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Two mannose-binding lectin homologues and an MBL-associated serine protease are expressed in the gut epithelia of the urochordate species *Ciona intestinalis*

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ABSTRACT

The lectin complement pathway has important functions in vertebrate host defence and accumulating evidence of primordial complement components trace its emergence to invertebrate phyla. We introduce two putative mannose-binding lectin homologues (*Cio*MBLs) from the urochordate species *Ciona intestinalis*. The *Cio*MBLs display similarities with vertebrate MBLs and comprise a collagen-like region, α-helical coiled-coils and a carbohydrate recognition domain (CRD) with conserved residues involved in calcium and carbohydrate binding. Structural analysis revealed an oligomerization through interchain disulphide bridges between N-terminal cysteine residues and cysteines located between the neck region and the CRD. RT-PCR showed a tissue specific expression of *Cio*MBL in the gut and by immunohistochemistry analysis we also demonstrated that *Cio*MBL co-localize with an MBL-associated serine protease in the epithelia cells lining the stomach and intestine.

In conclusion we present two urochordate MBLs and identify an associated serine protease, which support the concept of an evolutionary ancient origin of the lectin complement pathway.

1. Introduction

The immune system is traditionally divided into the adaptive (related to B- and T-lymphocytes and antibodies) and the innate immune system which together functions as a highly interactive and cooperative system to combat microorganisms. RAG based adaptive immunity seems to have arisen with the emergence of the jawed vertebrates, whereas innate immunity is more ancient and certain components and functions can be traced back to several different invertebrate phyla [1–5].

The collectins constitute one of the molecular groups of the innate immune system involved in pattern recognition of pathogens [6]. Mannose-binding lectins (MBL) are a group of collectins, which structurally are defined by the presence and of a carbohydrate recognizing domain (CRD) attached to a collagen region via an α-helical coiled-coil neck region. The structural unit is composed of three polypeptide chains, which further oligomerize through interchain disulphide bridges located in the N-termini. MBL has been isolated from several vertebrates including mammals [7–9], chicken [10], teleost fish [11,12] and lamprey [13]. Polymorphism and presence of different copy numbers/isoforms of MBL genes are a well-known phenomenon in vertebrates [12,14,15].

Reports on invertebrate collectins are sparse. One study describes a lectin with affinity for glucose isolated from the sessile ascidian *Halocynthia roretzi* [16]. This molecule was shown to have a C-type lectin CRD, but it lacks a collagen-like domain present in all vertebrate MBLs. Although the absence of a collagen-like region it was shown to be involved in C3 dependent phagocytosis and to be associated with a serine protease showing homology with other MBL-associated serine proteases (MASPs).

Vertebrate MBL recognizes patterns of complex carbohydrate structures designated pathogen-associated molecular patterns (PAMPs) on foreign surfaces and initiates the lectin pathway of the complement system. Complement activation through the lectin pathway is accomplished through activated MASPs. Three different MASPs (MASP-1, -2 and -3) have been identified in humans to be associated with MBL (or ficolins, which have also been demonstrated to activate complement via the lectin pathway [17]). MASP-2 activates and cleaves complement factors C4 and C2 to form the C3 convertase C4b2a [18]. Recent studies indicate various functional properties of MASP-1, including a direct

Abbreviations: MBL, mannose-binding lectin; MASP, MBL-associated serine protease; sMAP/MAp19, small MBL-associated protein19-kDa MBL-associated protein; RAG, recombination activating genes; IHC, immunohistochemistry.

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cleavage of C3 [19] and functions related to the coagulation system [20] whereas very little is known about the function of MASP-3.

A draft genome [21] and a large EST project [22] of the urochordate species *Ciona intestinalis* were published in 2002. These data allow quick, extensive *in silico* searches for immunorelevant molecules and makes *C. intestinalis* and other urochordates attractive models in comparative and evolutionary immunology. Based on these data we have isolated and characterized two MBL homologues with predicted specificity for mannose and galactose, respectively. The isolated *Ciona* MBLs were found to share structural characteristics with vertebrate MBLs and to localize to tissues lining the gut.

2. Materials and methods

2.1. Tissue preparation

Adult ascidians (*C. intestinalis*) were collected in Kerteminde Fjord, Denmark or kindly provided by Kerteminde Fjord & Baeltcenter, Denmark. Tissues from four distinct organs were selected and isolated from a pool of 20 *C. intestinalis*: intestine/stomach (gut), gonads, subneural gland and branchial sac. Tissues were immediately placed in sterile eppendorf tubes containing RNAlater™ (QIAGEN) and stored at −20 °C until used for RNA purification. Material containing "coelomic fluid" was obtained by removal of the fibrous tunica followed by mechanical homogenization (without anticoagulant additives), centrifugation and filtration through a 0.45 μm pore filter.

2.2. RT-PCR, cDNA cloning, sequencing, expression and purification of CioMBL

Total RNA was isolated using the RNeasy Mini-kit™ (QIAGEN) purification system. First-strand cDNA was synthesized by means of SuperScript II RNase H-Reverse Transcriptase (GibcoBRL 18064-022) and oligo dT priming, according to the manufacturer's instructions.

Specific primer sets based on an EST sequence from GenBank (*C. intestinalis* EST-database, accession nr: BW379942) were designed to obtain and amplify in frame cDNA products of both full-length *Cio*MBL and the neck-CRD region.

PCR was performed using Easy-A® High Fidelity PCR Cloning Enzyme (Stratagene) as recommended by the manufacturer with 25–30 cycles of 94 °C/30 s, 52 °C/30 s, 72 °C/2 min, and final extension of 72 °C/7 min. PCR products were cloned into the pBAD/TOPO® vector with a C-terminal His_6 tag (pBAD/TOPO® ThioFusion™ cloning and expression system (Invitrogen)) and sequenced by the dideoxy method using an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). Expression was induced with 0.02% (w/v) arabinose and r*Cio*MBL-Neck/CRD was affinity purified on a Ni-chelate-column (Ni-NTA Superflow, Invitrogen cat. no. 30410) and refolded according to the manufactures recommendations.

β-Actin normalized RT-PCR analysis was performed using *Cio*MBL specific primer sets (5′-TTATTGATGGGAAAGTTTGGT-3′ and 5′-TAACATCTCTGTTCTTGGGTC-3′) and *Cio* β-actin specific primer sets (5′-GGAAAAGATCTGGCATCACACTT-3′ and 5′-GCGTA-ACCCTCGTAGATTGG-3′) on cDNA templates from the four selected tissues preparations.

Eukaryotic cloning and expression of full-length recombinant *Cio*MBL-QPD and -EPN without His_6 tags were performed with CHO-S cells (Invitrogen) as described in the Flp-In™ System (Invitrogen) manual. For large-scale production r*Cio*MBL-CHO-QPD and -EPN were grown in separate culture flasks (NUNC™) in RPMI/10% FCS or in CHO cell serum-free medium (GIBCO™).

2.3. Generation and purification of monoclonal antibodies

Mice were immunized three times with 25 μg of purified recombinant *Cio*MBL-Neck/CRD or two 17 residue *Cio*MASP peptides from the N- and C-terminal part of the deduced MASP protein (MIDPEIAQWIERTSDLE and QQVIHRNDTHGILESPH, based on Genbank accession nr: XM_002128596) coupled onto PPD essentially as described by Lachmann et al. [23]. The antigens were adsorbed onto $Al(OH)_3$ and mixed in 1:1 ratio with Freunds incomplete adjuvant. Four days prior to the fusion the mice received an intravenous injection with 25 μg antigen administered with adrenalin. The fusion and selection was done essential as described by Kohler and Milstein [24] using the SP2/0-AG14 myeloma cell line as fusion partner. Positive clones were selected by differential ELISA screening against the MASP peptides coupled to ovalbumin or r*Cio*MBL-Neck/CRD and a non-related His_6-tagged protein produced with the same expression system. Cloning was performed by limited dilution and single clones were grown in culture flasks in RPMI + 10%. The mAbs were purified from culture supernatant by protein A affinity chromatography using the Äkta FPLC system according to the manufacturer's instructions (Amersham Pharmacia). Epitope mapping of the antibodies was performed using the method described by Andersen et al. [25]. We selected three anti-*Cio*MBL mAbs (mAb-8, -12 and -37) with different epitopes on the neck-CRD of r*Cio*MBL. They were found to react with both r*Cio*MBL-QPD and -EPN expressed in CHO cells (data not shown) and they were applicable for Western blot analysis, immunoprecipitation and immunohistochemical (IHC) analysis (data shown under results). Two anti *Cio*MASP mAbs reacting with two 17 residue peptides from the C- and N-terminal end, respectively of a deduced *Ciona* MASP protein were found to be applicable for IHC analysis.

2.4. ^{35}S biosynthetic labeling and immunoprecipitation of rCioMBL

CHO cells expressing either r*Cio*MBL-QPD or EPN were grown at 37 °C separately in 12 well culture plates (NUNC™) until approximately 80–90% confluence. The cells were washed five times in Dulbecco's Modified Eagle Medium (DMEM) without L-cysteine and L-methionine (GIBCO™). Two microlitre of ^{35}S-labeling medium (90% of DMEM without L-cysteine and L-methionine containing 10% dialyzed FCS mixed with 10% RPMI containing 10% FCS (all GIBCO™)) and 50 μl (20.5 MBq) Express Protein Label Mix-^{35}S-Easytag™ (PerkinElmer®) was added to each well. Cells were placed in a 37 °C CO_2 incubator over night where after the supernatant was harvested.

Immunoprecipitation was carried out as previous described [26] using 5 μg of each anti-*Cio*MBL mAbs or control mAb followed by analysis by SDS-PAGE and autoradiography.

2.5. Carbohydrate binding of rCioMBL

One microlitre of culture supernatant with either ^{35}S biosynthetic labeled r*Cio*MBL-QPD or EPN was incubated with 100 μl mannan-agarose beads (Sigma, St. Louis, USA, M-9917, Lot. 60K7041) and incubated for 4 h at 4 °C. The beads were washed with three times with 1 ml TBS/3 mM $CaCl_2$ and samples were eluted with 10 mM EDTA and analyzed by SDS-PAGE and autoradiography.

To analyze the carbohydrate specificity of r*Cio*MBL microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated o/n at 4 °C with 10 μg/ml of mannan (Sigma, St. Louis, USA, cat nr. 7250). After blocking in TBS/0.05%Tween/3 mM $CaCl_2$ r*Cio*MBL-QPD or EPN as serum-free culture supernatant were applied to the plates with different concentrations of carbohydrates (from 0 to 25 mM of glucose, GlcNAc, mannose and galactose) and incubated for 2 h at