Characterization of a Novel Drosophila melanogaster Galectin

EXPRESSION IN DEVELOPING IMMUNE, NEURAL, AND MUSCLE TISSUES*

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We have cloned and characterized the first galectin to be identified in Drosophila melanogaster. The amino acid sequence of Drosophila galectin showed striking sequence similarity to invertebrate and vertebrate galectins and contained amino acids that are crucial for binding β-galactoside sugars. Confirming its identity as a galectin family member, the Drosophila galectin bound β-galactoside sugars. Structurally, the Drosophila galectin was a tandem repeat galectin containing two carbohydrate recognition domains connected by a unique peptide link. This divergent structure suggests that like mammalian galectins, Drosophila galectin may mediate cell-cell communication or facilitate cross-linking of receptors to trigger signal transduction events. The Drosophila galectin was very abundant in embryonic, larval, and adult Drosophila. During embryogenesis, Drosophila galectin had a unique and specific tissue distribution. Drosophila galectin expression was concentrated in somatic and visceral musculature and in the central nervous system. Similar to other insect lectins, Drosophila galectin may function in both embryogenesis and in host defense. Drosophila galectin was expressed by hemocytes, circulating phagocytic cells, suggesting a role for Drosophila galectin in the innate immune system.

Many biological processes have been elucidated using Drosophila melanogaster as a model system. However, little is known about lectin-ligand interactions in Drosophila. Of the few Drosophila lectins that have been identified, a subset has been shown to be vital in embryogenesis and to function in innate immunity (1–5).

In embryonic Drosophila, two lectins, glicoelectin and a selectin homologue, have been identified and determined to play a role in embryogenesis (4, 5). Glicoelectin mediates cell-cell interactions that may be required for the formation of axonal commissures during nervous system development (4). Mutations in the selectin homologue lead to profound defects in eye and mechanosensory bristle development (5). Stage-specific regulation of the expression of specific glycoconjugates also occurs during Drosophila development, further suggesting important developmental roles for lectins (6–10).

Other lectins may have functions beyond development (1–3, 11). In larval and adult Drosophila, a C-type lectin has been identified (3). The expression of this C-type lectin is up-regulated following injury, suggesting that it has a role in the innate immune system. Other insects rely on lectins for recognition and phagocytosis of invading microorganisms and for the modulation of hemocyte aggregation during an immune response (1, 2, 11–13). Because Drosophila express diverse and complex oligosaccharide structures whose expression is spatially and temporally regulated (6), it is likely that these structures are recognized by additional specific lectins that remain to be identified. In support of this, a BLAST analysis of the Berkeley Drosophila Genome Project with consensus sequences from various lectin families has identified at least 21 putative Drosophila lectins, including a possible galectin homologue (6, 14, 15).

Galectins are an evolutionarily conserved lectin family that have been identified in mammals, birds, amphibians, reptiles, fish, nematodes, marine sponges, and multicellular fungi (15, 16). In some species there are a large number of galectin family members; 13 galectins have been identified in mammals (15, 17–19). However, a galectin homologue has not been definitively identified in D. melanogaster (15). In vertebrates, galectins are involved in a variety of cellular processes that determine cell fate, by mediating cell-cell interactions, inducing cell proliferation, or regulating cell death (20–23). For example, during mammalian brain development, galectin-1 promotes olfactory neuron fasciculation by cross-linking adjacent axons and promoting axonal adhesion to the extracellular matrix (24, 25). Galectins also maintain vertebrate immune system homeostasis and may be a vital component of the innate immune system in insects and mammals (12, 20, 22, 23, 26–28).

Given the large number of mammalian galectins, genetic approaches in mice may not elucidate all of the functions of specific galectins. The facile genetic analysis that is possible in Drosophila and the apparently small number of putative galectins in the Drosophila genome would simplify the examination of in vivo functions of galectins. We have cloned and characterized the first identified Drosophila galectin (Dmgal),1 and we have examined its distribution during embryogenesis and in the immune system.

1 The abbreviations used are: Dmgal, Drosophila melanogaster galectin; RACE, rapid amplification of cDNA ends; GSP, gene-specific primer; EST, expressed sequence tag; DTT, dithiothreitol; ECL, enhanced chemiluminescence; CRD, carbohydrate recognition domain.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF338142.

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**RESULTS**

Isolation of Dmgal cDNA—A partial EST with amino acid sequence similarity to the carbohydrate recognition domain of galectin family members was identified (15). To isolate the entire cDNA, 5'- and 3'-RACE were performed using gene-specific primers directed against the segments that showed greatest similarity to galectin, and *Drosophila* larval poly(A)⁺ RNA as a template. The Dmgal gene is located on chromosome 2L, 21A5 (32). The deduced amino acid sequence encoded by the complete cDNA is shown in Fig. 2.

The amino acid sequence contained important elements that are required for a protein to be defined as a galectin family member (33). Specifically, Dmgal contained two domains that had sequence similarity to the canonical carbohydrate recognition domains (CRD) of galectin family members (Fig. 1). Both CRDs contained the conserved sequence motifs H-NPR and WG-ER that are important for the binding of galectins to β-galactoside sugars (Fig. 1) (34), in contrast to the mammalian tandem repeat galectin, galectin-12, in which only one CRD contained these sequence motifs (18). The two CRDs were denoted following comparison of Dmgal with known mammalian galectin sequences and structures and by sequence comparison between each CRD. Other galectins that are classified in the tandem repeat family are galectins-4, -6, -8, -9.

**EXPERIMENTAL PROCEDURES**

*Generation of Full-length Dmgal cDNA—*To obtain a complete cDNA sequence, 3'-rapid amplification of cDNA ends (3'-RACE) was performed. 3'- and 5'-RACE-Ready cDNA was synthesized from larval poly(A)⁺ RNA (CLONTECH, Palo Alto, CA) using the SMART RACE cDNA Amplification kit according to the manufacturer’s instructions (CLONTECH). For 5'- and 3'-RACE, a gene-specific primer (GSP) was designed against a region of the expressed sequence tag (EST) (LP06039.5prime (15)) encoding a highly conserved amino acid sequence critical to saccharide binding. Touchdown PCR was performed with GSPs, GAGATGTTGGCTCACCATAATCC to according to the manufacturer’s instructions with the addition of a final cycle at 72 °C for 7 min to create poly(A) tails necessary for TOPO TA cloning. The PCR product was subcloned into the pCR4 TOPO vector (Invitrogen, Carlsbad, CA) and was sequenced by the Davis Sequencing Facility (Davis, CA). To ensure that the entire sequence was obtained, three additional GSPs were designed 174, 255, and 320 bp, respectively, downstream of GSPs. 3'-RACE was performed, the products subcloned into the pCR4 TOPO vector, and sequenced as described above.

To generate full-length cDNA, long distance PCR was performed with primers designed from the extreme 5'- and 3'-ends of the cDNA and 5'-RACE-Ready cDNA as a template, using the Advantage cDNA PCR kit according to the manufacturer’s instructions (CLONTECH). A final 12-min cycle at 72 °C was added to create poly(A) tails. The entire cDNA sequence was subcloned directly into the pTrcHis2 TOPO expression vector (Invitrogen) and sequenced to ensure orientation and completeness.

*Expression of Recombinant Dmgal—*Dmgal cDNA lacking a stop codon was subcloned into the pTrcHis2 TOPO expression vector to express the recombinant *Drosophila* protein with a C-terminal Myc-His tag. TOP10 One Shot cells (Invitrogen) were transformed with the expression vector and induced to express recombinant protein with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. The cells were harvested by centrifugation at 8000 rpm for 10 min. The bacterial supernatant was loaded directly on a β-lactosyl-Sepharose affinity column (Amersham Biosciences) for 1.5 h at 68 °C. Bound proteins were eluted with 0.1 M phosphate-buffered saline, 4 mM DTT, 0.02% sodium azide (wash buffer). Eluted fractions were separated by 10% SDS-PAGE and transferred to nitrocellulose for Western blotting.

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J. C. LeFebvre and L. G. Baum, unpublished observations.
and -12 and the Caenorhabditis elegans 32-kDa galectin (18, 35–39).

Examination of the CRDs of Dmgal suggested that each domain may differ with respect to the ability to bind sugar ligands. In the N-terminal CRD (CRD I), there was an Arg to Val substitution at amino acid 206. In most galectin family members this Arg stabilizes galectin-carbohydrate interactions (40). However, in other galectins this Arg is substituted with Lys (galectin-4, Xenopus galectin), Ile (galectin-8), and His (sponge galectin I), and these galectins retain the ability to bind β-galactoside sugars (36, 37, 41, 42). In CRD II there is a Val to Cys substitution at amino acid 406. This amino acid is also substituted with Gln and Ile in Conger eel Lec1 and C. elegans 32-kDa galectin, respectively. Interestingly, CRD I had the highest sequence similarity to galectin-4, -5, and -9, and CRD II had the highest sequence similarity to galectin-4 (36, 38, 39); differences between CRD similarity are also seen in the tandem repeat galectin-12 (18).

Characteristic of galectin family members, the amino acid sequence of Dmgal did not contain a classical secretion signal peptide or a transmembrane domain (33). However, the sequence did contain a 120-amino acid N-terminal domain that is not found in other galectins and shows no significant sequence identity with any other known protein (Fig. 1). This domain is likely to adopt a secondary helical structure (43). Characteristic of galectins, Dmgal did not contain a Ca$^{2+}$ binding domain.

Dmgal showed significant amino acid sequence similarity to galectin family members from various species (identity 23–35%). The alignment of Dmgal with galectins from selected species and the computation of sequence identities and similarity groups were generated using Genedoc (version 2.6.001) and ClustalX (version 1.8) (35, 36, 44–46) (Fig. 2). Dmgal had a great deal of sequence similarity with human galectin-4 and murine galectin-9, which are also tandem repeat galectins (38, 44). Interestingly, the similarity with these two mammalian tandem repeat galectins was slightly greater than that with the C. elegans tandem repeat galectin, demonstrating the strong conservation across vertebrate and invertebrate species.

A BLAST search of the Berkeley Drosophila Genome Project with the putative Dmgal amino acid sequence resulted in seven matches with a smallest sum probability value less than 0.5. One of the sequences was identical to the Dmgal sequence (GenBank™ accession number AE003590). However, five of the remaining six sequences lacked some of the amino acids considered critical for binding β-galactoside sugars (GenBank™ accession numbers AE003590, AE003799, AE003588, AE003713, and AE003583) (34). Only one sequence (GenBank™ accession number AE003514) contained amino acids involved in β-galactoside sugar recognition. However, more studies are necessary to determine whether this is a true galectin family member capable of binding β-galactoside sugars.

Biochemical Characterization of Recombinant Dmgal—The defining feature of galectin family members is the ability to bind β-galactoside sugars and confirmed its identity as a galectin family member. Because all galectin family members share structural similarities within their CRDs, we reasoned that a polyclonal rabbit antibody specific for human galectin-1 might cross-react with Dmgal. The anti-His blot was stripped and reprobed with polyclonal rabbit anti-human galectin-1. As shown in Fig. 3A, the anti-human galectin-1 cross-reacted with Dmgal, providing further evidence for strong structural similarities among galectins from different species.

Dmgal Is Expressed in Embryonic, Larval, and Adult Drosophila—Because the anti-human galectin-1 antibody bound Dmgal, we used this antibody as a reagent to determine the stage or stages of Drosophila development where Dmgal was expressed. 5 μg of total protein homogenate from embryonic, third instar larval and adult flies was separated by 10% SDS-PAGE and immunoblotted with anti-human galectin-1 antibody. As shown in Fig. 3B, a prominent 58-kDa band was expressed at all three stages. In addition, the $M_r$ of native Dmgal matched the $M_r$ of the predicted amino acid sequence. This suggests that Dmgal is not glycosylated, consistent with synthesis of galectin family members within the cytosol (47, 48).

We also examined expression of the Dmgal gene during specific developmental stages by Northern blot hybridization. Poly(A)$^+$ RNA from embryonic, larval, and adult Drosophila was probed with Dmgal cDNA. As shown in Fig. 3C, a single 1.5-kb mRNA for Dmgal was detected in embryonic, larval, and adult Drosophila.

Examination of Native Dmgal—To confirm that native Dmgal bound β-galactoside sugars and was recognized by anti-human galectin-1 antibody, we isolated native galectin from adult and third instar larvae protein homogenate by β-lactose affinity chromatography and eluted bound protein with β-lactose. Immunoblotting with rabbit anti-human galectin-1 polyclonal antibody revealed the 58-kDa Dmgal band (Fig. 3D, lanes 6 and 7), demonstrating that native Dmgal binds β-lactose. Two additional bands of 38 and 72 kDa, which were not seen on the anti-human galectin-1 Western blot of total protein homogenate (Fig. 3B), were enriched by β-lactose affinity chromatography. These may represent additional Drosophila galectins. Alternatively, these may represent modified (49) or degraded Dmgal.

The total adult homogenate prior to affinity chromatography, the unbound fraction, and the bound and eluted fraction were compared by SDS-PAGE analysis (Fig. 3D, lanes 1–3), Western blotting (Fig. 3D, lanes 4–6), and protein assay (data not shown). Western blot analysis with anti-human galectin-1 antibody showed Dmgal present in the load but not in the unbound fraction (Fig. 3D, lanes 4 and 5). There were no significant differences between the load and unbound fractions by Coomassie Blue staining (Fig. 3D) and or by quantification of total protein (data not shown). This implies that only a minor subset of proteins specifically bound to the affinity column. We were unable to detect protein in the eluate by Coomassie Blue staining (Fig. 3D, lane 3) or by protein assay. However, by Western blot, the 58-kDa Dmgal and two additional proteins that specifically bound to and eluted from the β-lactose column were evident (Fig. 3D, lane 6). In addition, stripping and reprobing the blot with a control antibody revealed that recognition of the proteins by the anti-human galectin-1 antibody was specific (data not shown).

Dmgal Expression during Embryogenesis—β-Galactoside-containing carbohydrate structures were expressed in neural tissue during specific stages of Drosophila embryogenesis (7, 8). The presence of oligosaccharide ligands for galectins suggested that Dmgal might also be present in the developing
FIG. 2. Amino acid sequence alignment of galectin family members from selected species. The alignment was generated using ClustalX (version 1.8). Identities (black shading) and similarity groups (gray shading) were computed using Genedoc (version 2.6.001) and the Blosum 62 scoring table. Percent identities are shown to the right. An asterisk denotes critical amino acids involved in saccharide binding. Drome, Drosophila melanogaster galectin; mouse_gal-9, Mus musculus galectin-9; human_gal-4, Homo sapien galectin-4; Caeel_32kD, C. elegans 32-kDa galectin; Bufar_gal-1, Bufo arenarum galectin-1; Conger lcl, Conger myriaster galectin-1.
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Because galectins participate in mammalian embryogenesis and brain development (24, 25), we performed whole mount in situ hybridization on wild type embryos to determine where and at which developmental stages Dmgal cDNA was expressed. As shown in Fig. 4, Dmgal mRNA was deposited maternally into the egg following fertilization. During gastrulation, the presumptive mesoderm showed enriched expression of Dmgal mRNA. In the elongated germ band embryo, Dmgal mRNA became strongly expressed in the mesodermal and neural layers and in the invaginating foregut and hindgut, whereas the epidermis showed weak Dmgal expression. In stages 13–15, Dmgal expression was concentrated in the central nervous system and in the somatic and visceral musculature. By stage 16, Dmgal was expressed in somatic musculature, enriched in the central nervous system (Fig. 4G), and not detected in the visceral musculature (data not shown).

Mammalian galectins mediate cell-cell interactions during mammalian brain and muscle development (24, 25, 50–52). In addition, mammalian galectins modulate cell proliferation and cell survival, two processes that are essential in the development of all organisms (22, 53–55). Because cell-cell adhesion, cell proliferation, and cell survival are regulated during embryogenesis and differentiation, Dmgal may participate in these processes during Drosophila development.

Dmgal Expression in the Drosophila Innate Immune System—In addition to developmental roles, galectins play key roles in adaptive and innate immunity (20–23, 27, 28). The Drosophila immune system consists of a humoral response of anti-microbial peptides from the fat body and a cellular response from circulating hemocytes (2). We did not detect expression of Dmgal in the fat body nor in larval lymph glands, the tissues that produce hemocytes (data not shown). However, as shown in Fig. 5, Dmgal was expressed in circulating larval hemocytes. Interestingly, it appeared that all isolated hemocytes displayed galectin labeling, suggesting that the three major types of hemocytes, lamellocytes, plasmatocytes/macrophages, and crystal cells expressed galectin. In addition, two distinct labeling patterns were observed (Fig. 5). In the majority of hemocytes (73 ± 4.5%), galectin labeling was localized in...
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A localized in a large patch at one pole of the cell. One 0.5-µm slice each of three different cells are shown. B, in the remainder of the hemocytes, Dmgal (green) was localized in multiple small concentrations throughout the cytosol. One 0.5-µm slice each of three different cells are shown. C, localization of Dmgal (green) on three consecutive 0.5-µm slices of a single hemocyte. D, control hemocytes, incubated with preimmune rabbit serum, showed no reactivity. Left, phase-contrast microscopy. Right, confocal microscopy. All cells were analyzed using the ×100 objective.

A large patch at one pole of the hemocyte (Fig. 5A). In the remaining hemocytes (27 ± 3.7%), galectin was concentrated in multiple small patches throughout the cytosol (Fig. 5B). Because more than one band was detected by affinity chromatography (Fig. 5D, lane 7), this staining pattern may represent the localization of different galectins or different forms of Dmgal (49). Alternatively, the staining pattern observed in Drosophila hemocytes may represent the synthesis and secretion pathway that has been described previously for mammalian galectins (47, 48). In mammalian cells, galectins have a unique synthesis and secretion pattern (47, 48). This unique secretion pathway is characterized by synthesis of galectin within the cytosol, concentration of galectin patches at the perimeter of the cell, and evagination of galectin into vesicles that are released from the cell (47, 48).

DISCUSSION

We have identified the first galectin in D. melanogaster. Dmgal shows striking sequence similarity to vertebrate galectins and possesses critical amino acids that are involved in carbohydrate binding (33). Dmgal binds β-galactoside sugars, confirming its identity as a galectin family member (33). Structurally, Dmgal contains two CRDs per molecule, suggesting that, like mammalian galectins, Dmgal may mediate cell-cell communication or trigger signal transduction events (22, 53–55). Dmgal had highest sequence similarity to mammalian galectin-4 and galectin-9. Galectin-9 can trigger apoptosis of cells in the immune system (38). Galectin-9 has also recently been shown to be identical to the mammalian urate transporter, implying that galectins may have multiple functions (56, 57). The conservation of galectins in species ranging from multicellular fungi (58) and sponges (59) to humans provides further evidence of a critical role for galectins in mediating cell-cell communication in all multicellular organisms.

Dmgal was abundant in embryonic, larval, and adult Drosophila. As shown in Fig. 3B, the 58-kDa Dmgal band was prominent, with no additional purification steps, in only 5 µg of embryonic, larval, and adult total protein homogenate. Galectins are abundant in the many tissues where they are expressed; in lizard, for example, a 14-kDa galectin consists of 25 µg/g of wet tissue (16). The abundant expression of the 58-kDa galectin in Drosophila suggests that it may have basic and important functions that are common to many cell types.

Cell surface carbohydrates have been proposed to function in cellular recognition events during embryogenesis, because the synthesis and presentation of diverse carbohydrate structures are spatially and temporally regulated during development (7, 8, 60, 61). In addition, carbohydrate modifications during Drosophila embryogenesis have been shown to regulate the activity of developmental proteins such as Notch, allowing Notch to be spatially activated along a discrete boundary of cells (62, 63). Lectins are postulated to participate in the cellular interactions necessary for development by recognizing complementary glycoconjugates expressed on cells or in the extracellular matrix.

Dmgal had a unique and specific tissue distribution during embryonic development. During gastrulation, galectin was expressed within the invaginating ventral furrow that forms the mesodermal layer. Later in embryogenesis, galectin was strongly expressed in the mesodermal and neural layer and in the invaginating foregut and hindgut. Interestingly, the mesoderm forms somatic and visceral muscle, connective tissue, and endothelium, tissues that are known to express galectins in mammals (21, 22, 64). In addition, the mesoderm also forms components of the Drosophila innate immune system, namely the fat body, lymph glands, and hemocytes (2). In mammals, galectins are expressed in lymphoid organs and play important roles in the innate and adaptive immune systems (20–23, 27, 28).

As embryogenesis continued, Dmgal expression was concentrated in the somatic and visceral musculature and in the central nervous system. The presence of galectin in Drosophila musculature suggests that it may mediate cell-cell and cell-matrix interactions that are required for muscle development. During chick muscle development, galectin appears at stages of myotome segregation and reaches high levels during muscle differentiation (51, 65). In chick muscle, galectin is also proposed to modulate adhesion to the extracellular matrix during myoblast fusion (51). Dmgal may have similar functions during Drosophila muscle development.

The expression of Dmgal was also enriched in the central nervous system. Studies of glycoconjugate expression during Drosophila development demonstrated that possible galactoside-containing carbohydrate ligands for Dmgal are present in the developing tracts of the ventral nerve cord and on axons and glia that ensheath neurons and oppose the axon tracts (7, 8). Dmgal could represent a receptor for these glycoconjugates and facilitate axon guidance or fasciculation. Furthermore, laminin is also present around developing glia and axons in the Drosophila nervous system (66). Laminin has been shown previously to bind galectin (67) and may be an extracellular matrix protein that Dmgal can bind during neural development. In galectin-1/− mice, intriguing deficits in olfactory axon pathology during neural development have been reported (24). In addition, galectin-1 and galectin-3 are transiently expressed by...
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A subset of murine dorsal root ganglia and have been proposed to mediate interactions necessary for axonal fascilitation (24, 68). Dmgal may also mediate cell-cell interactions and migration in Drosophila neural development.

Many insect and mammalian lectins play dual roles in development and in immune defense (1, 13, 22, 23). The Sarcoptes C-type lectin has a potential role in wing and leg imaginal disc development and is also secreted into the hemolymph following injury (13). The dual roles of lectins in development and immunity are similar to the dual roles of the pattern recognition receptor Toll in the establishment of the dorso-ventral axis in embryogenesis and in the immune response (69). In mammals, galectins participate in immune defense (68). Dmgal may also mediate cell-cell interactions and migration in Drosophila (14) will facilitate the elucidation of galectin functions in various organ systems during development and immune challenge.

The presence of a galectin homologue in Drosophila (15) was up-regulated in Anopheles mosquitoes following an immune challenge (12), suggesting that galectins participate in insect innate immunity.

Dmgal may also function in both development and in innate immunity. We found that Drosophila hemocytes express galectin. Hemocytes play vital roles in insect immunity by synthesizing anti-microbial peptides and phagocytosing microorganisms (2). On these cells, galectin was localized in cytoplasmic inclusions and in a large polar patch, suggesting that galectin may participate in recognition or phagocytosis of microorganisms. Whereas galectins are not typically thought to bind to sugars found on microbial cells, mammalian galectin-3 expressed by macrophages binds Candida albicans (70) and bacterial lipopolysaccharide (71, 72), and galectin-3 was present in phagocyte macrophages (73). Alternatively, because Dmgal is a tandem repeat galectin and thus divergent, Dmgal may modulate hemocyte aggregation during infection. In mammals and sponges, galectins can also trigger an alternative complement activation pathway, a host defense system that may be conserved in Drosophila (74). Intra cellular Dmgal may also be released from hemocytes upon immune challenge, because galectin-10 is released from eosinophils after stimulation (75) and galectin-3 is released from dendritic cell exosomes during antigen presentation (76).

We have cloned and characterized the first galectin family member from Drosophila (15). Genetic manipulation is a powerful tool for the study of protein function in vivo. The presence of a galectin homologue in Drosophila (15) will facilitate the elucidation of galectin functions in various organ systems during development and immune challenge.

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