

Galectin CvGal2 from the Eastern Oyster (*Crassostrea virginica*) Displays Unique Specificity for ABH Blood Group Oligosaccharides and Differentially Recognizes Sympatric *Perkinsus* Species

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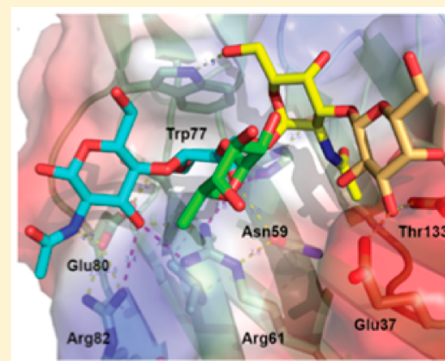
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S Supporting Information

ABSTRACT: Galectins are highly conserved lectins that are key to multiple biological functions, including pathogen recognition and regulation of immune responses. We previously reported that CvGal1, a galectin expressed in phagocytic cells (hemocytes) of the eastern oyster (*Crassostrea virginica*), is hijacked by the parasite *Perkinsus marinus* to enter the host, where it causes systemic infection and death. Screening of an oyster hemocyte cDNA library revealed a novel galectin, which we designated CvGal2, with four tandemly arrayed carbohydrate recognition domains (CRDs). Phylogenetic analysis of the CvGal2 CRDs suggests close relationships with homologous CRDs from CvGal1. Glycan array analysis, however, revealed that, unlike CvGal1 which preferentially binds to the blood group A tetrasaccharide, CvGal2 recognizes both blood group A and B tetrasaccharides and related structures, suggesting that CvGal2 has broader binding specificity. Furthermore, SPR analysis demonstrated significant differences in the binding kinetics of CvGal1 and CvGal2, and structural modeling revealed substantial differences in their interactions with the oligosaccharide ligands. CvGal2 is homogeneously distributed in the hemocyte cytoplasm, is released to the extracellular space, and binds to the hemocyte surface. CvGal2 binds to *P. marinus* trophozoites in a dose-dependent and β -galactoside-specific manner. Strikingly, negligible binding of CvGal2 was observed for *Perkinsus chesapeakei*, a sympatric parasite species mostly prevalent in the clams *Mya arenaria* and *Macoma balthica*. The differential recognition of *Perkinsus* species by the oyster galectins is consistent with their relative prevalence in oyster and clam species and supports their role in facilitating parasite entry and infectivity in a host-preferential manner.



Invertebrates display effective innate immunity for defense against microbial infection.^{1,2} Potentially pathogenic viruses, bacteria, fungi, and eukaryotic parasites are identified by a battery of soluble and cell-associated recognition factors, several of which have been structurally and functionally conserved along the lineages leading to vertebrates.^{3,4} Among them, a diversified lectin repertoire mediates the binding interactions with potential pathogens, resulting in agglutination, immobilization, and opsonization, leading to phagocytosis or encapsulation.^{5,6} However, a variety of microbial pathogens and parasites overcome the immune mechanisms of the host and establish successful infections that may lead to chronic or acute disease.^{7–10} Among these, the protozoan parasite *Perkinsus marinus* causes Dermo disease in the eastern oyster, *Crassostrea*

virginica, and is responsible for catastrophic losses in both native and farmed oyster populations, with a significant impact on the integrity of the estuarine environment.^{8–12} Another *Perkinsus* species, *Perkinsus chesapeakei* (= *Perkinsus andrewsi*), which is sympatric with *P. marinus* along most of its distribution range, preferentially infects clams.^{13–16} Although *P. chesapeakei* can also infect the eastern oyster,^{17,18} its pathogenicity for bivalves remains to be confirmed.¹⁹

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Table 1. Primers Used for CvGal2 Genomic DNA Analysis

primer	sequence	mRNA position	DNA position	mark ^a	seg.
CA1-F	CGGTTCAAGTCAAAGGCGAT	1296–1315	exon 10	amp	fifth
CA3-R	CCCTTCTGATGGTCGTGGAT	289–270	exon 4	amp	first
CA4-F	CATTTCAACGTTCCGAGGGG	1493–1512	exon 12	amp	sixth
CA4-R	AAGTTGCAGTCTCCGTCCAT	1737–1718	exon 13	amp	fifth
CA5-F	CTCCGTGTGGTTTTCTTCGC	24–43	exon 1	amp	first
CA5-R	ATTCGTATATTATCATAGTGCAGC	1922–1945	exon 13	amp	sixth
CA7-F	TTGTAACACTCGCACTTGGG		intron1	seq	first
CA7-R	TTTTCTGCCTCTCTGATCC	112–93	exon 2	seq	first
CA8-F	TTCACCCGATGCATGGTAGT		intron 3	amp	second
CA9-F	ACAACCAGACATGCATGACT		intron 3	seq	second
CA9-R	ATGTTCTCGGCCGTATTCTCT	1381–1400	exon 11	amp	fourth
CA10-F	CTCGCCATCTATCCACGTCT		intron 1	seq	first
CA10-R	ACCTTCCGTTTTCTCTGCCT	101–118	exon 2–intron 2	seq	first
CA11-F	GGGCGGGAAGTACCTCATC	468–487	exon 4	amp	third
CA11-R	CTAACCACGGTCTCTGTGC	702–683	exon 6	amp	second
CA12-F	GGCGAGGATGCAATGA	938–953	exon 8	amp	fourth
CA12-R	CCGCGGATTGAAGTGAAGA	1094–1075	exon 9	amp	third
CA14-R	GATGTTGTTCCGGTTGTCATTG	658–637	exon 6	seq	third
CA15-R	CGGATTTCCGCGGTGTGTAA	849–868	exon 7	seq	third
CA16-R	TTTCAGTGGGTTTACCAAGG		intron 4	seq	third

^aAmp, for segment amplification; seq, for sequence.

P. marinus trophozoites are phagocytosed by the oyster hemocytes present in various tissues exposed to the environment, such as the gills, gut, mantle, and palps.^{20,21} In a previous study,^{22,23} we identified a galectin of unique structure in *C. virginica*, which we designated CvGal (CvGal1 in a subsequent report^{22,23} and hereafter in this text) that facilitates parasite entry into the oyster hemocytes. Galectins are an evolutionarily conserved family of β -galactoside-binding lectins, the members of which have been identified in most eukaryotic organisms, from fungi to mammals, with the latter usually displaying a complex galectin repertoire.^{24,25} By binding to endogenous carbohydrate moieties, galectins exert not only diverse regulatory effects on early developmental processes^{26,27} but also tight homeostatic control of both innate and adaptive immune responses, including acute and allergic inflammation;²⁸ neutrophil adhesion and motility;²⁹ macrophage activation;³⁰ and development, activation, and apoptosis of B and T cells, among others.^{31–33} In addition, by binding exogenous glycans on the surface of potentially pathogenic microbes, parasites, and fungi, galectins can function as pattern recognition receptors (PRRs) in innate immunity.⁶ This apparent paradox in recognition of self and non-self ligands underscores the significant gaps in our knowledge about the structural and biophysical aspects of the interactions of galectins with endogenous and microbial carbohydrate moieties.³⁴

CvGal1 is expressed in the oyster hemocytes and released to the extracellular environment, where it recognizes carbohydrate moieties on both the hemocyte's cell surface and *P. marinus* trophozoites, promoting their phagocytosis. The phagocytosed trophozoites survive intracellular killing and proliferate. As the infected hemocytes migrate into the internal milieu of the oyster, they provide not only the means of parasite uptake and entry but also an environment favorable for parasite proliferation and dissemination.^{22,35}

To further elucidate the structural and functional diversity of the galectin repertoire of the eastern oyster and its potential role(s) in parasite recognition and host entry, we screened an oyster cDNA library to identify proteins containing potential

carbohydrate recognition domains (CRD) that display the galectin sequence motif. This screening yielded a novel galectin, which we designated CvGal2 (*C. virginica* galectin 2). In this study, we analyzed the gene organization and phylogenetic, structural, biochemical, and functional aspects of CvGal2, including its binding to *P. marinus* and *P. chesapeaki*. The results concerning the CvGal2-mediated differential recognition of these two *Perkinsus* spp. suggest that the oyster galectins facilitate parasite entry and infectivity in a host-preferential manner.

EXPERIMENTAL PROCEDURES

Reagents. Carbohydrates (monosaccharides, oligosaccharides, and glycoproteins, at the highest purity available), kanamycin, PMSF, lysozyme, and β -mercaptoethanol (2-ME) were purchased from Sigma-Aldrich (St. Louis, MO). Neoglycoproteins were obtained from Accurate Chemical & Scientific Corporation (Westbury, NY) (type 2 blood group A tetrasaccharide-BSA, 20 tetrasaccharide units/BSA polypeptide), V-laboratories Inc. (Covington, LA) (blood group A trisaccharide-BSA, 19 trisaccharide units/BSA polypeptide, and N-acetylgalactosamine-BSA, 28 GalNAc units/BSA polypeptide), or Sigma-Aldrich (GalNAc-BSA, 19 GalNAc units/BSA polypeptide). Other neoglycoproteins (GlcNAc-BSA, 35 GlcNAc units/BSA polypeptide, and Gal-BSA, 23 Gal units/BSA polypeptide) were a generous gift from Yuan Chuan Lee (Department of Biology, Johns Hopkins University, Baltimore, MD). HRP-labeled goat anti-rabbit IgG was obtained from Bio-Rad (Hercules, CA). Alexa 488-labeled goat anti-rabbit IgG was obtained from Invitrogen (Eugene, OR). Protein electrophoresis reagents were purchased from Bio-Rad (Hercules, CA). Restriction enzymes were acquired from New England Biolabs (Ipswich, MA). Revert aid first-strand cDNA synthesis kit and DreamTaq PCR master mix were from Thermo Fisher Scientific (Pittsburgh PA). Protease inhibitor cocktail was purchased from CalBiochem (La Jolla, CA). DNase was purchase from Novagen (Billerica, MA).

Oyster Hemocytes and Tissues. Adult eastern oysters (*C. virginica*), averaging 40–50 g each, were obtained from a local commercial firm, their shells were notched, and the animals were acclimated in 20 L tanks as reported elsewhere.^{22,23}

Hemolymph was drawn through the shell notch by puncture of the adductor muscle using an 18-gauge needle, and the hemolymph was collected in plastic tubes kept on wet ice for the preparation of hemocyte suspensions as previously described.^{22,23} Briefly, hemocytes from pooled hemolymph samples from 6 to 12 oysters were collected by centrifugation (800g for 10 min at 4 °C), washed three times in ice-cold HEPES-buffered saline (HBS; 10 mM HEPES, 430 mM NaCl, 10 mM CaCl₂, pH 7.3), and either used for protein or RNA extraction, as described below, or suspended in HBS containing glutaraldehyde (10 mM), incubated on wet ice for 10 min, washed three times in HBS, and stored at 4 °C until use. For collection of pallial fluid and various tissues, oysters were shucked open, the pallial fluid was collected, and the suspended cells were separated by centrifugation at 3000 rpm for 5 min at 4 °C. The soft parts of the oyster were dissected, and the selected tissues (mantle, gill, palp, rectum, digestive glands, heart, muscle, pallial fluid) were collected on ice for the preparation of cell extracts and RNA extraction. The dissected tissues, pallial fluid cell pellet, and hemocytes, freshly isolated as described above, were lysed by sonication in PBS containing protease inhibitor cocktail (Calbiochem), and the clear supernatant was collected after centrifugation at 3000 rpm for 10 min at 4 °C. For isolation of total RNA, the freshly isolated hemocytes, pallial fluid cell pellet, and the aforementioned tissues were extracted using TRIzol reagent (Invitrogen) as previously described.³⁶ RNA was quantified in a Nanodrop bioanalyzer at 260/280 nm.

Parasite Trophozoites. Clonal cultures of *P. marinus* and *P. chesapeaki* strains both established in our laboratory [*P. marinus* strain PRA 240,³⁷ *P. chesapeaki* (= *P. andrewsi*)¹⁴] and obtained from ATCC were propagated as reported earlier.³⁸ Cultures in log-phase growth were harvested and fixed with PFA as previously described^{22,23} for staining and flow cytometry analysis.

Analysis of CvGal2 Transcripts and Genomic Sequences. *Primers for PCR Amplification.* Primers used for all PCR are shown in Table 1 and were purchased from Sigma-Aldrich. Several primers were designed based on the cDNA sequence of CvGal2, whereas the others were designed based on the clone sequences obtained by genomic PCR described below.

Identification of CvGal2 Transcripts. For RT-PCR, first-strand cDNA was synthesized from 1 µg of total RNA using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). First-strand cDNA for 3'- and 5'-RACE (rapid amplification of cDNA ends) was synthesized from 5 µg of hemocytes' total RNA using a GeneRacer kit (Invitrogen). Each PCR amplification described below was conducted using a MyCycler thermal cycler (Bio-Rad) in a total volume of 20 µL with 0.5 U of Ex-TaqDNA polymerase (Takara), 800 µM dNTP, and the forward and reverse primers (500 nM each) indicated in Table 1.

Genomic PCR. Genomic DNA was purified using a DNeasy 96 blood and tissue kit (Qiagen) according to manufacturer's protocol. PCR amplification was conducted using 100 ng of genomic DNA in a total volume of 20 µL with forward and reverse primers (400 nM each) using DreamTaq PCR master mix (Thermo Scientific). During the first round, fragments 1–3 were amplified by PCR with primer sets CA5-F and CA3-R, CA1-F and CA2-R, and CA4-F and CA5-R, respectively. During

the second round, fragment 2 was amplified with primer set CA8-F and CA9-R (Table 1).

DNA Sequencing. PCR amplicons were isolated and purified with a gel extraction kit (Qiagen) and cloned into vector pGEM-T using the pGEM-T easy vector system (Promega). Positive clones were picked, and plasmids were extracted with a mini-prep kit (Qiagen) for sequencing. 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 pI from the deduced amino acid sequence were performed with ProtParam (www.expasy.org).

Phylogenetic Analysis. Outgroup sequences for both two- and four-CRD galectins were selected from the bivalves, *Argopecten irradians* and *Pinctada fucata*, and the gastropod snail, *Biomphalaria glabrata*. Additional two-CRD galectins were collected from *Crassostrea gigas*, and three other bivalve species. In the *C. gigas* genome, several identical or nearly identical sequences were found. All of these sequences were aligned against the CvGal1 sequence, and the two most complete sequences (EKC37204.1, EKC31760.1, both with four CRDs) were selected for further analysis. The CRDs from the tandem CRD galectin sequences were identified according to the domain predictions of blastp and used to create single CRD alignments. Thus, for each gene, the four (for four-CRD genes) or two (for two-CRD genes) CRDs formed individual alignment rows in the alignment and phylogenetic tree. Alignments were constructed using clustal Omega software with the -full-iter option.³⁹ Phylogenetic analysis was performed using RAxML with the optimal JTT model with gamma site to site rate variation and 100 rapid bootstrap replicates.⁴⁰

Recombinant Expression of CvGal2 and Production of Anti-CvGal2 Antibodies. *Construction of an Expression Vector for CvGal2.* A DNA fragment encoding the complete CvGal2 ORF was amplified by PCR with primers 5'-CAT ATG CGG ATC AGA GAG GCA GG and 3'-CTC GAG TTA GAT AAC AAC TTC CGG TTC G. The amplicon was subcloned into pGEM-T, and a plasmid with the correct sequence was digested with NdeI and XhoI (both from New England Biolabs), purified after 1% agarose gel electrophoresis, and subcloned into pET30a(+) (Novagen).

Expression and Purification of Recombinant CvGal2 (rCvGal2). The CvGal2 construct was transformed into *Escherichia coli* Rosetta (DE3) pLysS competent cells (Novagen). The expression of rCvGal2 was induced by 0.1 mM isopropyl β-D-thiogalactoside at 23 °C for 16 h in 3 L of LB medium containing 15 µg/mL kanamycin and 34 µg/mL chloramphenicol. The soluble proteins were extracted with BugBuster (Novagen) containing 1 mM PMSF, 1 mM protease inhibitor cocktail, 10 µg/mL of DNase, 10 µg/mL of lysozyme, and 0.07% 2-ME, which contained most of the recombinant CvGal2 (approximately 80%). This fraction was loaded onto a column packed with 4 mL of lactose-Sepharose (Sigma-Aldrich). After washing the column thoroughly with PBS containing 10 mM β-mercaptoethanol (PBS/2-ME), rCvGal2 was eluted with 0.1 M lactose in PBS/2-ME.

Protein Determinations. Protein concentrations were estimated with protein assay kit I (Bio-Rad) in 96-well flat-bottom plates as described by the manufacturer in the microassay format, using crystalline BSA as a standard. After 5 min of combining protein and dye, the reactions were read at

Table 2. Pairwise Percentage Identity between BaGal1 (Template) and CvGal1 and CvGal2 (Targets)

		BaGal1				CvGal1				CvGal2			
Chains			A	B	C	D	A	B	C	D			
BaGal1		100	18.6	22.8	18.8	22.8	19.9	23.7	21.6	21.5			
CvGal1	A	20.1	100	33.1	32.6	37.5	45.2	34.1	23.7	29.2			
	B	23.1	31	100	31.2	30.9	30.1	40	22.3	29.2			
	C	19.4	31	31.6	100	35.3	32.9	31.1	41	29.2			
	D	23.1	35.2	30.9	34.8	100	30.8	27.4	25.9	38.9			
CvGal2	A	21.6	45.5	32.4	34.8	33.1	100	27.4	23	27.8			
	B	23.9	31.7	39.7	30.4	27.2	25.3	100	25.9	28.5			
	C	22.4	22.8	22.8	41.3	26.5	21.9	26.7	100	27.8			
	D	23.1	29	30.9	30.4	41.2	27.4	30.4	28.8	100			

595 nm on SpectraMax340 plate reader (Molecular Devices, Sunnyvale, CA) controlled by SoftmaxPro software, version 1.

Production of Anti-rCvGal2 Antibodies. Anti-CvGal2 antisera were raised in New Zealand White rabbits following Tasumi and Vasta.²² The IgG was purified on Protein A-Sepharose, followed by immunoaffinity purification of the specific Ig's on the immobilized rCvGal2 as previously described.²² Cross-reactive antibodies were removed by passing the purified anti-CvGal1 and anti-CvGal2 antibodies through the immobilized rCvGal2 and rCvGal1, respectively, and the specific and unbound antibody fractions were collected and tested for specificity via western blot.

Analysis of CvGal2 Expression. RT-PCR Amplification. First-strand cDNA synthesis and PCR amplification were conducted as described above. For detection of CvGal2 expression levels, primers CA3-F and CA3-R were used (Table 1); for β -actin, primers ACTIN_F and ACTIN_R were used. PCR products were separated in 1.5% agarose gels, and DNA bands were visualized by staining with ethidium bromide.

Western Blot. Cells were lysed with ice-cold 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% v/v Triton X-100, protease inhibitor (1:100), 0.1 mM PMSF. Concentration of proteins in lysates was determined by standard BCA assay (Bio-Rad). Equivalent levels of protein from different oyster tissues were subjected to electrophoresis on 4–15% mini-protein TGX precast gels (Bio-Rad, no. 456-1084) and transferred onto PVDF membranes (PRC no. 88518). The membranes were blocked in 5% nonfat milk and probed with primary antibodies at 1:3000 dilutions. Next, membranes were washed in PBST followed by incubation with HRP-linked secondary rabbit polyclonal antibody (1:3000), and the bands were visualized with enhanced chemiluminescence (ECL) reagents following exposure of membranes to X-ray film (Kodak, Rochester, NY).

Immunofluorescence Analysis of Attached Hemocytes. Two-hundred microliters of hemolymph were added to each well of poly-D-lysine-coated 8-well chamber slides (Biocoat, BD Biosciences) and incubated at room temperature for 1 h. Each well was gently washed with HBS three times, and the attached cells were fixed with 200 μ L of 2% PFA in HBS for 30 min. After washing with HBS for 5 min three times, half of the wells were treated with 200 μ L of 0.05% Triton X-100 in HBS for 10 min at room temperature, whereas the remaining wells were incubated with buffer only. After washing all wells with HBS three times, blocking was conducted with 1% BSA in HBS for 1 h at room temperature. The fixed cells were stained with anti-CvGal2 (and anti-CvGal1 for comparative purposes) or preimmune rabbit IgG for 1 h at room temperature, followed by incubation with FITC-labeled anti-rabbit IgG for 1 h at room temperature. The

slides were mounted with ProLong antifade kit (Invitrogen) for observation by fluorescence microscopy (Carl Zeiss Microscopy GmbH, Germany). Images were captured and analyzed with ZEN 2011 software (Carl Zeiss Microscopy GmbH).

Characterization of the Carbohydrate Specificity of CvGal2. Enzymatic Treatment of Glycoproteins.

Porcine stomach mucin (PSM; 30 μ g/mL, final concentration) was incubated with PNGase F (25×10^3 U/mL final concentration in G7 reaction buffer, pH 7.5), O-glycosidase (2×10^6 U/mL in G7, pH 7.5), α -N-acetylgalactosaminidase (10^3 U/mL in G7, pH 7.5), α -L-fucosidase (1 U/mL in G6, pH 5.5), β 1,3-galactosidase (500 U/mL in G6, pH 5.5), or buffer only (G6 or G7 as control) at 37 °C for 48 h. To prepare desialylated glycoproteins, fetuin, PSM, ovine submaxillary mucin (OSM), or bovine submaxillary mucin (BSM) (10 μ g/mL in Tris buffer, pH 5.5) was incubated with neuraminidase (10 mU/mL final concentration) at 37 °C overnight. The neuraminidase-treated glycoproteins were boiled for 10 min to deactivate the enzyme before use. The untreated (fetuin, PSM, OSM, and BSM) and desialylated (asialofetuin, asialo-PSM, asialo-OSM, and asialo-BSM) glycoproteins as well as glycosidase-treated hemocyte extracts were diluted in PBS and used for coating 96-well plates for binding and binding-inhibition assays, as described below.

Solid-Phase Binding, Binding-Inhibition, and Competitive Inhibition Assays. Solid-phase lectin binding assays were carried out as described elsewhere.^{22,41} Briefly, 96-well microtiter plates were coated with authentic untreated or enzyme-treated glycoproteins or neoglycoproteins (20 μ g/mL in PBS) for 3 h at 37 °C. The plates were washed with 0.1% Tween in PBS (PBST) and blocked overnight with 3% BSA in PBS at 4 °C. The plates were washed three times with cold PBS/2-ME and stored at 4 °C. For binding studies, rCvGal2 (100 μ L; 0.5 μ g/mL in PBS/2-ME) was dispensed into the coated plates and incubated for 1 h at 4 °C. For binding-inhibition studies, rCvGal2 (0.2 μ g/mL) was preincubated with serial dilutions of PSM, asialo-PSM, OSM, asialo-OSM, BSM, or asialo-BSM (1:3 serial dilutions starting at 10 μ g/mL) for 1 h at 4 °C in PBS/2-ME. The rCvGal-inhibitor mixtures were delivered into the coated plates and incubated for 1 h at 4 °C. For controls, the glycan inhibitor solution was substituted by PBS, and purified lectin, by PBS. After three washes with PBS/2-ME, the plates were fixed with 2% formaldehyde in PBS for 30 min at 37 °C. The wells were washed twice with 100 mM glycine in PBS and three times with PBST. Binding of rCvGal2 was detected by purified rabbit anti-CvGal2 IgG, followed by an HRP-conjugated anti-rabbit antibody. The plates were developed with TMB substrate for 5 min, and the reaction was stopped by adding 1 M HCl. Optical densities at 450 nm were measured on SpectraMax340 plate reader (Molecular Devices) controlled by

SoftmaxPro software, version 1. All experiments were carried out in triplicate and repeated at least twice.

Glycan Array Analysis. Glycan array analysis was carried out at Core H of the Consortium for Functional Glycomics (CFG) at Emory University, on version 5.0 of the array printed with 611 glycans in replicates of six. rCvGal2 (1 mg/mL) was dialyzed against PBS/2-ME and diluted to 20 and 200 μ g/mL before adding it onto the array for analysis. The bound rCvGal2 was detected with rabbit anti-CvGal2 purified IgG, followed by a goat anti-rabbit IgG labeled with AlexaFluor 488 (Invitrogen).

Comparative Homology Modeling. The structure of CvGal2 was modeled using toad (*Bufo arenarum*) ovary galectin-1 (BaGal1) as template (PDB ID: 1GAN⁴²). The four CRDs of CvGal2 (A, 1–144; B, 145–26; C, 287–419; and D, 420–556) showed sequence identities with 1GAN and CvGal1 in the lower 20% range (Table 2). The initial automatic alignment between template and target CRDs was performed using the program Molecular Operating Environment (MOE, v 2013.8001, Chemical Computing Group Inc.) and, after inspection, was adjusted to maintain galectin's archetypical recognition of galactoside, which, using BaGal1 numbering, is Trp69–Gal, (Asn62, Glu72)–O6–Gal, (Arg49, His45, Asn47)–O4–Gal, and (Arg49, Glu72)–O4- or O3–GlcNAc in core types 1 and 2, respectively (Supporting Information Figure S1A).

Initial structural models based on the alignment were generated with MOE homology modeling routines and manually adjusted afterward by minimization to locally improve the interaction with the modeled ligands. An Amber12HT force field (Amber12 parametrization⁴³ 2012 and EHT⁴⁴) was used for all energy calculations in MOE. The antigens were built and optimized using the Glycam.org server with their glycam force field.

Surface Plasmon Resonance (SPR) Measurements. Surface plasmon resonance measurements were carried on a Biacore T100 (GE Healthcare) instrument at 25 °C. rCvGal2 and neoglycoproteins (blood group A tetrasaccharide-BSA, blood group A trisaccharide-BSA, GalNAc-BSA, Gal-BSA, and GlcNAc-BSA) were immobilized on individual CMS chips in duplicate cells until 3000 response units were reached by using an amine coupling kit provided by the manufacturer. A reference channel was immobilized with ethanolamine. Binding analyses were performed by injecting a solution of analytes (PSM, asialofetuin, and oligosaccharides) over four cells at 2-fold increasing concentration in 10 mM HEPES, pH 7.4, containing 150 mM NaCl and 0.005% surfactant P20 at a flow rate of 20 μ L/min for 2 min and allowed to dissociate for another 5 min. The surface was regenerated after each cycle by injecting a 3 M MgCl₂ (aq) solution for 3 min at a flow rate of 30 μ L/min. Data were collected at a rate of 10 Hz. T100 Biacore evaluation software was utilized to subtract the appropriate blank reference and to fit the sensorgram globally by applying a 1:1 Langmuir model. GraphPad Prism software was used to deduce EC₅₀ values for analytes PSM and ASF. Mass transfer effects were checked using the t_c values displayed by the T100 Biacore evaluation software. No significant mass transport effects were observed.

Analysis of Interactions among CvGal2, Oyster Hemocytes, and Perkinsus Trophozoites. *Enzymatic Treatment of Hemocyte Extracts and Intact Hemocytes.* Hemocyte extracts (100 μ g/mL) were incubated with PNGase F (25 \times 10³ U/mL final concentration in G7 reaction buffer, pH 7.5), O-glycosidase (2 \times 10⁶ U/mL in G7, pH 7.5), α -N-

acetylgalactosaminidase (10³ U/mL in G7, pH 7.5), α -L-fucosidase (1 U/mL in G6, pH 5.5), β 1,3-galactosidase (500 U/mL in G6, pH 5.5), or buffer only (G6 or G7 as control) at 37 °C for 48 h. Fixed intact hemocytes were incubated with PNGase F (25 \times 10³ units/mL), O-glycosidase (2 \times 10⁶ units/mL), α -N-acetylgalactosaminidase (10³ U/mL), or buffer only at 37 °C for 72 h.

Solid-Phase Binding of CvGal2 to Hemocyte Extracts and Competitive Inhibition Assays. Solid-phase binding assays to examine binding of CvGal2 to hemocyte extracts were carried out as described above for glycoproteins, following Ahmed et al.⁴¹ Briefly, 96-well microtiter plates were coated with untreated or enzyme-treated hemocyte extracts (0.5 μ g/mL in PBS) for 3 h at 37 °C. The plates were washed with PBST and blocked overnight with 3% BSA in PBS at 4 °C. The plates were washed three times with cold PBS/2-ME and stored at 4 °C. For binding studies, rCvGal2 (100 μ L; 0.5 μ g/mL in PBS/2-ME) was dispensed into the coated plates and incubated for 1 h at 4 °C. For competitive binding studies, the wells were incubated with either anti-A or anti-B blood group antibodies (100 μ L; 0.5 μ g/mL in PBS) for 1 h at 4 °C, and after three washes with PBS/2-ME, rCvGal2 (100 μ L; 0.5 μ g/mL in PBS/2-ME) was dispensed and incubated as above. After three washes with PBS/2-ME, the plates were fixed with 2% formaldehyde in PBS for 30 min at 37 °C. The wells were washed twice with 100 mM glycine in PBS and three times with PBST. Binding of rCvGal2 was detected by purified rabbit anti-CvGal2 IgG, followed by an HRP-conjugated anti-rabbit antibody. The plates were developed with TMB substrate for 5 min, and the reaction was stopped by adding 1 M HCl. Optical densities at 450 nm were measured on SpectraMax340 plate reader (Molecular Devices) controlled by SoftmaxPro software, version 1. All experiments were carried out in triplicate and repeated at least twice.

Flow Cytometry Analysis of Galectin Binding to Oyster Hemocytes and Perkinsus spp. Fixed hemocytes or fixed *P. marinus* trophozoites were incubated with 3% BSA in HBS 1 h and subsequently incubated with 0–100 μ g/mL of rCvGal1 or rCvGal2 for 1 h, both at room temperature. The cells were washed three times and stained with 100 μ L of rabbit anti-CvGal1 or anti-CvGal2 IgG, respectively, for 1 h, followed by incubation with Alexa 488-labeled anti-rabbit IgG for 1 h, both at room temperature. After three washes in HBS, the stained cells were analyzed with an Accuri C6 flow cytometer. For competition experiments, cells were preincubated with 0–100 μ g/mL of rCvGal1 or rCvGal2, anti-A, or anti-B antibody for 30 min before adding 2 μ g/mL of rCvGal2 or rCvGal1, respectively, followed by antibody detection.

Statistical Analyses. Numerical results were quantified using ImageJ software. Comparison of two groups was performed by Student's *t* test for nonpaired samples. All results with *p* < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

The roles of galectins in the recognition of carbohydrate moieties on the surface of viruses, bacteria, and eukaryotic parasites as a first step of the innate immune response have been firmly established in recent years (reviewed in ref 6). Recent studies have corroborated not only their roles in recognition of potential pathogens but also their key functions as effector factors in microbial clearance from the internal milieu.^{45–48} However, some eukaryotic parasites have adapted to subvert the roles of lectins in recognition and defense to facilitate their infectivity for the hosts^{22,23,49} or vectors.⁵⁰ Among these, the

role in feeding by mediating hemocyte uptake and intracellular digestion of phytoplankton.²² CvGal1 also recognizes a wide variety of environmental bacteria, some of which are well-established pathogens, and therefore would play a key role in immune recognition and defense by phagocytosis and intracellular oxidative killing of the potentially infectious microbes.²² Most notably, however, is that *P. marinus* trophozoites are strongly recognized by CvGal1 and that the immune/feeding functions of CvGal1 are subverted to become a parasite receptor that facilitates passive host entry by phagocytosis.²² The observation that pre-exposure of the oyster hemocytes to a specific anti-CvGal1 antibody only partially reduced phagocytosis, even at the highest antibody concentrations tested,²² was intriguing and led us to propose the existence of additional hemocyte receptor(s) for *Perkinsus* entry.²²

As mammals express a very diverse and complex galectin repertoire comprising over a dozen distinct galectin species,⁵ we hypothesized that additional oyster galectins might also function as hemocyte receptors for *P. marinus* uptake. Screening an oyster hemocyte cDNA library yielded only one distinct galectin transcript sequence, which we designated CvGal2. In this study, we proceeded to characterize the molecular, structural, and binding properties of CvGal2 with the goal of gaining further understanding of the functional aspects of the oyster's galectins as hemocyte receptors for *P. marinus* infection.

Gene Structure and CRD Organization of CvGal2. Using degenerate PCR primers, we identified in the eastern oyster a novel galectin cDNA sequence, which we named CvGal2, that spanned 1975 nt (Figure 1A) and contained a 1674 nt open reading frame. This sequence encoded a protein of 557 amino acid residues that included 4 potential CRDs, with a calculated molecular mass of 64.1 kDa and a predicted isoelectric point of 8.11. This sequence was verified by amplifying the full-length coding region, which yielded a product of identical sequence that included the 5'- and 3'-UTRs, 88 and 213 nt, respectively. To determine the structure of the gene that encodes CvGal2, we used a PCR amplification approach on genomic DNA with primers designed from the cDNA sequence; this enabled the identification of the intron sequences (Table 1). The CvGal2 gene sequence (~9 kb), obtained by the alignment of multiple sequences of overlapping PCR products, is composed of 13 exons (lengths in downstream order: 58, 60, 89, 328, 86, 202, 120, 89, 205, 120, 89, 205, and 114 nt) separated by 12 introns (2.382, 0.125, 0.351, 2.189, 0.295, 0.153, 0.138, 0.308, 0.324, 0.115, 0.482, and 0.389 kb), a structure similar to that of CvGal1 (Figure 1B). None of the introns is present within the regions encoding the individual CRDs.

Oyster galectin CvGal2 carries four tandemly arrayed CRDs, which are similar, but not identical, in sequence. Thus, CvGal2 shares a similar gene structure and CRD organization with that of CvGal1. The number and organization of the CRDs in oyster galectins CvGal1 and CvGal2 represent a unique feature in the galectin family that suggests unique binding properties. Since the affinity of interactions between lectins and their carbohydrate ligands is relatively weak, the simultaneous binding of multiple CRDs to multivalent ligands can significantly increase the avidity of the interaction.⁵⁸ In lectins carrying a single CRD, such as the mannose binding lectin, high avidity for the target is achieved by the association of peptide subunits into oligomeric structures in which multiple CRDs simultaneously interact with ligands.^{59,60} The presence of tandemly arrayed CRDs encoded within a single polypeptide, such as in the oyster galectins,

would lead to cooperative binding independent of oligomer formation and may be critical for the recognition, agglutination, and immobilization of microbial pathogens.^{22,61} Furthermore, the binding to hemocyte glycans of multiple CRDs in the oyster galectins would facilitate cross-linking and lattice formation at the hemocyte surface, which may trigger signaling pathways leading to phagocytosis and cell activation, as described for other lectins.⁶²

Phylogenetic Analysis of CvGal2. The CvGal2 gene encodes four tandemly arrayed CRD structures and thus is similarly organized as the gene encoding CvGal1 (Figure 1). Alignment of the CRD sequences from CvGal2 and CvGal1 revealed high conservation of the primary structure of the domains responsible for ligand binding, particularly of the amino acid residues critical for β -galactoside recognition (Figure 2A). Therefore, we analyzed the phylogenetic relationships of CvGal2 to CvGal1, as well as to all available galectin sequences from other bivalve mollusk species, using the galectins from the Atlantic bay scallop, *Argopecten irradians*, the pearl oyster, *Pinctada fucata*, and the planorbid freshwater gastropod, *Biomphalaria glabrata*, as the outgroups. The Pacific oyster, *C. gigas*, genome contains at least five duplicate partial galectin sequences randomly named, which converge to two genes (in this study, named gal-1 and gal-2) with high identity to CvGal1 and CvGal2, respectively (Supporting Information Table S2). These two genes shared by the two *Crassostrea* species (*C. virginica* and *C. gigas*) each contained four tandemly repeated CRDs, here labeled A–D based on their order from the N- to the C-terminus. Each of the domains formed a single monophyletic clade in the phylogeny, with the lowest bootstrap support for the first domain (A) (<60%), 71% support for the fourth domain (D), and high support for domains B (96%) and C (100%) (Figure 2B). The two *C. gigas* galectins, gal-1 and gal-2, were distinct, so within the clades corresponding to the individual CRD domains, gal-1 and gal-2 from the two *Crassostrea* species formed two distinct well-supported groups (Figure 2B). However, the individual domains from the two outgroup species, *A. irradians* and *P. fucata*, had different relationships within each domain to *C. gigas* gal-1 and gal-2, albeit mostly with poor bootstrap support. Finally, there was a distinct division between the two- and four-CRD galectins with 99% bootstrap support. Within the two-CRD galectins, again two distinct clades were formed, corresponding to the first and second domains with 62 and 79% bootstrap support, respectively. While the branching order of the species was not consistent between the first (A) and second (B) domains, *B. glabrata* was a consistent, well-supported outgroup.

The distance-based tree was consistent with phylogenetic analysis of galectins reported elsewhere.^{22,63,64} However, the phylogenetic analysis of the individual CRDs of CvGal2 and CvGal1, and galectins from other bivalve species, revealed that each CRD formed a single monophyletic clade. Thus, the results suggest that, within the genus *Crassostrea*, the duplication of the four-CRD galectins into gal-1 and gal-2 (or CvGal1 and CvGal2) likely occurred prior to speciation into *C. gigas* and *C. virginica* because gal-1 and gal-2 were consistently distinct in the CRD phylogeny. Likely, the most recent common ancestor of the two species contained the two four-CRD genes and a single two-CRD gene. Furthermore, the four- and two-CRD galectins do not appear to be the result of simple duplication or reduction. The reason is that between the two possible scenarios, one involving the duplication (or reduction) of an entire multi-domain gene and the second involving duplication of individual

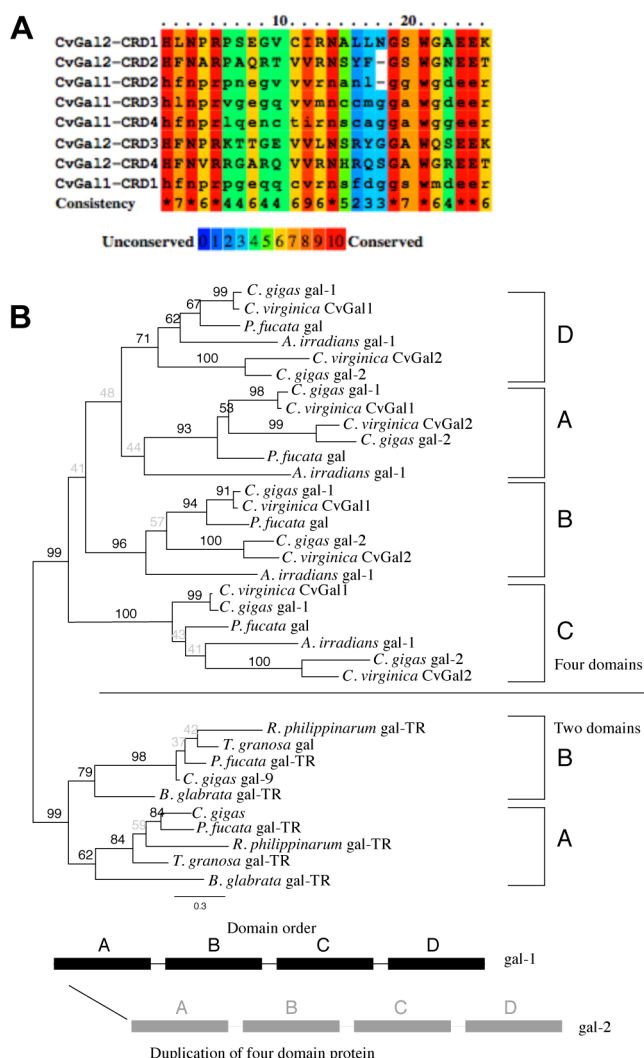


Figure 2. Phylogenetic analysis of CvGal2. (A) Alignment of the four CRDs of CvGal2 with those of CvGal1. The color code for the amino acid conservation is illustrated below. (B) Phylogenetic analysis of the individual CRDs of CvGal2 with those of galectins from various mollusk species. Four-CRD galectins include the following: *C. virginica* CvGal1 (ABG75998.1), *C. gigas* gal-1 (EKC37204.1), *C. gigas* gal-2 (EKC31760.1), *Pinctada fucata* gal (ACO36044.1), and *Argopecten irradians* gal-1 (ACS72240). Two-CRD galectins include the following: *C. gigas* gal-9 (EKC40501.1), *P. fucata* gal-TR (ADU60337.1), *Ruditapes philippinarum* gal-TR (ACA09732.1), *Tegillarca granosa* gal (AGH06131.1), and *Biomphalaria glabrata* gal-TR (ABQ09359.1).

domains within a gene, based on the phylogenetic analysis the former must be ruled out. In other words, under the first scenario, a two-CRD gene could be duplicated into a four-CRD gene. However, the individual domain phylogeny shows that the four-CRD galectins are not interleaved with the two-CRD galectins, so the two- and four-CRD genes are distinct and not the product of simple duplication (or reduction).

Tissue Expression and Distribution of CvGal2. To examine the spatial distribution of CvGal2 transcripts in oyster tissues, we used RT-PCR with primers based on the CvGal2 cDNA sequence. CvGal2 transcripts were present in all tissues tested, which included gill, heart, digestive gland, mantle, palp, rectum, hemocytes, and cells present in the pallial fluid (Figure 3A). To examine the presence and subcellular localization of the mature CvGal2 protein, we cloned and expressed recombinant

CvGal2 and raised antiserum against rCvGal2. Because the amino acid sequences of CvGal1 and CvGal2 are similar (albeit not identical), we further passed the anti-CvGal2 antibody through an rCvGal1 affinity column to absorb any potential cross-reactive antibodies against CvGal1 and, reciprocally, the anti-CvGal1 antibody through an rCvGal2 affinity column. The specificity of these anti-rCvGal1 and anti-rCvGal2 antibodies was verified by western blot, testing crude hemocyte extracts and plasma, with purified rCvGal1 and rCvGal2 serving as specificity controls. Single bands of the expected electrophoretic mobility were detected in crude extracts from circulating unattached hemocytes, hemocytes that had attached to a plastic surface, and the supernatant plasma from the latter, as well as the purified rCvGal1 and rCvGal2 controls, respectively (Figure 3B). No signal was observed in the preimmune IgG controls (Figure 3B). The results confirmed the specificity of the antibodies for the authentic CvGal1 and CvGal2, supported the RT-PCR results indicating that CvGal2 is transcribed in hemocytes, and revealed that CvGal2 is secreted into the extracellular space. Subsequently, we examined the subcellular distribution of CvGal2 in attached hemocytes by immunofluorescence, comparing the fluorescence signal distribution in intact cells with that from cells that had been permeabilized by Triton X-100 (Figure 3C). In addition to highly fluorescent sparse granular structures, the CvGal2 signal was detected on the plasma membrane of the intact attached hemocytes, although it was weaker than the mostly homogeneous stain observed in the cytoplasm of the permeabilized hemocytes (Figure 3C). No fluorescence was observed in the preimmune IgG controls (Figure 3C).

Although the CvGal2 transcripts were detected in all oyster tissues tested, these are abundant in both circulating and resident hemocytes. Thus, it is likely that, as we observed for CvGal1,²² the CvGal2 transcripts identified in these tissues actually originate in the infiltrating hemocytes. The *P. marinus* trophozoites enter the oyster through filter-feeding and initially come in contact with the gills, palps, gut, or mantle, where they are phagocytosed by resident hemocytes⁶⁵ and survive intracellular oxidative stress by their effective antioxidative mechanisms.^{53,54,66,67} The trophozoites proliferate in phagosome-like structures, eventually lysing the host hemocytes and causing systemic infection and death of the oyster, which, by releasing large numbers of parasites into the water column, spreads the infection to neighboring oysters.^{20,35} Thus, the expression of CvGal2, as well as CvGal1,^{22,23} is associated with the cells and tissues that have been proposed to be the entry sites for *P. marinus* trophozoites.^{20,35,37} Furthermore, although CvGal2 is abundant in the hemocyte cytoplasm, it is also secreted into the extracellular space and binds to the hemocyte membrane's glycocalyx. Because CvGal2 lacks a signal peptide, its presence in the extracellular space suggests that the unconventional secretion mechanism reported for mammalian galectins may also be involved in CvGal2 secretion.⁶⁸ In contrast with CvGal1, however, which was expressed in only about 30% of the hemocytes and is mostly associated with granular cells, CvGal2 is homogeneously distributed in all hemocytes examined, both granulocytes and hyalinocytes, suggesting a broader array of biological activities, such as cross-linking of potential microbial pathogens, cell adhesion, and activation of hemocyte signaling pathways.^{69–72}

Glycan Array Analysis. The glycan array binding profile of rCvGal2 (20 and 200 μ g/mL) (<http://www.functionalglycomics.org/glycomics/publicdata/home.jsp>) re-

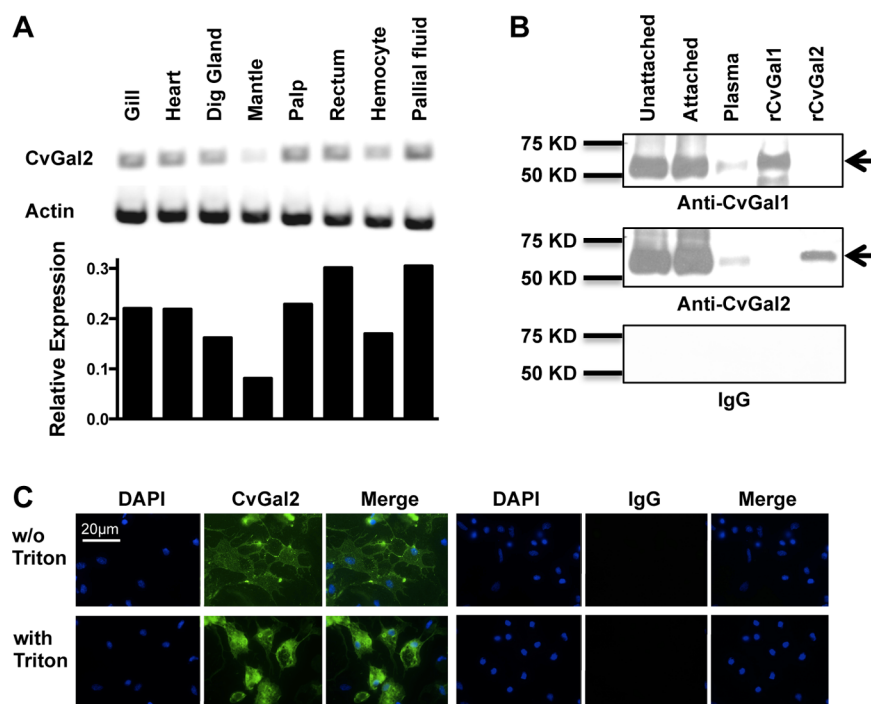


Figure 3. Expression of CvGal2 in eastern oyster tissues. (A) mRNA expression of CvGal2 in different tissues of the eastern oyster assessed by RT-PCR. Relative expression levels relative to actin are shown. (B) CvGal1 and CvGal2 proteins in unattached hemocytes, attached hemocytes, or plasma detected by western blot. Recombinant CvGal1 (rCvGal1) and CvGal2 (rCvGal2) were included as specificity controls for the antibody, and a membrane exposed to preimmune IgG is included as a control for IgG specificity. (C) Hemocytes were allowed to attach for 1 h, and the presence of CvGal2 on cell surface (without Triton) or total (with Triton) was revealed by fluorescence microscopy after antibody staining. Images from cells stained with preimmune IgG (IgG) are included as controls for IgG specificity. Scale bar: 20 μ m.

vealed that CvGal2 recognizes carbohydrates containing nonreducing terminal Gal and GalNAc with high affinity (Figure 4 and Supporting Information Table S1). The strongest binder in the glycan array was the complex biantennary structure GalNAc α 1-3(Fuca1-2)Gal β 1-4GlcNAc β 1-6(GalNAc α 1-3(Fuca1-2)Gal β 1-4GlcNAc β 1-3)GalNAc (Figure 4A, 1), followed by Gal α 1-3(Fuca1-2)Gal β 1-4GlcNAc β 1-2Man α 1-6(Gal α 1-3(Fuca1-2)Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc (Figure 4A, 2), Gal α 1-3(Fuca1-2)Gal β 1-4Glc β (Figure 4A, 3), and six monoantennary and three biantennary glycans with core structure (Gal/GalNAc) α 1-3(Fuca1-2)Gal β 1-4/3GlcNAc (Figure 4A, 4–12). This suggested that the anomeric linkage β 1-4 or β 1-3 of the subterminal Gal to the GlcNAc affects the binding and that CvGal2 prefers type-2 backbone structures Gal β 1-4GlcNAc in *N*-acetylactosamine and galactose. In addition, the Fuc linked in α 1-2 to the subterminal Gal was present in all preferred ligands. Thus, rCvGal2 recognizes array elements displaying blood group A/B type 2 tetrasaccharides with high specificity, followed by the type 1 structures (Figure 4). This is in contrast with the clear preference for blood group A over blood group B oligosaccharides that we observed in a similar analysis of CvGal1.^{23,57} As in CvGal1, however, the Fuc linked α 1-2 to the subterminal Gal appeared to further strengthen the binding. Thus, unlike most described galectins that recognize oligosaccharides exhibiting galactosyl units at the nonreducing end, particularly *N*-acetylactosamine moieties,^{45,73–75} this study together with previous reports from our laboratory^{22,23,57} reveals that oyster galectins CvGal1 and CvGal2 display specificity for ABH blood groups. The sequence alignment of CvGal2 CRDs with those of vertebrates and bivalve species showed that two amino acid residues (His53 and Asp55), which

interact with the nitrogen of the *N*-acetyl group, are missing in all four CvGal2 CRDs. In the *C. elegans* galectin Lec-6, the absence of these two residues confers distinct carbohydrate specificity relative to that of the mammalian prototype galectins.⁴¹ A few other galectins, from fungi to mammals, share similar binding properties for blood group oligosaccharides. Galectin CGL2 from inky cap mushroom *Coprinus cinereus* is specific for blood group A moieties,^{76,77} whereas mammalian galectins 2, 3, 4, and 8 can recognize A and B oligosaccharides.^{74,75}

Comparative Modeling. To rationalize the structural basis for the binding properties of CvGal2 described above, we modeled its structure using *Bufo arenarum* galectin-1 (BaGal1) as template (PDB ID: 1GAN⁴²) and analyzed the interactions of the CRDs with the carbohydrate structures identified in the glycan array as being CvGal2 ligands. The four CRDs of CvGal2 (A, 1–144; B, 145–286; C, 287–419; and D, 420–556) show sequence identities with BaGal1 and CvGal1 in the lower 20% range, from 19 to 23% (Table 2). Large sequence variations, insertions, and deletions, with respect to the template, were accommodated in permissive regions of the template structure such as solvent-exposed loops (Figure 5A). Modeling the CvGal2 structure revealed interesting features in the quaternary structure of this galectin. The sequence alignment of CRDs of CvGal2 with those in the BaGal1 template revealed longer N-terminal regions of CRD A and D that may form an additional N-terminal β -strand, homologous to the first β -strand of *C. cinereus* galectin-2 CGL2.⁷⁷ This extra strand places the N- and C-termini of the CRD beside each other at one side of the β -sandwich opposite the binding site. These additional strands could interfere with the typical homodimerization by an extended β -sheet observed in galectin-1. Instead, the intervening

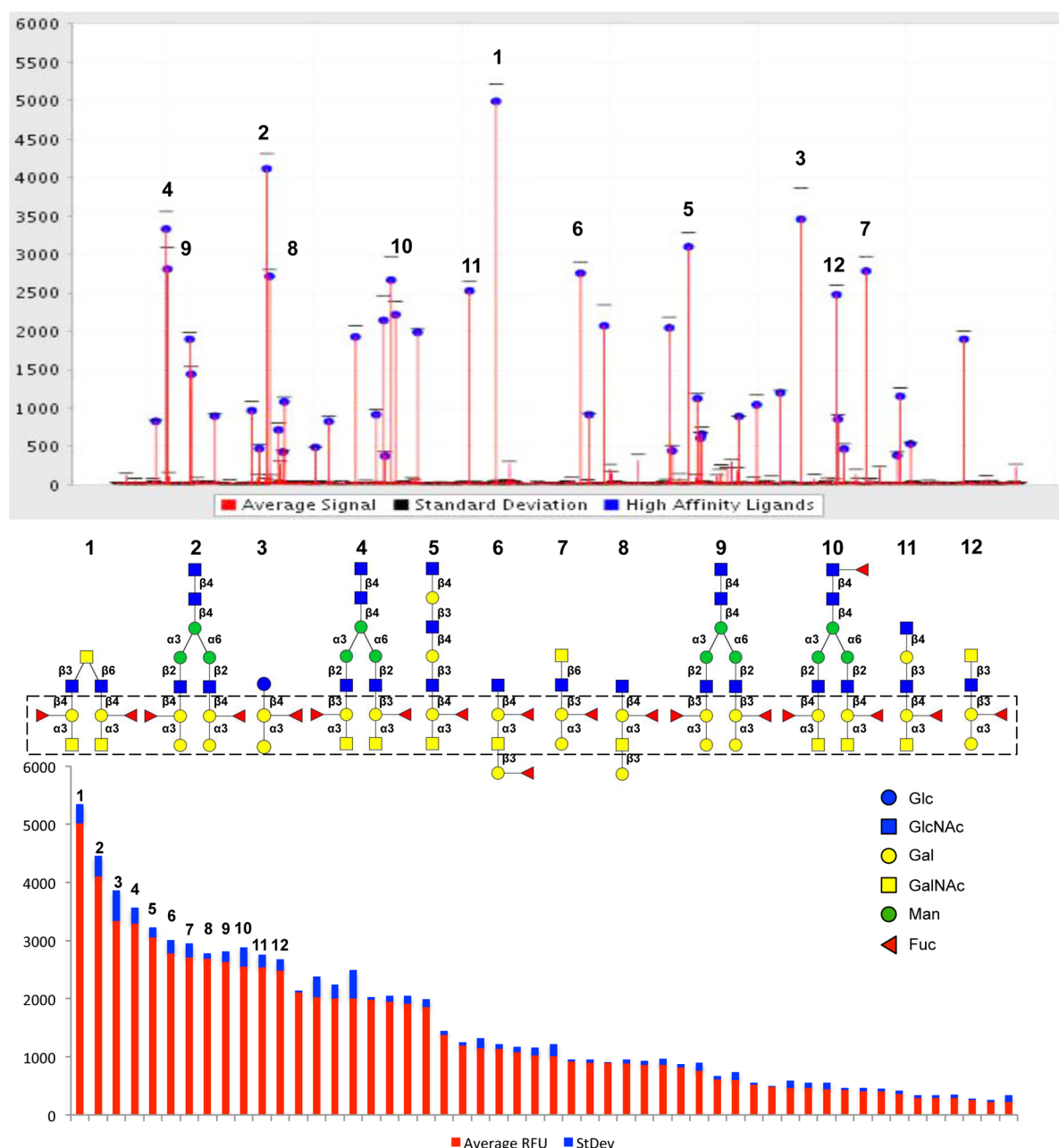


Figure 4. Glycan array analysis of CvGal2 specificity. Recombinant CvGal2 was subjected to glycan array analysis (Consortium for Functional Glycomics). The fluorescent signals along the glycan array are shown, with the top-ranked signals labeled (top) and rearranged by signal strength as follows: high to low from left to right (bottom). The 12 top-ranked glycan CvGal2 ligands are shown with their core structures boxed (middle).

stretches between CRDs suggest a quaternary arrangement in which the A and D CRDs are displayed in the side opposite that of B and C.

The model of the CRDs (A–D) also provided the structural basis of the carbohydrate specificity revealed by the glycan array and biochemical analyses. The modeling alignment between BaGal1 and the four CRDs of CvGal2 preserves the interactions that recognize the β -galactoside ligand: Trp69—Gal, (Asn62, Glu72)—O6-Gal, (Arg49, His45, Asn47)—O4-Gal, and (Arg49, Glu72)—O4- and O3-GlcNAc in core types 1 and 2, respectively (Figure 5B). This alignment identifies a shorter sequence between strands β 4 and β 5 that results in a smaller loop 4 compared to that in prototype galectins (i.e., galectin-1) such as BaGal1.⁴² In contrast, loop 3 (except in the CRD B) is longer than that in BaGal1. At loop 4, a conserved histidine

residue (His 53 in BaGal1, Figure S1) makes an apolar contact with the C2 and O2 atoms of the disaccharide's Gal moiety. This histidine residue, which is highly conserved in most galectin-1's, is missing in CvGal1²³ and CvGal2 (Figure 5A) as well as in CGL2, a galectin of fungal origin.⁷⁷ The main feature in the alignment between template and targets is that, like CvGal1²³ and CGL2,⁷⁷ CvGal2 shows a short loop 4. In addition, β 3 and β 11 strands show specificity-relevant modifications (Figure 5C).

Recognition of the α (1-2)Fucose of H Type 2 Structures. The 2'-fucosyl moiety may be loosely accommodated in the space left over by the shortening of loop 4 and may be in contact only with its 6-methyl group on the aliphatic side of the galactoside-binding arginine. Although it is tempting to associate the small size of the loop 4 with the recognition of H antigens, human galectin-1 (hGal-1), which has a long loop 4,

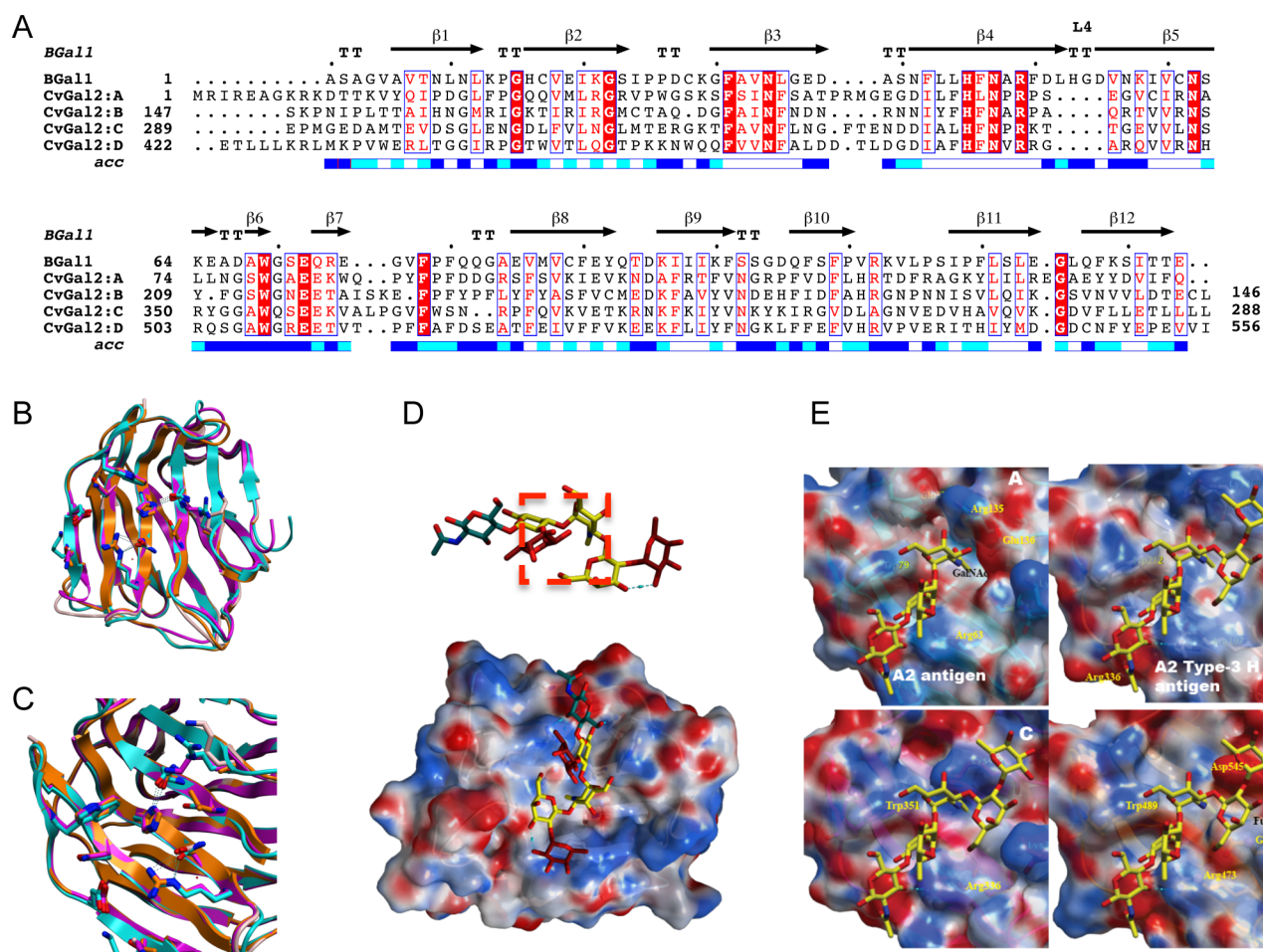


Figure 5. Models of individual CvGal2 CRDs. (A) Alignment with toad galectin1 (BaGal1) as template (PDB ID: 1GAN⁴²). (B) Overlay of the four CRDs (CRD1, CRD2, CRD3, CRD4) based on the model. (C) Residues of the glycoside recognition site and other in $\beta 3$ and $\beta 11$ with possible glycan interactions. (D) Accessible surface of the CvGal2–A colored by electrostatic potential A2 type-3 antigen [Fuc α 1-2Gal β 1-3GlcNAc α 1-3(Fuc α 1-2)Gal β 1-4GlcNAc] in this galectin's extended site. (E) Electrostatic surface of the four CvGal2's CRD models with an A2 antigen (CRD A) and A2 type-3 antigen (CRDs B–D) bound.

recognizes galactosides better than $\alpha(1-2)$ fucosylated H antigens (Supporting Information Table S1). The docking simulation of an H antigen in hGal-1 is shown in Figure S1B. The required Fuc conformation (Figure S1B) is not compatible with the $\alpha(1-3)$ linked pyranose of A and B oligosaccharides, explaining the lack of affinity of hGal-1 for these moieties (see hGal-1 in Supporting Information Table S1). This short loop opens the site to easily accommodate simultaneously the 2'-fucosyl group and the $\alpha(1-3)$ Gal[NAc] of A and B oligosaccharides, with an apparent gain of affinity with respect to hGal-1. hGal-1, with a long loop 4, recognizes H epitopes well (Supporting Information Table S1), but it does so with a conformation of the $\alpha(1-2)$ fucosyl not achievable by A or B antigens. Loop 4 in the conformation observed in galectin-1 (galectin-3 also has a long loop, but in a different conformation) selects against A and B moieties (hGal-1 in Supporting Information Table S1 and Figure S1B). No direct polar interactions with protein groups are predicted with Fuc (Figure SD). Water-mediated interactions with Fuc can explain the observed high affinity for H-containing groups (Supporting Information Table S1).

Recognition of the $\alpha(1,3)$ Galactose of Blood Group A and B Antigens. The 6-OH of the $\alpha(1,3)$ -linked galactose may H-bond the 5-NH group of the binding site tryptophan in

CvGal2 (Trp69 in BaGal1). Tryptophan's 5-NH at the galactoside binding site (A, 79; B, 212; C, 351; and D, 489 in the respective CRDs) H-bonds to the hydroxymethyl OH group of the $\alpha 3$ -linked pyranose in a gt (O6 gauge to O5 and trans to C4) conformation. The 2-NAc group of the $\alpha 3$ -linked GalNAc of A oligosaccharides docks in a hydrophobic pocket at strand $\beta 3$ on top of A, Ser48; B, Ala180; C, Ala315; and D, Val453 (Figure SE). A similar coordination was observed in the complex of CGL2 with an A2 oligosaccharide (PDB ID: 1UFL).⁷⁷ Two strategically placed small residues on the N-terminus of $\beta 3$ (Figure SB), [G/A/S/T]F[A/S]hN (h stands for hydrophobic), open a pocket that docks the methyl group of the 2-NAc moiety. Variations in sequences, like in CvGal2 CRD D that shows bulkier residues (Q and V), may disrupt this recognition.

Recognition of Biantennary Glycans, Sulfated Moieties, and Additional Carbohydrate Units at the Reducing or Nonreducing End. CvGal2 strongly recognizes, up to an order of magnitude higher than the corresponding core glycoside, epitopes displaying blood group A and B antigens in the glycan array (Supporting Information Table S1), which closely agrees with the predicted model. Due to the multivalency associated with this four-CRD lectin, several biantennary glycans appear in the first 10 ranked epitopes (Figure 4). Figure 6A shows how two CRDs, from the same or different CvGal2

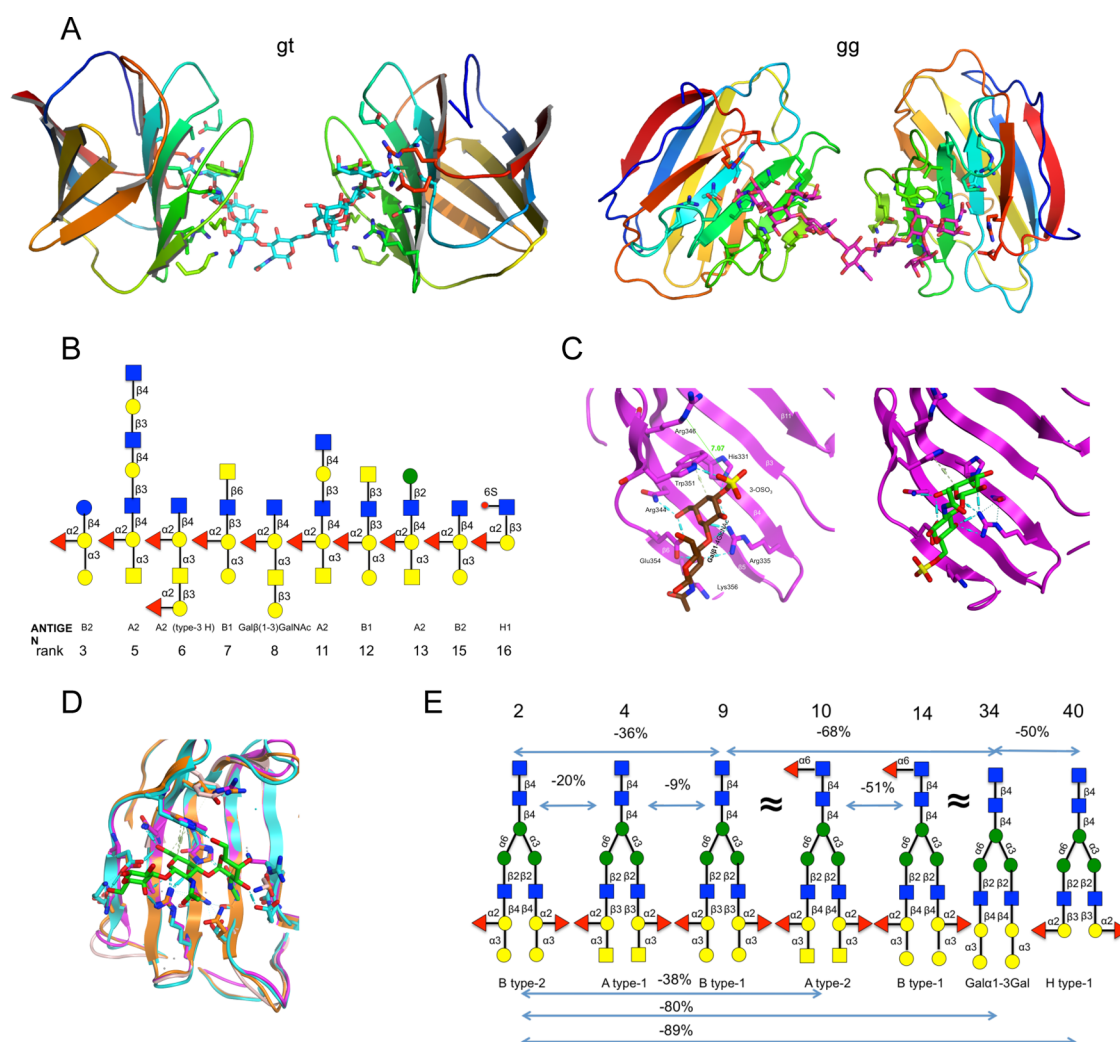


Figure 6. Model of glycan recognition by CvGal2. (A) Multiple recognition of the first ranked biantennary glycan (gt and gi: two lowest-energy structures of O-GalNAc's extended core 2) recognition by two CRDs). (B) Glycans with a common core structure on the strong binding list. (C) Sulfation of the 3-OH (c1) or 6-OH (c2) group of the nonreducing end galactose. (D) Model of the recognition of oligosaccharide A type-4 epitope by CvGal2's CRDS (light blue, orange, magenta, and pink). (E) Selected biantennary N-glycans of complex-type. CvGal2 recognizes A and B of type 1 and 2 epitopes. The negative percentages are the evaluation of the percent change in the fluorescent signal ($((F_j - F_i)/F_i \times 100\%)$). $\alpha(1-3)$ Galactosylation and $\alpha(1-2)$ fucosylation provide apparent 10- and 5-fold affinity enhancements, respectively.

molecules, may recognize any of the two lowest-energy structures (gt and gg) of the O-GalNAc extended core 2 that was ranked first for the glycan array (Figure 4). CvGal2 binds core type 1 (Galβ(1-3)GlcNAc) and core type 2 (Galβ(1-4)GlcNAc). Despite the predominance of core 2 epitopes in the array (48.5% of the array glycans), a significant number (seven) of core 1 structures (only 12% of all glycans in the array) are among the first 20 ligands ranked, suggesting a preference for core 1 (*p*-value for a random selection of 0.0005) (Figure 6B).

CvGal2 recognizes sulfated core glycosides 20-fold stronger than nonsulfated ones (Supporting Information Table S1). Sulfation of 3-OH at the nonreducing end of core 2 or of 6-OH at the reducing end of core 1 galactoside is recognized for CvGal2 (Supporting Information Table S1). Our model suggest that 3-OH sulfation at the galactose is recognized by the NH-5 group of the binding side Trp by forming an H-bond with a sulfate O (Figure 6C). However, the glycan array results from different galectins (Supporting Information Table S1) suggest that such interaction alone is not sufficient to explain the galectins' binding-pattern: all of them share the same Trp. A

comparison among glycan array profiles of galectins in Supporting Information Table S1 shows that the presence or absence of a positively charged residue at the C-terminus of strand β5 divides strong binders (CvGal2 B and C, CGL2, and hGal1) from weak binders (CvGal1 and Gal-3). Sulfation of the 6-OH group at the reducing end of glycosides type 1 are better recognized by CvGal2 than by the other galectins in Supporting Information Table S1; apparently, smaller (Thr in B and D) or more flexible positively charged residue (Lys in the CDRs A and C) better accommodate the substitution than the more rigid and bulkier Arg of the other galectins.

Interestingly, CvGal2 also strongly recognizes A type-4 and Forssman glycotopes (Supporting Information Table S1), although it would appear to be physically impossible to accommodate the $\alpha(1-4)$ galactosylation at the nonreducing end of the galactoside without affecting its recognition. A plausible explanation of the observed affinity is the shift in recognition of a carbohydrate moiety at the nonreducing end to the GalNAcα(1-3)GalNAcβ(1-3)Galα1-4 segment of the glycotope (Figure 6D). In this alternative interaction, the

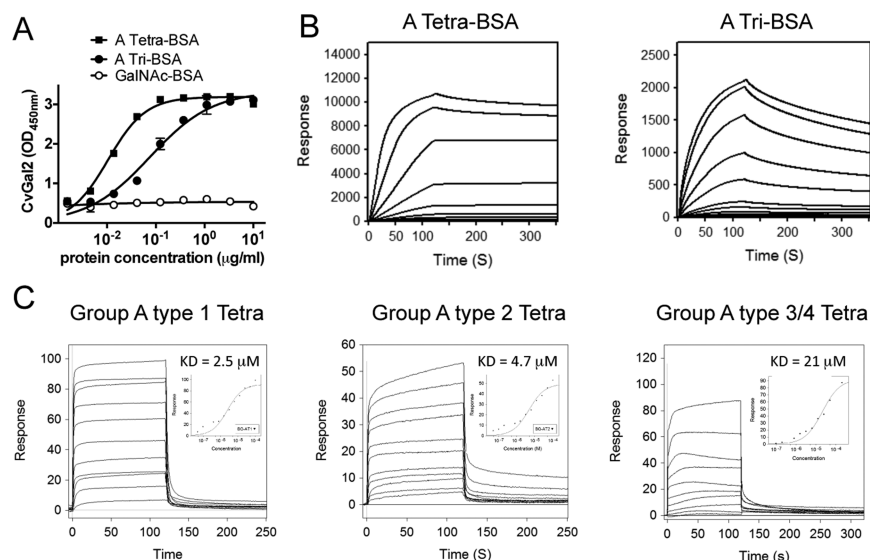


Figure 7. Recognition of blood group A oligosaccharide by CvGal2. (A) Blood group A tetrasaccharide-BSA (A Tetra-BSA), blood group A trisaccharide-BSA (A Tri-BSA), or *N*-acetylgalactosamine-BSA (GalNAc-BSA) was delivered at the concentrations indicated (serial dilution starting from 10 µg/mL; 100 µL/well) into 96-well plates, and the binding of CvGal2 (0.2 µg/mL) was assessed by ELISA. Data show optical density at 450 nm (OD_{450nm}) in triplicates with standard error (SEM). (B) Neoglycoproteins were immobilized up to 1000 response units on CM5 chips, and the binding of rCvGal2 was assessed by SPR, flowing through rCvGal2 as the analyte. The SPR sensorgrams were recorded with 2-fold serial dilutions of the analyte starting from 100 µg/mL. Sensorgrams for the binding of rCvGal2 onto blood group A tetrasaccharide-BSA (A Tetra-BSA) and trisaccharide-BSA (A Tri-BSA) are shown. Negligible responses were observed on sensorgrams for GalNAc-BSA (not shown). Biacore T100 evaluation software was used to deduce *K_D* values. (C) SPR was performed with immobilized rCvGal2 and the indicated oligosaccharides as analyte. SPR sensorgrams were recorded with 2-fold serial dilutions starting from 100 µg/mL. SPR sensorgram is shown in the inset with a 1:1 steady-state fitting curve.

third Gal moiety takes the place of the glycoside Glc[NAc], only losing an H-bond to the binding site Glu (Glu 4-OH of the GlcNAc in the core type 2).

The CvGal2 CRD cannot recognize extensions from the galactoside reducing end (Figure 5D). Thus, with the exception of (1-6) linkages to the glucosamine that may fold back to contact the protein, no affinity trends for CvGal2 were detected for additions at the reducing end (Figure 6B and Supporting Information Table S1). Hydroxyls at the 3 and 4 positions of the β(1-3)-linked Gal of H type-3 precursors can be recognized by polar residues at β11: A, R135–E136; B, K269; C, K402; and D, D545 (Figure 5C). The β11 residues of CvGal2 CRD A may select for a nonsubstituted 3-OH group, H type-3 oligosaccharides (Figure 5A,E), whereas CRDs B, C, and D can accommodate additional terminal carbohydrates (Figure 5B–D,E). Binding to the array oligosaccharides with short linkers like Sp0 (a two carbons chain), however, shows different results than the slightly longer Sp8 linker (Supporting Information Table S1), suggesting interference with the recognition of the glycoside's Glc[NAc] moiety at the reducing end. To analyze the effect of α(1-3)galactosylation and α(1-2)fucosylation at the nonreducing end independent of linkage effects, we compared a selection of biantennary complex-type N-glycans from the glycan array elements (Figure 6E). This comparison among glycans of the same basic structure at the nonreducing end would exclude any contributions from different backbone and linkages to the interaction, isolating the recognition of particular features of the glycotope. CvGal2 strongly recognizes A and B oligosaccharides of types 1 and 2, with a weaker preference for B moieties. Lack of α(1-2)fucosylation or α(1-3)galactosylation weaken this recognition 5- and 10-fold, respectively (Figure 6E).

The structural model of CvGal2 enabled a comprehensive interpretation of the glycan array binding profile, including its

preference for blood group oligosaccharides, the relative high prevalence of biantennary structures, and the recognition of sulfated moieties and Forssman antigens. While the archetypical galectin carbohydrate-binding site recognizes core disaccharides Galβ(1-[3/4])Glc [or GlcNAc], the glycan array results indicated that CvGal2 binds to oligosaccharides displaying blood group A and B oligosaccharides, in agreement with the structural model. The modeling revealed that, like in CvGal1²³ and CGL2,⁷⁷ the shorter loop 4 and a longer loop 3 in CvGal2 relative to the typical prototype galectins^{23,42} provide the structural basis for the preference for blood group oligosaccharides. The recognition of the *N*-acetyl group at the nonreducing GalNAc in blood group A is established by a pocket formed on the *N*-terminus that docks the methyl group of the *N*-acetyl moiety, a coordination similar to that observed in the CGL2–A2 antigen complex (PDB ID: 1ULF).⁷⁷ The shorter loop 4 opens the site to accommodate simultaneously the 2'-fucosyl group and the α(1-3)Gal[NAc] of A and B antigens, with an apparent gain of affinity with respect to the mammalian prototype galectins such as hGal-1 that possess the typical long loop 4.⁴² In this regard, the four CvGal2 CRDs (A–D) show sequence variations that suggest differences in their fine specificity for A, B, and H oligosaccharides. Recognition of the latter, however, cannot be attributed solely to the shorter loop 4 since hGal-1 recognizes α(1-2)fucosylated H antigens better than galactosides.⁷⁴ However, in contrast with CvGal2, hGal-1 recognizes H epitopes with a conformation of the α(1-2)fucosyl that is not achievable by A or B antigens.⁷⁴

The presence of several core 1 biantennary glycans within the top-ranked bound oligosaccharides strongly suggests that the potential multivalency of CvGal2 may contribute by cross-linking the *O*-GalNAc in extended core 1 (and possibly core 2) structures. The model also revealed the structural basis for the

strong binding of CvGal2 to sulfated oligosaccharides as being established not only by the H-bond between the sulfate O and the NH-5 group of the Trp common to other galectins but also by the smaller or more flexible positively charged residues in the CvGal2 CRDs that can better accommodate the sulfation of the 6-OH group at the reducing end of the type 1 structures. Finally, the recognition by CvGal2 of $\alpha(1-4)$ galactosylation at the nonreducing end of the oligosaccharide revealed by the binding to A type-4 and Forssman glycotopes in the glycan array, which initially appears to be structurally incompatible with the CvGal2 model, can be explained by a shift in the recognition of the nonreducing end moiety to the GalNAc $\alpha(1-3)$ GalNAc $\beta(1-3)$ Gal $\alpha(1-4)$ segment of the glycotope, in which the third Gal unit takes the place of the Glc[NAc].

Binding of CvGal2 to Neoglycoproteins and Natural Glycoproteins in a Solid-Phase Assay and Binding Affinity Measurements (SPR). To quantitatively assess the binding of CvGal2 to blood group oligosaccharides, we evaluated the binding of CvGal2 to neoglycoproteins that display blood group A tetrasaccharide, the A trisaccharide, or GalNAc. CvGal2 effectively bound to the blood group A tetrasaccharide-BSA, with lower affinity to the A trisaccharide-BSA, and showed no binding to GalNAc-BSA (Figure 7A). SPR analysis revealed that the K_D 's for CvGal2 binding to blood group A tetrasaccharide-BSA (20 units/BSA) and trisaccharide-BSA (19 units/BSA) are 4.8 and 60 nM, respectively (Figure 7B). We further assessed the binding affinity of CvGal2 with various blood group A/B/H oligosaccharides by SPR. The results revealed that CvGal2 bound effectively ($K_D = 2.5$ –21 μ M) to three types (types 1, 2, and 3/4) of blood group A tetrasaccharides. BSA linked tetra- and trisaccharide bind in the nanomolar range due to avidity resulting from multivalent sites (19 or 20 saccharide units/BSA) of neoglycoproteins.

We further evaluated, by a solid-phase assay, the inhibition of the binding of CvGal2 to asialofetuin by glycoproteins that display some of the oligosaccharides identified as being CvGal2 ligands in the glycan array (Figure 8A). These include glycoproteins that contain abundant blood group oligosaccharides, like PSM,⁷⁸ and the desialylated forms of PSM and fetuin that are rich in nonreducing terminal galactose.⁷⁹ Asialo-PSM ($IC_{50} < 0.01$ μ g/mL) and PSM ($IC_{50} < 0.01$ μ g/mL) behaved as the strongest inhibitors, at least >40-fold better than any other glycoprotein tested. These were followed by asialo-BSM (IC_{50} : 0.037–0.708 μ g/mL), BSM (0.089–3.997), asialo-OSM (0.571–0.949), and OSM (IC_{50} : 5.13–44.79 μ g/mL), all of which were stronger than asialo-fetuin (5.86–348.2 μ g/mL) itself (Table 3).

We subsequently assessed, by SPR, the binding kinetics of rCvGal2 to the glycoproteins identified above as CvGal2 ligands, such as PSM and asialofetuin. The results showed that, in agreement with the binding-inhibition studies, PSM behaved as the strongest binding ligand with an EC_{50} value of 0.28 μ g/mL (Figure 8B), whereas for asialofetuin, the binding of CvGal2 was significantly weaker ($EC_{50} \geq 680$ μ g/mL) (Figure 8B). Next, we evaluated the binding ligands on PSM by enzymatically removing GalNAc. The results showed a 70% reduction in CvGal2 binding to GalNAc α -ase-treated PSM compared to that of the untreated control. On the other hand, binding was reduced by PNGase F treatment significantly but not completely (Figure 8C).

These results not only corroborated the glycan array analysis and validated the structural models but also provided information about the specificity of CvGal2 for blood group

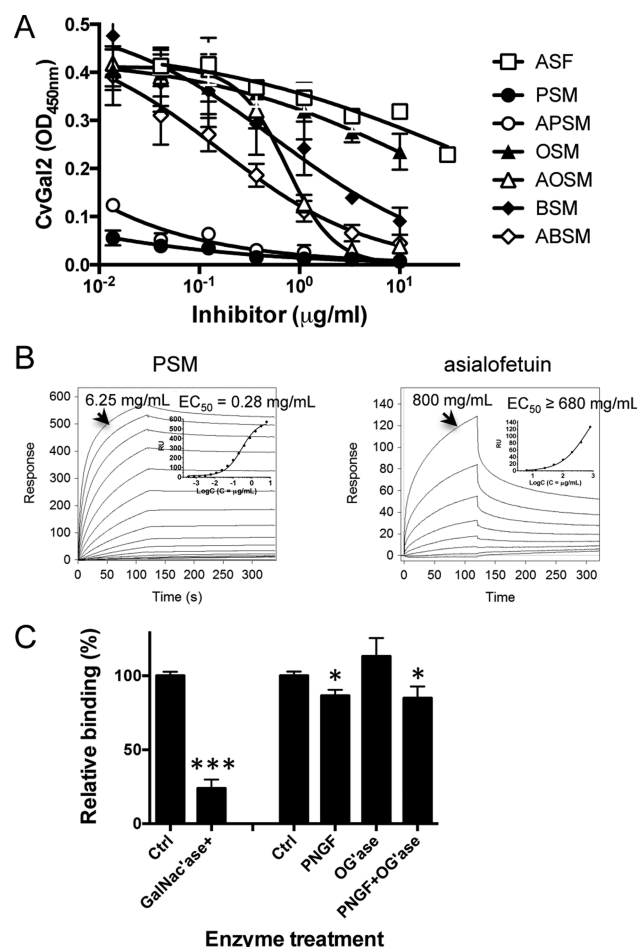


Figure 8. Binding of CvGal2 to natural glycoproteins. (A) Binding of rCvGal2 to asialofetuin (20 μ g/mL) in the presence of serial dilutions of asialofetuin (ASF), PSM, asialo-PSM (APSM), OSM, asialo-OSM (AOSM), BSM, or asialo-BSM (ABSM) was measured in a solid-phase binding assay. Data show optical density at 450 nm (OD_{450nm}) in triplicates with standard error (SEM). IC_{50} values were calculated (Table 3). (B) Binding of CvGal2 to natural glycoproteins was measured by SPR with immobilized rCvGal2, using PSM (left) or asialo-fetuin (right) as analytes. The sensorgrams were recorded with 2-fold serial dilutions starting at the highest concentration indicated. SPR sensorgram is shown in the inset with a 1:1 binding curve. GraphPad Prism software was used to deduce EC_{50} values. (C) PSM (50 ng/mL) coated wells were treated with neuraminidase (Ctrl) plus N-acetylgalactosidase (GalNAc α -ase), PNGase F (PNGF), O-glycosidase (OG α -ase), or both. Binding of rCvGal2 to treated wells was measured in a solid-phase binding assay. Relative binding to untreated wells was calculated, and the mean with SEM from triplicates is shown. *, $p < 0.05$; ***, $p < 0.001$ vs control.

oligosaccharide moieties that are covalently bound to polypeptide scaffolds and presented in distinct displays, revealing notable differences in the binding properties of CvGal1. For example, the recognition of blood group A tetra- and trisaccharides on neoglycoproteins by CvGal2 observed in solid-phase binding assays and SPR analysis was in sharp contrast with that of CvGal1, which recognizes only the tetrasaccharide.²³ The binding affinity of CvGal2 for the type 1, 2, and 3/4 blood group A oligosaccharides also confirmed the above results from the glycan array analysis and the structural modeling. Furthermore, binding-inhibition and SPR measurements confirmed that glycoproteins that display abundant ABH blood group oligosaccharides and nonreducing terminal

Table 3. Inhibition of CvGal2 Binding to Asialofetuin by Glycoproteins

inhibitor	IC ₅₀ (95% CI, μ g/mL)
asialofetuin	5.856 to 348.2
PSM	<0.01 ^a
asialo-PSM	<0.01 ^a
OSM	5.131 to 44.79
asialo-OSM	0.571 to 0.949
BSM	0.089 to 3.997
asialo-BSM	0.037 to 0.708

^aToo low to be calculated accurately (see Figure 8A).

galactose, like PSM and asialo-PSM,^{78,79} are the preferred ligands for CvGal2. While fetuin displays abundant N-acetyllactosamines [Sia α 2-3Gal β 1-4GlcNAc β],⁷⁹ PSM is rich in oligosaccharides that display nonreducing terminal GalNAc and L-Fuc, namely, blood group H [Fuc α 1-2Gal β 1-4GlcNAc β] and A [GalNAc α 1-3(Fuc α 1-2)Gal β 1-4GlcNAc β] moieties.⁷⁸ The similar binding of CvGal2 to intact and asialo-PSM and the significantly weaker binding to asialofetuin indicate that CvGal2 recognizes only relatively weakly those subterminal sugars, such as galactose, that are exposed by the cleavage of nonreducing terminal sialic acids. In contrast, CvGal1 showed significantly stronger binding to asialo-PSM than to PSM and effectively recognized asialofetuin, suggesting that subterminal sugars unmasked by sialidases also contribute to the binding of CvGal1 to asialo-PSM and asialofetuin.²³ The comparative assessment of CvGal2 binding to untreated PSM and PSM that

had been treated with PNGase F and O-glycosidase not only verified that CvGal2's binding to PSM is carbohydrate-mediated but also revealed that the oligosaccharide ligands for CvGal2 in PSM are N-linked. This result identifies a significant difference in the binding properties of CvGal1 and CvGal2, since the PSM ligands recognized by the former are both N- and O-linked.²³ Furthermore, pretreatment of PSM with GalNAc'ase clearly showed that GalNAc at the nonreducing end is required for the binding of CvGal2 to PSM's blood group A oligosaccharides, in agreement with the glycan array and modeling studies.

Binding of rCvGal2 to Hemocyte Surface Glycans. On the basis of the results above, we tested whether CvGal2 recognizes glycans on the surface of hemocytes, as we had previously observed for CvGal1.^{22,23} First, we used ELISA to evaluate the presence of CvGal2 ligands in hemocyte extracts, and the results showed that CvGal2 binds to the hemocyte extract in a dose-dependent manner (Figure 9A). Pretreatment of hemocyte extract with GalNAcase decreased the binding over 80% (Figure 9B), whereas no effect was observed with either PNGase F or O-glycosidase treatment.

Next, we evaluated if the CvGal2 ligands in the hemocyte extract are recognized by anti-A or anti-B monoclonal antibodies by a competitive ELISA. Surprisingly, neither the anti-A nor anti-B antibody blocked the binding of CvGal2 to the hemocyte extract up to 1:25 dilution, whereas anti-A antibody reduced the binding to PSM at 1:100 and 1:25 dilutions by 20 and 40%, respectively (Figure 9C). We tested the binding of CvGal2 to intact circulating hemocytes by flow cytometry and the binding of CvGal2 to hemocytes attached and spread on a plastic surface by immunofluorescence. rCvGal2 bound to both the circulating

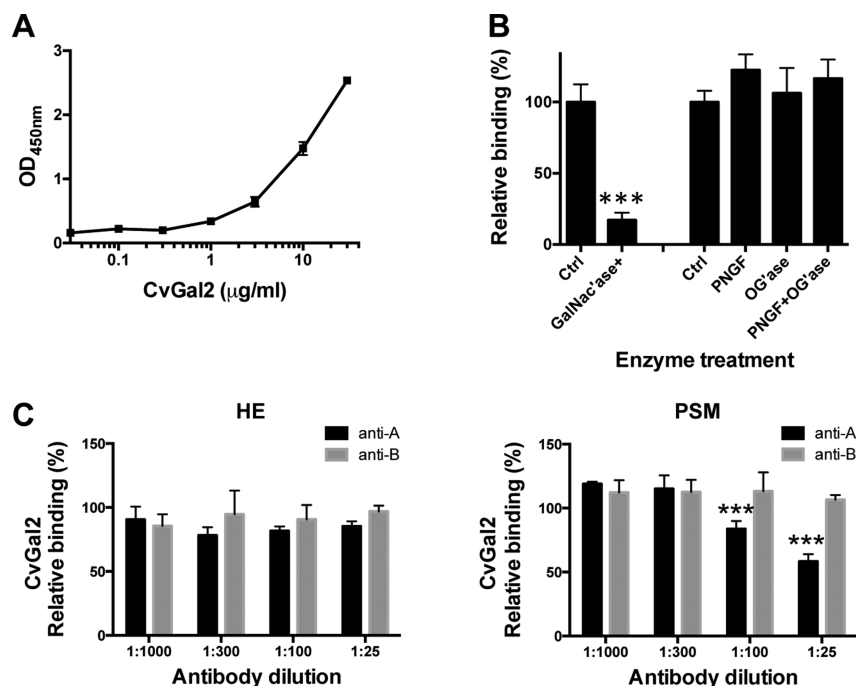


Figure 9. Recombinant CvGal2 binds to hemocyte extract. (A) Hemocyte extract (1 μ g/mL) coated wells were incubated with different concentration of rCvGal2 (0–30 μ g/mL). Binding of rCvGal2 was measured in a solid-phase binding assay, and the mean optical density at 450 nm (OD_{450nm}) of triplicates with SEM is shown. (B) Hemocyte extract (1 μ g/mL) coated wells were treated with neuraminidase (Ctrl) plus N-acetylgalactosidase (GalNAc'ase), PNGase F (PNGF), O-glycosidase (OG'ase), or both PNGF and OG'ase. Binding of rCvGal2 to treated wells was measured as above. Relative binding to untreated cells was calculated, and the mean from triplicates with SEM is shown. *, $p < 0.05$; ***, $p < 0.001$ vs control. (C) Hemocyte extract (1 μ g/mL) (left panel) or PSM (50 ng/mL) (right panel) coated wells were preincubated with different concentrations of anti-A or anti-B (1:25 to 1:1000 dilution) before incubation with rCvGal2 (2 μ g/mL). Binding of rCvGal2 to treated wells was measured as above. Relative binding to untreated cells was calculated, and the mean from triplicates with SEM is shown. ***, $p < 0.001$ vs control.

and attached hemocytes in a dose-dependent (Figure 10A,B) and carbohydrate-specific (Figure 10C) manner. In contrast to

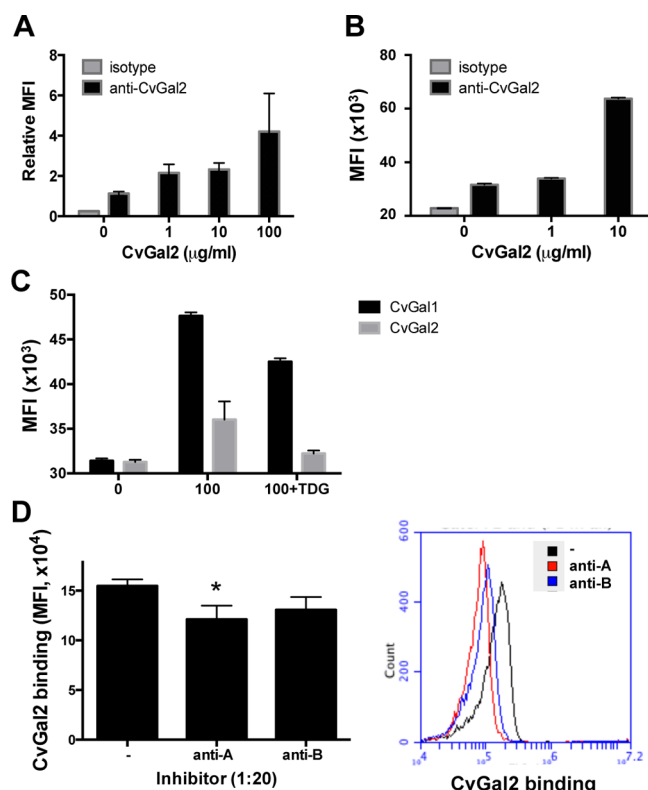


Figure 10. Recombinant CvGal2 binds to oyster hemocytes. (A) Attached hemocytes were incubated with different concentrations of rCvGal2 for 1 h at RT. The binding of rCvGal2 to attached hemocytes was detected and revealed under a fluorescent microscope as described in the [Experimental Procedures](#). The fluorescence intensity from at least five randomly selected views was quantified and normalized to that of samples without exogenous rCvGal2 (0). Data are shown as relative mean fluorescent intensity (MFI) \pm SD. (B) Freshly isolated hemocytes were fixed while unattached and incubated with different concentrations of rCvGal2 (0–10 μ g/mL) for 1 h at RT. The cells were stained with anti-CvGal2 antibody followed by FITC-conjugated secondary antibody, and the binding of recombinant proteins was revealed by flow cytometry. Data are shown as MFI \pm 95% CI. (C) Fixed hemocytes were incubated with CvGal1 or CvGal2 (100 μ g/mL) with or without thiodigalactose (TDG) for the binding assay and were examined by flow cytometry as above. (D) Fixed cells were preincubated with anti-A or anti-B antibody to outcompete rCvGal2 binding before adding rCvGal2 (2 μ g/mL) to the binding assay and examined by flow cytometry as above. Data are shown as MFI \pm 95% CI. *, $p < 0.05$ vs control (–).

what we observed with the hemocyte extract, the anti-A antibody reduced the binding of CvGal2 to the intact hemocytes by 20% ($p < 0.05$), whereas the anti-B antibody reduced the binding only marginally ($p = 0.10$) (Figure 10D).

By the use of glycosidase treatments, we subsequently investigated the type of glycan structures on hemocytes responsible for CvGal2 binding. Both PNGase F and O-glycosidase reduced CvGal2 binding by 15% ($p < 0.05$) and 40% ($p < 0.05$), respectively, suggesting that the CvGal2 ligands were displayed on both N- and O-linked cell surface glycans. GalNAc'ase and fucosidase treatments reduced the binding by at least 30% (Figure 11A). For comparative purposes, the binding of the anti-A antibody to the glycosidase-treated

hemocyte surface was evaluated. PNGase F treatment did not reduce the anti-A binding, but O-glycosidase treatment reduced binding by at least 20%, confirming the presence of O-linked blood group A moieties on the hemocyte's surface. Both GalNAc'ase and fucosidase reduced anti-A binding to the hemocytes by 20% (Figure 11B).

Next, we evaluated whether CvGal2 and CvGal1 share the same carbohydrate ligands on the hemocyte surface. Preincubation with CvGal1 (100 μ g/mL) reduced CvGal2 binding up to 90% in a dose-dependent manner (Figure 11C, left panel). In the reciprocal experiment, preincubation with CvGal2 (100 μ g/mL) reduced CvGal1 binding up to 60%, also in a dose-dependent manner (Figure 11C, right panel). The results suggest that the carbohydrate ligands for CvGal1 and CvGal2 on the hemocyte surface overlap only partially.

The analysis of binding of CvGal2 to oyster hemocyte glycoproteins was consistent with those obtained with purified mammalian glycoproteins in that GalNAc is a key sugar recognized by CvGal2 on the surface of the oyster hemocytes. As the binding of CvGal2 to PSM could be effectively outcompeted by pretreatment with a monoclonal anti-A antibody, the lack of a similar, strong inhibitory effect of the same monoclonal antibody with oyster hemocyte extract or intact hemocytes suggests that CvGal2 can recognize not only blood group A oligosaccharides on the surface of hemocytes but also other distinct N- or O-linked GalNAc-bearing moieties that lack the blood group A structure. Significant reduction in CvGal2 binding by pretreatment of the hemocytes with GalNAc'ase and fucosidase, however, confirmed that blood group A oligosaccharides on the surface of hemocytes partially contribute to CvGal2 binding. This observation also points to differences in binding between CvGal1 and CvGal2, since the binding of the former to the hemocyte surface is mostly attributable to blood group A carbohydrate moieties.²³ Finally, the reciprocal partial competitive binding to the hemocyte surface of both CvGal1 and CvGal2 clearly shows that although ligands common to both galectins are exposed they also bind to nonoverlapping surface ligands, suggesting distinct biological roles for each galectin.

In a previous study, we established that CvGal1 is expressed and secreted by a hemocyte subpopulation, most likely granulocytes, and binds to β -integrin and dominin on the hemocyte surface.^{23,57} It is possible that CvGal2, which is expressed equally in both granular hemocytes and hyalinocytes, also binds to either one or both CvGal1 ligands, but the results suggest that additional hemocyte sugar moieties are recognized by CvGal2. In either case, the partial sharing of glycan ligands on the hemocyte cell surface, together with the observation that CvGal1 and CvGal2 possess multiple CRDs that exhibit differences in specificity as shown by the modeling study, suggests a complex interplay of opsonic properties for potential pathogens of both galectins together with the effects of their selected CRDs binding to the hemocyte surface, leading to cell activation by synergistic activities or downregulation by competitive inhibition of galectin binding to the hemocyte surface.

Differential Recognition of Sympatric *Perkinsus* Species by rCvGal2. As CvGal1 binds to *P. marinus* cell surface and promotes parasite entry,^{22,23} we examined the potential binding of CvGal2 to *P. marinus* and compared it to *P. chesapeakei*, a sympatric *Perkinsus* species that is mostly prevalent in clam species. CvGal2 bound strongly to *P. marinus* in a dose-dependent and carbohydrate-specific manner (Figure 12), but

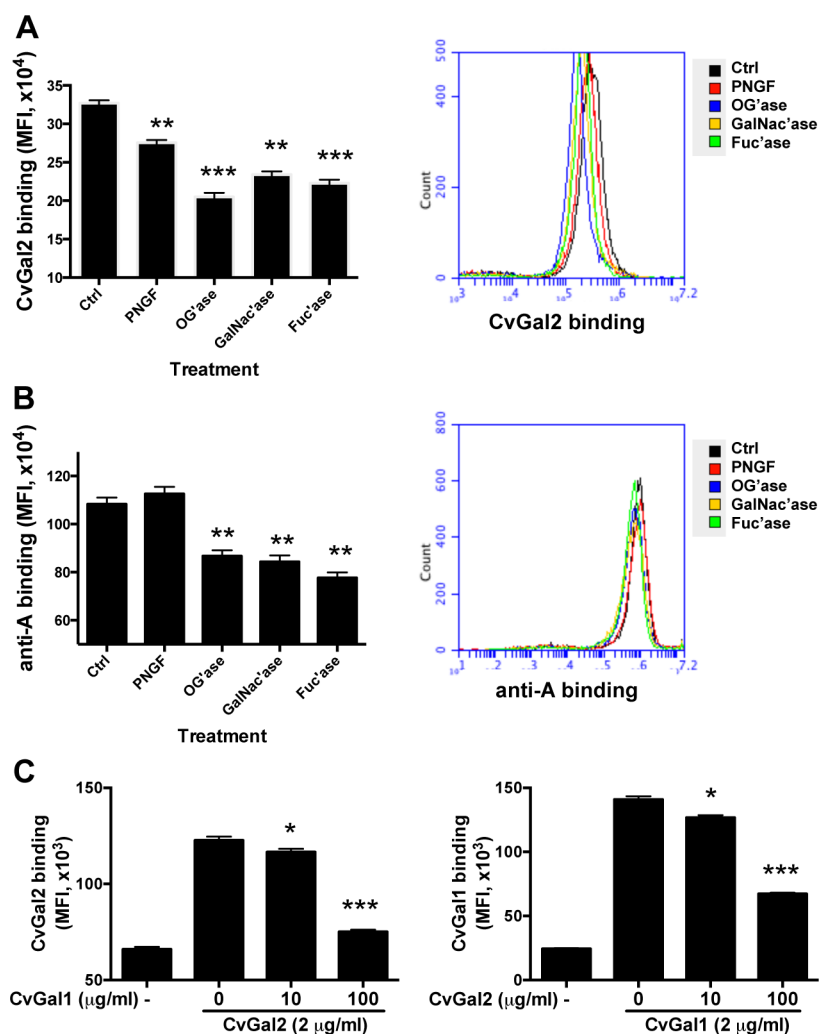


Figure 11. Binding of rCvGal2 and anti-A/B to intact and Gal'ase- and GalNAc'ase-treated hemocytes. (A) Fixed hemocytes were treated with PNGase F (PNGF), O-glycosidase (OG'ase), N-acetylgalactosidase (GalNAc'ase), or fucosidase (Fuc'ase) and incubated with CvGal2 to determine the binding by flow cytometry. (B) The fixed cells were treated as above and stained with anti-A antibody for flow cytometry analysis. (C) Fixed cells were preincubated with rCvGal1 (0–100 μg/mL) or rCvGal2 (0–100 μg/mL) before adding rCvGal2 (2 μg/mL) or rCvGal1 (2 μg/mL), respectively, for the binding assay and examined by flow cytometry as above. Data are shown as MFI \pm 95% CI. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs control.

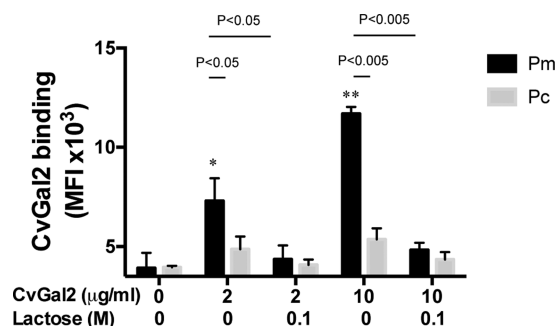


Figure 12. Binding of rCvGal1 and rCvGal2 to *Perkinsus* parasites. *P. marinus* (Pm) or *P. chesapeakei* (Pc) were fixed and incubated with 0–10 μg/mL of rCvGal2 (bottom), which was preincubated with (0.1) or without (0) 0.1 M lactose, for 1 h at RT. The cells were stained with anti-CvGal2 antibody followed by FITC-conjugated secondary antibody, and the binding of recombinant proteins was revealed by flow cytometry. Data are shown as MFI \pm 95% CI. *, $p < 0.05$; **, $p < 0.005$ vs no rCvGal2 (0).

only negligible binding to *P. chesapeakei* was observed. This striking selectivity in recognition properties of CvGal2 for *Perkinsus* species may have functional implications in parasite infectivity and pathogenicity. Along the mid-Atlantic coast of the U.S., and particularly in the Chesapeake and Delaware Bays, *P. chesapeakei* is a species sympatric with *P. marinus*.¹⁷ Although, as mentioned above, *P. marinus* is prevalent and pathogenic for the eastern oyster,¹⁷ *P. chesapeakei* is mostly prevalent in sympatric clam species, such as *Mya arenaria*, *Macoma balthica*, *Tagelus plebeius*, *Mulinia lateralis*, *Rangia cuneata*, *Cyrtopleura costata*, and *Mercenaria mercenaria*.^{10,14,15,17–19} Furthermore, the pathogenicity of *P. chesapeakei* for the eastern oyster has not been rigorously demonstrated, and it remains unclear if the relatively lower prevalences observed in the eastern oyster are due to this species being a less suitable or permissive host for *P. chesapeakei* as compared to *P. marinus*.^{17,18} As CvGal1 and CvGal2 strongly bind to the *P. marinus* cell surface and promote parasite entry into the host,^{22,23} the negligible binding of CvGal2 to *P. chesapeakei* may explain its low prevalence relative to *P. marinus* in the eastern oyster and perhaps the reduced pathogenicity. The diversified binding specificity and biological

functions of CvGal1 and CvGal2 may provide an evolutionary advantage to the oyster against infectious challenge, but *P. marinus* has coevolved to subvert their role(s) to gain entry into the host. In this regard, *P. chesapeakei* would have instead coevolved with other bivalve hosts, including the aforementioned clam species. Thus, on the basis of our results, it is tempting to speculate that host preference and pathogenicity of *Perkinsus* species for sympatric bivalve species may be the result of a close coevolutionary process between a parasite species and a bivalve host. This would be determined by the adaptation of the parasite glycocalyx to specific host recognition factors, such as galectins CvGal1 and CvGal2, that would facilitate parasite entry into the preferred host such as the eastern oyster.⁶ Current studies in our laboratory are aimed at assessing potential differences in the binding of CvGal1 and CvGal2 to glycotopes on the hemocyte surface, *P. marinus* trophozoites, phytoplankton food, and microbial pathogens to address these unanswered questions.

■ ASSOCIATED CONTENT

■ Supporting Information

Figure S1: Toad galectin-1 (BaGal1) archetypical recognition of the galactoside and the model of the recognition of H2 epitope by human galectin-1. Table S1: Glycans profile of CvGal2 and other selective galectins. Table S2: Sequences used for phylogenetic analysis. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00362.

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Notes

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■ ABBREVIATIONS

CvGal2, *Crassostrea virginica* galectin 2; BaGal1, *Bufo arenarum* galectin-1; hGal-1, human galectin-1; CGL2, *Coprinus cinereus* galectin-2; CRD, carbohydrate recognition domain; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; LacNAc, N-acetyllactosamine; TDG, thiodigalactose; BSM, bovine submaxillary mucin; OSM, ovine submaxillary mucin; PSM, porcine stomach mucin; SPR, surface plasmon resonance; PRR, pattern recognition receptors; 2-ME, β -mercaptoethanol; PBS/2-ME, PBS containing 10 mM β -mercaptoethanol; PBST, 0.1% Tween in PBS; HBS, HEPES buffered saline; RACE, rapid

amplification of cDNA ends; FC, flow cytometry; PFA, paraformaldehyde

■ REFERENCES

- (1) Bachere, E., Gueguen, Y., Gonzalez, M., de Langeril, J., Garnier, J., and Romestand, B. (2004) Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. *Immunol. Rev.* 198, 149–168.
- (2) Boehm, T. (2012) Evolution of vertebrate immunity. *Curr. Biol.* 22, R722–732.
- (3) Janeway, C. A., Jr., and Medzhitov, R. (2002) Innate immune recognition. *Annu. Rev. Immunol.* 20, 197–216.
- (4) Vasta, G. R., Ahmed, H., and Odom, E. W. (2004) Structural and functional diversity of lectin repertoires in invertebrates, protochordates and ectothermic vertebrates. *Curr. Opin. Struct. Biol.* 14, 617–630.
- (5) Vasta, G. R., and Ahmed, H. (2008) *Animal Lectins: A Functional View*, CRC Press, Boca Raton, FL.
- (6) Vasta, G. R. (2009) Roles of galectins in infection. *Nat. Rev. Microbiol.* 7, 424–438.
- (7) Casadevall, A., and Pirofski, L. A. (2000) Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. *Infection and immunity* 68, 6511–6518.
- (8) Dame, R., Bushek, D., Allen, D., Lewitus, A., Edwards, D., Koepfler, E., and Gregory, L. (2002) Ecosystem response to bivalve density reduction: management implications. *Aquat. Ecol.* 36, 51–65.
- (9) Harvell, C. D., Kim, K., Burkholder, J. M., Colwell, R. R., Epstein, P. R., Grimes, D. J., Hofmann, E. E., Lipp, E. K., Osterhaus, A. D., Overstreet, R. M., Porter, J. W., Smith, G. W., and Vasta, G. R. (1999) Emerging marine diseases—climate links and anthropogenic factors. *Science* 285, 1505–1510.
- (10) Dungan, C. F., Hamilton, R. M., Hudson, K. L., McCollough, C. B., and Reece, K. S. (2002) Two epizootic diseases in Chesapeake Bay commercial clams, *Mya arenaria* and *Tagelus plebeius*. *Dis. Aquat. Org.* 50, 67–78.
- (11) Kennedy, V. S., Newell, R. I. E., and Eble, A. F., (1996) *The Eastern Oyster: Crassostrea virginica*, 2nd ed., University of Maryland Sea Grant Publications, College Park, MD.
- (12) Andrews, J. D. (1996) History of *Perkinsus marinus*, a pathogen of oysters in Chesapeake Bay 1950–1984. *Journal of Shellfish Research* 15, 13–16.
- (13) McLaughlin, S. M., Tall, B. D., Shaheen, A., Elsayed, E. E., and Faisal, M. (2000) Zoosporulation of a new *Perkinsus* species isolated from the gills of the softshell clam *Mya arenaria*. *Parasite* 7, 115–122.
- (14) Coss, C. A., Robledo, J. A., Ruiz, G. M., and Vasta, G. R. (2001) Description of *Perkinsus andrewsi* n. sp. isolated from the Baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of a species-specific PCR-based diagnostic assay. *J. Eukaryotic Microbiol.* 48, 52–61.
- (15) Bureson, E. M., Reece, K. S., and Dungan, C. F. (2005) Molecular, morphological, and experimental evidence support the synonymy of *Perkinsus chesapeakei* and *Perkinsus andrewsi*. *J. Eukaryotic Microbiol.* 52, 258–270.
- (16) Coss, C. A., Robledo, J. A., and Vasta, G. R. (2001) Fine structure of clonally propagated in vitro life stages of a *Perkinsus* sp. isolated from the Baltic clam *Macoma balthica*. *J. Eukaryotic Microbiol.* 48, 38–51.
- (17) Pecher, W. T., Alavi, M. R., Schott, E. J., Fernandez-Robledo, J. A., Roth, L., Berg, S. T., and Vasta, G. R. (2008) Assessment of the northern distribution range of selected *Perkinsus* species in eastern oysters (*Crassostrea virginica*) and hard clams (*Mercenaria mercenaria*) with the use of PCR-based detection assays. *J. Parasitol.* 94, 410–422.
- (18) Reece, K. S., Dungan, C. F., and Bureson, E. M. (2008) Molecular epizootiology of *Perkinsus marinus* and *P. chesapeakei* infections among wild oysters and clams in Chesapeake Bay, USA. *Dis. Aquat. Org.* 82, 237–248.
- (19) Bushek, D., Landau, B., and Scarpa, E. (2008) *Perkinsus chesapeakei* in stout razor clams *Tagelus plebeius* from Delaware Bay. *Dis. Aquat. Org.* 78, 243–247.

- (20) Bushek, D., Ford, S. E., and Chintala, M. M. (2002) Comparison of in vitro-cultured and wild-type *Perkinsus marinus*. III. Fecal elimination and its role in transmission. *Dis. Aquat. Org.* 51, 217–225.
- (21) Ford, S. E., Chintala, M. M., and Bushek, D. (2002) Comparison of in vitro-cultured and wild-type *Perkinsus marinus*. I. Pathogen virulence. *Dis. Aquat. Org.* 51, 187–201.
- (22) Tasumi, S., and Vasta, G. R. (2007) A galectin of unique domain organization from hemocytes of the Eastern oyster (*Crassostrea virginica*) is a receptor for the protistan parasite *Perkinsus marinus*. *J. Immunol.* 179, 3086–3098.
- (23) Feng, C., Ghosh, A., Amin, M. N., Giomarelli, B., Shridhar, S., Banerjee, A., Fernandez-Robledo, J. A., Bianchet, M. A., Wang, L. X., Wilson, I. B., and Vasta, G. R. (2013) The galectin CvGal1 from the eastern oyster (*Crassostrea virginica*) binds to blood group A oligosaccharides on the hemocyte surface. *J. Biol. Chem.* 288, 24394–24409.
- (24) Cooper, D. N. W. (2002) Galectinomics: finding themes in complexity. *Biochim. Biophys. Acta, Gen. Subj.* 1572, 209–231.
- (25) Vasta, G. R., Quesenberry, M., Ahmed, H., and O'Leary, N. (1999) C-type lectins and galectins mediate innate and adaptive immune functions: their roles in the complement activation pathway. *Dev. Comp. Immunol.* 23, 401–420.
- (26) Liao, D. I., Kapadia, G., Ahmed, H., Vasta, G. R., and Herzberg, O. (1994) Structure of S-lectin, a developmentally regulated vertebrate beta-galactoside-binding protein. *Proc. Natl. Acad. Sci. U. S. A.* 91, 1428–1432.
- (27) Ahmed, H., Du, S.-J., and Vasta, G. R. (2009) Knockdown of a galectin-1-like protein in zebrafish (*Danio rerio*) causes defects in skeletal muscle development. *Glycoconjugate J.* 26, 277–283.
- (28) Liu, F. T. (2000) Galectins: a new family of regulators of inflammation. *Clin. Immunol.* 97, 79–88.
- (29) Sato, S., Ouellet, N., Pelletier, I., Simard, M., Rancourt, A., and Bergeron, M. G. (2002) Role of galectin-3 as an adhesion molecule for neutrophil extravasation during streptococcal pneumonia. *J. Immunol.* 168, 1813–1822.
- (30) Barrionuevo, P., Beigier-Bompadre, M., Illarregui, J. M., Toscano, M. A., Bianco, G. A., Isturiz, M. A., and Rabinovich, G. A. (2007) A novel function for galectin-1 at the crossroad of innate and adaptive immunity: galectin-1 regulates monocyte/macrophage physiology through a nonapoptotic ERK-dependent pathway. *J. Immunol.* 178, 436–445.
- (31) Rabinovich, G. A., Rubinstein, N., and Toscano, M. A. (2002) Role of galectins in inflammatory and immunomodulatory processes. *Biochim. Biophys. Acta, Gen. Subj.* 1572, 274–284.
- (32) Novelli, F., Allione, A., Wells, V., Forni, G., and Mallucci, L. (1999) Negative cell cycle control of human T cells by beta-galactoside binding protein (beta GBP): induction of programmed cell death in leukaemic cells. *J. Cell. Physiol.* 178, 102–108.
- (33) Perillo, N. L., Pace, K. E., Seilhamer, J. J., and Baum, L. G. (1995) Apoptosis of T cells mediated by galectin-1. *Nature* 378, 736–739.
- (34) Vasta, G. R., Ahmed, H., Nita-Lazar, M., Banerjee, A., Pasek, M., Shridhar, S., Guha, P., and Fernandez-Robledo, J. A. (2012) Galectins as self/non-self recognition receptors in innate and adaptive immunity: an unresolved paradox. *Front. Immunol.* 3, 199.
- (35) Chu, F.-L. E. (1996) Laboratory investigations of susceptibility, infectivity, and transmission of *Perkinsus marinus* in oysters. *Journal of Shellfish Research* 15, 57–66.
- (36) Feng, C., Zhang, L., Nguyen, C., Vogel, S. N., Goldblum, S. E., Blackwelder, W. C., and Cross, A. S. (2013) Neuraminidase reprograms lung tissue and potentiates lipopolysaccharide-induced acute lung injury in mice. *J. Immunol.* 191, 4828–4837.
- (37) Fernández-Robledo, J. A., Lin, Z., and Vasta, G. R. (2008) Transfection of the protozoan parasite *Perkinsus marinus*. *Mol. Biochem. Parasitol.* 157, 44–53.
- (38) Gauthier, J. D., and Vasta, G. R. (1995) *In vitro* culture of the Eastern parasite *Perkinsus marinus*: optimization of the methodology. *J. Invertebr. Pathol.* 66, 156–168.
- (39) Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J. D., and Higgins, D. G. (2014) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7, 539.
- (40) Stamatakis, A. (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analysis with thousands of taxa and mixed models. *Bioinformatics* 22, 2688–2690.
- (41) Ahmed, H., Bianchet, M. A., Amzel, L. M., Hirabayashi, J., Kasai, K., Giga-Hama, Y., Tohda, H., and Vasta, G. R. (2002) Novel carbohydrate specificity of the 16-kDa galectin from *Caenorhabditis elegans*: binding to blood group precursor oligosaccharides (type 1, type 2, Talpha, and Tbeta) and gangliosides. *Glycobiology* 12, 451–461.
- (42) Bianchet, M. A., Ahmed, H., Vasta, G. R., and Amzel, L. M. (2000) Soluble beta-galactosyl-binding lectin (galectin) from toad ovary: crystallographic studies of two protein-sugar complexes. *Proteins: Struct., Funct., Genet.* 40, 378–388.
- (43) Case, D. A., et al. (2012) *AMBER 12*, University of California, San Francisco, CA.
- (44) Gerber, P. R., and Muller, K. (1995) MAB, a generally applicable molecular force field for structure modelling in medicinal chemistry. *J. Comput.-Aided Mol. Des.* 9, 251–268.
- (45) Stowell, S. R., Arthur, C. M., Dias-Baruffi, M., Rodrigues, L. C., Gourdiere, J. P., Heimburg-Molinaro, J., Ju, T., Molinaro, R. J., Rivera-Marrero, C., Xia, B., Smith, D. F., and Cummings, R. D. (2010) Innate immune lectins kill bacteria expressing blood group antigen. *Nat. Med.* 16, 295–301.
- (46) Levrony, E. L., Aguilar, H. C., Fulcher, J. A., Kohatsu, L., Pace, K. E., Pang, M., Gurney, K. B., Baum, L. G., and Lee, B. (2005) Novel innate immune functions for galectin-1: galectin-1 inhibits cell fusion by Nipah virus envelope glycoproteins and augments dendritic cell secretion of proinflammatory cytokines. *J. Immunol.* 175, 413–420.
- (47) Toledo, K. A., Fermino, M. L., del Cistia Andrade, C., Riul, T. B., Alves, R. T., Muller, V. D., Russo, R. R., Stowell, S. R., Cummings, R. D., Aquino, V. H., and Dias-Baruffi, M. (2014) Galectin-1 Exerts Inhibitory Effects during DENV-1 Infection. *PLoS One* 9, e112474.
- (48) Wang, X.-W., Xu, J.-D., Zhao, X.-F., Vasta, G. R., and Wang, J.-X. (2014) A shrimp C-type lectin inhibits proliferation of the hemolymph microbiota by maintaining the expression of antimicrobial peptides. *J. Biol. Chem.* 289, 11779–11790.
- (49) Okumura, C. Y., Baum, L. G., and Johnson, P. J. (2008) Galectin-1 on cervical epithelial cells is a receptor for the sexually transmitted human parasite *Trichomonas vaginalis*. *Cell. Microbiol.* 10, 2078–2090.
- (50) Kamhawi, S., Ramalho-Ortigao, M., Pham, V. M., Kumar, S., Lawyer, P. G., Turco, S. J., Barillas-Mury, C., Sacks, D. L., and Valenzuela, J. G. (2004) A role for insect galectins in parasite survival. *Cell* 119, 329–341.
- (51) Hager, K. M., and Carruthers, V. B. (2008) MARveling at parasite invasion. *Trends Parasitol.* 24, 51–54.
- (52) Petri, W. A., Jr., Haque, R., and Mann, B. J. (2002) The bittersweet interface of parasite and host: lectin-carbohydrate interactions during human invasion by the parasite *Entamoeba histolytica*. *Annu. Rev. Microbiol.* 56, 39–64.
- (53) Fernandez-Robledo, J. A., Schott, E. J., and Vasta, G. R. (2008) *Perkinsus marinus* superoxide dismutase 2 (PmSOD2) localizes to single-membrane subcellular compartments. *Biochem. Biophys. Res. Commun.* 375, 215–219.
- (54) Schott, E. J., and Vasta, G. R. (2003) The PmSOD1 gene of the protistan parasite *Perkinsus marinus* complements the *sod2Δ* mutant of *Saccharomyces cerevisiae*, and directs an iron superoxide dismutase to mitochondria. *Mol. Biochem. Parasitol.* 126, 81–92.
- (55) Vasta, G. R., Sullivan, J. T., Cheng, T. C., Marchalonis, J. J., and Warr, G. W. (1982) A cell membrane-associated lectin of the oyster hemocyte. *J. Invertebr. Pathol.* 40, 367–377.
- (56) Vasta, G. R., Cheng, T. C., and Marchalonis, J. J. (1984) A lectin on the hemocyte membrane of the oyster (*Crassostrea virginica*). *Cell. Immunol.* 88, 475–488.
- (57) Kurz, S., Jin, C., Hykollari, A., Gregorich, D., Giomarelli, B., Vasta, G. R., Wilson, I. B., and Paschinger, K. (2013) Hemocytes and plasma of the eastern oyster (*Crassostrea virginica*) display a diverse

repertoire of sulfated and blood group A-modified N-glycans. *J. Biol. Chem.* 288, 24410–24428.

(58) Dam, T. K., and Brewer, C. F. (2008) Effects of clustered epitopes in multivalent ligand-receptor interactions. *Biochemistry* 47, 8470–8476.

(59) Ng, K. K., Drickamer, K., and Weis, W. I. (1996) Structural analysis of monosaccharide recognition by rat liver mannose-binding protein. *J. Biol. Chem.* 271, 663–674.

(60) Holmskov, U., Thiel, S., and Jensenius, J. C. (2003) Collections and ficolins: humoral lectins of the innate immune defense. *Annu. Rev. Immunol.* 21, 547–578.

(61) Davicino, R. C., Elicabe, R. J., Di Genaro, M. S., and Rabinovich, G. A. (2011) Coupling pathogen recognition to innate immunity through glycan-dependent mechanisms. *Int. Immunopharmacol.* 11, 1457–1463.

(62) Vasta, G. R., Ahmed, H., Bianchet, M. A., Fernandez-Robledo, J. A., and Amzel, L. M. (2012) Diversity in recognition of glycans by F-type lectins and galectins: molecular, structural, and biophysical aspects. *Ann. N. Y. Acad. Sci.* 1253, 14–26.

(63) Houzelstein, D., Goncalves, I. R., Fadden, A. J., Sidhu, S. S., Cooper, D. N. W., Drickamer, K., Leffler, H., and Poirier, F. (2004) Phylogenetic analysis of the vertebrate galectin family. *Mol. Biol. Evol.* 21, 1177–1187.

(64) Lensch, M., Lohr, M., Russwurm, R., Vidal, M., Kaltner, H., Andre, S., and Gabius, H.-J. (2006) Unique sequence and expression profiles of rat galectins-5 and -9 as a result of species-specific gene divergence. *Int. J. Biochem. Cell Biol.* 38, 1741–1758.

(65) Allam, B., Ashton-Alcox, K. A., and Ford, S. E. (2002) Flow cytometric comparison of haemocytes from three species of bivalve molluscs. *Fish Shellfish Immunol.* 13, 141–158.

(66) Lin, Z., Fernandez-Robledo, J. A., Cellier, M. F., and Vasta, G. R. (2011) The natural resistance-associated macrophage protein from the protozoan parasite *Perkinsus marinus* mediates iron uptake. *Biochemistry* 50, 6340–6355.

(67) Schott, E. J., Pecher, W. T., Okafor, F., and Vasta, G. R. (2003) The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species. *Exp. Parasitol.* 105, 232–240.

(68) Hughes, R. C. (1999) Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim. Biophys. Acta, Gen. Subj.* 1473, 172–185.

(69) Partridge, E. A., Le Roy, C., Di Guglielmo, G. M., Pawling, J., Cheung, P., Granovsky, M., Nabi, I. R., Wrana, J. L., and Dennis, J. W. (2004) Regulation of cytokine receptors by Golgi N-glycan processing and endocytosis. *Science* 306, 120–124.

(70) Camby, I., Le Mercier, M., Lefranc, F., and Kiss, R. (2006) Galectin-I: a small protein with major functions. *Glycobiology* 16, 137R.

(71) Ohtsubo, K., and Marth, J. D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* 126, 855–867.

(72) Zick, Y., Eisenstein, M., Goren, R. A., Hadari, Y. R., Levy, Y., and Ronen, D. (2002) Role of galectin-8 as a modulator of cell adhesion and cell growth. *Glycoconjugate J.* 19, 517–526.

(73) Di Lella, S., Sundblad, V., Cerliani, J. P., Guardia, C. M., Estrin, D. A., Vasta, G. R., and Rabinovich, G. A. (2011) When galectins recognize glycans: from biochemistry to physiology and back again. *Biochemistry* 50, 7842–7857.

(74) Stowell, S. R., Arthur, C. M., Mehta, P., Slanina, K. A., Blixt, O., Leffler, H., Smith, D. F., and Cummings, R. D. (2008) Galectin-1, -2, and -3 exhibit differential recognition of sialylated glycans and blood group antigens. *J. Biol. Chem.* 283, 10109–10123.

(75) Stowell, S. R., Arthur, C. M., Slanina, K. A., Horton, J. R., Smith, D. F., and Cummings, R. D. (2008) Dimeric Galectin-8 induces phosphatidylserine exposure in leukocytes through poly(lactosamine) recognition by the C-terminal domain. *J. Biol. Chem.* 283, 20547–20559.

(76) Cooper, D. N., Boulianne, R. P., Charlton, S., Farrell, E. M., Sucher, A., and Lu, B. C. (1997) Fungal galectins, sequence and specificity of two isolectins from *Coprinus cinereus*. *J. Biol. Chem.* 272, 1514–1521.

(77) Walser, P. J., Haebel, P. W., Kunzler, M., Sargent, D., Kues, U., Aebi, M., and Ban, N. (2004) Structure and functional analysis of the fungal galectin CGL2. *Structure* 12, 689–702.

(78) Thomsson, K. A., Karlsson, H., and Hansson, G. C. (2000) Sequencing of sulfated oligosaccharides from mucins by liquid chromatography and electrospray ionization tandem mass spectrometry. *Anal. Chem.* 72, 4543–4549.

(79) Baenziger, J. U., and Fiete, D. (1979) Structure of the complex oligosaccharides of fetuin. *J. Biol. Chem.* 254, 789–795.