Molecular characterization and oligosaccharide-binding properties of a galectin from the argasid tick *Ornithodoros moubata*

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The argasid tick *Ornithodoros moubata* is a vector of various viral and borrelian diseases in animals and humans. We report here molecular characterization and oligosaccharide-binding properties of a novel galectin (*OmGalec*) from this tick. *OmGalec* consisted of 333 amino acids with a predicted molecular weight of 37.4 kDa. Its amino acid sequence did not contain a signal peptide or transmembrane domain. It possessed tandem-repeated carbohydrate recognition domains, in which the typical motifs important for carbohydrate affinity were conserved. *OmGalec* was expressed both transcriptionally and translationally at all stages of the tick life cycle and in multiple organs and was abundant in hemocytes, midguts, and reproductive organs, which are of importance in immunity, interaction with pathogens, and development, respectively, suggesting that *OmGalec* is a multifunctional molecule. The oligosaccharide affinity profile analyzed by applying an automated frontal affinity chromatography system revealed that r*OmGalec* showed a general feature of the galectin family, i.e. significant affinity for lactosamine-type disaccharides, Gaβ1-3(4)Glc(NAc), via recognition of 4-OH and 6-OH of galactose and 3 (4)-OH of Glc(NAc). Its preference for type I saccharides and α1-3GalNAc-containing oligosaccharides might provide clues for identifying its ligands and its potential multiple functions. Our results may contribute to the elucidation of galectin functions in the development and immunity of arthropods and/or vector and pathogen interaction and provide valuable information for the development of novel tick control strategies.

Key words: frontal affinity chromatography/galectin/ligand–receptor interaction/oligosaccharide affinity/*Ornithodoros moubata*

Introduction

*Ornithodoros moubata*, a hematophagous arachnid, is one of the most important argasid ticks found in eastern and western Africa (Walton 1962). There are egg, larva, four instars of nymphs, and adult stages in its life cycle, with multiple hosts at the various stages. *O. moubata* may transmit a variety of human and animal diseases during blood sucking, including tick-borne relapsing fever, Q-fever, African swine fever, and arboviral encephalitis caused by *Borrelia duttoni*, *Coxiella burnetii*, African swine fever virus, and West Nile virus, respectively (Plowright et al. 1970; Weyer 1975; Malatre et al. 1991; Dupont et al. 1997; Lawrie et al. 2004). It may also mechanically transmit hepatitis B virus and HIV (Humphery-Smith et al. 1993). Currently, chemical acaricides are widely used as the major tick control strategy, but it has caused various problems, such as acaricide resistance and environmental pollution. It is necessary to develop alternative tick control strategies, which requires better understanding of tick immunity, development, and the interaction between the vector and pathogens molecularly.

Previous studies have shown that arthropod hemolymph contains various plasma proteins involved in innate immunity, including pattern recognition lectins (Kanost et al. 2004). With an interest in novel genes possibly related with tick innate immunity, we performed immunoscreenings on cDNA expression library from *O. moubata*, using monoclonal antibody against plasma protein of the tick hemolymph. As a result, a novel gene with high homolog with galectin family was isolated and designated as *OmGalec* (GenBankTM/EBI Data Bank accession no. AB255165).

Galectins are characterized by an evolutionally conserved sequence motif in their carbohydrate recognition domain (CRD) and a specific affinity for β-galactosides and are widely recognized to be multifunctional molecules. By binding to a wide array of glycoconjugates, they mediate cell–cell and cell–extracellular matrix interactions and are thereby involved in diverse biological phenomena, such as development, differentiation, morphogenesis, tumor metastasis, apoptosis, and immunity (Perrillo et al. 1997; Cortegano et al. 1998; Liu and Rabinovich 2005). However, less is known about the number, nature, and function of galectins in arthropods; a few reported studies showed the presence of galectins in insects’ function in immunity, development, and
interaction between vectors and pathogens (Dimopoulos et al. 1998; Pace et al. 2002; Kamhawi et al. 2004; Pace and Baum 2004). Herein, we describe molecular characterization of OmGalec and its oligosaccharide-binding properties determined using an automatic frontal affinity chromatography (FAC) system, which has been shown to be rapid, sensitive, systematic, reliable, and quantitative, to analyze lectin and saccharide interaction (Hirabayashi et al. 2002; Hirabayashi et al. 2003; Nakamura et al. 2005). To our knowledge, this is the first study in the specific oligosaccharide-binding profile of a galectin from a disease-transmitting vector, which may provide valuable information for the elucidation of galectin functions in tick physiology and/or interaction between the tick and pathogens.

Results
Identification of OmGalec as tandem-repeat galectin by sequence analysis
The deduced amino acid sequence of OmGalec consists of 333 amino acid residues with a predicted molecular weight of 37.4 kDa and exhibited an identity of 36% to a midgut galectin-like protein from an ixodid tick Rhipicephalus appendiculatus (Figure 1A). As is characteristic of galectin family members, the sequence did not contain a classical secretion signal peptide, a transmembrane domain, or a Ca\(^{2+}\) domain. As depicted in Figure 1B, OmGalec contains two CRDs connected by a link peptide, classifying OmGalec as a tandem-repeat galectin (Hirabayashi and Kasai 1993). Both CRDs possess a typical motif containing amino acid residues conserved evolutionally in the galectin family and involved in carbohydrate-binding activity, which are His, Asn, Arg, Val, Asn, Trp, and Glu aligned as indicated in Figure 1B. The similarity between two CRDs is 32%, suggesting that they might have different sugar-binding specificities. The phylogenetic tree indicated that OmGalec is closely related to galecins from arthropods (Figure 1C).

Expression of OmGalec mRNA
As shown in Figure 2, OmGalec mRNA was detected at all developmental stages. The expression level was higher in larva, fourth instar nymph, and adult than in other stages. OmGalec mRNA expression was also found in multiple tissues and organs, including the hemocytes, midguts, ovaries, and salivary glands, and in the whole carcasses. It was more abundant in hemocytes, midguts, and ovaries than in other organs.

Identification of endogenous OmGalec protein
Endogenous OmGalec protein was examined by western blot analysis with murine antibody against rOmGalec. Murine antibody against glutathione S-transferase (GST) was used as a control. The result indicated that endogenous OmGalec was expressed at all developmental stages. Figure 3 shows the result of western blot analysis of the endogenous OmGalec in the first instar nymphs. The specific band of 37.4 kDa in lane 1 but not in the control, lane 2, indicated that the molecular mass of endogenous OmGalec corresponded to the predicted molecular mass.

Localization of endogenous OmGalec
The paraffin sections from both male and female ticks at the fourth instar nymph stage were prepared and examined by immunohistochemical (IH) analysis. As shown in Figure 4, specific reactions to murine antibody against rOmGalec were found in multiple organs (panels 1–4 in Figure 4), mainly in the midgut epithelia and lumen (panels 5 and 6), and in male and female reproductive organs, such as testes (panels 7 and 8), vasa deferentia, ovary, and oviduct (panels 9–12). Hemocytes were isolated from the tick hemolymph and detected by immunofluorescent antibody test (IFAT) with murine antibody against rOmGalec. As shown in panels 13 and 14 in Figure 4, strong specific reaction to antibody against rOmGalec was found in all hemocytes detected, suggesting that there was a high-level expression of endogenous OmGalec in hemocytes.

Hemagglutination activity of recombinant OmGalec and lactose inhibition
Hemagglutination activity is a requirement for categorization as a lectin, while the ability to bind β-galactoside oligosaccharides is the defining feature of galectin family members. Therefore, the hemagglutination assay was first carried out using trypsin-treated rabbit erythrocytes. The results suggested that the minimum concentration of rOmGalec that caused agglutination was 0.5 μM. Lactose inhibited hemagglutination caused by rOmGalec in a dose-dependent manner (Figure 5).

Lactose affinity of recombinant and endogenous OmGalec
As shown in Figure 6, both rOmGalec and endogenous OmGalec were pulled down by lactose–agarose but not by fucose–agarose, suggesting that both recombinant OmGalec and endogenous OmGalec possess specific affinity for lactose, which preliminarily identified OmGalec as a galectin member with respect to its bioactivity.

Oligosaccharide-binding specificity
An automated FAC system was used to further explore the oligosaccharide affinity properties of rOmGalec. In this study, 96 oligosaccharides with various structures (Figure 7) were analyzed. Using p-methoxyphenyl-β-Forssman pentasaccharide (Figure 8A), effective content (B\(_t\)) of rOmGalec was determined via concentration-dependence analysis (Figure 8B and C). The affinity constants (K\(_a\)) toward these 96 oligosaccharides were calculated according to the equation K\(_a\) = 1/K\(_d\) = (V – V\(_0\))/B\(_t\) (Arata et al. 2001) and are shown in Figure 8D, which could be summarized as follows:

1. In total, 23 oligosaccharides had detectable affinity for rOmGalec, all of which contained Galβ1-3/4Glc(NAc) or Galβ1-3GalNAc structures, suggesting that these structures are necessary for affinity between rOmGalec and glycans.
2. rOmGalec had no detectable affinity for glycans of high-mannose type (002 ~ 017), agalacto-type (101 ~ 201), and others (906 ~ 908), because they did not contain β-galactosides.
3. rOmGalec had no detectable affinity for any glycan containing Lewis (Le)-type chains, Galβ1-3(Fucα1-4) GlcNAc or Galβ1-4(Fucα1-3)GlcNAc. Comparisons in
Amino acids are numbered on both sides. Identical and similar amino acids are shaded in black and gray, respectively. (B) Schematic structure of OmGalec shows N- and C-terminal CRDs (N-CRD and C-CRD, respectively). They are located in tandem and connected by a link peptide. The residues marked with * are part of a typical motif conserved in the galectin family which is involved in carbohydrate-binding activity. (C) A phylogenetic tree comparing OmGalec with the selected tandem-repeat galectins from invertebrate and vertebrate animals. Om (AB255165) from O. moubata; Ra (AAO60051) from R. appendiculatus; Dm (NP_608487.1) from D. melanogaster; Pp (AA11557) from P. papatasi, Hs4 (NP_006140), Hs8 (AAF19370), Hs9 (NP_033665), and Hs12 (NP_149092) are galectins from Homo sapiens; Ce1 (NP_496801) from Caenorhabditis elegans; Mm4 (NP_034836) and Mm9 (NP_034838) from Mus musculus; Ag (EAA06231) from Anopheles gambiae str. PEST; Rn4 (NP_037107) and Rn9 (NP_037109) from Rattus norvegicus; Hc (AAD11972) from Haemonchus contortus; and Ss4 (Q29058) from Sus scrofa.

Fig. 1. Identification of OmGalec as a tandem-repeat galectin by sequence analysis. (A) Amino acid sequence of OmGalec and its ClustalW alignment with some other tandem-repeat galectins from arthropods and humans. Amino acids are numbered on both sides. Identical and similar amino acids are shaded in black and gray, respectively. (B) Schematic structure of OmGalec shows N- and C-terminal CRDs (N-CRD and C-CRD, respectively). They are located in tandem and connected by a link peptide. The residues marked with * are part of a typical motif conserved in the galectin family which is involved in carbohydrate-binding activity. (C) A phylogenetic tree comparing OmGalec with the selected tandem-repeat galectins from invertebrate and vertebrate animals. Om (AB255165) from O. moubata; Ra (AAO60051) from R. appendiculatus; Dm (NP_608487.1) from D. melanogaster; Pp (AA11557) from P. papatasi, Hs4 (NP_006140), Hs8 (AAF19370), Hs9 (NP_033665), and Hs12 (NP_149092) are galectins from Homo sapiens; Ce1 (NP_496801) from Caenorhabditis elegans; Mm4 (NP_034836) and Mm9 (NP_034838) from Mus musculus; Ag (EAA06231) from Anopheles gambiae str. PEST; Rn4 (NP_037107) and Rn9 (NP_037109) from Rattus norvegicus; Hc (AAD11972) from Haemonchus contortus; and Ss4 (Q29058) from Sus scrofa.

Pairs of 419 and 313, 420 and 323, 721 and 720, 726 and 724, 730 and 728, and 731 and 729 suggested α1-3 (4) fucosylation to GlcNAc diminished the affinity of rOmGalec to the glycans.

rOmGalec had no detectable affinity for α2-6 sialylated glycans. The comparisons between 313 and 504, 313 and 506, and 701 and 704 suggested that α2-6 sialylation diminished the glycans’ affinity for rOmGalec. For
were used as controls. The transcription profiles of 18S rRNA (lane 1), midguts (lane 2), ovaries (lane 3), salivary glands (lane 4), and the PCR was carried out on mRNA from different organs, including hemocytes and in different organs. (316 stages.

Fig. 2. Transcriptional profiles of OmGalec at different developmental stages and in different organs. (A) RT-PCR was performed on mRNA extracted from O. moubata at different developmental stages. Lanes 1–7 represent egg, larva, 1–4 instars of nymph, and adult (female) stages, respectively. (B) RT-PCR was carried out on mRNA from different organs, including hemocytes (lane 1), midguts (lane 2), ovaries (lane 3), salivary glands (lane 4), and the remaining of the carcasses (lane 5). The transcription profiles of 18S rRNA were used as controls.

Identification of endogenous OmGalec. Lysate of ticks from first instar of nymphs was analyzed by western blot analysis using murine antibody against rOmGalec (lane 1). Murine antibody against GST was used as a control (lane 2). Lane 1 shows that endogenous OmGalec with a molecular mass of 37.4 kDa was detected. Standard molecular masses are shown on the left. The same result was also seen at all other developmental stages.

The glycolipid-type glycans, in which 4-OH of Gal were substituted by GalNAc or Gal (702, 708, and 715), Kₐ was not detectable. The results suggest that 4-OH and 6-OH groups of Gal are necessary for the affinity.

5 Kₐ of rOmGalec for type I (Galβ1-3GlcNAc) oligosaccharides was much higher than that for type II (Galβ1-4GlcNAc) ones, as shown by comparisons of Kₐ between isomers (314 > 313, 728 > 724, and both 732 and 733 > 734). These results suggest that rOmGalec bound preferentially to glycans with type I chains. Moreover, Kₐ for 302 ~ 307 was below the limit of detection, which might be due to the fact that rOmGalec showed rather low affinity for the complexity-type N-glycans with type II chains. However, the Kₐ values of samples 313 and 323, which are triantennary and tetraantennary glycans with type II chains, were both detectable, suggesting that the affinity was enhanced as the number of Galβ1-4GlcNAc branches increased, the so-called “glycoside clustering effect” (Hirabayashi et al. 2002).

6 rOmGalec had detectable affinity for sample 722, Fucox1-2(Galα1-3)Galβ1-4Glc (B-tetrasaccharide), but not 723, Fucox1-2(Galα1-3)Galβ1-4(Fucα1-3)Glc (B-pentasaccharide). Structural comparison between them indicated that the third hydroxyl group of GlcNAc is required for the binding, which is also evident from the comparison between 728 and 730.

7 rOmGalec had detectable affinity for some oligolactosamines (901 ~ 903), with the highest Kₐ for that with 2 N-acetyllactosamine units (902). However, the affinity decreased when the repeat number of N-acetyllactosamine unit reached 3. No detectable affinity was found for oligolactosamine with 5 N-acetyllactosamine units.

8 rOmGalec had detectable affinity for single Galβ1-3Lac structure (735). However, it had no detectable affinity to oligosaccharides with repetition of the Galβ1-3 unit (736 ~ 738).

9 The affinity of rOmGalec was not increased by α1-2 fucosylation of the nonreducing terminal galactose of LMT (729 = 728). However, modification with α1-3GalNAc at this galactose (720) clearly enhanced the affinity for rOmGalec. The preference of rOmGalec for α1-3GalNAc-containing glycolipid-type glycans was further demonstrated by the extremely high affinity for Forssman (717).

10 rOmGalec had moderate affinity for both A- and B-tetrasaccharides (719 and 722, respectively).

Discussion

In this study, we describe a tick galectin isolated from cDNA library of O. moubata. Its native expression and localization in the tick were confirmed at both transcription and translation levels. Its molecular characterizations and oligosaccharide-binding properties identified OmGalec as a member of galectin family and suggested that it might, like mammalian galectins, mediate cell–cell communication or trigger signal transduction events (Baum et al. 1995; Liu 2000; Pace et al. 2000).

OmGalec was found at all developmental stages of the tick and in multiple organs, as detected in adult ticks, suggesting that OmGalec may have essential and important functions that are common to many cell types. A galectin from Drosophila melanogaster was suggested to be involved in the regulation of embryogenesis (Pace et al. 2002). A putative galectin homolog was upregulated significantly in the human malaria parasite Plasmodium berghei (Dimopoulos et al. 1997; Richman et al. 1997; Dimopoulos et al. 1998), suggesting that the galectin participates in mosquitoes’ innate immunity. In addition, a galectin from the gut of the sand fly Phlebotomus papatasii was reported to mediate the interaction between the vector and Leishmania protozoan parasites, which was specific and thought to facilitate the adherence of the parasites to the epithelial cells of the midgut (Kamhawi et al. 2004). The distribution of endogenous OmGalec in embryos, hemocytes, and midguts will encourage us to carry out further studies to test whether it plays roles in embryogenesis, innate immunity, and interaction with pathogen.

Previous studies showed that the first reported tick lectin Dorin M was also isolated from the hemolymph plasma of this tick and presented in the hemocytes (Kovar et al. 2000; Rego...
et al. 2006). The other lectin-like protein presented in the hemocytes is OMFREP. Both of them are fibrinogen-related proteins and have similarity with tachylectins-5. They are thereby considered to play a role in innate immunity (Rego et al. 2005, 2006). OmGalec has no significant similarity with these two lectin or lectin-like proteins. However, by the recognition of different patterns of glycan epitopes, they might all play similar roles in innate immunity. OmGalec contains two

**Fig. 4.** Localization of endogenous OmGalec. Panels with odd numbers show the results detected using murine antibody against rOmGalec. The other specimens were probed using antibody against GST as a control. (1)–(12), tick paraffin sections examined by IH, OmGalec was stained brown; (1) and (2), female tick sections; (3) and (4), male tick sections; (5) and (6), midgut (MG); (7) and (8), testes (TS); (9) and (10), ovaries (OV) and muscles (MS); (11) and (12), oviduct and salivary gland (OD and SG, respectively); (13) and (14), localization of OmGalec in hemocytes isolated from adult tick. OmGalec was stained green with FITC conjugate, whereas nuclei were stained blue with DAPI for background. The results suggested that OmGalec was expressed in multiple organs and was especially abundant in the midguts and reproductive organs, as well as hemocytes.
functional elucidation of OmGalec. In this study, an automated FAC system was used to screen the specific binding of rOmGalec to 96 oligosaccharides of various structures, including N-linked glycoprotein type, glycolipid type, and some other types. The affinity profile of rOmGalec revealed that OmGalec had the general feature of the galectin family, that is, significant affinity for lactosamine-type disaccharides, Galβ1-3(4)GlcNAc, via recognition of 4-OH and 6-OH of galactose and 3 (4)-OH of GlcNAc (Hirabayashi et al. 2002). The affinity profile also showed a preference for OmGalec for type I structure and extremely high affinity for α1-3GalNAc-containing glycolipid type glycans, such as Forssman, A-hexa, and A-tetra, suggesting that OmGalec has some specialized features similar to those of galectins clone 1 and clone 2 from the sponge Geodia cydonium (Peifer et al. 1993; Hirabayashi et al. 2002). In sponges, galectins can trigger an alternative complement activation pathway, a host defense system (Lagueux et al. 2000), which may be conserved in the argasid tick O. moubata. All these features might provide clues for our searches for in vivo ligands and functions of this galectin.

It is noteworthy that rOmGalec had no detectable affinity for poly Galβ1-3 structures (736 ~ 738) although it showed detectable affinity for the single unit of Galβ1-3 (735). This property is different from those of human galectin 3, galectin 9 (Pelletier et al. 2003), and possibly a galectin from the sand fly P. papatasi, which possess high affinity for the (Galβ1-3)3Lac structure (Kamhawi et al. 2004). Moreover, this poly-β-galactosyl structure on lipopolysaccharide of Leishmania major was reported to be an epitope for the interactions between the parasite and its vector, P. papatasi, and between the parasite and its host, human beings. Our unpublished data also revealed that a galectin from an ixodid tick Haemaphysalis longicornis, a vector of the hemaprotozoan Babesia parasite, has affinity for (Galβ1-3)3Lac structures, which is enhanced proportionately with the increasing number of Galβ1-3 units. It is also interesting that rOmGalec had detectable affinity for some polylactosamines (901 ~ 903), an epitope required for galectin-1-induced T-cell death after HIV-1 infection (Lanteri et al. 2003). Since O. moubata is not a vector of protozoa such as Babesia parasites but a vector for some virus diseases, more in vivo experiments on the tick–virus interaction should be carried out to test whether the sugar-binding specificity of OmGalec plays a role in the tick–pathogen interaction and the host specificity.

In conclusion, we characterized a novel galectin and analyzed its oligosaccharide-binding properties in detail by FAC analysis in this study, which have provided valuable information for studies on OmGalec and ligand interaction in the tick or between the tick and pathogens. Further in vivo experiments are necessary to obtain direct data for the better understanding of the possible functions of this interaction in tick development, immunity, and vector–pathogen interaction, so as to facilitate the control of the disease vector and the blockage of disease transmission.

**Materials and methods**

**Maintenance of ticks**

The argasid tick *O. moubata* was maintained at 25 °C and 50–60% relative humidity in continuous darkness (Inoue...
et al. 2001). Blood feeding was performed on ears of rabbits. The engorged ticks were then returned to the containers at 25 °C for development.

**Animals**

All animals used in the study were acclimatized to the experimental conditions for 2 weeks prior to the experiment. Animal experiments were conducted in accordance with the protocols approved by the Animal Care and Use Committee, National Institute of Animal Health (Approval nos 441, 508, 578), or the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (Approval nos 6-42, C-2).
Production and purification of murine monoclonal antibody

Briefly, plasma proteins from hemolymph of the fourth instar nymphs 6 days postengorgement were mixed with the Freund complete adjuvant (Difco, Detroit, MI) at a dose of 100 μg and used for an intraperitoneal injection of a BALB/c mouse (7 weeks old, female). Successive immunizations were carried out intraperitoneally 2, 3, 4, 6, and 8 weeks later using the same dose of proteins mixed with the Freund incomplete adjuvant (Difco). An additional injection of the tail vein of the mouse with the same proteins was performed 74 days after the primary immunization. Three days after the last injection, mouse spleen cells were fused with SP2/myeloma cells for hybridoma production. Hybridoma culture, screening for hybridoma-producing antibodies, and purification of monoclonal antibodies were performed as described previously (Liao et al. 2005).
Construction and immunoscreening of cDNA expression library, cDNA sequencing, and homology searching

Total RNA was extracted from the fourth instar nymphs 6 days postengorgement by using the acid guanidinium–phenol–chloroform method. Poly (A)+ RNA was purified from the total RNA, using Oligotex-dT30 (Takara Bio Inc., Otsu, Japan). A cDNA expression library was constructed from 5 μg of the purified mRNA, as described previously (Liao et al. 2005). The library was screened by using murine monoclonal antibody against plasma proteins. For the positive clones, the cDNA insert was excised in vivo from lambda phage and inserted into the plasmid pBluescript SK (+) according to the manufacturer’s instructions (Stratagene, La Jolla, CA). DNA was sequenced on ABI PRISM 377 DNA sequencer, using a Dye primer cycle sequencing kit (Perkin Elmer, Foster, CA). Homology searching was carried out using the BLAST program (National Center for Biotechnology Information, Bethesda, MD). The inserts with high homology to insect galectin cDNA were designated as OmGalec. Signal peptide and transmembrane domain prediction were carried out by using the CBS program Signal P 3.0 (Bendtsen et al. 2004) and TMHMM v. 2.0 (Center for Biological Sequence Analysis, Technical University of Denmark, Copenhagen, Denmark).

Subcloning of OmGalec into bacterial expression vector pGEX-4T-3

The OmGalec gene was amplified by polymerase chain reaction (PCR) using a set of oligonucleotides, 5’ACGAATTCCTAG TATCGTATGCCCTCT3’ and 5’GACTGAAGTCACAGGA CTCACAGAG3’, as sense and antisense primers. The nucleotide sequences of the primers included EcoRI or Xhol restriction enzyme site (as indicated by underline) and their corresponding positions on the cDNA. The OmGalec was ligated into the EcoRI–Xhol site of a bacterial expression vector, pGEX-4T-3, and transformed into Escherichia coli DH5α strain. The resultant recombinant plasmid was designated as pGEX-4T/OmGalec.

Expression of recombinant OmGalec in E. coli

A clone of E. coli transformed with the recombinant plasmid was cultured in Luria broth medium and then induced to express recombinant OmGalec (rOmGalec) by the addition of 0.5 mM isopropyl-S-β-thiogalactopyranoside. rOmGalec was a fusion protein of OmGalec with GST. It was extracted and purified from the soluble fraction of the bacterial lysate by using glutathione Sepharose 4B (GE Healthcare Bio-Science AB, Uppsala, Sweden), as described previously (Chang et al. 2003).

Production of murine polyclonal antibody against rOmGalec

BALB/c CrSlc mice (SLC Japan, Hamamatsu, Japan) were divided into two groups. Mice in the first group were inoculated intraperitoneally once with rOmGalec at a dose of 80 μg/mouse mixed with an equal volume (200 μL/mouse) of the Freund complete adjuvant (Difco). Two booster immunizations were performed at an interval of 14 days with the same antigens mixed with Freund incomplete adjuvant (Difco). Mice in a second group were immunized with GST as a control, using the same procedures as those for the first group. All mice were bled 10 days after the second boost.

Examination of OmGalec mRNA expression

For detecting the expression at different developmental stages, mRNA was extracted from ticks at each stage, using a Quick Prep Micro mRNA Purification kit according to the manufacturer’s instructions (GE Healthcare). To detect the OmGalec distribution in different organs, mRNA was extracted from hemocytes, midguts, ovaries, salivary glands, and carcasses of 10 adult female ticks. Reverse transcription (RT)-PCR was performed using an RNA amplification kit according to the manufacturer’s instructions (Takara Bio Inc.) with 15 ng of mRNA as a template for each 10 μL of reaction mixture. The reaction mixture was incubated at 30 °C for 10 min, 50 °C for 30 min, 95 °C for 2 min, and 5 °C for 5 min. The PCR conditions were 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s, followed by 10 min of extension at 72 °C. 18S rRNA was used as a control.

Western blot analysis

The ticks from each stage were homogenized separately in a small mortar frozen in liquid nitrogen, and the homogenate was then subjected to sonication. The tick lysate was then analyzed by western blot analysis (Chang et al. 2003) with murine antibody against rOmGalec or against GST (1:100) as the primary antibody and alkaline phosphatase-conjugated goat antimouse IgG (ICN Biomedicals, Irvine, CA) as the secondary antibody (1:1000). The reaction was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Promega, Madison, WI).

Immunohistochemistry

Paraffin sections were prepared from the fourth instar nymphs, as described previously (You et al. 2003). After the sections were deparaffinized and rehydrated, the IH test was performed using the Pathostain ABC-POD (M) kit (Wako) according to the manufacturer’s instructions. The primary antibody was murine antiserum against rOmGalec diluted (1:100) in PBS containing 3% normal goat serum. Murine antiserum against GST was used as a negative control. The color was developed by incubation with 3,3’-diaminobenzidine (Sigma, St Louis, MO) solution containing 0.03% H2O2. After dehydration and clearance, the sections were observed under a Leica IM500 microscope (Leica, Wetzlar, Germany).

Immunofluorescent antibody test

Hemocytes isolated from the hemolymph of adult tick were detected using murine antibodies against rOmGalec or GST (diluted 1:100 in PBS containing 3% fetal bovine serum). Fluorescein isothiocyanate-conjugated antiserum IgG (H + L) (1:400) (Jackson Immunoresearch, West Grove, PA) was used as the secondary antibody. The samples were mounted in mounting medium with DAPI (Vector, Burlingame, CA) and observed under a Leica DMI 6000B fluorescence microscope. Images were collected by using Leica FW4000 software.

Hemaggulination assay and lactose inhibition

The hemaggulination assay was carried out as described previously (Dunphy et al. 2002). Briefly, rabbit erythrocytes were treated with 0.1% trypsin for 30 min at 37 °C and fixed in 0.05% glutaraldehyde solution for 30 min. The cells were suspended in PBS containing 2 mM ethylenediaminetetraacetic...
acid and 2 mM dithiothreitol as a 4% suspension. Serial 2-fold dilutions of rOmGalec were added to 96-well U-bottom microtiter plates (BM Bio, Tokyo, Japan), 25 μL/well. Then, 0.5% bovine serum albumin (BSA) (50 μL/well) and 4% erythrocytes (25 μL/well) were added and mixed gently. The result was recorded after incubation at RT for 1 h. For the detection of lactose inhibition, serial 2-fold dilutions of lactose were mixed with rOmGalec and subjected to the same protocol, as described for the hemagglutination assay. Four disaccharides, trehalose, cellobiose, sucrose, and maltose, and a monosaccharide fucose were used as negative control in the inhibition assay.

Detection of lactose affinities of recombinant and endogenous OmGalec
Bacterial lysate of E. coli-expressing rOmGalec and tick lysate homogenized from the first instar nymphs in PBS containing 1 mM dithiothreitol (Nacalai, Kyoto, Japan) and complete protease inhibitor (Roche, Mannheim, Germany) were centrifuged at 10 000 g for 10 min and then each supernatant was divided into two aliquots, each of which was incubated with lactose–agarose and fucose–agarose (Seikagaku, Tokyo, Japan), respectively, at 4°C for 1 h. After three washes with PBS, materials bound to the agarose were eluted with 200 mM lactose and fucose (Nacalai), respectively. The eluted fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by silver staining and western blot analysis using antisera against rOmGalec and GST, respectively.

Preparation of column for FAC
The purified rOmGalec was dialyzed against coupling buffer (0.1 M NaHCO3, pH 8.3, containing 0.5 M NaCl) and coupled to N-hydroxysuccinimide-activated Sepharose according to the manufacturer’s instructions (GE Healthcare). Then, the Sepharose was blocked with an excess amount of ethanolamine and subjected to three cycles of washes with 0.1 M acetic acid (pH 4.0) containing 0.5 M NaCl and coupling buffer alternately. The resultant Sepharose was resuspended in 10 mM Tris–HCl (pH 7.4) containing 0.8% NaCl and packed into a stainless steel miniature column (inner diameter 2 mm, length 10 mm, and bed volume 31.4 μL).

Sources of oligosaccharides
Of 77 pyridylaminated (PA)-oligosaccharides, 63 (002–014, 103, 105, 107, 307, 313, 314, 323, 405, 410, 418, 419, 420, 503, 701–703, 705–713, 715–721, 724, 726, and 728–731) were from Takara Bio Inc. and 14 from Seikagaku Co. Of 19 nonlabeled glycans, 727 was from Funakoshi Co. (Tokyo, Japan), 906 and 907 were from Seikagaku Co., 733, 734, and 908 from Dextra Laboratories, Ltd (Reading, UK), 725, 909, and 910 from Calbiochem (Darmstadt, Germany); and 722, 723, 732, and 735–739 were generous gifts from Dr K. Yoshida (Seikagaku) and Dr T. Urashima (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan). These nonlabeled glycans were pyridylaminated with GlycoTag (Takara Bio Inc.).

FAC analysis
The principles and procedures of FAC analysis were described previously (Hirabayashi and Kasai 1993; Nakamura et al. 2005). In brief, when a fluorescence-labeled glycan is continually applied to a rOmGalec column, if it has no affinity for rOmGalec, it will flow out of the column at its original speed, with no change in fluorescent signals. However, if it has affinity for rOmGalec, the interaction will cause the fluorescent signal to be retarded. By measurement of the retarded volume in terms of $V - V_0$ and detection of the effective concentration of rOmGalec, the affinity constant can be calculated. The analysis was performed by using a FAC-1 (Shimadzu, Kyoto, Japan), a recently developed machine for automated FAC. In our preliminary test, Forsmann was proved to have strong affinity to rOmGalec column at the concentration of 5 nM, whereas it showed no significant binding to a BSA-agarose column. Therefore, for the determination of the effective ligand content ($B_0$), concentration-dependence analysis and Woolf–Hofstee-type plot analysis were performed with p-methoxyphenyl-β-Forsmann pentasaccharide (Tokyo Chemical Industry Co. Ltd, Tokyo, Japan) at various concentrations ranging from 5 to 20 μM, as described previously (Hirabayashi et al. 2003). The elution was monitored using a UV detector (Shimadzu, SPD-10A VP) at 280 nm. PA-oligosaccharides, including 45 N-linked type (2.5 nM) and 38 glycolipid type and 13 others (5 nM), were dissolved in tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.4, containing 0.8% NaCl) and used as ligands. They were successively injected into the rOmGalec-column using the auto-sampling system. The flow rate and the column temperature were kept at 0.125 mL/min and 25°C, respectively. TBS was used as the washing buffer. The elution of PA-oligosaccharides was monitored using a fluorescence detector (Shimadzu, RF10AXL) with excitation and emission wavelengths of 310 and 380 nm, respectively. The results were analyzed using FAC ASCII DATA analyzer version 5.1.2. Affinity constants ($K_a$) were calculated according to the equations $K_a = B_0/(V - V_0)$ ($A_0 << K_0$) and $K_a = 1/K_0$ ($K_0$, dissociation constant; $B_0$, effective ligand content in the column; $V - V_0$, retarded volume of elution; and $A_0$, original concentration of glycan). Considering the data collection interval (1 s) and the flow rate (0.125 mL/min), the theoretical experimental error should be 2 μL in the $V - V_0$. However, we estimated the experimental error, using the average value of $V - V_0$ of rOmGalec for 20 glycans with no galactosides plus 2 times of standard deviation. It was 3.3 μL. So, when $K_a$ was calculated, the value of $V - V_0$ was the difference of the reading value and the estimated experiment error.

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Conflict of interest statement
None declared.

Abbreviations
BSA, bovine serum albumin; CRD, carbohydrate recognition domain; FAC, frontal affinity chromatography; GST, glutathione S-transferase; IFAT, immunofluorescent antibody test; IH, immunohistochemical assay; OmGalec, galectin from *Ornithodoros moubata*; PA, pyridylamminated; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; TBS, Tris-buffered saline.

References


