

Isolation, cloning and functional characterization of porcine mannose-binding lectin

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SUMMARY

Binding of mannose-binding lectin (MBL), a C-type lectin, and its associated serine proteases, MASP-1 and MASP-2, to cell surface carbohydrates activates the lectin complement pathway. As MBL plays an important role in innate immunity, it has been cloned and characterized in several species. While the pig may be used as a source of organs/tissues for xenotransplantation, little is known about its MBL, thus, we report the isolation of three monomeric forms of MBL from porcine serum. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Coomassie staining of reduced porcine MBL revealed the presence of three monomeric forms with approximate molecular masses of 30 000, 32 000 and 34 000. Protein sequencing identified these monomeric forms as one single protein, suggesting post-translational modification. Western blot analysis demonstrated the cross-reactivity of anti-human MBL polyclonal antibody with porcine MBL. A full-length porcine liver MBL cDNA was isolated and the predicted amino acid sequence exhibited 64.9% identity with human MBL and 50.2% and 56.7% identity with rat A and C MBL, respectively. Furthermore, Northern blot analysis demonstrated the presence of a single (~1.4–1.6 kilobase pair) transcript in porcine liver. Addition of purified porcine MBL to MBL-deficient human sera augmented *N*-acetylglucosamine inhibitable C3 deposition to mannan-coated plates in a dose-dependent manner. Taken together, these data demonstrate that porcine and human MBL are highly conserved, sharing structural and functional characteristics.

INTRODUCTION

The human complement system is a host defence mechanism and can be activated by three separate pathways: the classical, the alternative and the lectin pathways. The classical pathway is activated following antibody–antigen interactions leading to deposition of C1q and the activation of C1s and C1r. The alternative pathway can be triggered by a number of mechanisms, such as haemodialysis, cardiopulmonary bypass, tissue plasminogen activator and yeast cell walls (zymosan). The lectin pathway is activated upon binding of mannose-binding lectin (MBL) and its two-associated serine proteases (i.e. MASP-1 and MASP-2) to a carbohydrate ligand which then cleave C2 and C4 to form the classical C3 convertase.^{1,2} The three pathways merge at C3 and cleavage of C3 culminates in the activation of the terminal complement pathway and

ultimately in formation of the terminal complement complex (C5b-9).³

MBL is a C-type lectin and can specifically bind to terminal-reducing sugars such as *N*-acetylglucosamine (GlcNAc), mannose, fructose and glucose present on micro-organisms. Human MBL circulates in the blood as large (200 000–650 000 MW) homo-oligomers consisting of 9–18 monomers of about 32 000 MW each. Each monomer consists of an amino-terminal region rich in cysteine residues, a collagen-like domain composed of 18–20 tandem repeats of a Gly-X-Y sequence, a neck region and a carboxy-terminal carbohydrate recognition domain region.^{4–6} Human MBL is present in a single form. However, it has been reported that rats and mice present two structurally and functionally distinct MBL proteins, MBL-A and MBL-C.^{6,7} MBL is believed to be an important constituent of innate immunity, with deficiencies in MBL being associated with recurrent infections in infants.⁸

While MBL is known to have a wide array of binding activity to carbohydrate moieties on micro-organisms, lectins are quite selective in respect to host glycoproteins. The pig may, in the near future, be a source of xenotransplantable tissues/organ, yet little is known about porcine MBL. Thus, to further the characterization of this interesting animal lectin, we have characterized porcine MBL protein and cDNA (GenBank

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for 3–4 hr at 42°. A dimer of the 370-bp fragment of porcine MBL cDNA was radiolabelled using the Prime-a-Gene labelling system (Promega) and purified sequentially through two Sephadex G-50 spin columns. The probe (5 ng/ml at 2×10^9 counts per min/ μ g) was denatured by boiling and was hybridized overnight at 42°. The blot was washed twice in $2 \times$ SSC/0.1% SDS for 10 min at room temperature, followed by two washes in 0.2 SSC/0.1% SDS for 10 min at room temperature. The blot was exposed to Kodak MS autoradiography film and developed.

MBL-dependent C3 deposition enzyme-linked immunosorbent assay (ELISA)

Radioimmunoassay 96-well plates were coated with 100 μ l of 0.5 mg/ml mannan in sodium carbonate/bicarbonate buffer pH 9.6 and incubated overnight at 4° as described. Plates were then washed three times with PBS buffer containing 0.5% Tween-20 and washed once with PBS buffer followed by a second wash with veronal-buffered saline (VBS: 141 mmol/l NaCl and 1.8 mmol/l sodium barbital). Five per cent MBL-deficient human serum containing purified porcine MBL (0–250 ng/ml; 100 μ l) or human MBL (0–60 ng/ml; 100 μ l) was added to each well and incubated for 0.5, 1, or 2 hr at 37°. Plates were then washed four times with PBS buffer containing 0.5% Tween-20. Human C3 deposition was detected by addition of 50 μ l of 1:3000 dilution of horseradish peroxidase-conjugated goat anti-human C3 (ICN, Costa Mesa, CA) to each well and incubated at 20° for 1 hr. The plates were washed four times and 50 μ l of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic) acid (ABTS) was added to each well and the plates were read at 405 nm using a microplate reader (Molecular Devices, Sunnyvale CA). GlcNAc (50 mmol/l), an inhibitor of the lectin complement pathway, was added to some wells to inhibit lectin pathway activation. Non-specific background absorbance consisted of wells containing VBS buffer without sera. All ELISA experiments were performed in triplicate.

RESULTS

Purification of porcine MBL

Purified porcine (2 μ g) and human (2 μ g) MBL were fractionated on a 9% SDS–polyacrylamide gel electrophoresis (PAGE) under partially reduced conditions (Fig. 1). Coomassie staining of human MBL demonstrated monomeric (32 000), dimeric (64 000), trimeric (96 000) and higher molecular mass forms of human MBL. Porcine MBL demonstrated three monomeric forms with approximate molecular masses of 30 000, 32 000, and 34 000.

Purified porcine (1 μ g) and human (500 ng) MBL were fractionated on a 9% SDS–PAGE under non-reduced conditions (Fig. 2). Rabbit anti-human polyclonal antibody (R2.2) recognized non-reduced human and porcine MBL by Western blot analysis. Cross-reactivity of the anti-human polyclonal antibody confirmed the presence of similar epitopes between porcine and human MBL.

Cloning of porcine MBL cDNA

In order to clone porcine MBL, an initial RT-PCR reaction was carried out on porcine liver mRNA using oligonucleotides

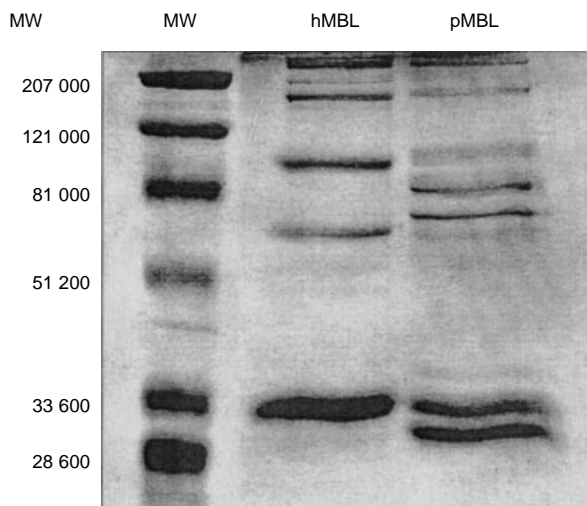


Figure 1. SDS–PAGE analysis of human and porcine MBL. Protein samples (2 μ g each) were analysed under reduced conditions on a 9% polyacrylamide gel: molecular weight marker (MW); human MBL (hMBL); porcine MBL (pMBL).

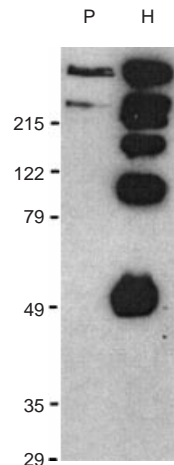


Figure 2. Western blot analysis of affinity-purified porcine MBL. Human (H; 500 ng) and porcine MBL (P; 1 μ g) were resolved on a 9% gel via SDS–PAGE under non-reducing conditions, transferred onto a nitrocellulose membrane and reacted with horseradish peroxidase-conjugated rabbit anti-human MBL polyclonal antibody (R2.2).

based on the conserved cDNA regions of human and bovine MBL cDNA sequence. A 370-bp PCR band was obtained which, following nucleotide sequencing was positively identified as a MBL homologue. This PCR product corresponded to position 300–670 bp of human MBL cDNA. A full-length porcine liver MBL cDNA of 723 bp and an open reading frame of 241 amino acids was obtained (data not shown; GenBank accession number AF164576) using RACE. The deduced amino acid sequences of porcine and human MBL were aligned and demonstrated a high degree of identity (Fig. 3). Microcapillary reverse-phase HPLC nano-electrospray tandem mass spectroscopy (μ LC/MS/MS) analysis confirmed that the three isoforms of pMBL present on the Coomassie-stained gel



Figure 3. Amino acid sequence alignment of human MBL (hMBL) and porcine MBL (pMBL). Shaded boxes represent amino acid residues that differ between porcine and human forms. Amino acid numbering is indicated to the right of the figure. Asterisks depict the position of cysteine residues and underlined residues represent the sequenced porcine peptide fragments.

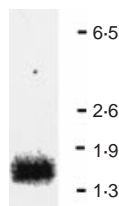


Figure 4. Northern blot analysis of porcine liver MBL. Total RNA (10 µg) was fractionated on a 0.9% formaldehyde gel, transferred onto a membrane and hybridized with a 370-bp porcine-specific cDNA probe. A single transcript was observed. Numbers to the right of the figure of MW markers in kilobases.

were the same protein, suggesting post-translational modification (see underlined residues in Fig. 3). Interestingly, the 30 000 band also contained a human MASP-2 homologue.

Northern blot analysis

To investigate the presence of porcine MBL transcript(s), liver poly(A)⁺ RNA was subjected to Northern blot analysis. Upon hybridization of liver mRNA with a 370-bp radio-labelled porcine MBL cDNA probe, a single transcript with an approximate molecular weight of 1.4–1.6 kbp was detected (Fig. 4).

MBL-dependent C3 deposition ELISA

C3 deposition onto mannan-coated plates was measured by ELISA to evaluate whether porcine MBL could functionally restore lectin complement pathway activation in human MBL-deficient human sera. Addition of purified human MBL (top panel, Fig. 5) to MBL-deficient human sera restored C3 deposition in a dose-dependent manner. Maximum C3 deposition was obtained with approximately 50 ng/ml of

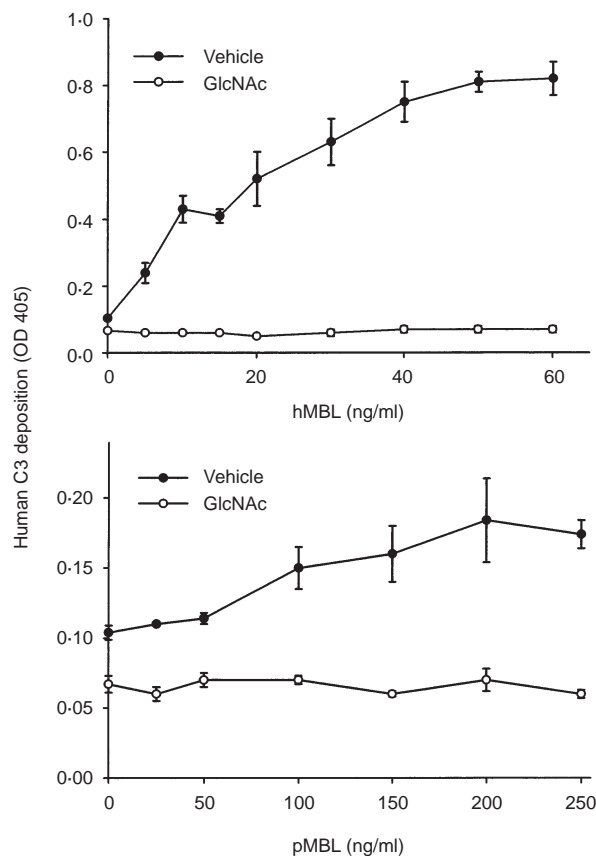


Figure 5. Pharmacodynamics of porcine and human MBL. Various concentrations of purified human (top panel) or porcine (bottom panel) MBL were added to MBL-deficient human serum. Addition of human or porcine MBL increased C3 deposition in a dose-dependent manner. *N*-acetylglucosamine (GlcNAc; 50 mmol/l) inhibited C3 deposition. Values are mean ± SEM of three separate experiments.

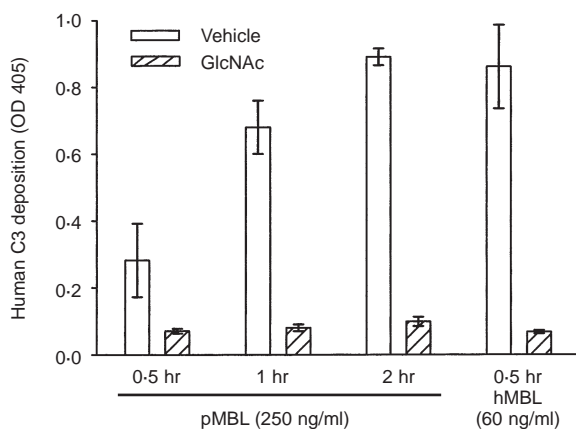


Figure 6. Pharmacokinetics of porcine MBL. Porcine MBL (250 ng/ml) was added to MBL-deficient human sera and incubated on mannan-coated plates for 0.5, 1 or 2 hr and then analysed for human C3 deposition. C3 deposition increased with the incubation period and was similar to human MBL (60 ng/ml) incubated for 0.5 hr. *N*-acetylglucosamine (GlcNAc; 50 mmol/l) inhibited C3 at all time-points. Values are mean \pm SEM of three separate experiments.

human MBL and this is equivalent to the amount of human MBL present in human sera (1–2 μ g/ml).^{2,11} Addition of purified porcine MBL (bottom panel, Fig. 5) was less effective than human MBL in restoring C3 deposition, but the effect was dose-dependent with maximum C3 deposition observed at approximately 200 ng/ml porcine MBL. Addition of GlcNAc (50 mmol/l) inhibited C3 deposition, demonstrating that porcine or human MBL can be inhibited by GlcNAc in this assay. Thus, these data indicate that purified porcine MBL functionally restores the lectin complement pathway in MBL-deficient human sera.

Since porcine MBL-induced C3 deposition was less, we speculated that pharmacokinetics may influence human C3 cleavage. As shown in Fig. 6, increasing the incubation period from 0.5 to 2 hr increased GlcNAc-inhibitable C3 deposition. Equivalent C3 deposition was observed when MBL-deficient human serum was supplemented with human MBL (60 ng/ml) and incubated for 30 min compared with porcine MBL (250 ng/ml) supplementation and incubation for 1 or 2 hr. These data demonstrate that longer periods of incubation are required for efficient C3 deposition by porcine MBL-mediated human lectin pathway activation compared to the intact human system.

DISCUSSION

MBL homologues have been identified in several species including monkey, cow, rabbit, rat and mouse.^{7,12–14} The primary sequence of human MBL has been elucidated and the four domains have been characterized based on their homology with other lectins.⁵ Unlike human MBL, two structurally and functionally distinct MBL isoforms, MBL-A and MBL-C, are known to be present in rats and mice.¹⁵ The ability of rat MBL-A to activate complement may be related to the cysteine content of the amino-terminal region of the MBL protein backbone.¹⁵ The amino terminus of MBL-C in rat and mouse has two cysteines, whereas MBL-A, human MBL and, as we

now describe, porcine MBL have three cysteines. The presence of these cysteines allows the formation of functionally active multimeric MBL forms.¹⁵

Porcine liver MBL cDNA unveiled the striking overall structural homology of porcine MBL to human MBL. Porcine MBL shares an overall identity to rat MBL-A and -C proteins of 50.2% and 56.7%, respectively. Human (248 amino acids; Accession #4557739) and porcine MBL (241 amino acids) share an overall identity of 64.9% at the amino acid level (allowing for eight gaps). Thus, at the amino acid level, porcine MBL is more similar to human than to rat MBL. Purified porcine MBL functionally replaced human MBL and increased C3 deposition of MBL-deficient human sera in the mannan assay. C3 deposition was inhibited by GlcNAc, demonstrating that complement activation was MBL-dependent. Furthermore, these data demonstrate that GlcNAc can functionally inhibit porcine MBL.

SDS-PAGE demonstrated the presence of three monomeric forms of porcine plasma MBL under reducing conditions. The presence of three monomeric forms of porcine MBL supports the previously published observation by Storgaard and colleagues.¹⁶ However, microcapillary reverse-phase HPLC nano-electrospray tandem mass spectroscopy (μ C/MS/MS) analysis confirmed that the three isoforms of porcine MBL were the same protein, suggesting post-translational modification. These data are different from those presented by Storgaard and colleagues.¹⁶ Furthermore, we established the presence of a single (~1.4–1.6 kbp) liver transcript. While we cannot categorically rule out the possible presence of another porcine cDNA for MBL, our data suggest that the three porcine monomeric isoforms isolated by our procedures are the same protein with post-translational modifications. We observed functional inhibition of porcine MBL-mediated lectin complement pathway activation in MBL-deficient human sera using GlcNAc, whereas a previous study demonstrated that porcine MBL did not bind to GlcNAc-agarose.¹⁶ The inability of porcine MBL to bind GlcNAc-agarose may be a result of inefficient coupling of GlcNAc to agarose. Furthermore, we used GlcNAc to elute the proteins from a maltose column that were then identified by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectroscopy (μ C/MS/MS) to be porcine MBL isoforms. Thus, the porcine MBL that was isolated by our methods reveals a single protein with post-translational modifications.

Activated complement is known to induce tissue-mediated injury by several mechanisms. Although a collection of studies has demonstrated the involvement of complement in a number of disease states, the specific role of MBL still remains unknown. Identification of the important residues in the carbohydrate recognition domain of the protein could potentially unveil the structural interactions and the binding selectivity of MBL. The observation that porcine MBL can functionally activate the lectin pathway in MBL-deficient human sera may have importance in xenotransplantation or other diseases involving MBL.

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