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First evidence of protein G-binding protein in the most primitive vertebrate: Serum lectin from lamprey (*Lampetra japonica*)

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ABSTRACT

The intelectins, a recently identified subgroup of extracellular animal lectins, are glycan-binding receptors that recognize glycan epitopes on foreign pathogens in host systems. Here, we have described NPGBP (novel protein G-binding protein), a novel serum lectin found in the lamprey, *Lampetra japonica*. RT-PCR yielded a 1005 bp cDNA sequence from the lamprey liver encoding a 334 amino acid secretory protein with homology to mammalian and aquatic organism intelectins. Gene expression analyses showed that the NPGBP gene was expressed in the blood, intestines, kidney, heart, gill, liver, adipose tissue and gonads. NPGBP was isolated by protein G-conjugated agarose immunoprecipitation, and SDS–PAGE analyses showed that NPGBP migrated as a specific band (~35 and ~124 kDa under reducing and non-reducing conditions, respectively). These results suggested that NPGBP forms monomers and tetramers. NPGBP gene expression was induced by *in vivo* bacterial stimulation, and NPGBP showed different agglutination activities against pathogenic Gram-positive bacteria, Gram-negative bacteria and fungi. The induction of NPGBP suggested that it plays an important role in defense against microorganisms in the internal circulation system of the lamprey. When incubated with an unrelated antibody, the specific binding between NPGBP and protein G was competitively inhibited, indicating that NPGBP and the Fc region of Ig bind to the same site on protein G. We thus assume that the tertiary structure of NPGBP is similar to that of the Fc region of Ig. Additionally, NPGBP can effectively promote endothelial cell mitosis. These findings suggest that NPGBP plays a role in the immune defense against microorganisms, and this study represents one of the few examples of the characterization and functional analysis of an aquatic organism intelectin.

1. Introduction

Protein–carbohydrate interactions mediated by lectins have been recognized as key components of innate immunity in vertebrates and invertebrates; these interactions facilitate the recognition of potential pathogens and downstream effector functions, such as pathogen agglutination and immobilization and complement-mediated opsonization and killing (Vasta et al., 2011; Yan et al., 2012). C-type lectins are proteins that contain a carbohydrate recognition domain (CRD) and bind to carbohydrate structures in a Ca^{2+}-dependent manner. Calcium ions are directly involved in ligand binding and in maintaining the structural integrity of the CRD, which is necessary for the lectin activity, but many C-type lectins are Ca^{2+}-independent and may not necessarily bind to sugar ligands (Lopes-Ferreira et al., 2011; Drickamer, 1999; Weis et al., 1998).

The intelectin family, also called the X-lectin family, is a newly discovered lectin family whose members are involved in development and innate immunity (Lee et al., 2004; Lin et al., 2009). Since the discovery of intelectins, homologues have been identified in protochordates, agnathans, fish, amphibians, mice (intelectins 1 and 2) (Datta et al., 2005; Tsuji et al., 2001) and humans (HL-1 and HL-2 intelectins (Lee et al., 2001; Tsuji et al., 2007), omentin (Yang et al., 2006), and lactoferrin receptor (Suzuki et al., 2001)). Intelectins are Ca^{2+}-dependent soluble oligomers of 34–45 kDa glycosylated subunits. Each unit has a fibrinogen-like domain that functions as the CRD, with specificity for alpha-galactosides (Chang et al., 2004; Shin et al., 2008). Several intelectin homologues have been identified in fish, including the rainbow trout (expressed in liver) and the grass carp (gcIntL, approximately 55% identity to HL-1, expressed in multiple tissues, including head, kidney, spleen, heart, gill, intestine, and brain) (Vasta et al., 2004). Bin Lin et al. reported the identification of seven intelectins in zebrafish (Lin et al., 2009). Intelectin protein was also detected on the surface

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of catfish skin and was distributed in the skin, gill, renal tubules and blood plasma of this fish (Tsutsui et al., 2011). However, only 5 years have passed since the initial identification of fish intelectin by Chang and Nie (2007), and the available information about intelectins remains far from exhaustive.

The lamprey *Lampetra japonica* is a typical migratory species whose members migrate to fast-flowing stretches of clear streams to spawn during late spring; by autumn, the young of anadromous populations make their way towards the sea (Hardisty, 2006; Lennon, 1954). Compared to air, water generally contains a greater number of microorganisms, such as bacteria, fungi, and protozoa. Lamprey are continuously exposed to this dangerous environment, but they do not have a stratum corneum, and their body surfaces are composed of live cells. These animals are therefore at high risk for constant attack from microorganisms. In host defense systems, the recognition of bacterial components is important for the induction of immune responses (Russell and Lumsden, 2005). Pathogen cell wall components have various biological activities and contain bacteria-specific carbohydrate chains that do not exist in mammals. The recognition of these carbohydrate chains is useful for the induction of cellular and humoral immune responses to eliminate pathogens (Tsuji et al., 2001). It is likely that extracellular and soluble serum lectins act as a first line of defense in the host by recognizing specific carbohydrate moieties on pathogen surfaces.

Recently, a novel protein G-binding protein (NPGBP) belonging to the family of intelectins was isolated from the lamprey, *L. japonica*. Here, we report the first interaction of lectins with protein G. The goal of this study was to characterize this lamprey intelectin protein. We report here the primary structure, gene expression, and histological localization of the lamprey intelectin NPGBP.

2. Materials and methods

2.1. Animals

Adult *L. japonica* (length: 36.4–58.4 cm, weight: 112–274.5 g) were obtained from the Tongjiang Valley of Songhua River, Heilongjiang Province, China in December 2011.

Bacterial injection was performed as follows: six lampreys were selected and randomly divided into two groups (3 individuals per group). The bacterial injection group ($n = 3$) fish were injected intraperitoneally with 100 μL of the bacterial suspension (10 mg/ml), which was repeated three times (once every 5 days, on average). The control group ($n = 3$) received the same volume of physiological saline. On the 15th day post-injection, the fish were sacrificed, and the expression of NPGBP in various tissues was detected by RT-PCR and western blot.

The cDNA library construction and EST sequencing of liver were previously reported by our research group (Gao et al., 2005; Xue et al., 2011). Trizol reagent, 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE), MTT, Lipofectamine™ LTX and Plus Reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The reverse transcription (RT) PCR kit, *Bam*HI and *Eco*RI were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The vector pET32a and *Escherichia coli* strains DH5α and BL21 were prepared in our lab. Human umbilical vein endothelial cells (HUVECs) were prepared in our lab. Metal affinity columns for purifying His-tagged proteins were purchased from Novagen (Madison, WI, USA). The BCA protein assay kit was purchased from Beyotime (Nantong, Jiangsu, China).

2.2. NPGBP discovery

Blood samples (20 ml) were collected by cutting off the lamprey's tail. The samples were incubated for 1 h at 37 °C and then centrifuged 20 min at 14,000*g*, 4 °C to collect serum. The serum was transferred to a new tube and frozen. Then, 20 μl of lamprey serum was mixed with 10 μl protein G and 270 μl PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 50 mM NaCl, 2.7 mM KCl, pH 7.4) and incubated at 4 °C with rocking overnight. The samples were centrifuged at 10,000*g* for 15 s in a microcentrifuge. Supernatants were then carefully discarded. The beads were washed twice with 1 ml RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin) to remove nonspecifically associated proteins and then were washed three times with 1 ml PBS to remove detergents. Finally, the beads were resuspended in 60 μl sample buffer (65 mM Tris–Cl, pH 8.0, 10% (v/v) glycerol, 2.3% (w/v) SDS, 0.01% bromophenol blue, 1% DTT) and boiled at 100 °C for 10 min. The samples were centrifuged at 10,000*g* for 15 s in a microcentrifuge before being loaded on SDS–PAGE.

MALDI-TOF mass spectrometry was carried out on a Bruker workstation. Protein spot excision and in-gel digestion were conducted according to the instructions for sample preparation. Briefly, protein spots were washed twice with ddH_2O, destained with 50 μl destaining solution (50 mM NH_4HCO_3/CH_3CN, 1:1), and then digested for 24 h with trypsin. Mass spectra were obtained after the samples were crystallized with the matrix 2,5-dihydroxybenzoic acid onto AnchorChip™ (Bruker Daltonics, Billerica, MA) target plates, using a ReflexIV™ (Bruker Daltonics) MALDI-TOF MS instrument (Zheng et al., unpublished data).

2.3. Amino acid sequencing and bioinformatic analyses

Intelectin homolog sequences were obtained from GenBank (NCBI, http://www.ncbi.nlm.nih.gov/) and the Ensembl Genome Browser (http://www.ensembl.org) by blastx, tblastn or blastn using the NPGBP cDNA or NPGBP protein sequence as a query. The amino acid sequences of the NPGBP proteins in lamprey and other species were aligned in ClustalX 1.81 using the default settings. The result of the multiple sequence alignment was converted into mega format and directly imported to MEGA 4.1 to construct a phylogenetic tree. A neighbor-joining (NJ) tree was constructed based on the pairwise deletion of gaps/missing data and the p-distance matrix of the amino acids model with 500 bootstrap replications (Xue et al., 2012).

2.4. The purification of NPGBP by protein G-affinity chromatography

A 20 ml sample of the lamprey serum was diluted with 180 ml TBS (Tris-buffered saline; 50 mM Tris, 150 mM NaCl, 0.05% NaN_3, pH 7.4). Then, the diluted lamprey serum was added to the protein G-agarose affinity column (BCA, Shanghai, China) at a flow rate of 0.5 ml per minute; this procedure was repeated twice. The column was washed with TBS until $A_{280} < 0.008$, and then the elution buffer (50 mM glycine, pH 2.7) was added to the column at a rate of 0.5 ml/min until all protein was eluted from the column. The eluted NPGBP was collected in 1.5 ml microcentrifuge tubes, to which 100 μl of neutralization buffer (1 M Tris, 1.5 M NaCl, 1 mM EDTA, 0.5% NaN_3, pH 8.0) was added and placed on ice to prevent protein denaturation. Then, 10 ml of elution buffer, pH 1.9, was added to the column as described in the step above. Protein was collected until $A_{280} < 0.008$.

SDS–PAGE was performed on a 12.5% separating gel. In brief, 10 μl of each affinity-purified fraction was added to 10 μl SDS sample buffer with or without 10% β-mercaptoethanol. Electrophoresis was performed using a constant current of 20 mA for 1.5 h, and the protein was visualized by staining with Coomassie Brilliant Blue (CBB) R-250.