Lectin from the Manila Clam *Ruditapes philippinarum* Is Induced upon Infection with the Protozoan Parasite *Perkinsus olseni*

Received for publication, February 8, 2006, and in revised form, June 19, 2006 \* Published, JBC Papers in Press, June 19, 2006 \*

Young Mee Kim\*, Kyung-Ill Park\*, Kwang-Sik Choi\*, Richard A. Alvarez\*, Richard D. Cummings\*, and Moonjae Cho\*\*

From the \*Department of Medicine, \*School of Applied Marine Science, Cheju National University, Jeju 690-756, Korea, \*Department of Biochemistry, Emory University School of Medicine and O. Wayne Rollins Research Center, Atlanta, Georgia 30322

Glycan-binding proteins (lectins) are widely expressed in many invertebrates, although the biosynthesis and functions of the lectins are not well understood. Here we report that Manila clam (Ruditapes philippinarum) synthesizes a lectin termed Manila clam lectin (MCL) upon infection with the protozoan parasite Perkinsus olseni. MCL is synthesized in hemocytes as a ~74-kDa precursor and secreted into hemolymph where it is converted to 30- and 34-kDa polypeptides. The synthesis of MCL in hemocytes is stimulated by one or more factors in Perkinsus-infected hemolymph, but not directly by Perkinsus itself. MCL can bind to the surfaces of purified hypospores and zospores of the parasite, and this binding is inhibitable by either EDTA or GalNAc. Fluorescent beads coated with purified MCL were actively phagocytosed by hemocytes from the clam. Immunohistochemistry showed that secreted MCL is concentrated within cyst-like structures. To define the glycan binding specificity of MCL we examined its binding to an array of biotinylated glycans. MCL recognizes terminal non-reducing β-linked GalNAc as expressed within the LacdiNAc motif GalNAcβ1–4GlcNAcβ1–R and glycans with terminal, non-reducing β-linked Gal residues. Our results show that the synthesis of MCL is specifically up-regulated upon parasite infection of the clams and may serve as an opsonin through recognition of terminal GalNAc/ Gal residues on the parasites.

Marine invertebrates have an innate immune system that is similar in some ways to the innate immune system in vertebrates. The invertebrate innate immune system is constitutive with pre-existing or immediately expressed immune functions that serve to prevent and limit invading microbes and pathogens. In both the invertebrate and vertebrate innate immune systems carbohydrate-binding proteins (lectins) play crucial roles, with involvement in processes such as non-self-recognition, inflammation, opsonization, cell-cell and cell to extracellular matrix interactions, fertilization, development, and regeneration (1–5). Lectins have been identified in many marine invertebrates, including tunicates (6), sponges (7, 8), crustaceans (9, 10), echinoderms (11–15), actinidae (16), and marine bivalves (17–21).

These lectins may be classified into two general groups. One group is Ca\(^{2+}\)-dependent and includes the C-type lectin family. Recently, C-type GalNAc-specific lectins from Cucumaria echinata were shown to interact with target membranes and exhibit strong hemolytic activity and cytotoxicity through pore formation (22). In the ascidian, Halocynthia roretzi, mannos-binding lectin has been reported (23). It is associated with mannos-binding lectin-associated serine proteases 1 and 2, which are the enzymes responsible for complement activation initiated by mannos-binding lectin in the lectin pathway (24).

The second group of lectins comprises the galactosyl-binding lectins, which have structural homologies with galectins. The sponges Geodia cydonium and Suberites domuncular express galectin-related lectins that are similar in structure to molecules that are involved mammalian immune responses (25). In marine bivalve host defense, a heterogeneous sialic acid-binding lectin with affinity for bacterial lipopolysaccharides has been isolated and partially characterized from the hemolymph of the horse mussel Modiolus modiolus (21). The isolated horse mussel lectin exhibited strong antibacterial activity against tested Vibrio strains, which suggests that the lectin plays a role in the elimination of bacteria and participates in an innate immunity.

We recently discovered a novel lectin (MCL)\* from the Manila clam (26). The lectin has an apparent molecular mass of 138 kDa in non-reducing SDS-PAGE and consists of 74-, 34-, and 30-kDa species. Inhibition assays showed that mucins containing GalNAc inhibit MCL binding (26). To better understand the biological roles of MCL in clams, we have explored MCL biosynthesis, carbohydrate-binding specificity, and its potential function in host defense.

Here we report that the infection of clams by the protozoan parasite Perkinsus olseni induces the synthesis of MCL by clam hemocytes. Newly synthesized MCL is secreted into hemolymph as a 74-kDa precursor protein that is processed to the 30- and 34-kDa species. We have also defined the glycan-binding

* This work was supported by a Korea Research Foundation Grant (KRF-2003-042-F00006), by the Basic Research Program of the Korea Science & Engineering Foundation (Grant R01-2006-000-11316-0), and by NiGMS, National Institutes of Health Grant GM62116 to the Consortium for Functional Glycomics. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\* To whom correspondence should be addressed. Tel.: 82-64-754-3837; Fax: 82-64-725-2593; E-mail: moonjcho@cheju.ac.kr.

\* The abbreviations used are: MCL, Manila clam lectin; FITC, fluorescein isothiocyanate; TBS, Tris-buffered saline; TTBS, TBS containing Tween; PBS, phosphate-buffered saline; PBS-T, PBS containing EDTA; MS, mass spectrometry.
the presence of ligand (GalNAc) to reduce the likelihood of labeling the active binding site. After labeling only the active lectin is purified by affinity chromatography and used for the experiments. Fluoresceinated MCL was incubated in the triplicate wells for 2 h at 4 °C in Tris-buffered saline (TBS, 25 mM Tris-Cl, pH 7.8, 150 mM NaCl, 25 mM CaCl₂, 0.1 mg/ml bovine serum albumin) and washed three times in the same buffer, without added albumin, before measuring fluorescence intensity in a fluorescence plate reader.

**Phagocytosis Assay**—The opsonizing ability of MCL was tested by a flow-cytometric assay that measures the phagocytosis of MCL-coated fluorescent beads by hemocytes. Fluorescent latex beads (φ 2.0 μm, 20 μl, Polyscience, Inc., Warrington, PA) were prepared by incubating them for 2 h with either 20 μl of MCL (10,000 titer/50 μl to human blood group O) in 1 ml of TBS-Ca²⁺; 20 μl of MCL with 2 mg/ml GalNAc in 1 ml of TBS-Ca²⁺; or 1 ml of filtered seawater (control group). After incubation the beads were washed 3 times with filtered seawater, and then 30 μl of a suspension of fluorescent beads from each preparation was added to a suspension of hemocytes prepared from Manila clams. Hemocytes were prepared from clam hemolymph (100 μl). The cells were incubated with different bead preparations at 20 °C in the dark on a gentle shaker for 1 h. An equal volume of 6% neutral formalin was added to the fix hemocytes, which were then analyzed by flow cytometry. Another control group was prepared where beads that had been incubated with 1 ml of filtered seawater were added to the hemocyte suspension and then immediately mixed with 3 μl of cytochalasin B at 0.1% final concentration to block further phagocytosis. Phagocytosis was then analyzed by two parameters, log amplifications of the complexity and cell size, and the log fluorescence frequency distribution histogram of the hemocyte population was determined. The data were collected for 100 s. The percentage of cells phagocytosing beads was noted. Statistical analyses were performed using statistical software with possible differences among groups defined by one-way analysis of variance followed by Duncan’s multiple range tests. Differences were considered significant at a probability level of 0.05.

**SDS-PAGE**—To determine the molecular sizes of the proteins that were eluted from the columns, 10% polyacrylamide gel electrophoresis was used under non-reducing and reducing conditions. The molecular masses of the proteins were compared using high and low molecular weight markers (Sigma-Aldrich).

**Lectin Activity Assay**—The direct hemagglutination assay was performed by a standard procedure using trypsin-treated human blood group O erythrocytes in TBS-Ca²⁺ (26). Aliquots of 50 μl of an erythrocyte suspension, which were diluted 10-fold with TBS-Ca²⁺, were added to microplate wells and mixed with 100 μl of serially diluted MCL with or without inhibitors. The mixture was shaken for 10 min on a microplate well shaker, and hemagglutination was scored by microscopic observation of visible cell clumps.

**Western Blotting**—After electrophoresis on 12.5% polyacrylamide gels, proteins were transferred to a 0.45-μm polyvinylidene difluoride membrane (Pierce). The transfer was carried out at 110 V for 2 h using 25 mM Tris (pH 8.3), 192 mM glycine, 20% MeOH, and 1% SDS as the transfer buffer. The gel was

---

**Induction of Manila Clam Lectin by Perkinsus olseni**

**Materials and Methods**

**Purification of Perkinsus Trophozoites from Manila Clam**—Trophozoites of *P. olseni* were extracted from the hemolymph withdrawn from the blood sinus of the adductor muscle of Manila clams, which were collected at Wando Island off the southern coast of Korea, where a high incidence of the parasite infection has been reported (27). *Perkinsus* in the hemolymph were propagated for 1 week in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:2) with Hepes buffer and 5% fetal bovine serum according to Ordas and Figueras (49).

**Perkinsus Purification and Culture**—One milliliter of hemolymph containing trophozoites of *Perkinsus* was withdrawn from the blood sinus of the adductor muscle of Manila clams with an insulin syringe and seeded in 24-well polystyrene plates. One milliliter of 35% sterilized artificial sea water prepared with Marine Mix (Sigma-Aldrich) with 4000 IU/ml penicillin/streptomycin was added to each well, and after 12 h of incubation at 26 °C, the contents of the wells were pooled and centrifuged for 10 min at 1500 × g. The pellet containing parasites was resuspended and incubated in artificial sea water with 4000 IU/ml penicillin/streptomycin and incubated for another 12 h at 26 °C. Parasites were centrifuged again, and the supernatant was carefully removed from each well. To each well was added 1 ml of Dulbecco’s Modified Eagle Medium/Ham’s F-12 (1:2) with 50 mM Hepes buffer, 3.5 mM sodium bicarbonate, and 5% fetal bovine serum in 35% artificial sea water. During the first week the media was changed twice, and when the number of *Perkinsus* cells was >10⁷ cells/ml, another subculture was initiated.

**Biosynthesis of MCL in the Hemocytes**—The hemolymph (1 ml) was withdrawn from the *Perkinsus*-infected clam and centrifuged (1000 × g). The supernatant was analyzed for a hemocyte-free hemolymph, and the pellet contained the hemocytes. The hemocytes were resuspended and incubated in media, as described above, for the indicated times at 37 °C and subjected to Western blot analysis for MCL.

**Hemolytic Assay**—The potential hemolytic activity of MCL was assessed using human erythrocytes. Erythrocytes were isolated from heparinized blood by centrifugation (1000 × g, 5 min) and washed three times with phosphate-buffered saline (PBS). The cell suspension was adjusted to 1 × 10⁹ ml⁻¹ in PBS. The cell suspensions (20 μl) were incubated at 37 °C for 30 min with equal volumes of MCL. The incubation media was centrifuged (1000 × g, 5 min). The absorbance of the solution was measured at 420 nm. The absorbance obtained after treating erythrocytes with only PBS and 2% SDS were taken as 0 and 100%, respectively.

**Glycan Binding Specificity of MCL**—MCL was purified as described previously (26) and was dialyzed into 150 mM NaCl containing 100 mM Na-HEPES, pH 7.8, and 10 mM CaCl₂, and reacted with fluorescein isothiocyanate (FITC) (50 μg of FITC/mg of protein). Procedures for probing the full glycan arrays are available at www.functionalglycomics.org/static/consortium/. Biotinylated glycans were bound to streptavidin-coated plates (Pierce) by incubation at 4 °C overnight at a concentration of 100 μM. Purified MCL was labeled with FITC in
Induction of Manila Clam Lectin by Perkinsus olseni

placed in Coomassie Blue to verify that the transfer was successful. The membrane was incubated in a blocking solution of TBS-containing Tween (TBS, 100 mM Tris-HCl, 0.9% NaCl, 0.1% Tween-20) plus 1% bovine serum albumin for 1 h at room temperature. The membrane was washed three times for 10 min each in TTBs and incubated with anti-MCL antibody (1:500 dilution in TTBs plus 1% bovine serum albumin) for 1 h at room temperature. After three washes, the membrane was incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG (BioDom Co.), which was diluted 1:500 in TTBs. The membrane was re-washed, and the antigen-antibody complexes were detected using enhanced chemiluminescence (ECL, Amersham Biosciences).

Immunofluorescence Staining—Hypnospores and zoospores of Perkinsus were washed with PBS plus 20 mM EDTA (PBS-T), incubated with 100 ml of MCL in TBS-Ca\(^{2+}\) for 1 h, then incubated in 5% bovine serum albumin in PBS-T and a mono-specific antibody against MCL (1:100 dilution in PBS-T) for 1 h. The cells were then washed three times in PBS-T and incubated with a 1:200 dilution in PBS-T of FITC-conjugated goat antibody against rabbit immunoglobulins (Sigma-Aldrich) for 1 h. The cells were incubated with pre-immune rabbit IgG as a control. For immunohistochemical staining, Perkinsus-infected clams collected from Wando Island were embedded in Jung Tissue Freezing Medium (Jung, Germany). Cryostat sections were fixed in ice-cold acetone and air-dried for 30 min. Tissues were equilibrated to 0.1 M PBS, and then incubated in 1% goat serum in PBS-T for 30 min to block nonspecific antibody binding. Samples were then incubated with rabbit polyclonal anti-MCL (1:200) diluted in 0.1 M PBS-T for 1 h at room temperature in a humidified chamber. A negative control was performed by replacing antibody by pre-immune rabbit IgG. Specific binding of the primary antibodies was visualized using a FITC-labeled goat anti-rabbit IgG (1:1000). The immunofluorescence images were captured on a microscope (BX50, Olympus, Tokyo, Japan).

Diagnosis of Perkinsus Infection—Two populations of the Manila clam were collected from Gomso and Gimyong on the west and south coasts of Korea. According to Park and Choi (27), Gomso is known to be a Perkinsus-infected area, whereas Gimyong is Perkinsus-free. Clams were incubated in fluid thioglycollate medium, according to Ray (28). After incubation for 1 week, the tissues were stained with Lugol's iodine and examined under a light microscope. Incidence of Perkinsus in clams was also confirmed by digesting the fluid thioglycollate medium-incubated clam tissues in 2 M NaOH, as described by Choi et al. (29) and examining Perkinsus hypnospores using a light microscope.

In-gel Digestion and Peptide Sample Preparation—All solvents used in this procedure were high-performance liquid chromatography grade. The SDS-polyacrylamide gels were stained with Coomassie Brilliant Blue. Protein bands were excised from the stained gel. Then the excised bands were washed three times with 1:1 (v/v) solution of acetonitrile/deionized water for 10 min and dehydrated with 100% acetonitrile. The bands were finally washed with 1:1 (v/v) solution of 100% acetonitrile/100 mM ammonium bicarbonate and dried using a SpeedVac. Proteins contained in the gel pieces were reduced by using 10 mM tris(carboxyethyl)phosphine in 0.1 M ammonium bicarbonate at 56 °C for 45 min and then alkylated with 55 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 30 min. Next, the washing step above was repeated on the alkylated sample. After the washing step, the gel pieces were dried and soaked in sequencing-grade trypsin solution (500 ng) on ice for 45 min. Then the gel pieces were immersed in 100 μl of 50 mM ammonium bicarbonate, pH 8.0, at 37 °C for 14–18 h. The resulting peptides were extracted sequentially for 20 min with 45% acetonitrile in 20 mM ammonium bicarbonate, 45% acetonitrile in 0.5% trifluoroacetic acid, and 75% acetonitrile in 0.25% trifluoroacetic acid with agitation. The extracts containing tryptic peptides were pooled together and evaporated under vacuum.

Micro Liquid Chromatography-Mass Spectrometry/MS Spectrometry Analysis and Protein Data Base Search—In-gel digested proteins were loaded onto fused silica capillary columns (100-μm inner diameter, 360-μm outer diameter) containing 8 cm of 5-μm particle size Aqua C18 reverse-phase column material. The column was placed in-line with an Agilent Technologies (Palo Alto, CA) HP 1100 quaternary liquid chromatography pump, and a splitter system was used to achieve a flow rate of 250 nl/min. Buffer A (5% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile and 0.1% formic acid) were used to make a 90-min gradient. The gradient profile started with 5 min of 100% buffer A, followed by a 60-min gradient from 0 to 55% buffer B, a 25-min gradient from 55 to 100% buffer B, and a 5-min gradient of 100% buffer B. Eluted peptides were directly electrosprayed into a LTQ linear ion trap mass spectrometer (ThermoFinnigan, Palo Alto, CA) by applying 2.3 kV of direct current voltage. A data-dependent scan consisting of one full mass spectrometry (MS) scan (400–1400 mass/charge) and five data-dependent MS/MS scans were used to generate MS/MS spectra of eluted peptides. A normalized collision energy of 35% was used throughout the data acquisition. MS/MS spectra were searched against the National Center for Biotechnology Information rat protein sequence data base using Bioworks version 3.1 (Beckman Coulter, Fullerton, CA) and Sequest Cluster System (14 nodes, Thermo Electron, San Jose, CA). DTASelect was used to filter the search results, and the following Xcorr values were applied to different charge states of peptides: 1.8 for singly charged peptides, 2.2 for doubly charged peptides, and 3.2 for triply charged peptides. Manual assignments of fragment ions in each MS/MS spectra were performed to confirm the protein data base search results.

RESULTS

Induction of MCL upon Perkinsus Infection—MCL was purified from Manila clams as described previously (26). The protein has an apparent molecular mass of 138 kDa in non-reducing SDS-PAGE and consists of 74-, 34-, and 30-kDa species. However, during the purification of MCL from mucus fluid, we found that different batches of clams gave different yields, even when the amounts of starting material were similar. The different MCL yields appeared to correlate with the degree of infection of the Manila clam with Perkinsus. As shown in Table 1, body lysate from a Manila clam that was heavily infected with Perkinsus showed strong hemagglutinating activity, whereas...
that from a non-infected clam showed very little hemagglutinating activity. The mucus fluids from *Perkinsus*-infected or non-infected clams were analyzed by Western blotting using anti-MCL antibody (Fig. 1). The mucus fluid from the infected clams showed strong expression of MCL, which migrated as three species (apparent molecular masses of 74, 34, and 30 kDa). Non-infected clams hardly express MCL if any. The presence of MCL in the infected clams suggested two obvious possibilities; either *Perkinsus* itself produced the MCL during infection, or clams produced it upon infection, possibly for host defense. It seemed unlikely that *Perkinsus* makes large amounts of MCL and secretes it into mucus fluid, because *Perkinsus* is an intracellular endoparasitic protozoan.

To test the hypothesis that MCL might be induced by *Perkinsus* as part of the host defense mechanism in the clam, we purified *Perkinsus* trophozoites from infected clams and analyzed MCL production by Western blot analysis. A photograph of *Perkinsus* is shown in Fig. 2A. The infected clams contained high levels of MCL (Fig. 2B, lanes 1 and 4), whereas purified *Perkinsus* appeared to contain only a small amount of the 74-kDa species of MCL (Fig. 2B, lane 2). When *Perkinsus* were washed with 1 M NaOH for 2 min before Western blotting, the 74-kDa MCL band was lost (Fig. 2B, lane 3). The wash with NaOH did not affect the integrity of *Perkinsus* (data not shown), indicating that no MCL was contained within the parasite. These results indicate that MCL is bound to the surface of *Perkinsus* and suggest that MCL is acquired rather than synthesized by *Perkinsus*.

**MCL Is Synthesized in Hemocytes and Secreted as a 74-kDa Protein**—Because it has been reported that invertebrate hemocytes can produce lectins (30–32), we tested whether MCL is synthesized by Manila clam hemocytes. Hemocytes were purified from *Perkinsus*-infected Manila clams and analyzed by Western blotting analysis for MCL. Body lysates from *Perkinsus*-infected clam, mucus fluid from *Perkinsus*-infected clam, and hemocytes from *Perkinsus*-infected clam are shown.

**TABLE 1**

| Hemagglutination titera | Infected | Non-infected |
|-------------------------|----------|--------------|
|                         |          |              |
| 1024                    | 2        |              |
| 8192                    | 0        |              |
| 2048                    | 2        |              |
| 2048                    | 4        |              |

a Fresh clam meat (100 × g) was measured and added to 100 ml of distilled water. The mixture was blended three times for 30 s at maximum speed, incubated 1 h at 4 °C, and then centrifuged at 5000 × g. The supernatant (100 μl) was used for hemagglutination. The titer was determined by serial 2-fold dilution and defined as the dilution factor. The blood type did not affect the hemagglutination titer.

**FIGURE 1. Western blot analysis of mucus fluid from *Perkinsus*-infected and non-infected clams.** Manila clams were collected from different locations, and the hemagglutination activity and degree of *Perkinsus* infection were assayed as described under "Materials and Methods." Western blot analysis with anti-MCL was performed on mucus fluids from two separate *Perkinsus*-infected and two separate non-infected clams following SDS-PAGE.

**FIGURE 2. Western blot analysis of MCL in purified *Perkinsus*.** *Perkinsus* were purified and cultured as described under "Materials and Methods." Cultured *Perkinsus* were photographed under light microscopy (A) and analyzed for MCL by Western blot using anti-MCL (B). *Perkinsus* were purified (lane 2) from infected animals and washed briefly with 1 M NaOH (lane 3) and analyzed by Western blot. As positive controls, lysates of whole clam (lane 1) and hemocyte (lane 4) from *Perkinsus*-infected clam were analyzed, respectively.

**FIGURE 3. MCL production in hemocytes from infected Manila clams.** The hemocytes, body lysate, and mucus fluid were obtained from *Perkinsus*-infected Manila clams and analyzed by Western blotting analysis for MCL. Body lysates from *Perkinsus*-infected clam, mucus fluid from *Perkinsus*-infected clam, and hemocytes from *Perkinsus*-infected clam are shown.
Induction of Manila Clam Lectin by Perkinsus olseni

FIGURE 4. Synthesis and secretion of MCL. The hemolymph (1 ml) was withdrawn from a Perkinsus-infected clam and centrifuged (1000 \( \times \) g). The supernatant was taken for hemocyte-free hemolymph, and the pellet contained the hemocytes. A, the hemocytes were incubated in media for the indicated times at 37 °C and subjected to Western blot analysis for MCL. B, the indicated amounts of hemocyte-free hemolymph and hemocytes after centrifugation (0 h) were analyzed by Western blotting for MCL.

TABLE 2

Common peptide sequences obtained from 74-, 34-, and 30-kDa polypeptides by tryptic digest and tandem mass spectrometry

| M<sub>a</sub> (with charge) | M<sub>b</sub> (without charge) | Amino acid sequences |
|---------------------------|-----------------------------|---------------------|
| 544.3 (+2)                | 1086.6                      | LAELEEQK            |
| 589.8 (+2)                | 1177.6                      | NALHVYR             |
| 609.3 (+2)                | 1216.6                      | SNNLDGLEK           |

* Amino acids I/L or Q/K could not be distinguished by mass.

FIGURE 5. Perkinsus does not directly induce MCL expression in isolated hemocytes. Hemocytes were purified from uninfected or Perkinsus-infected clams. Hemocytes from uninfected clams were incubated with cultured Perkinsus hypnospores for 12 h at room temperature. The mixtures were precipitated with trichloroacetic acid and analyzed by Western blotting against MCL. Lane 1, hemocyte (10⁴ cells) from non-infected clam; lane 2, Perkinsus hypnospore (10⁴ cells) alone; lane 3, as in lane 1 but treated with 10⁴ cells of Perkinsus hypnospore; lane 4, as in lane 1 but treated with 2 × 10⁶ cells of Perkinsus hypnospore; lane 5, as in lane 1 but treated with 3 × 10⁶ cells of Perkinsus hypnospore; lane 6, hemocytes (10⁴ cells) from an infected clam.

from infected Manila clams also contained MCL (Fig. 3, lane 3), but only the 74-kDa band was predominant, and the 30- and 34-kDa bands were present at only very low levels. These results suggest that MCL is synthesized as a 74-kDa precursor that may be processed to the 30- and 34-kDa species.

To test this possibility hemocytes were purified from infected clams and incubated in culture media for up to 6 h, and the MCL was probed by Western blot analyses. At the initial time isolated hemocytes contained predominantly the 74-kDa form of MCL, but upon incubation both the 30- and 34-kDa species were generated (Fig. 4A). These results indicate that the 74-kDa form of MCL is a precursor to the 30- and 34-kDa forms and that their production of the latter forms occurs by proteolytic cleavage after secretion. We also performed proteomic analyses of 74-, 34-, and 30-kDa polypeptides. MCL was purified and analyzed by reducing SDS-PAGE. Each peptide was digested with trypsin and analyzed by liquid chromatography-MS/MS. As shown in Table 2, we found the identical tryptic peptides from 74-, 34-, and 30-kDa proteins, which confirmed 34- and 30-kDa proteins came from the 74-kDa precursor. MCL seems to be a new protein, because BLAST searches of proteomic peptides showed no significant homology with any known protein. Interestingly, identical tryptic peptides were found from the 34- and 30-kDa bands. This result may be interpreted either that the 74-kDa protein is degraded to 34 then 30 kDa or the 74-kDa peptide comprises a tandem-repeated gene structure.

To test whether the cleavage occurs after secretion, we examined the form of MCL in fresh hemolymph and hemocytes from Perkinsus-infected clams. Only the 74-kDa species was detected in both samples, confirming that the MCL is generated as 74-kDa species that is secreted and circulates as a 74-kDa protein prior to proteolytic cleavage (Fig. 4B). The nature of the protease(s) responsible for cleavage and their sites of action within the infected clam will be the subject of future studies.

Factors in the Hemolymph but Not in Perkinsus Induce MCL Synthesis by Hemocytes—Because the Perkinsus infection of the clam correlated with the synthesis of MCL in hemocytes, we tested whether live Perkinsus could trigger the synthesis of MCL in isolated hemocytes from uninfected clams. The live Perkinsus were purified and incubated for 12 h with hemocytes purified from an uninfected clam, and whole reaction mixtures were subjected to Western blotting. No MCL was detected in hemocytes incubated in the presence of Perkinsus (Fig. 5), whereas hemocytes from the infected clams contained the 74-kDa MCL. These results demonstrate that Perkinsus parasites do not directly induce MCL synthesis in hemocytes.

To explore factors that might induce MCL synthesis in hemocytes from infected clams, we tested the cell-free hemolymph from Perkinsus-infected clam. For these studies, we purified hemocytes from the Perkinsus-free clam or the Perkinsus-infected clam and incubated them with hemolymph from Perkinsus-infected clams. Hemocytes from Perkinsus-infected clams contained MCL, whereas hemocytes from Perkinsus-free clams lacked MCL (Fig. 6, left panel, 0 time). However, when hemocytes from Perkinsus-free clams were incubated with hemolymph from Perkinsus-infected clam, large amounts of MCL were generated (Fig. 6, right panel, 12 h). These results indicate that one or more unknown factors present in hemolymph from Perkinsus-infected clams induces MCL synthesis by hemocytes.

26858 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 37 • SEPTEMBER 15, 2006
MCL Binds to GalNAc-containing Glycans with High Affinity—Previous hapten inhibition experiments showed that the lectin activity of MCL was not inhibited by a large variety of mono- and oligosaccharides, but it was inhibited weakly by GalNAc and glycans from the bacterial species *Vibrio fisheri*is and *Pseudoalteromonas haloplanktis*. Interestingly, highly branchedmannans from the marine bacteria *Alteromonas aurantica*, *Alteromonas atlanticus*, *Vibrio fluvialis*, and *Marinomonas communis* also showed high inhibitory activity (26). Because of the confusing nature of these results, we explored direct binding of fluorescein-labeled MCL to a defined glycan array containing biotinylated glycans captured on streptavidin-coated microtiter plates. The detailed structures of glycans used in this experiment are listed in the home page of the Protein-Carbohydrate Interaction Core H in the National Institutes of Health-sponsored Consortium for Functional Glycomics (www.functionalglycomics.org/static/consortium/). As shown in Fig. 7, glycans containing terminal, non-reducing GalNAc were well recognized by MCL. Table 3 shows the detailed structures of glycans well recognized by MCL. Interestingly, glycans containing the LacdiNAc sequence GalNAcβ1–4GlcNAcβ1–R, which is a determinant that is abundantly expressed by many invertebrates and parasites, populated the list of highest binders to MCL, followed by glycans containing non-reducing terminal β-linked Gal. These results show that MCL is a highly specific lectin recognizing terminal β-linked GalNAc and Gal residues in glycoconjugates.

MCL Bind to Surface of Perkinsus—The studies above suggest the possibility that infection of clams by *Perkinsus* triggers the synthesis of MCL, which may act as a defensive molecule and/or opsonin by directly binding to *Perkinsus*. To investigate this possibility we examined MCL binding to purified *Perkinsus* hypnospores and zoospores using immunochemistry with the anti-MCL antibody. MCL bound well to both *Perkinsus* hypnospores and zoospores (Fig. 8, A and C, respectively) and gave typical patterns of cell-surface staining. By contrast, in control studies *Perkinsus* hypnospores and zoospores treated with the pre-immune IgG showed only dim fluorescence (Fig. 8, B and D, respectively). These results demonstrate that MCL can bind to the surfaces of both *Perkinsus* hypnospores and zoospores.

To investigate the property of the ligands on the hypnospores, we performed binding assay in the presence of various sugars (Fig. 9). Glucose did not affect binding and mannose showed some effect on binding (Fig. 9A). Binding of MCL to hypnospores was

![FIGURE 6. Induction of MCL from hemocytes by unknown factors in the hemolymph.](image1)

![FIGURE 7. Binding of MCL to glycan array of MCL.](image2)
Induction of Manila Clam Lectin by Perkinsus olseni

**TABLE 3**
Glycans well recognized in the glycan array by MCL

| Glycan no. | Glycan  | Trivial name | Avg. S/N |
|-----------|---------|--------------|----------|
| 98        | GalNAcβ1-4GlcNAc-PAA-Sp1 | LacdiNAc | 10.83    |
| 91        | β-d-Gal-PAα Sp1 | β-d-GalNAc | 7.92     |
| 94        | β-GalNAc-PAA-Sp1 | β-d-GalNAc | 7.90     |
| 22        | GalNAcβ1-4GlcNAcβ-Sp2 | LacdiNAc | 7.29     |
| 112       | GalNAcβ1-4GlcNAcβ1-2Manβ1-6(GalNAcβ1-4GlcNAcβ1-2Manβ1-3) | Bi-LDN (remodeled from human fibronogen) | 6.75     |
| 93        | β-GalNAc-PAA-Sp1 | β-GalNAc (Tn) | 6.50     |

*a Glycan number refers to the designated number of the biotinylated glycan in the Glycan Array (version 2.8) of the Consortium for Functional Glycomics.

*b Glycans are ranked in the table according to their S/N ratio ranking. See raw data in Fig. 7.

**FIGURE 8.** Binding of MCL to Perkinsus. Cultured Perkinsus hypnospores (A and B) and zoospores (C and D) were incubated with purified MCLs, and bound MCL was detected with monospecific anti-MCL (A and C) or pre-immune IgG (B and D), followed by the addition of FITC-labeled secondary antibodies. E, F, G, and H are the phase-contrast images of A, B, C, and D, respectively.

inhibited by EDTA, indicating the Ca$^{2+}$ dependence of the lectin, and by GalNAc and mannose, demonstrating that MCL is recognizing unusual glycan epitopes containing both GalNAc and mannose on the hypnospore surface. Based on glycan array (Fig. 7) and inhibition of MCL binding to hypnospores (Fig. 9), we suggest that the structure of MCL ligand in hypnospores contains LacdiNAc as a major binding epitope and mannose as a minor contribution. However, we could not completely block all binding of MCL by these reagents, suggesting that residual binding may result from nonspecific interactions or interactions that are carbohydrate-independent.

**MCL Does Not Contain Hemolytic Activity**—The potential hemolytic activity of MCL was determined using human erythrocytes. Erythrocytes were isolated from heparinized blood and incubated with MCL. The absorbance of incubation media was measured at 420 nm. MCL did not cause hemolysis of human erythrocytes (data not shown), indicating that MCL does not contain intrinsic hemolytic activity.

**MCL May Be Involved in Innate Immunity to Parasites**—We explored whether MCL might have an alternative activity and could induce phagocytosis of MCL-coated particles. To this end fluorescent beads were coated with MCL, and their phagocytosis by hemocytes was investigated. Phagocytosis of fluorescent beads reacted with MCL was approximately twice that of the control group containing beads treated only with saline ($p < 0.05$, Fig. 10, A and B). In addition, fluorescent beads that were incubated with MCL and its inhibitor free GalNAc showed reduced phagocytosis that was similar to the control saline group, indicating that GalNAc blocked phagocytosis (Fig. 10, A and B). Soybean lectin (commercially available GalNAc-binding lectin) also triggered opsonization and phagocytic uptake but not as efficiently as MCL (Fig. 10B). These data suggest that MCL may act as an opsonin by potentially binding to Perkinsus or other parasites expressing appropriate glycan targets and by inducing their adhesion and/or phagocytosis by hemocytes.

**MCL Is Localized in Encapsulated Perkinsus**—Because we observed that MCL binds to Perkinsus and that MCL-coated beads enhance the phagocytosis, we investigated the actual localization of MCL in Perkinsus-infected clam tissue using immunohistochemistry. Frozen sections of infected clams were incubated with monospecific anti-MCL antibody and stained with FITC-labeled secondary antibody. Negative controls with secondary antibody only (Fig. 11, A and B) did not show fluorescence staining. However, a strong signal was detected around Perkinsus (Fig. 11, C and D). The infiltrated hemocytes that produce MCL were stained strongly and were found around Perkinsus trophozoite. MCL appears to be highly enriched inside encapsulated bodies. These results strongly suggest MCL binding to Perkinsus may be involved in the encapsulation.

**DISCUSSION**

Our studies have explored the immunological responses of the commercially important bivalve the Manila clam (R. philippinarum) to an invading pathogen. Our results show that Perkinsus is a major pathogen of the Manila clam (R. philippinarum) and that MCL expression is a marker of Perkinsus infection. Infection of clams by Perkinsus induces the synthesis of MCL in the hemocyte as a 74-kDa precursor, which can be secreted into hemolymph where it is converted to 30- and 34-kDa species. MCL binds well to glycans containing terminal, non-reducing LacdiNAc sequences (GalNAcβ1-4GlcNAcβ1- R). Mature MCL binds to Perkinsus surfaces and may be involved in recruitment of hemocytes to Perkinsus. These stud-
ies significantly extend our earlier discovery of MCL and the finding that it exhibits Ca\(^{2+}\)-dependent carbohydrate binding activity to mucins and Perkinsus hypnospore (26). Perkinsus is a protozoan parasite of several commercially important marine mollusks, including the American oyster, Crassostrea virginica, the Manila clam, R. philippinarum, and the Australian black rib abalone, Haliotis rubra (33). In clams, cellular defensive activities to Perkinsus often induce pathological symptoms such as hemocytic infiltration, digestive tubule atrophy, and nodule formation on mantle and foot due to inflammation (27, 29, 34, 35). Perkinsus is also believed to be responsible for mass mortalities in clam populations (27, 34, 35).

It has been reported that bacterial infection causes changes in the components of plasma or hemolymph in mollusks, such as agglutinins, anti-bacterial peptides, and lysosomal enzymes (36–41). However, little is known about the nature of these changes and the factors responsible for their synthesis. In the crustacean horseshoe crab, a group of lectins termed tachylectins were found in the hemocytes and shown to recognize pathogen-associated molecular patterns. These lectins have different carbohydrate-binding specificities, and they were induced in response to lipopolysaccharide (42). In the tunicate Styela plicata in vivo challenge with the inflammatory agent zymosan causes generation of a collectin-like protein that is secreted into hemolymph after 96 h (30).

Ordas et al. (43) assayed defensive parameters of non-infected clams (Ruditapes decussatus) and clams heavily infected with Perkinsus atlanticus and found that the serum anti-bacterial activity and the agglutination titer were significantly different between infected and non-infected clams. In our study, lysates from non-infected clams (Ruditapes decussatus) had minimal agglutination activity, whereas lysates from Perkinsus-infected clams had high titer agglutinating activity (Table 1). Thus, the hemolymph of the infected clam contains a hemagglutinin that is only lowly expressed in uninfected clams, and we have identified at least one of these hemagglutinins as being the MCL lectin.

We found that Perkinsus itself does not directly induce the synthesis of MCL from hemocyte when they were incubated together; rather, hemolymph from infected clams was active in stimulating hemocytes to synthesize MCL (Figs. 5 and 6). These results suggest that infection of clams by Perkinsus leads to secretion of immune related factors, possibly cytokines, which trigger the synthesis of MCL in hemocytes. In the tunicate, S. plicata, an inflammatory cytokine, interleukin 1-like protein, has been reported (44). Our results strongly suggest the MCL may be involved in defense mechanisms and lectin synthesis is triggered by the infection.

MCL is synthesized as a 74-kDa protein by hemocytes from infected clams and secreted into hemolymph, and it is eventually cleaved to 30- and 34-kDa species. We previously reported that MCL consist of three subunits when purified from whole lysate in the presence of protease inhibitors (30, 34, and 74 kDa) (26). Our present results show that MCL is synthesized as a 74-kDa precursor protein that is processed to 30- and 34-kDa species after secretion into hemolymph. In the non-reducing SDS-PAGE, MCL behaves as a dimer (138 kDa) before and after cleavage of the 74-kDa species to the 30- and 34-kDa species (data not shown). Thus, MCL is synthesized in hemocytes where it is dimerized by disulfide bonds and secreted. Apparently, cleavage of the 74-kDa subunit does not affect carbohydrate-binding activity, based on the observation that MCL in body lysates binds to the affinity absorbents equivalently to pro-

\[\text{Relative Fluorescence Intensity (AU)}\]

![Image](https://example.com/image.png)

**FIGURE 9. Hapten inhibition of MCL binding to Perkinsus.** A, purified MCL was incubated with cultured Perkinsus hypnospore and detected as in Fig. 8 in the presence of PBS, 40 mM of glucose, mannose, GalNAc, and EDTA. B, the control was conducted in the absence of added MCL. The image was opened and quantified in ImageJ, a Java-based image-processing program.\(^3\)

\(^3\) W. S. Rasband (1997–2006) ImageJ, National Institutes of Health, Bethesda, MD, rsb.info.nih.gov/ij/.
Induction of Manila Clam Lectin by Perkinsus olseni

A

MCL treated beads

26.99%

MCL treated beads with inhibitor

14.76%

B

Phagocytosis rate (%)

MCL  MCL+GαNac  Normal  Soybean lectin  Soybean lectin + GαNac  Cytochalasin B

20  15  10  5  0
Induction of Manila Clam Lectin by Perkinsus olseni

cessed MCL (data not shown). It is still uncertain where the conversion of the 74-kDa species to the 30- and 34-kDa species is occurring, either in the hemolymph or in a terminal destination such as tissue or surface of pathogens. Future studies will address this issue.

We reported earlier that mucin types 1 and 2, which contain GalNAc residues, exhibited the best inhibition of MCL-mediated hemagglutination and that highly branched mannan structures from marine-originated bacteria also showed strong inhibition (26). However, because these glycan structures are not well characterized, and high branched mannan from Escherichia coli did not inhibit hemagglutination of MCL, the results were not conclusive whether the general high mannan structure inhibited agglutination. In glycan array analyses MCL showed the highest binding to glycans containing the Lacto-N-ac motif (GalNAcβ1-4GlcNAcβ1-R) and to glycans with terminal β-Gal and β-GalNAc structures. Many lectins from marine invertebrates have affinity toward to GalNAc residues (45). Interestingly, a mannose-binding lectin-like lectin from invertebrates has also been reported to have binding specificity toward to GalNAc-containing glycans instead of mannose (48).

Thus, the recognition of LactoN-ac-type glycans, which are abundantly expressed in many invertebrates and parasites, is consistent with our predicted role of MCL in innate immune responses in the clam.

Broad specificity of innate immune-related lectins may be a general biological phenomenon. A previous hapten inhibition assay showed that lipopolysaccharides derived from bacteria-infecting marine organisms but not lipopolysaccharides from other sources are potent inhibitors of MCL (26). The tachylectin-2 from horseshoe crab binds to both ν-GlcNAc and ν-GalNAc and lipopolysaccharide from Gram-negative bacteria (42). The C-type lectin perlucin from the abalone has been reported to have broad binding specificity with the ability to bind glycoproteins containing galactose or mannose/glucose (46). In the absence of immunoglobulin molecules, broad binding specificity of lectin may be required for effective humoral non-self-recognition in the marine invertebrate.

MCL can act as an opsonin to stimulate in vitro phagocytosis (Fig. 10, A and B). We also made the novel observation that MCL synthesis is induced by Perkinsus infection of clams. It has been reported that many lectins from marine invertebrates show antibacterial activity. The lectin from the horse mussel M. modiolus (21) exhibited strong antibacterial activity against tested Vibrio strains. The gigalin H and gigalin E lectins from oyster (Crassostrea gigas) acted as opsonins to stimulate in vitro phagocytosis of the marine bacterium Vibrio anguillarum (47). However, the induction of lectin involvement in defense against parasitic invasion, as we have seen for Perkinsus, has not been reported previously. Our present study shows that only the hemocyte from Perkinsus-infected clams produces MCL and that MCL is able to bind to the Perkinsus (Figs. 2 and 8). However, Perkinsus trophozoite is probably too large to allow direct engulfment by hemocytes. MCL may function to recruit hemocytes around Perkinsus and/or function in phagocytosis of parasite-derived material. The infiltration of hemocytes (Fig. 11) around Perkinsus is a well known symptom of Perkinsus-infected clam in the gill (50). Clusters of Perkinsus trophozoites were observed encapsulated in amorphous eosinophilic material, forming cyst-like structures (Fig. 11) (50). Parasite encapsulation is well characterized in insects (51). For example, the C-type lectin immulectin has...
Induction of Manila Clam Lectin by Perkinsus olseni

been reported to trigger encapsulation of parasites as a pattern recognition receptor (51). MCL may act as a pattern recognition receptor to activate lectin pathways of complement or to trigger encapsulation of parasites. In summary, our studies indicate that MCL is a parasite-inducible lectin that can bind parasites and promote their opsonization or encapsulation.

Acknowledgment—We thank the Protein Network Research Center (Seoul, Korea) for proteomics analysis.

REFERENCES

1. Vasta, G. R. (1990) in Defense Molecules (Marchalonsis, J., and Reinisch, C. L., eds) pp. 183–199, Wiley-Liss, New York
2. Dodds, A. W., and Day, A. J. (1996) in New Sirections in Invertebrate Immunology (Soderhall, K., Iwanaga, S., and Vasta, G. R. eds) pp. 303–341, SOS Publications, Fair Haven, NJ
3. Soderhall, K. (1992) Dev. Comp. Immunol. 6, 601–611
4. Faye, I. (1990) J. Biol. Chem. 265, 1–9
5. Soederhall, K. (1982) J. Biochem. 139, 151–159
6. Nair, S. V., Pearce, S., Green, P. L., Mahajan, D., Newton, R. A., and Raftos, D. A. (2000) Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 125, 279–289
7. Gamilu, V., Rinkevich, B., Schacke, H., Kruse, M., and Wener, E. G. (1994) J. Biochem. Hoppe-Seyler 375, 583–588
8. Miarons, P. D., and Fresno, M. (2000) J. Biol. Chem. 275, 29283–29289
9. Takahashi, Y., Itami, T., and Kondo, M. (1995) Fish Pathol. 30, 141–150
10. Margues, M. R. F., and Barrao, M. A. (2000) Aquaculture 191, 23–44
11. Giga, Y., Ikai, A., and Takahashi, K. (1987) J. Biol. Chem. 262, 6197–6203
12. Himeshima, T., Kawabata, S., Hirata, M., and Iwanaga, S. (1995) J. Biol. Chem. 270, 14493–14499
13. Matsui, T., Ozeki, M., Suzuki, M., Hino, A., and Titani, K. (1994) J. Biol. Chem. 269, 1127–1133
14. Hatakeyama, T., Ohuchi, K., Kuroki, M., and Yamasaki, N. (1995) Biosci. Biotechnol. Biochem. 59, 1314–1317
15. Oda, T., Tsuru, M., Hatakeyama, T., Nagatomo, H., Muramatsu, T., and Yamasaki, N. (1997) J. Biochem. 121, 560–567
16. Gaphurov, J. M., Bulgakov, A. A., Galkin, V. V., and Rasskazov, V. A. (1999) Toxicol 37, 1591–1604
17. Renwanz, L., and Stahmer, A. (1983) J. Comp. Physiol. B149, 535–546
18. Suh-Chae, Y. A., Jeune-Chung, K. H., and Chung, S. R. (1988) Korean J. Biochem. 21, 46–52
19. Dam, T. K., Sarkar, M., Ghosal, J., and Choudhury, A. (1992) Mol. Cell. Biochem. 107, 1–9
20. Fisher, W. S. (1992) J. Exp. Mar. Biol. Ecol. 162, 1–13
21. Tunkijjanukij, S., and Olafsen, J. A. (1998) Dev. Comp. Immunol. 22, 139–150
22. Hatakeyama, T., Furukawa, M., Nagatomo, H., Yamasaki, N., and Mori, T. (1996) J. Biol. Chem. 271, 16915–16920
23. Sekine, H., Kenjo, A., Azumi, K., Ohi, G., Takahashi, M., Kasukawa, R., Ichikawa, N., Nakata, M., Mizuochi, T., Matushita, M., Endo, Y., and Fujita, T. (2001) J. Immunol. 167, 4504–4510
24. Endo, Y., Nonaka, M., Saiga, H., Kakinuma, Y., Matushita, A., Takahashi, M., Matushita, M., and Fujita, T. (2003) J. Immunol. 170, 4701–4707
25. Muller, W. E., Blumberg, B., and Muller, I. M. (1999) Transplantation 68, 1215–1227
26. Bulgakov, A. A., Park, K. I., Choi, K. S., Lim, H. K., and Cho, M. (2004) Fish Shellfish Immunol. 16, 487–499
27. Park, K. I., and Choi, K. S. (2001) Aquaculture 203, 9–22
28. Ray, S. M. (1966) Proc. Natl. Shellfish. Assoc. 54, 55–69
29. Choi, K. S., Park, K. L., Lee, K. W., and Matsuoka, K. (2002) J. Shellfish Res. 21, 119–125
30. Green, P. L., Nair, S. V., and Raftos, D. A. (2003) Dev. Comp. Immunol. 27, 1–9
31. Goto, A., Kadowaki, T., and Kitagawa, Y. (2003) Dev. Biol. 264, 582–591
32. Jenny, M. J., Ringwood, A. H., Lacy, E. R., Lewitus, A. J., Kempton, J. W., Gross, P. S., Warr, G. W., and Chapman, R. W. (2002) Mar. Biotechnol. (NY) 4, 81–93
33. Hine, P. M. (1996) Rev. Sci. Tech. 15, 563–577
34. Navas, J. I., Castillo, M. C., Vera, P., and Ruiz-Rico, M. (1992) Aquaculture 107, 193–199
35. Montes, J. F., Durfort, M., and Garcia-Valero, J. (1996) Dis. Aquat. Org. 26, 149–157
36. Dyrnyda, E. A., Pipe, R. K., and Ratcliffe, N. A. (1997) Cell Tissue Res. 289, 527–536
37. Wootton, E. C., Dyrnyda, E. A., Pipe, R. K., and Ratcliffe, N. A. (2003) Aquat. Toxicol. 65, 13–25
38. Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J. A., and Bulet, P. (1996) J. Biol. Chem. 271, 21808–21813
39. Asokan, R., Arumugam, M., and Mullainadhan, P. (1997) Dev. Comp. Immunol. 21, 1–12
40. Mitta, G., Hubert, F., Noel, T., and Roch, P. (1999) Eur. J. Biochem. 265, 71–78
41. Mitta, G., Vandenbulcke, F., Hubert, F., and Roch, P. (1999) J. Cell Sci. 112, 4233–4242
42. Kedavata, S., and Iwanaga, S. (1999) Dev. Comp. Immunol. 23, 391–400
43. Ordas, M. C., Ordas, A., Beloso, C., and Figueras, A. (2000) Fish Shellfish Immunol. 10, 597–609
44. Nair, P. V., Burandt, M., Hutchinson, A., Raison, R. L., and Raftos, D. A. (2001) Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 129, 11–24
45. Beisel, H. G., Kedavata, S., Iwanaga, S., Huber, R., and Bode, W. (1999) EMBO J. 18, 2133–2132
46. Mann, K., Weiss, I. M., Andre, S., Gabis, H. J., and Fritz, M. (2000) Eur. J. Biochem. 267, 5257–5264
47. Olafsen, J. A. (1995) Adv. Exp. Med. Biol. 371, 343–348
48. Kenjo, A., Takahashi, M., Matushita, M., Endo, Y., Nakata, M., Mizuochi, T., and Fujita, T. (2001) J. Biol. Chem. 276, 19959–19965
49. Ordas, M. C., and Figueras, A. (1998) Dis. Aquat. Org. 33, 129–136
50. Cremonte, F., Balseiro, P., and Figueras, A. (2005) Dis. Aquat. Org. 64, 85–90
51. Ling, E., and Xu, Y.-Q. (2005) Dev. Comp. Immunol. 30, 289–299