate in methanol:acetone (2:1)). Western blots in C and D were developed using HRP-conjugated anti-mouse IgG and ECL (Amersham).

**Figure 2. Immunohistochemical detection of the halibut GlcNAc-binding protein in early ontogeny.** **A)** Mucosal cells in gut at 119 °d are highlighted by arrows, (mu) indicates muscle; **B)** Sacchiform cells in skin at 206°d are indicated by arrows, (mu) indicates muscle; **C)** Liver hepatocytes at 408 °d; **D)** Medulla oblongata (mdo) of brain at 495 °d; **E)** Optic lobe (ol) of brain at 495 °d; **F)** Goblet cells in oesophagus are strongly specific as indicated by arrows; **G)** Mucosal cells in gills at 655 °d are indicated by arrows; **H)** Arteries of gills and gill mucosa at 860 °d; **I)** Myeloma (my) of kidney at 860 °d is strongly positive but tubuli (tu) not; **J)** Eye shows strong positive in plexiform layer (pl) at 860 °d; **K)** Skin mucosal cells (arrows) at 1050 °d; **L)** Goblet cells in the intestinal tract are strongly positive at 1050 °d. The scale bars indicate 50 μm in all figures.

**Fig.1**


A)
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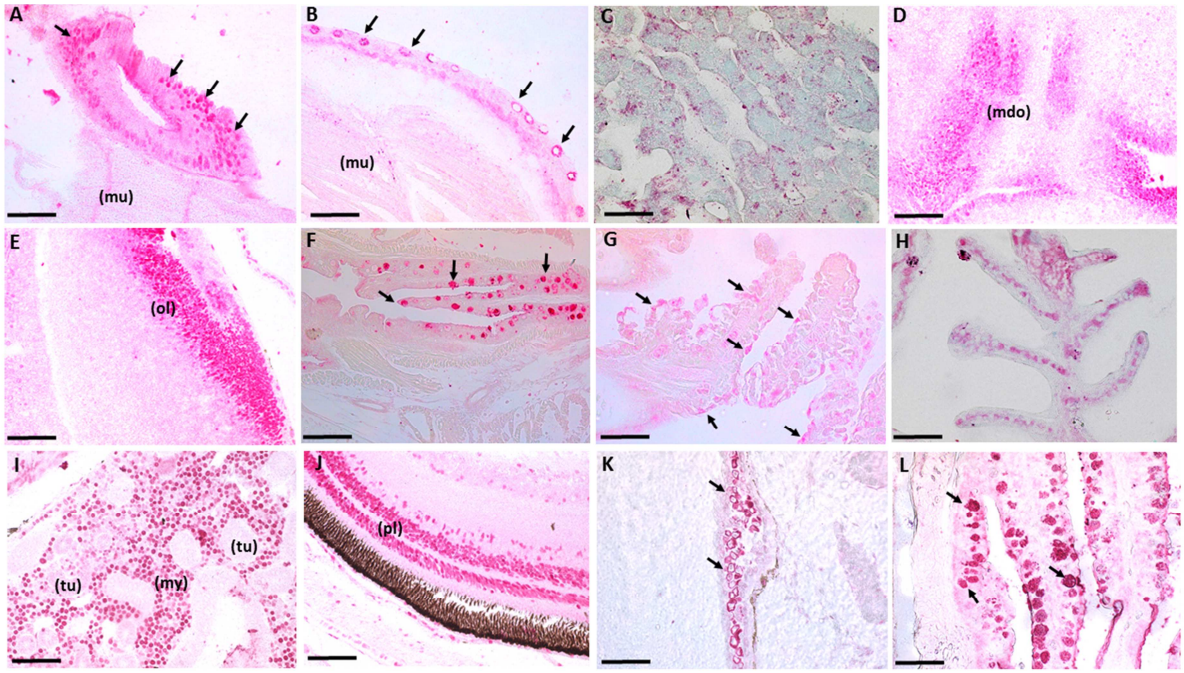

**Highlights**

- A novel 27 kDa ladder-like lectin protein was isolated from halibut serum by binding to N-acetyl glucosamine (GlcNAc)
- The GlcNAc-binding halibut protein showed a multimeric ladder-like structure under non-reducing conditions
- The GlcNAc-binding protein was detected in mucosal surfaces of skin, gills and gut as well as liver and kidney throughout early halibut ontogeny
- Ontogeny detection in neuronal tissue of brain and eye indicates roles in tissue remodelling and neurogenesis