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A Galectin of Unique Domain Organization from Hemocytes of the Eastern Oyster (Crassostrea virginica) Is a Receptor for the Protistan Parasite Perkinsus marinus$^{1,2}$

Satoshi Tasumi and Gerardo R. Vasta$^3$

Invertebrates display effective innate immune responses for defense against microbial infection. However, the protozoan parasite Perkinsus marinus causes Dermo disease in the eastern oyster Crassostrea virginica and is responsible for catastrophic damage to shellfisheries and the estuarine environment in North America. The infection mechanisms remain unclear, but it is likely that, while filter feeding, the healthy oysters ingest P. marinus trophozoites released to the water column by the infected neighboring individuals. Inside oyster hemocytes, trophozoites resist oxidative killing, proliferate, and spread throughout the host. However, the mechanism(s) for parasite entry into the hemocyte are unknown. In this study, we show that oyster hemocytes recognize P. marinus via a novel galectin (C. virginica galectin (CvGal)) of unique structure. The biological roles of galectins have only been partly elucidated, mostly encompassing embryogenesis and indirect roles in innate and adaptive immunity mediated by the binding to endogenous ligands. CvGal recognized a variety of potential microbial pathogens and unicellular algae, and preferentially, Perkinsus spp. trophozoites. Attachment and spreading of hemocytes to foreign surfaces induced localization of CvGal to the cell periphery, its secretion and binding to the plasma membrane. Exposure of hemocytes to Perkinsus spp. trophozoites enhanced this process further, and their phagocytosis could be partially inhibited by pretreatment of the hemocytes with anti-CvGal Abs. The evidence presented indicates that CvGal facilitates recognition of selected microbes and algae, thereby promoting phagocytosis of both potential infectious challenges and phytoplankton components, and that P. marinus subverts the host’s immune/feeding recognition mechanism to passively gain entry into the hemocytes. The Journal of Immunology, 2007, 179: 3086–3098.

I
nvertebrate and vertebrate species are endowed with efficient innate immune recognition and effector mechanisms that are successful in fighting infectious disease (1). Recognition of potential pathogens is achieved by either humoral factors present in plasma and other body fluids or by cell-associated receptors that lead to various effector functions, such as activation of complement and coagulation cascades, opsonization, and phagocytosis (2). Phagocytosis is usually followed by intracellular killing and destruction of the microorganism (3). Among the various receptors present on the surface of phagocytic cells of both vertebrates and invertebrates, lectins are critical factors for defense against potential microbial pathogens (4). Lectins are carbohydrate-binding proteins that are ubiquitous in body fluids, tissues, and cells. Based on their structure and binding properties, they have been classified in several families, such as C-, P-, F-, and I-types, ficolins, pentraxins, and galectins (5). In mammals, lectins from the C-type, ficolins, and pentraxins mostly recognize exogenous ligands, such as glycans on the surface of virus, bacteria, fungi, and protozoa, thereby acting as nonself recognition molecules and engaging effector functions such as immobilization, opsonization, and phagocytosis of the potential pathogens (6–9). In contrast, galectins mostly recognize endogenous ligands and indirectly participate in inflammation and adaptive immunity by mediating chemotaxis, apoptosis, and developmental and regulatory aspects of adaptive immune responses (10–17). However, in invertebrates, our knowledge of the biological roles of galectins in innate immunity is very limited and fragmentary (5).

Although like most invertebrate species, oysters possess effective innate immune mechanisms, they become readily infected when exposed to Perkinsus marinus trophozoites. This parasite causes Dermo disease, which is responsible for the catastrophic decline of natural and farmed oyster populations, and, thus, significant damage to the estuarine ecosystem along the Atlantic and Gulf coasts of North America (18). The infection mechanism(s) are still unknown, but it is likely that while filter-feeding, the healthy oysters ingest P. marinus trophozoites released to the water column by the infected neighboring individuals (19–21). Because unlike the biflagellated zoospores the trophozoite is not motile, entry into the host cells is passive, and mediated by recognition and phagocytosis by the host’s hemocytes. Uptake of live P. marinus trophozoites fails to invoke an oxidative response, although hemocytes are equipped with the pathways that can lead to a strong respiratory burst. This is due to the parasite’s anti-oxidative machinery that catalytically prevents the formation and/or degrades the reactive oxygen intermediates produced by the hemocyte upon phagocytosis (22). Thus, the parasite survives the oxidative attack, and proliferates intracellularly, leading to systemic infection and eventually overwhelming the host (19–23).
However, the molecular mechanisms for recognition and phagocytosis of *P. marinus* trophozoites by the oyster hemocytes are unknown.

In this study, we identified and characterized in oyster hemocytes a galectin (*Crassostrea virginica* galectin (CvGal)) of unique carbohydrate recognition domain (CRD) organization that, unlike most mammalian galectins, recognizes exogenous carbohydrate ligands. CvGal binds to a variety of potential microbial pathogens and phagolysosomal components, thus representing not only a nonself recognition component of the host cellular defense mechanisms, but also for feeding and intracellular digestion. Furthermore, we show that CvGal strongly interacts with *Perkinsus* trophozoites, functions as a hemocyte surface receptor for the parasite, and mediates its phagocytosis. Therefore, we propose that the parasite successfully competes with potential pathogens and algal food for binding to CvGal, to gain entry into the host phagocytic cells.

### Materials and Methods

#### Reagents

Carbohydrates (monosaccharides, oligosaccharides, and glycoproteins, at the highest purity available), LPS, and lipoteichoic acid were purchased from Sigma-Aldrich. DNA primers were obtained from Invitrogen Life Technologies. Protein electrophoresis reagents were purchased from Bio-Rad. Restriction enzymes were acquired from New England Biolabs. Rabbit erythrocytes were obtained from Immucor. Ex-Taq DNA polymerase and dNTPs were from Takara.

#### Animals

Adult eastern oysters (*C. virginica*), averaging 40–50 g each, were obtained from Mook Sea Farm and maintained in 2000-liter tanks at 30 ppt salinity; 20°C, and fed daily with live algae (*Tetraselmis sp.* and *Isochrysis sp.*), each species on alternate days. Oysters were confirmed as *Tetraselmis* sp. and *Isochrysis* sp., each species on alternate days). Oysters were provided by the algal culture facility at Aquaculture Research Center (NR10, and TXsc, and (25). Unicellular algae (*V. anguillarum* sp., *V. mimicus* and *V. parahaemolyticus* (CGSC 5158) was obtained from the Coli Genetic Stock Collection at the American Type Culture Collection.

#### Microbial, algal, and parasite species and strains

*Hemicentrotus pulcherrimus*, and *E. tarda* were cultured in tryptic soy broth medium, whereas the others were designed based on the clones sequences obtained by 3′- and 5′-RACE or genomic PCR described below.

### Table 1. Primers used for amplification of DNA fragments

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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</thead>
<tbody>
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<td>F2</td>
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</tr>
<tr>
<td>F4</td>
<td>CAAAATCTCCACACCTTCCA</td>
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<tr>
<td>GENE_R2</td>
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<td>GeneRacer 5′-primer</td>
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<tr>
<td>AP-2</td>
<td>ACTATAGGGCCACGCTGTGTT</td>
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</table>

**Genomic PCR**

Genomic DNA was purified using DNeasy 96 Tissue kit (Qiagen). PCR amplification was conducted using 100 ng of genomic DNA in a total volume of 20 µl with 0.5 U of Ex-Taq DNA polymerase (Takara), 800 µM dNTP, and forward and reverse primers (500 nM each) otherwise mentioned. For CvGal, F2 and R3 primers were used. For actin, ACTIN_F and ACTIN_R primers were used. PCR products were separated in 2% agarose gels, and DNA bands were visualized by staining with ethidium bromide.

**Immune challenge, extraction of RNA and DNA, and cDNA synthesis**

Oysters were notched at the anterior side of the shell, close to the adductor muscle, allowed to acclimate for 3 days, and injected with 100 µl of LPS (*E. coli* 0111:B4, 100 µg/ml in artificial sea water (ASW)) into the adductor muscle sinus using a 30-gauge hypodermic needle. After 24 h, oysters were bled from the adductor muscle using 18-gauge needle, and subsequently dissected. Palp, gill, mantle, and rectum tissues were collected and stored in −80°C until use for RNA or genomic DNA extraction.

### Table 1. Primers used for amplification of DNA fragments

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</tr>
<tr>
<td>AP-2</td>
<td>ACTATAGGGCCACGCTGTGTT</td>
</tr>
</tbody>
</table>

**RT-PCR**

Each PCR amplification described below was conducted using a thermal cycler PCR Express (Hybaid) in a total volume of 20 µl with 0.5 U of Ex-Taq DNA polymerase (Takara), 800 µM dNTP, and forward and reverse primers (500 nM each) otherwise mentioned. For CvGal, F2 and R3 primers were used. For actin, ACTIN_F and ACTIN_R primers were used. PCR products were separated in 2% agarose gels, and DNA bands were visualized by staining with ethidium bromide.

### 3′- and 5′-RACE

To determine the sequence of the region containing the 3′-untranslated region (UTR), PCR was conducted with primer F2 and GeneRacer 3′-primer (1.5 µM). To determine the sequence of the region containing 5′-UTR, PCR was conducted with primer R3 and GeneRacer 5′-primer (1.5 µM).

#### Genomic PCR

Genomic DNA was purified using DNeasy 96 Tissue kit (Qiagen). PCR amplification was conducted using 100 ng of genomic DNA in a total volume of 20 µl with 0.4 U of KOD HiFi DNA polymerase (Novagen), 200 µM of dNTPs, 1 mM MgCl2, and forward and reverse primers (400 nM each). At first, PCR was conducted with sets of GENE_F1 and GENE_R1, GENE_F2 and GENE_R2, GENE_F3 and GENE_R3, GENE_F1 and GENE_R2, GENE_F2 and GENE_R3, GENE_F1 and GENE_R3, and with F4 and GENE_R1 primers. PCR amplification of the genomic region containing 3′-UTR was conducted with GENE_F3 and R3 primers.
Analysis of cDNA and genomic sequences

DNA sequencing was conducted from both directions using dye termination reactions, and analyzed on an Applied Biosystems model 373 Stretch sequencer. Sequence assembly was performed manually. Calculations of theoretical molecular mass and pl from the deduced amino acid sequence were performed with ProtParam (www.expasy.ch). Nucleotide sequence of CvGal cDNA was analyzed by National Center for Biotechnology Information (NCBI) protein-protein BLAST (blastp; http://www.ncbi.nlm.nih.gov/BLAST/). Identification of the CvGal putative promoter was predicted by NNPP, version 2.2, program (http://www.fruitfly.org/seq_tools/promoter.html).

Genomic Southern hybridization

Ten micrograms of the same genomic DNA used for genomic PCR was digested by BglII, EcoRI, EcoRV, HindIII, NdeI, or NsiI at 37°C for 18 h. Digested DNA was resolved with 0.8% agarose gel at 15 V for 7 h in TAE buffer, and blotted onto Hybond-XL membrane (Amersham Biosciences). After cross-linking by baking at 80°C for 2 h, the membrane was rinsed with 5× SSC, and prehybridized with ULTRAhyb (Ambion) at 42°C for 30 min. The probe was prepared by PCR amplification using 10 ng of DNA fragment in a total volume of 100 μl with 2 U of KOD HiFi DNA polymerase, and GENE_F1 and GENE_R2 primers (400 nM each). The amplification was used for homology modeling by the SWISS-Model software, version 1.83 (http://align.genome.jp/), using slow/accurate mode above. These selected and trimmed sequences were aligned by clustal W (http://www.ddbj.nig.ac.jp/index.jsp), and only sequences predicted by NNPP, version 2.2, program (http://www.fruitfly.org/seq_tools/promoter.html).

Homology modeling

Based on results of a conserved domain search from NCBI Conserved Domain Search program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), four galectin domains were isolated from the full-length CvGal cDNA sequence and aligned with the bovine spleen galectin-1 (26), zebrafish Drgal1-L2 (27), and Caenorhabditis elegans 16-KDa galecin, N16 (28) by the T-COFFEE program (http://www.ch.embnet.org/software/TCoffee.html). The alignment was used for homology modeling by the SWISS-MODEL program (http://swissmodel.expasy.org/) using the structure of bovine spleen galecin-1 and N-acetyllactosamine (LacNAc) complex (1SLT_B) as a template.

Phylogenetic analysis

Amino acid sequences of invertebrate galectins were obtained using DDBJ ARSA software (http://arsa.ddbj.nig.ac.jp/index.jsp), and only sequences containing full-length of open reading frame were selected (Table II). Galectin-domain(s) were identified and isolated from the alignment described above. These selected and trimmed sequences were aligned by clustal W software, version 1.83 (http://align.genome.jp), using slow/accurate mode with matrix of GONNET for both pairwise and multiple alignment parameters. Other parameters were left as default. Relationships were analyzed by the neighbor-joining (N-J) distance method, and an unrooted tree was constructed.

Characterization of the carbohydrate specificity of rCvGal

Hemagglutination assays. Microhemagglutination tests were conducted in BSA-blocked, 96-well Terasaki plates (Robbins Scientific) as reported earlier (29). Briefly, 5 μl of a 5 × 10^5 cells/ml suspension of rabbit erythrocytes (previously fixed with 0.05% glutaraldehyde in PBS for 10 min at room temperature) in PBS were added to equal volumes of 2-fold dilutions of rCvGal in PBS. The plates were gently vortex-mixed for 10 s and incubated at room temperature for 1 h. Agglutination was read under a light microscope (BH-2; Olympus), and scored from 0 (negative) to +3. Relative activity was calculated by dividing the score of each sample by the score of the positive control (only CvGal and rabbit erythrocytes). Negative controls were conducted by adding PBS instead of rCvGal dilution. rCvGal was induced by 0.1 mM isopropyl β-D-thiogalactoside at 23°C for 16 h in 3 liters of LB medium containing 30 μg/ml kanamycin. Soluble proteins extracted by BugBuster (Novagen) with 1 mM PMSF and 0.07% mercaptoethanol, contained most of the recombinant CvGal (~80%). This fraction was loaded onto a column packed with 5 ml of Lactose-Gel (EY Laboratories). After washing the column thoroughly (washing buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.07% mercaptoethanol), CvGal was eluted with 0.2 M lactose in washing buffer. This fraction was further purified with MonoQ 5/5 column (Amersham Biosciences) connected to an Akta HPLC system (Amersham Biosciences). From a 3-liter E. coli culture, ~20 mg of recombinant CvGal were purified.

Protein determinations

Protein concentrations were estimated with the Bio-Rad Protein Assay kit (Novagen). This kit was performed with 5 μl of a 5 × 10^5 cells/ml suspension of rabbit erythrocytes (previously fixed with 0.05% glutaraldehyde in PBS for 10 min at room temperature) in PBS were added to equal volumes of 2-fold dilutions of rCvGal in PBS. The plates were gently vortex-mixed for 10 s and incubated at room temperature for 1 h. Agglutination was read under a light microscope (BH-2; Olympus), and scored from 0 (negative) to +3. Relative activity was calculated by dividing the score of each sample by the score of the positive control (only CvGal and rabbit erythrocytes). Negative controls were conducted by adding PBS instead of rCvGal dilution.
or proteoglycans. The carbohydrate used were as follows: α-fucose, α-galactose, α-glucose, α-mannose, galactosamine, α-N-acetyl-α-galactosamine (GalNAc), α-N-acetyl-α-glucosamine (GlcNAc), melibiose, lactose, lactulose, α-D-galactopyranosyl-α-D-arabinose, 4-O-(α-D-galactopyranosyl)-α-D-mannopyranose, LacNAc, thiodigalactose (TDG), transferrin, fetuin, asialofetuin, fibronectin, lactoferrin, laminin, thyroglobulin, human α₁- acid glycoprotein (orosomucoid) bovine submaxillar mucin, ovine submaxillar mucin, arabinogalactan, chondroitin sulfate, and hyaluronic acid. Controls were the substitutions of the carbohydrate solution by PBS, and substitution of purified lectin by PBS. Results were expressed as the reciprocal percentage agglutinating activity relative to the no-carbohydrate-added control (100%; no sugar, glycoprotein, mucin, or proteoglycan added). For glycoproteins, the actual sugar concentrations in the inhibition tests were calculated by multiplying the percentage agglutinating activity relative to the no-carbohydrate-added control (100%; no sugar, glycoprotein, mucin, or proteoglycan added). For glycoproteins, the actual sugar concentrations in the inhibition tests were calculated by multiplying the percentage agglutinating activity relative to the no-carbohydrate-added control (100%; no sugar, glycoprotein, mucin, or proteoglycan added). For glycoproteins, the actual sugar concentrations in the inhibition tests were calculated by multiplying the percentage agglutinating activity relative to the no-carbohydrate-added control (100%; no sugar, glycoprotein, mucin, or proteoglycan added).

Preparation of rabbit anti-CvGal antisera and purification of specific antibodies

Anti-CvGal antisera were raised by Open BioSystems. IgG was purified by a HiTrap Protein A HP 1 ml column (Amersham Biosciences). Anti-CvGal-specific IgG was further purified on an Ag-bound affinity column prepared by coupling 2 mg of rCvGal to a HiTrap NHS-activated HP 1-ml column. The flow-through fraction of this column (absorbed IgG) and preimmune IgG, purified as described above, were used as negative controls.

Western blot analysis

Specificity analysis of purified anti-CvGal IgG in hemocyte extracts. Hemocyte extracts were prepared by treating the packed cells with 1× SDS-sample buffer at 100°C for 5 min, and clearing the supernatant by centrifugation. rCvGal (10 ng) and hemocyte extract (40 μg of total protein) were resolved in 10% SDS-PAGE gel, and blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) using a TE 22 Mini Tank Trans- fer Unit (Amersham Biosciences) with Towbin buffer (36) at 0.4 A for 90 min. After blocking with blocking buffer (5% skim milk in PBS containing 0.05% Tween 20 (PBST)), membranes were incubated with 200 ng/ml anti-CvGal, preimmune, or absorbed IgGs in blocking buffer at room temperature for 1 h. After washing with PBS for 5 min, the membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) at room temperature for 5 min, and signals were detected on CL-X Posure Film (Pierce) after a 10-s exposure.

Examination of CvGal secretion. One hemolymph aliquot containing 2.50×10⁵ trophozoites was centrifuged at 800× g for 5 min immediately after collection, the plasma was separated from the hemocytes, and both were maintained on wet ice. A second hemolymph aliquot was plated in a 24-well tissue culture-treated polystyrene plate (BD Biosciences). After removing the supernatant, 500 μl of ASW were added to each well and incubated at room temperature for 1 h. For each experiment, three replicate wells were used. After incubation, supernatants were removed and cleared by centrifugation, and the proteins were precipitated by trichloroacetic acid. The attached cells were lysed by addition to each well of 160 μl of 1× SDS-sample buffer, and incubation for 5 min at room temperature. Proteins from unattached and attached hemocytes (equivalent to 2.50×10⁵ cells/well) were resolved in 10% SDS-PAGE gel, and blotted onto polyvinylidene difluoride membrane. Detection of CvGal was conducted as described above, with exception of primary Ab concentration as 50 ng/ml and exposure time as 1 min.

In vivo challenge of hemocytes with bacterial wall and P. marinus.

Attachment of hemocytes to the bottom of the wells. After removing the supernatant, 500 μl of ASW containing 100 μg/ml bacterial wall mixture (LPS from E. coli 026:B6, 055:B5, 0111:B4, 0127: B8, 0128:B12; E. coli H100 (Re mutant), P. aeruginosa PAO1 (RfD2) mutant, P. syringae pv. syringae, RfD2 mutant), P. aeruginosa PA01 (RfD2) mutant, P. syringae pv. syringae, RfD2 mutant) were added to each well of 24-well tissue culture-treated polystyrene plate (BD Biosciences). After removing the supernatant, 500 μl of ASW were added to each well. After incubation at room temperature for 1 h, three different fields were randomly selected, and the number of total cells and cells engulfing P. marinus trophozoites were counted. The phagocytosis ratio was calculated dividing the number of phagocytosing trophozoites.
cells by total number of cells. The result was expressed as phagocytosis ratio relative to that in ASW.

Results

The oyster hemocyte galectin (CvGal) is a novel protein of unique CRD organization and distinct carbohydrate specificity. Recent information from public genomic and expressed sequence tag databases from *C. virginica* and the Asian oyster *Crassostrea gigas* confirmed our earlier findings (37, 38) by revealing the presence of multiple lectins with potential galactosyl-binding properties, either belonging to the C-type or galectin families (http://www.ifremer.fr/GigasBase/ and http://www.marinegenomics.org).

To examine the spatial distribution of galectin transcripts in oyster hemocytes and selected tissues, we used RT-PCR with primers based on a partial galectin sequence (accession number CD526748), and detected galectin transcripts in all tissues tested (Fig. 1).

In the next step, we elucidated the primary structure of the protein by cloning the full-length cDNA encoding the galectin’s sequence, obtained by overlapping partial sequences determined by 3′- and 5′-RACE. The CvGal cDNA spanned 2005 nt (Fig. 2), containing a 1665-nt open reading frame encoding 555 aa residues, with a calculated molecular mass of 63,360.50 Da, and predicted isoelectric point of 4.83. This sequence was verified by amplifying full-length of coding region, which yielded a product of identical sequence. The 5′- and 3′-UTRs were 46 and 294 nt, respectively.

To determine the structure of the gene that encode for CvGal, we used a PCR amplification approach on genomic DNA. The CvGal gene (H11011 6.7 kb), obtained by the alignment of multiple sequences of overlapping PCR products is composed of 12 exons (lengths in downstream order: 0.024, 0.089, 0.328, 0.086, 0.202, 0.120, 0.089, 0.205, 0.120, 0.086, 0.205, and 0.114 kbp) separated by 11 introns (2.35, 0.698, 0.318, 0.202, 0.094, 0.167, 0.365, 0.375, 0.186, 0.154, and 0.136 kbp), none of which is present within the regions encoding the individual CRDs (Fig. 3A). The nearest predicted promoter region was from H11002 124 to H11002 75 bp and predicted transcription initiation site was H11002 84 bp (score, 0.81). Southern blot analysis of the genomic DNA digests with various restriction enzymes revealed bands of approximate sizes as follows: BglII, 6.5; EcoRI, 5.0; EcoRV, 3.0; HindIII, 2.8; NdeI, 5.7; NsiI, 6.1 kbp (Fig. 3B), in good agreement with expected sizes based on genomic DNA sequence and the identified restriction sites.

The sequence alignment of CvGal with galectins from vertebrates and invertebrate species revealed that from the 9 aa residues that in bovine galectin-1 participate in ligand binding, 7 are conserved in all four CvGal CRDs (His44, Arg46, Arg48, Asn61, Trp68, Glu71, and Arg73); numbers corresponding to the bovine galectin 1 are circled in Fig. 2). This is due to the lack of four consecutive amino acid residues that are present in the mammalian prototype galectins and include His53 and Asp55, residues that participate in ligand binding by interacting with the nitrogen of the NAc group via a water molecule (Fig. 3C). We examined the potential consequences of this deletion on the structural aspects of the CvGal by homology modeling of the C-backbones of all four CvGal CRDs on the structure of a mammalian prototype galectin CRD (26). In the model (Fig. 3D), it becomes clear that the seven conserved residues from all four CvGal CRDs maintain their positions and

FIGURE 1. CvGal is expressed in hemocytes and various tissues. cDNAs were synthesized from total RNA from hemocytes, palps, gills, mantle, midgut, and rectal tissues. Results of PCR amplification using CvGal-specific primers (upper) and that of actin-specific primers (lower) are shown.

FIGURE 2. Nucleotide sequence of cDNA and deduced amino acid sequence of CvGal. Numbers on the left and the right indicate the nucleotide and amino acid positions, respectively. Asterisks mark the stop codon, and the polyadenylation signal is shown in italics. Conserved amino acid residues that are supposed to bind the sugar ligand are circled.
orientations in the binding cleft, relative to the bovine spleen galectin, whereas the lack of four residues results in a dramatically shortened loop (indicated by the solid arrow for CRD-2, -3, and -4, and by a dashed arrow for CRD-1).

To experimentally test this possibility, we prepared the rCvGal and characterized its carbohydrate-binding specificity by hemagglutination-inhibition assays using a panel of mono-, oligo-, poly-saccharides, and glycoproteins (Fig. 3, E–H; the 50% inhibition values are shown in Table III). Among the monosaccharides, galactosamine and N-acetylgalactosamine showed high inhibition relative to others such as L-fucose. Disaccharides containing non-reducing terminal D-galactose showed high inhibitory capacity relatively to the monosaccharides, particularly lactose (100% at 50 mM), LacNAc (100% at 25 mM), and TDG (100% at 50 mM). Similarly, among the glycoproteins, lactoferrin, laminin, thyroglobulin, and asialofetuin showed relatively high inhibition. In comparison, most mucins and proteoglycans tested showed low inhibition capacity, except for hyaluronic acid (78% at 250 μg/ml).

Although mucin and fibronectin behave as good ligands for the avian and amphibian galectins, it was not the case for CvGal.
To examine the possible ancestry of the individual CvGal CRDs, we constructed a tree based on genetic distance that included most galectins from invertebrate species described to date. These consisted of 28 sequences, and because most of them were from the tandem-repeat type, they provided a total of 50 CRDs, of which 48 were included in the analysis (Table II). The unrooted N-J tree (Fig. 4) revealed three clusters of single CRD galectins and two clusters of tandem-repeat galectins, the first including the C-terminal CRD, and the second clustering the N-terminal CRDs and CvGal group, with only a few exceptions. All four CvGal CRDs clustered relatively closer to the single CRD galectins from Anopheles stephensi, C. elegans, Geodia cydonium, and Haemonchus contortus.

Upon hemocyte attachment and spreading, CvGal is translocated to the periphery, secreted to the extracellular space, and remains associated to the cell surface.

To examine the subcellular localization of the mature CvGal protein, we raised an antiserum against the rCvGal, and validated the specificity of purified anti-rCvGal Abs by Western blot. Single bands of the expected size were detected for the crude hemocyte extract and rCvGal (Fig. 5A), whereas no signal was observed in the absorbed IgG and preimmune IgG controls (data not shown). These results showed that the Ab is specific for the authentic CvGal, supported RT-PCR results indicating that the gene is transcribed in the hemocyte, and revealed that significant amounts of monomeric CvGal remain associated to these cells.

Because the oyster hemocytes become motile and avidly phagocytic upon attachment and spreading on a foreign surface,
we examined by Western blot the presence of CvGal in circulating hemocytes, in plasma, and in the attached and spread cells. Both the unattached and attached hemocytes revealed a strong band, whereas only the attached cells released soluble CvGal (Fig. 5B). Subsequently, we examined the subcellular distribution of CvGal in unattached and attached hemocytes by immunofluorescence (Fig. 5, C and D). In the unattached hemocytes, CvGal was observed in the cytoplasm of ~20–30% of the saponin-permeabilized cells, but none in the untreated cells. In contrast, intense diffuse staining was observed in both permeabilized and untreated attached cells, approximately in the same proportion as in the unattached saponin-permeabilized hemocytes (20–30%). We identified these cells as granulocytes, as judged from their distinct morphology under phase contrast microscopy (39). On the untreated attached hemocytes, a high concentration of CvGal was detected on the plasma membrane, particularly in the filopodia. No fluorescence was observed in the absorbed IgG (Fig. 5, C and D) and preimmune IgG controls (data not shown). We also examined the possibility that the lectin released by the attached granulocytes binds to the hemocyte surface moieties. For this, we exposed unattached hemocytes to rCvGal and tested its potential binding by immunofluorescence. Intense staining was observed in ~100% of the cells (Fig. 6), indicating that CvGal can strongly bind to both attached and unattached hemocytes. Furthermore, this suggests that, as CvGal is secreted by the attached granulocytes, it first binds to the cell surface, and after saturation the remaining galectin is released to the environment.

CvGal binds to a variety of microorganisms and phytoplankton components, but preferentially to Perkinsus spp. trophozoites

In addition to binding endogenous ligands from the hemocyte surface, CvGal may bind exogenous ligands, namely surface sugars on potential bacterial pathogens, parasites, or phytoplankton. To examine this possibility, we used a lectin adsorption assay to test the binding capacity of CvGal to a variety of ligands relative to rabbit erythrocytes. These included bacteria, both Gram-positive and -negative, Perkinsus spp. parasites including P. marinus, and unicellular algae. Most bacterial strains that were tested failed to adsorb the rCvGal over 20% of the control. However, A. veronii bv sobria and Carnobacterium sp. adsorbed ~90%, and S. faecalis, B. subtilis, and V. mimicus adsorbed between 30 and 50% of the lectin activity (Fig. 7A). Most interestingly, all tested Perkinsus spp. and isolates consistently adsorbed between 95 and 100% of the lectin activity, indicating that CvGal preferentially binds to the surface of the Perkinsus spp. parasites over most microbes tested. This was in sharp contrast with the poor binding of CvGal to

**FIGURE 6.** Binding of rCvGal to unattached hemocytes. Fixed unattached hemocytes were incubated with rCvGal and detected by anti-CvGal IgG. Scale bar, 10 μm.

**FIGURE 7.** CvGal binds to a variety of microorganisms, especially to P. marinus trophozoites. A, Adsorption test of rCvGal with selected microorganisms. Results are shown as relative absorption. Bars show SDs of quadruplicate experiments. B and C, Adsorption test of rCvGal with Isochrysis sp. (B) or P. marinus trophozoites (C) at various cell pellet-to-lectin solution ratios. Bars show SDs of quadruplicate experiments. D, Binding of rCvGal to P. marinus trophozoites. Scale bar, 10 μm.

*Isochrysis* sp. (4% absorption), a unicellular alga that is a main component of the phytoplankton on which oysters feed. This observation was further confirmed with a quantitative adsorption assay; P. marinus adsorbed ~75% of the lectin activity at
CvGal is up-regulated by challenge with *P. marinus* trophozoites, and surface-associated CvGal is a receptor for *P. marinus*. A. Western blotting of in vitro-challenged hemocytes with bacterial cell wall components or *P. marinus*. Relative intensities of bands to ASW pellet band were calculated and plotted. Bars show SDs from three independent experiments. The inserted image is from a representative experiment. B. Phagocytosis assay. Results are shown as relative phagocytosis ratio to that of ASW. Statistical analysis was conducted by one-way ANOVA, followed by Turkey’s multiple-comparison test. Bars are SDs.

Expression of CvGal is up-regulated by challenge with *P. marinus* trophozoites, and the hemocyte surface-associated CvGal is a receptor for *P. marinus*

Because CvGal preferentially binds *P. marinus* trophozoites, and is present on the surface of the actively phagocytic hemocytes, it may have a role as a receptor for parasite phagocytosis. We therefore tested the effect of exposure of attached and spread oyster hemocytes to *P. marinus* trophozoites in vitro, compared with exposure to a mix of cell walls from various bacterial species, using untreated hemocytes as controls. Exposure of hemocytes to *P. marinus* trophozoites consistently resulted in a slightly higher release of CvGal to the supernatant than from those exposed to bacterial cell walls, or from the untreated hemocytes (Fig. 8A). Similarly, as the exposure time increased to 2 h, the content of CvGal in attached hemocytes was consistently higher in those exposed to *P. marinus* trophozoites (results not shown).

Finally, we examined the potential role of the hemocyte-associated CvGal in phagocytosis of *P. marinus* trophozoites. For this, we treated hemocytes with increasing concentrations of the anti-CvGal Abs or anti-CvGal-depleted Igs, and compared phagocytosis of *P. marinus* trophozoites between the treated hemocytes and untreated controls (Fig. 8B). The increase in concentration of the anti-CvGal Abs was concomitant with an increased inhibition of phagocytosis, revealing that the hemocyte surface-associated CvGal is a receptor for *P. marinus* that mediates phagocytosis. However, only partial inhibition of phagocytosis was observed (≈40%) at the highest concentration tested, suggesting that other surface receptors may also participate in phagocytosis of *Perkinsus* trophozoites.

Discussion

It is widely accepted that recognition of exposed glycans on the cell surface of potential pathogens by host humoral or cell-associated lectins is a key component of the innate immune response of vertebrates and invertebrates. During earlier studies, we partially characterized multiple lectins in the oyster plasma and hemocytes, which recognize glycoproteins that display terminal galactose and N-acetylated sugars (37, 38). Although the family identity of these lectins remained unresolved, further studies revealed that some of the sugars recognized coincided with the sugars present on the surface of the *P. marinus* trophozoites (40). These observations led us to hypothesize that the parasite may have adapted to exploit the host recognition mechanisms to gain entry into the hemocytes. A search of public genomic and expressed sequence tag databases from the oysters *C. virginica* and *C. gigas* yielded multiple lectin sequences, either belonging to the C-type or galectin families, with signature motifs indicative of galactosyl-binding properties. In contrast to C-type lectins, galectins constitute a relatively homogeneous lectin family and virtually all members bind β-galactosyl residues (5, 14, 26). Therefore, we considered the oyster galectin a good candidate as a hemocyte receptor for *P. marinus* trophozoites. By the use of a PCR approach based on a galectin sequence, we identified transcripts in oyster hemocytes and all selected tissues tested, and based on the similar intensity of the amplicons, it is likely that the signals observed arise from the hemocytes that infiltrate these tissues. Infections by *Perkinsus* species take place through trophozoites that are released by infected oysters into the environment that become in contact with or are ingested through filter-feeding by healthy neighboring oysters (19). The trophozoites are phagocytosed by hemocytes located in the mantle, palps, gills, or gut epithelium (41) and reside in a phagosome-like structure, where they proliferate by multiple fission and budding. Infected hemocytes eventually lyse and the released trophozoites are phagocytosed by other hemocytes, which migrate throughout the tissues and disseminate the parasite. Thus, it is noteworthy that hemocytes,
which are the main cellular components of the oyster’s immune system, and tissues expressing galectin transcripts such as gills, palps, gut, and mantle, are all in direct contact with the external environment, and have been proposed as portals for *P. marinus* infection (20, 21).

The CvGal sequence revealed that it is a novel member of this family, of unique CRD organization. Sequence comparison of CvGal to multiple galectins from various organisms revealed the presence of four canonical galectin CRDs connected by linkers ranging from 8 to 17 amino acids, a domain organization that does not fit any of the known galectin types. Proto- and chimera-type galectins contain one CRD per subunit, whereas tandem-repeat galectins contain two CRDs joined by a linker peptide (5, 15). Elucidation of the gene organization of CvGal confirmed the presence of four tandemly arrayed CRDs and that the position of first intron of CvGal, between eighth and ninth amino acid residues, is identical with that of Lec-6, a 16-kDa galectin from *C. elegans* (42), and different from the mammalian prototype galectins, where it is located between second and third, or third and fourth amino acid residues (43). Southern blot analysis was in good agreement with expected sizes based on genomic DNA sequence and the identified restriction sites, and suggested that the gene encoding CvGal is present as a single copy. The presence of four similar, albeit distinct tandemly arrayed CRDs in CvGal, represents a unique feature for a member of the galectin family, and its structure and specificity pose novel questions about its binding properties. Lectin-ligand interactions are relatively weak compared with other immune recognition molecules, but high avidity for the target is achieved by the association of peptide subunits into oligomeric structures in which multiple CRDs simultaneously interact with ligand (44). In the soluble collectins (45) such as the mannose-binding lectin, the subunits possessing a single CRD associate to form a bouquet-like oligomer with all CRDs facing a potential target surface. In contrast, the cruciform subunit organization of the conglutinins, enables the single CRDs to cross-link the target surfaces. A less frequent organizational plan is the presence of tandemly arrayed CRDs encoded within a single polypeptide such as in F-lectins (46), immulectins (47), and tandem-type galectins (48). In this context, the four-CRD CvGal may have the potential for cooperative binding as well as cross-linking of the recognized glycans.

The sequence alignment of CvGal CRDs with those of vertebrates and invertebrate species showed that two amino acid residues (His53 and Asp55) that participate in ligand binding by interacting with the nitrogen of the NAc group via a water molecule, are missing in all four CvGal CRDs. In the *C. elegans* galectin Lec-6, the absence of these two residues confers broader carbohydrate specificity relative to that of the mammalian prototype galectins (28). The structural analysis of the CvGal CRDs by homology modeling strongly suggested that, although CvGal CRDs bind β-galactosyl residues, their specificity for N-acetylated disaccharides and oligosaccharides may differ from the mammalian galectins. Carbohydrate specificity studies on rCvGal confirmed that the relative inhibitory efficiency of each one of these sugars is different, and that CvGal has a broader sugar specificity, although its inhibition profiles are similar to that for the mammalian prototype galectins.

A distance-based tree, which was consistent with phylogenetic analysis of galectins reported elsewhere (49, 50), revealed that all four CvGal CRDs clustered relatively closer to the single-CRD galectins, suggesting not only that they may have originated by repeated duplication of a primordial CRD, rather than a single duplication of a tandem-repeat galectin gene, but also their possible orthology with galectin CRDs from the earliest metazoans. However, in contrast with galectins from vertebrate species, those from invertebrates and parazoa are quite divergent both at the amino acid and genomic structure levels, and predictions about their phylogenetic relationships should be interpreted cautiously.

Studies on the subcellular localization of the mature CvGal protein in both circulating and attached hemocytes revealed that in the former CvGal is present in the cytoplasm of about one-third of the hemocytes, but upon attachment and spreading, of the granulocyte subpopulation, is translocated to the periphery, and is secreted to the extracellular space. Galectins lack a signal peptide, and are secreted by an unconventional mechanism (51). Because CvGal also lacks a signal peptide, its presence in the extracellular space suggests that a similar secretion mechanism may be involved. Immunofluorescence studies also revealed that, as CvGal is secreted by the attached granulocytes, it binds to the cell surface, and the remaining galectin is released to the environment. The soluble CvGal can then bind to all other circulating (nonactivated) cells, both granulocytes and hyalinocytes. A variety of biological functions may result from the cis- and trans- galectin-glycocalyx interactions, such as increased half-life of surface receptors, cell adhesion, and signaling (52–54). A similar role may be proposed for CvGal upon binding to cell surface moieties of the cells that secrete it. Furthermore, although hyalinocytes may lack the mechanisms to synthesize CvGal, they express cell surface β integrins (39), which are well-known galectin ligands (15, 55). Thus, binding of CvGal to hyalinocytes may lead to their phagocytic activation.

However, the most surprising observation in this study was that CvGal also binds to exogenous ligands, including a variety of microorganisms and phytoplankton components, and preferentially, to *Perkinsus* spp. trophozoites, in a carbohydrate-specific manner. Galectins bind to endogenous ligands and have been proposed to mediate developmental processes in various animal models including sponges and other invertebrates (15) and, more recently, to participate in regulation of adaptive immune mechanisms by modulating inflammation (10, 15), apoptosis (11, 12), and B cell development (13, 14). Only a few examples of recognition of exogenous ligands by galectins have been reported, namely the binding of mammalian galectins-3 and -9 to polygalactose moieties of *Leishmania major* (56, 57), and the binding of galectin-3 to soluble and egg shell-associated glycans of the parasite helminth *Schistosoma mansoni* displaying GalNACβ1–4GlcNAc (58). Our finding that CvGal is produced by hemocytes and secreted to the surrounding plasma by the activated, attached, and spread cells, and directly binds not only to the oyster hemocytes but also to a wide variety of microbes and phytoplankton components is intriguing in terms of its binding to both endogenous and exogenous ligands. CvGal recognized with various degrees of intensity most bacteria, algae, and *Perkinsus* spp. tested, suggesting a direct role in recognition of potential microbial pathogens, as well as algal food. The secreted CvGal was also found strongly bound to the hemocyte surface, suggesting that it may either function as a soluble opsonin for potential pathogens entering the oyster’s circulatory system, or as a hemocyte surface receptor for both microbial pathogens, and algal food ingested into the digestive ducts of the alimentary canal. Furthermore, the observation that expression of CvGal in hemocytes is up-regulated by exposure to *P. marinus* trophozoites, suggests that the surface-associated CvGal may also activate signaling pathways leading to the synthesis of additional CvGal. Although the experimental time
course is substantially different, our results are consistent with those from Tanguy et al. (59) in which gene expression profiling in oysters revealed a 4.6-fold increase in galectin-like transcripts 10 days after challenge with *Perkinsus* spp.

The partial inhibition of phagocytosis of *P. marinus* trophozoites by pretreatment of hemocytes with anti-CvGal revealed that the hemocyte surface-associated CvGal is a phagocytosis receptor for *P. marinus*, albeit most likely not the only one. These results are noteworthy in the context of the oyster’s feeding and digestion mechanisms. Oysters filter-feed on plankton and other particles in suspension, which enter the gut and are either extracellularly digested, or are phagocytosed by the numerous granulocytes that are present in the digestive ducts and are subject to intracellular killing and digestion (60). During this process, hemocytes may migrate from the gut lumen into the internal milieu (61). Oyster hemocytes can phagocytose biotic or abiotic particles, via nonspecific or specific mechanisms. In other invertebrate species, wettability, hydrophobicity, or charge of the particle, have been proposed as surface properties that determine nonspecific recognition leading to phagocytosis or encapsulation (62). However, a variety of pattern recognition receptors including peptidoglycan recognition proteins (63–65), β integrins (39), and lectins (66, 67) have been shown to mediate specific recognition leading to phagocytosis. Oysters have a very diversified lectin repertoire, which includes C- and F-type lectins, ficolins, and galectins, some of which are present on the hemocyte surface (37, 38), but their potential roles as phagocytosis receptors have not been explored. The present study shows that the four-CRD galectin CvGal is present on the cell surface of attached and spread hemocytes, most likely along other specific and nonspecific receptors. Nonetheless, CvGal is able to recognize at various degrees of avidity potenti-}

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**FIGURE 9.** Schematic model of CvGal binding to *P. marinus* and CvGal function(s) in the oyster alimentary and circulatory systems. *A*, Schematic model of CvGal binding to *P. marinus*. *B* and *C*, Large numbers of hemocytes, particularly granulocytes, are present in the lumen of the digestive ducts of the alimentary canal, where they phagocytose plankton, food particles and migrate to the hemocoele. Stronger recognition of *Perkinsus* spp. trophozoites by the hemocyte CvGal provides a selective advantage to the parasite over algal cells for uptake, and results in an efficient host entry mechanism. *D*, Trophozoites survive intrahemocytic killing mechanisms and proliferate. Once carried into tissues and the circulatory system, the heavily burdened hemocytes lyse and trophozoites are released. Circulating uninfected hemocytes that initially lack CvGal at cell surface, become activated upon recognition of released trophozoites or by surface binding of CvGal released to the plasma by activated granular hemocytes. This process may contribute to infection of the surrounding uninfected hemocytes, either by CvGal acting as an opsonin and/or cross-linking the parasite to hemocytes displaying CvGal at their surface, leading to phagocytosis.
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