Biochemical and molecular characterization of galectins from zebrafish (Danio rerio): notochord-specific expression of a prototype galectin during early embryogenesis

Hafiz Ahmed, Shao-J. Du, Nuala O’Leary, and Gerardo R. Vasta

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202

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Galectins are a family of β-galactoside-binding lectins that on synthesis are either translocated into the nucleus or released to the extracellular space. Their developmentally regulated expression, extracellular location, and affinity for extracellular components (such as laminin and fibronectin) suggest a role in embryonic development, but so far this has not been unequivocally established. Zebrafish constitute an ideal model for developmental studies because of their external fertilization, transparent embryos, rapid growth, and availability of a large collection of mutants. As a first step in addressing the biological roles in zebrafish embryogenesis, we identified and characterized members of the three galectin types: three proto-galectins (Drgal1-L1, Drgal1-L2, Drgal1-L3), one chimera galectin (DrgaL3), and one tandem-repeat galectin (Drgal9-L1). Like mammalian prototype galectin-1, Drgal1-L2 preferentially binds to N-acetyllactosamine. Genomic structure of Drgal1-L2 revealed four exons, with the exon–intron boundaries conserved with the mammalian galectin-1. Interestingly, this gene also encodes an alternatively spliced form of Drgal1-L2 that lacks eight amino acids near the carbohydrate-binding domain. Zebrafish galectins exhibited distinct patterns of temporal expression during embryo development. Drgal1-L2 is expressed postbud stage, and its expression is strikingly specific to the notochord. In contrast, Drgal1-L1 is expressed maternally in the oocytes. Drgal1-L3, Drgal3, and Drgal9-L1 are expressed both maternally and zygotically, ubiquitously in the adult tissues. The distinct temporal and spatial patterns of expression of members of the zebrafish galectin repertoire suggest that each may play distinct biological roles during early embryogenesis.

Key words: Danio rerio/developmental expression/notochord/specificity/zebrafish galectins

Introduction

There is growing evidence that complex carbohydrate structures encode information that modulate interactions between cells or between cells and the extracellular matrix

by specifically binding to cell surface–associated or soluble carbohydrate-binding receptors (Bakkers et al., 1997; Hathaway and Shur, 1997; Laine, 1997). The expression patterns of such carbohydrate-binding receptors are of particular interest in the context of development because the structures of potential ligand molecules, such as glycoproteins, glycolipids, and other glycans, are subject to change by glycosidases and glycosyltransferases (Shur, 1993; Wassarman, 1995). The rapidity and complexity of the changes in exposed carbohydrate residues during early development suggest that they may play important roles during embryogenesis.

Galectins, a family of β-galactoside-binding developmentally regulated proteins formerly known as S-type lectins, have been proposed to participate in a variety of biological functions, including cell–cell and cell–extracellular matrix interactions that mediate developmental processes (Barondes et al., 1994; Hirabayashi, 1997). However, the detailed mechanisms of their biological role(s) remain unclear. Unlike other lectin families, such as C-type lectins, galectins are a relatively homogeneous, evolutionarily conserved and ubiquitous group, with representatives identified in most animal taxa examined so far, including the parazoa and both protostome and deuterostome lineages (Cooper, 2002; Hirabayashi, 1997; Vasta et al., 1999). Furthermore, from the biochemical, structural, and genetic standpoints, galectins constitute one of the best-characterized lectin families (Ahmed et al., 1996a; Cho and Cummings, 1995; Liao et al., 1994; Ohyama and Kasai, 1988; Schwarz et al., 1998; Sparrow et al., 1987).

Based on structural features, galectins have been classified in three types: proto, chimera, and tandem-repeat (Hirabayashi and Kasai, 1993). Prototype galectins contain one carbohydrate-recognition domain (CRD) per subunit and are usually homodimers of noncovalently linked subunits. In contrast, chimera-type galectins are monomeric with a C-terminal CRD similar to the proto type, joined to an N-terminal peptide of yet unknown functional properties. Tandem-repeat galectins, in which two CRDs are joined by a linker peptide, are also monomeric. Proto- and tandem-repeat types make up several distinct galectin subtypes. Galectin subtypes have been numbered following the order of their discovery (Barondes et al., 1994), and so far, 14 have been described in mammals (Dunphy et al., 2002).

Substantial efforts have been invested toward the elucidation of the role(s) of galectins in embryogenesis and development using mammalian models (Colnot et al., 1997), but because of the diversity of the galectin repertoire and the technical difficulties in working ex utero with mammalian embryos, results have been inconclusive (Colnot et al., 1998). Lower vertebrates and invertebrates appear to have
smaller galectin repertoires (Cooper, 2002). Thus species currently used as model organisms for genetic and developmental studies, such as the zebrafish (Danio rerio), fruitfly (Drosophila melanogaster), and nematode (Caenorhabditis elegans) may be more suitable alternatives for studies on the functional roles of galectins. Galectins have been identified and characterized in these species by biochemical and molecular approaches (Cooper, 2002; Hirabayashi et al., 1997; Pace et al., 2002) as well as current genome projects (C. elegans Sequencing Consortium, 1998; Berkley Drosophila Genome Project, NIH Zebrafish Genome Project).

Zebrafish should constitute a particularly useful animal model for investigating biological roles of galectins in early embryo development of vertebrates because this species offers a number of advantages over mammalian systems. First, fertilization is external: the embryos develop rapidly in vitro and are transparent, making it possible to visualize the expression of putative genes mediating cell adhesion and/or migration. Second, the early expression of those gene(s) can be easily manipulated in zebrafish embryos, enabling visualization of the phenotypic consequences of their misexpression. Third, a growing collection of interesting mutations that affect early embryonic development are being generated and mapped, providing an excellent resource for genetic studies of the function and mechanisms of action of galectin(s) genes. Fourth, zebrafish cell lines have been established that might be very useful for studying gene expression and protein export at a cellular level. Finally, because zebrafish are evolutionarily closer to mammals than the above-mentioned invertebrate models, structural and functional information obtained in this organism may be more applicable to the interpretation of similar observations in mammalian systems.

In this study we report for the first time the identification and characterization of members of the three galectin types in zebrafish and their differential expression during early development.

Results

Purification and characterization of galectins from zebrafish

Lactose-binding lectins were purified from whole adult zebrafish extracts by affinity chromatography on lactosyl-Sepharose as described under Materials and methods. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the lactose eluate resolved in four bands corresponding to 14.5, 15.5, 22, and 24 kDa, of which the 14.5 kDa (designated as Drgal1-L2) was separated by anion-exchange chromatography on DEAE-Sepharose, based on its acidic properties (Figure 1Ab). On western blot, only the 14.5-kDa component of the lactose eluate cross-reacted with an anti–[striped bass proto galectin] antibody (Figure 1B). Drgal1-L2 behaved as a dimer in gel permeation chromatography (Figure 1C). The yield of the purified Drgal1-L2 was approximately 2 μg/g adult fish tissue and had a specific activity of 1–2 × 10⁴ mg/ml with protease-treated rabbit erythrocytes. To determine the amino acid sequence of Drgal1-L2, the protein was trypsin-digested, and selected peptides sequenced. From the 38 peaks separated by high-performance liquid chromatography (HPLC) from the tryptic digest, 9 were predominant (data not shown). Four of these fractions were further purified, and the peptides were sequenced (a total of 56 amino acids) by using a combination of tandem mass spectrometry and Edman degradation. The selected peptides yielded the following amino acid sequences: peptide 16 (mass 1168.4): YMHFEGEVR; peptide 23 (mass 1238.4): DNNFPFIQDK; peptide 32 (mass not confirmed): VGQTILTITGVPKD; and peptide 38 (mass 2677.8): ITFTNEFVLTLPDGSEIHFPNR. Based on these peptide sequences, molecular probes were synthesized for cloning the Drgal1-L2 cDNA.

Carbohydrate specificity of Drgal1-L2

The carbohydrate-binding specificity of Drgal1-L2 was determined by analyzing the binding of the Drgal1-L2-HRP conjugate to asialofetuin (ASF) in the presence of several saccharides in a solid phase assay under the optimal conditions established (Ahmed et al., 1996a, 2002). For each test saccharide, a complete inhibition curve was established, and the molar concentrations that inhibited the binding of the lectin conjugate to ASF by 50% (I₅₀) were calculated and normalized with respect to lactose, which was included in each plate as a standard (Table I). The binding of the Drgal1-L2 to Galβ1,4GlcNAc and thiogalactoside were about 8–12-fold higher than that to lactose. The binding of Drgal1-L2 to Galβ1,3GalNAc was negligible. The overall binding-inhibition pattern of Drgal1-L2 suggests a specificity very similar to that observed in mammalian 14 kDa galectins exhibiting...
conserved (Type I) CRDs (Ahmed and Vasta, 1994). Binding of Drgal1-L2 to plastic-coated laminin was linear up to approximately 0.6 µg/well of laminin (Figure 2).

cDNA cloning and sequence analysis of Drgal1-L2

The full-length cDNA for Drgal1-L2 (GenBank accession number AY421704) was obtained by PCR followed by 5′- and 3′-rapid amplification of cDNA ends (RACE) (Figure 3). The cDNA was approximately 850 bp with a conserved polyadenylation signal sequence at nucleotide positions 816–821. The cDNA encodes a polypeptide of 134 amino acids with a calculated molecular weight of 15,254 and theoretical pI of 5.25. Comparison of the protein sequence deduced from the cDNA with the sequences obtained for the tryptic peptides revealed full sequence identity. Comparison of the Drgal1-L2 amino acid sequence with sequences of selected galectins (Figure 4) confirmed that the D. rerio lectin is a member of the galectin family. When considering the sequence of the CRD and its molecular size (subunit or native), it clearly belongs to the prototype galectins and is more closely related to the galectin-I group than to any other galectin family members. When compared to galectins from ectothermic vertebrates, the highest percent identity was observed with electric eel (64%; Paroutaud et al., 1987), followed by conger eel (32%; Muramoto and Kamiya, 1992), and toad Bufo arenarum (32%; Ahmed et al., 1996a). The percent identities with galectin-I from homeotherm vertebrates, such as human (Gitt and Barondes, 1986) and bovine (Ahmed et al., 1996b), ranged from 30% to 35%, whereas it was 28% identical to the 16-kDa prototype galectin from C. elegans (Hirabayashi et al., 1996).

Recently, several galectin sequences from the zebrafish genome project became available in the GenBank database (Cooper, 2002). Analyses of these sequences revealed two additional prototype galectin-I like lectins (Drgal1-L1 and Drgal1-L3), a chimera galectin (Drgal3), and two tandem-repeat galectin9-like lectins (Drgal9-L1 and Drgal9-L2). Drgal1-L1 (calculated molecular weight of 15,556 and pI of 4.93) is 79% identical to Drgal1-L2 (Figure 4). Drgal1-L3 (calculated molecular weight of 14,804 and pI of 6.64) is only 49% identical to Drgal1-L2 and 45% to Drgal1-L1. Because the cDNA sequences of Drgal3 and Drgal9-L1 in the database are incomplete, we used 5′- and 3′-RACE to determine their full-length sequences. The cDNA for Drgal3 (GenBank accession number AY421705) had an open reading frame (ORF) (45–728) and a potential initiation codon (Figure 5). The ORF encoded 228 amino acids with a predicted molecular weight of 25,017 and calculated pI of 5.23. It is noteworthy that the N-terminal portion (90 amino acids) of Drgal3 was significantly shorter relative to the known chimera galectins from higher vertebrates, such as human (114 aa; Robertson et al., 1990) and rat (128 aa; Oda et al., 1993). The full-length cDNA of Drgal9-L1 (GenBank accession number AY421706) was also cloned by 5′- and 3′-RACE (Figure 6). The cDNA contained an ORF (53–1012) that encoded 320 amino

<table>
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<th>Compound</th>
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<th>Relative inhibitory activityb</th>
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<tr>
<td>Galβ1,4Glc</td>
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<td>Galβ1,4Glcβ-OPhNH2 (p)</td>
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<td>1.4</td>
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<tr>
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<td>2.3</td>
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<td>&lt;0.01</td>
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Table 1. Inhibition of Drgal1-L2 binding to asialofetuin by a series of saccharidesa

a Each saccharide was tested in duplicate and average value was taken.
b Lactose was included in each plate as a standard and I50 for each saccharide was normalized with respect to lactose.
c I50 varied from 4.4–6.5 µM in 10 experiments.
d % Inhibition of binding was indicated in parenthesis because I50 was not achieved at the highest concentration tested.

Fig. 2. Binding of 14.5 kDa galectin to laminin. Binding of the galectin (10 µg/100 µl/well) to variable amount of laminin (0.02–0.3 µg/well) with and without lactose. After washing the plate, the bound galectin was detected with rabbit anti-galectin antibody, followed by goat anti-rabbit IgG peroxidase and development with ABTS substrate.
acids with a predicted molecular weight of 36,097 and calculated pI of 8.67. The sequence revealed two CRDs (38% identical): N-terminal (147 aa) and C-terminal (140 aa) connected by a 33-aa-long linker peptide.

Gene organization and linkage analysis of Drgal1-L2

A genomic clone for Drgal1-L2 was isolated by polymerase chain reaction (PCR) from a Genome Walker genomic library. The Drgal1-L2 gene contains four exons (see Figure 3 for positions of the introns), with highly conserved exon/intron boundaries as in mammalian galectin-1 (Hirabayashi and Kasai, 1993). Linkage analysis using the LN54 hybrid panel, placed the Drgal1-L2 gene in the linkage group 3, at 5.66 centiRay (1 cR is ~148 kb) from the marker fc23e05.

Identification of a Drgal1-L2 isoform (Drgal1-L2d)

During sequencing of Drgal1-L2, we identified a cDNA clone encodes a galectin isoform (Drgal1-L2d) that was identical to the Drgal1-L2 except that it lacks eight amino acids (FAINIGHS) corresponding to part of the exon III (see Figure 3). Examination of the genomic sequence around the deleted region revealed a cryptic splicing sequence at the deleted site, suggesting that the isoform Drgal1-L2d probably arises from alternate splicing of Drgal1-L2 gene (Figure 3). To rule out the presence of a second gene encoding for the Drgal1-L2d isoform, PCR amplification of zebrafish genomic DNA was carried out with primers 5 and 6, which would amplify both genes. The PCR product was subcloned, and multiple clones were sequenced. All sequences were identical and represent a single gene corresponding to Drgal1-L2. No clones corresponding to Drgal1-L2d were identified.

Galectin expression in developing and adult zebrafish

The temporal expression of each galectin was analyzed in zebrafish oocytes and embryos by reverse transcription PCR. Drgal1-L1 was expressed in 0 h postfertilization, but declined in blastula and post-blastula stage embryos. These data suggest that Drgal1-L1 is maternally expressed. In contrast, little or no Drgal1-L2 expression could be detected in newly fertilized eggs and embryos at 3–9 h postfertilization, but become evident at 12 h postfertilization, and remained

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**Fig. 3.** Nucleotide and deduced amino acid sequence from cDNA for the Drgal1-L2. Sequences for the peptides are underscored. Sequence for the clone (350 bp) generated from the degenerate primers starts at nucleotide 92 and terminates at 433. The stop codon is marked by an asterisk. The putative polyadenylation signal sequence in the 3'-untranslated region of the transcript is in italics. The position of introns are indicated by vertical lines. The cryptic site for alternate splicing of Drgal1-L2 gene is shown in the beginning of exon III.
high in later stages (14–19 h postfertilization; 1–5 day(s) postfertilization (Figure 7A). Interestingly, Drgal1-L3 was expressed both maternally and zygotically with a sharp decline at 6 h postfertilization (Figure 7A). The expression of Drgal3 was detected at 0 h postfertilization and increased at 3 h postfertilization. Its expression declined at 6 h postfertilization but increased at 9 h postfertilization and remained high at later stages (Figure 7A). Unlike all galectins, the expression of Drgal9-L1 was strong in all stages tested (Figure 7). Figure 7B shows organ expression in adult zebrafish. Drgal1-L2 is expressed in brain, spleen, liver, intestine, and muscle as analyzed by reverse transcription PCR. For Drgal1-L1, weak expression was observed only in oocytes and brain. Drgal1-L3 was strongly expressed in liver, intestine, and muscle and weakly in oocytes and spleen. But Drgal3 and Drgal9-L1 were expressed in all organs tested (oocytes, brain, spleen, liver, intestine, muscle, and eye). The isoform Drgal1-L2d was developmentally expressed (Figure 7C).

The spatial expression of Drgal1-L2 was examined by whole mount in situ hybridization. Results on 16- and 24-h postfertilization embryos confirmed that Drgal1-L2 is expressed in early embryonic stages, particularly in the notochord (Figure 8A, C, E, F). The embryos probed with sense RNA did not show any staining (Figure 8B, D).

The in situ hybridization results were further corroborated by whole mount antibody staining using anti-Drgal1-L2 antibodies on 24 h postfertilization embryos (Figure 8H). The antibody was validated as specific for Drgal1-L2 on western blot: only a single band corresponding to 14.5 kDa (Drgal1-L2) was detected in both the crude zebrafish extract and the lactose eluted galectin mix (Figure 8G). Immunostaining results revealed that the Drgal1-L2 protein is localized in the notochord (Figure 8H). The preimmune serum controls did not show comparable staining in notochord (not shown).

Discussion
In this study we characterize the diversity and developmental expression of the zebrafish galectin repertoire. We purified, biochemically characterized, and cloned Drgal1-L2 and its isoform Drgal1-L2d, and cloned the full-length cDNAs of the zebrafish chimera and tandem-repeat galectins Drgal3 and Drgal9-L1. Furthermore, we identified from the database two additional closely related yet distinct protogalactins Drgal1-L1 and Drgal1-L3. All zebrafish galectins characterized in this study showed remarkable structural similarities with mammalian galectins, and this enabled their unambiguous classification within the three well-established galectin groups. Galectin repertoires of protostome invertebrates and lower vertebrates appear to be limited to one or two galectin types (Cooper, 2002). For example, C. elegans and Drosophila have proto- and tandem-repeat type galectins (Cooper, 2002; Hirabayashi et al., 1997; Pace et al., 2002). In this aspect, it is noteworthy that at the evolutionary level of teleosts all three galectin groups are already represented. Nevertheless, based on our studies and the information currently available from the...
zebrafish genome project, it seems quite clear that the zebrafish galectin repertoire is smaller than that of mammals. Therefore, this species should be a more suitable model organism for the study of biological roles of galectins than the mammalian model systems, in which 14 distinct galectins have been already identified (Dunphy et al., 2002) and additional galectin members are evident from GenBank databases (Cooper, 2002).

Like mammalian protogalectins, Drgal1-L2 is a homodimer with a single CRD per subunit. The primary structure of the zebrafish Drgal3 revealed the typical organization of the mammalian tandem-repeat galectins, where the two CRDs are connected by a linker peptide. Similarly, despite a shorter N-terminal peptide region, the zebrafish Drgal3 exhibited the typical organization of the chimera type mammalian galectins. Alignment of amino acid sequences for Drgal1-L2, Drgal1-L1, and Drgal1-L3 with the sequences of selected galectins; the presence of conserved critical residues in their CRDs; and their subunit and native molecular sizes clearly indicate that they belong to the prototype galectins and are more closely related to galectins-1 than to any other subgroup. All three prototype galectins are acidic and showed galectin-1-like sequences that contain conserved (Type I) CRDs (Ahmed and Vasta, 1994). To our knowledge, this is the first observation of the fact that three closely related yet distinct prototype galectins are present in a given organism (Cooper, 2002). Two prototype galectins (galectin-1-like) have been described in chicken, but they are substantially divergent: they are only 39% identical, and one is acidic, whereas the other is almost neutral. When the amino acid sequence of Drgal1-L2 sequence was compared with galectins from vertebrates and invertebrates, the highest identity was observed with electric eel (64%), whereas lower percent identities were observed with mammalian...
Molecular characterization and developmental expression of zebrafish galectins

Galectins-1 from human, bovine, mouse, and amphibians, and an invertebrate homolog (C. elegans) ranged from 28% to 35%. The observed cross-reactivity with an anti-(proto)galectin from M. saxatilis, another teleost fish, further confirmed the structural similarities observed.

Despite some difference in intron sizes, the gene encoding for Drgal1-L2 showed an organization similar to the mamalian galectin-1 genes. The gene organization of galectins from human, mouse, and chicken is remarkably similar. Two galectins-1 from human and mouse (Chiariotti et al., 1991; Gitt and Barondes, 1991), galectin-2 (Gitt et al., 1992), and chicken 14-kDa galectin (Ohyama and Kasai, 1988) have identical numbers of exons, and their sizes are similar. Each gene (about 3.2 kb) contains four exons (from the 5' end to the 3' end: exon I, 6–9 bp; II, 80–83; III, 160–172 bp; IV, 144–150 bp), of which the largest (exon III)

Fig. 6. Nucleotide and deduced amino acid sequence from cDNA for the Drgal9-L1. The two carbohydrate-binding domains are connected by a link peptide (underscored). The stop codon is marked by an asterisk. The putative polyadenylation signal sequence in the 3'-untranslated region for the transcript is in italics. The critical amino acid residues that are known to interact with N-acetyllactosamine (Liao et al., 1994) are shown in bold.
encodes the CRD. The gene structures of galectins-3 from mouse and rat show that the exon–intron structure of their CRD domain is similar to the 14-kDa galectins, whereas their N-terminal domains are encoded by two exons (Grützmacher et al., 1992). The genomic structure of the C. elegans 32-kDa galectin revealed a unique insertion pattern consisting of two introns, of which the first one is conserved with mammalian galectins (Arata et al., 1997).

Based on our studies, the presence of a second gene that may encode for the Drgal1-L2d isoform was ruled out, so it is likely that its transcripts originate by alternate splicing of the Drgal1-L2 gene. Alternate splicing of galectin genes has been described for galectin-9 in the linker peptide only (Wada and Kanwar, 1997) and for galectin-3 between the N-terminal Gly/Pro-repeat domain and the CRD (Gorski et al., 2002).

Binding-inhibition profiles clearly indicate that Drgal1-L2 shares all the binding properties of galectin-1 and other galectins with conserved (Type I) CRDs (Ahmed and Vasta, 1994), including strong binding to laminin, and different from those with variable ones, such as the recently described C. elegans galectin (Ahmed et al., 2002). Like all galectins, equatorial OH at C-4 of Gal residue (in Lac/LacNAc), and substitutions of 4'-OH, 6'-OH (Gal residue) and 3-OH (Glc/GlcNAc residue) are not favorable for Drgal1-L2 binding, because compounds Glcβ1,4Glc, GlcNAcβ1,4Galβ1,4Glc, NeuAcα2,6Galβ1,4Glc, and Fucα1,3Galβ1,4Glc were very poor inhibitors. Like all galectins-1 containing conserved CRDs, axial OH at C-3 of Glc/GlcNAc is not favorable for binding because Galβ1,3GalNAc was found to be very poor inhibitor. Among the saccharides tested, N-acetyllactosamine and thiogalactoside were the most potent inhibitors. The overall inhibition data for Drgal1-L2 were similar to those of galectins-1 and fully consistent with the primary structure of the binding site. Based on the primary structures of Drgal1-L1 and Drgal1-L3, a similar carbohydrate specificity profile is expected. However, because Drgal1-L2d lacks several amino acid residues corresponding to exon III that encodes the carbohydrate-binding cleft, its carbohydrate-binding properties are most likely qualitatively and quantitatively different from Drgal1-L2. Homology modeling and sugar binding-inhibition assays will be carried out in the near future to examine this possibility.

Our results on the expression of zebrafish galectins in oocytes and during embryogenesis suggest that these are developmentally regulated and that their functions take place at different developmental stages. During embryogenesis, Drgal1-L1 is expressed maternally, whereas the expression of Drgal1-L2 occurs zygotically. Interestingly, Drgal1-L3, Drgal3, and Drgal9-L1 were expressed both maternally and zygotically. Differential expression of several galectin types was also observed in human and mouse. In human embryogenesis, galectins-1 and -3 were found differentially expressed during the first trimester (Van den Brule et al., 1997). In mouse embryogenesis, galectins-1 and -3 are expressed in the trophectoderm of the blastocyst, and following gastrulation galectin-1 is first detected in somite myotomes, whereas galectin-3 is confined to the notochord (Colnot et al., 1997).
The strong expression of Drgal1-L2 in the notochord may indicate its role in early embryo development. Several scenarios are possible. First, Drgal1-L2 may be involved in notochord development by regulating adhesion or migration of notochord cells during embryogenesis. This study demonstrated that Drgal1-L2 strongly binds to laminin, and the presence and distribution of this glycan in the notochord has been characterized in detail (Parsons et al., 2002). The presence of both the galectin and its ligand in the notochord, a critical structure for further patterning of the embryo, suggests a relevant role for Drgal1-L2 in the zebrafish embryogenesis. It is known that Hedgehog signal secreted from the notochord plays an important role in slow muscle formation (Blagden et al., 1997; Currie and Ingham, 1996; Du et al., 1997). Like Hedgehog, Drgal1-L2 may also be involved in muscle cell differentiation. Hedgehog from the floor plate is also involved in slow muscle formation (Coutelle et al., 2001; Du et al., 1997). Drgal1-L2

Fig. 8. Whole mount in situ hybridization and immunostaining. (A–F) In situ hybridization of whole zebrafish embryo showing Drgal1-L2 expression in notochord (A, C, E, F). (A) Lateral view of 24-h postfertilization embryo; (B) 24-h postfertilization embryo with sense probe (−) control; (C) 24-h postfertilization embryo showing trunk; (D) 24-h postfertilization embryo with sense probe showing trunk; (E) lateral view of 16-h postfertilization embryo; (F) cross-section of trunk after whole mount in situ hybridization; (G) specificity of anti-Drgal1-L2 antibodies. (I) Crude zebrafish extract and (II) the affinity-purified galectin mix were tested with the antibodies on western blot. (H) Whole mount immunostaining with anti-Drgal1-L2 antibodies. Lateral view of trunk of zebrafish embryo (24-h postfertilization) showing staining in notochord.
may influence notochord formation and consequently the expression of Hedgehog signals, and thus Drgal1-L2 may indirectly be involved in muscle cell differentiation.

Because the notochord is critical for the neural tube patterning, Drgal1-L2 may be involved in neural tube formation and expression of Wnt and bone morphogenetic proteins (BMPs). It has been demonstrated that Wnt and BMPs expressed in the neural tube are critical for muscle development (Stern et al., 1995; Tajbakhsh et al., 1998). Thus Drgal1-L2 may indirectly be involved in muscle development. During myogenesis, glycoproteins or proteoglycans are directly involved in transducing Wnt and Hedgehog and epidermal growth factor signaling (Lin and Perrimon, 1999; Perrimon and Bernfield, 2000), and it is possible that Drgal1-L2 plays a role in modulating the activity of these glycans. Moreover, during development of skeletal muscle a population of myoblasts migrate to their destinations through the extracellular matrix and then differentiate, so it has been suggested that lectins might control the interaction between myoblasts and the extracellular matrix by selective modulating the interaction of integrin with fibronectin and laminin and thus regulates the migration of myoblasts (Gu et al., 1994). In mammalian models it has been shown that in skeletal muscle galectin-1 is secreted during differentiation (Goldring et al., 2002; Gu et al., 1994) and accumulates with laminin in the basement membrane surrounding each myofiber (Cooper and Barondes, 1990).

**Materials and methods**

**Reagents**

The protein assay reagent was from BioRad (Hercules, CA). Size exclusion chromatography molecular weight standards were purchased from Amersham Pharmacia (Piscataway, NJ). The peroxidase substrate diammonium 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Sequencing-grade reagents and solvents for protein sequencing, amino acid analysis, and HPLC were from Applied Biosystems (Foster City, CA). Sequencing-grade trypsin was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Horseradish peroxidase (HRP), and carbohydrates used in binding-inhibition assays were from Sigma (St. Louis, MO). All other reagents were of the highest grade commercially available.

**Purification of D. rerio 14.5-kDa galectin**

The galectin was purified following the procedure described elsewhere (Ahmed et al., 1996a), with some modifications. Briefly, approximately 400 adult zebrafish (D. rerio; 200 g total weight) were homogenized with a PRO 400 tissue homogenizer using 59 mm × 150 mm generator (PRO Scientific, Monroe, CT) in cold (4°C) phosphate buffered saline (PBS)/0.01 M 2-mercaptoethanol (ME) containing 0.1 M lactose and 0.1 mM phenylmethylsulfonyl fluoride, at 2 ml buffer/g wet tissue. The homogenate was centrifuged at 27,000 × g for 1 h at 4°C, and the clear supernatant was dialyzed against PBS/ME and absorbed on a lactosyl-Sepharose column preequilibrated with PBS/ME/0.002 M EDTA/0.5 M NaCl. The column was thoroughly washed with equilibrating buffer followed by five bed volumes of PBS (1:10)/ME, and the bound protein was eluted with 0.1M lactose in PBS (1:10)/ME. To isolate the 14.5-kDa galectin (D. rerio protogalectin; Drgal1-L2) from other galectin species, the eluted fractions containing protein were pooled and absorbed on a DEAE-Sepharose column (1 ml bed volume) preequilibrated with PBS (1:10)/ME. After washing the column with PBS (1:10)/ME, the bound protein was eluted with PBS (1:10)/ME/0.5 M NaCl.

**Analytical procedures**

Agglutination tests with protease-treated rabbit erythrocytes were carried out in bovine serum albumin (BSA)-coated 96-well Terasaki plates (Robbins Scientific, Mountain View, CA) as reported earlier (Vasta et al., 1986). Protein concentrations were determined on 96-well flat-bottom plates with the BioRad Protein Assay following a modification of the manufacturer's protocols, using BSA as standard (Ahmed et al., 1996a). Analytical PAGE in the presence of SDS (2%) was carried out on 15% (w/v) acrylamide gels under reducing conditions as reported elsewhere (Laemmli and Favre, 1973). The molecular weight of the native protein was estimated by gel permeation chromatography carried out on a Pharmacia Superose 6 column (1 × 30 cm) as described elsewhere (Vasta et al., 1986).

**Preparation of rabbit anti-Drgal1-L2 antiserum**

The anti-Drgal1-L2 antiserum was prepared in New Zealand white rabbits at Duncroft (Lovetsville, FL) by multiple subcutaneous and intramuscular injections of affinity-purified Drgal1-L2 (100 µg/injection), and the antibody titer was determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Vasta et al., 1986). The specificity of the antiserum was assessed by western blot. For this purpose, the affinity-purified galectins or zebrafish crude extract were electrotransferred onto polyvinyl difluoride membranes, and blocked with PBS/0.05% Tween 20/3% fish gelatin (Sigma) for 1 h at room temperature (RT) and incubated with anti-Drgal1-L2 antibody (1:1000 dilution) for 1 h. After three washes with PBS/0.05% Tween 20, membranes were incubated with goat anti-rabbit IgG-HRP conjugate (1:2000 dilution) in PBS (azide free)/0.05% Tween 20/3% gelatin for 1 h, washed three times with PBS (azide free)/TWEEN 20, and developed with chloronaphthol for peroxidase activity.

**Characterization of the carbohydrate specificity of Drgal1-L2**

**Preparation of the Drgal1-L2-HRP conjugate.** For conjugation, the DEAE-Sepharose column containing Drgal1-L2 was washed with azide-free PBS (1:10) to remove lactose and ME, and the bound protein was eluted with 1.5 ml PBS (azide-free)/0.5 M NaCl/0.1 M lactose. The galectin was conjugated to activated HRP at 4°C overnight with stirring following the procedure previously described (Ahmed et al., 2002). Briefly, activation of HRP (4 mg in 0.5 ml PBS, pH 7.2) was performed with 1 mg sulfonated succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate followed by conjugation of the activated HRP with the Drgal1-L2 (100 µg). After overnight incubation at 4°C, the conjugation
mixture was diluted 50-fold with cold water and adsorbed onto DEAE-Sepharose (0.5 ml) preequilibrated with azide-free PBS (1:10). The column was washed to remove lactose, and the conjugate was eluted with 4 ml PBS (azide-free)/1 M NaCl, followed by purification by affinity chromatography on lactosyl-Sepharose as indicated. The purified galectin-HRP conjugate was dialyzed with azide-free PBS and stored in 1% BSA/50% glycerol at −20°C.

Solid phase binding-inhibition assay. Binding of the Drgal1-L2 to ASF, and its inhibition by sugars were assessed and optimized as reported elsewhere (Ahmed et al., 1996a, 2002). Briefly, ASF (0.5 μg/100 μl/well) in 0.1 M Na₂CO₃/0.02% NaN₃ (pH 9.6) was adsorbed onto the wells of microtiter plates (Immulon; Dynatech Laboratories, Chantilly, VA) at 37°C for 3 h, and the bound glycoprotein was fixed with 2% formaldehyde in PBS at 37°C for 30 min. The plates were washed three times with PBS (azide-free)/0.05% Tween 20, and incubated with the galectin-HRP conjugate (10 ng/100 μl/well for binding assays) or with preincubated mixture of equal volume of conjugate and varying concentrations of test ligands (for binding-inhibition assays). After incubation for 1 h at 4°C, the plates were washed with ice-cold azide-free PBS-Tween 20 buffer, and the bound peroxidase activity was assayed with ABTS. Binding of Drgal1-L2 to laminin and its inhibition by lactose were examined by ELISA, as previously described (Ahmed et al., 1990).

Peptide sequencing of Drgal1-L2

The purified Drgal1-L2 was subjected to SDS–PAGE as described before and after staining with Coomassie blue the band was cut out and in-gel digested with trypsin in 0.05 M Tris–HCl (pH 8.5)/1 M guanidine hydrochloride (EIS = 1:50, w/w, at 30°C, 10–20 h). The tryptic digests were acidified to pH ~ 2 using 10% trifluoroacetic acid, and the fragments were purified by HPLC, analyzed by matrix-assisted laser desorption/ionization mass spectrometry, and sequenced using Edman degradation as previously described (Ahmed et al., 1996a).

Galectins primers

The oligonucleotides (degenerated and gene-specific) were designed and synthesized at BioAnalytical Services, Center of Marine Biotechnology, University of Maryland Biotechnology Institute (Baltimore, MD). The galectin primers used are defined by numbers as follows. Drgal1-L2 primers:

1. 5' - GCCACCTGAGGCGGCCGCA - 3' (forward)
2. 5' - CACAGCCCTCAAAAGTTTGGGCT - 3' (reverse)
3. 5' - CCTGTTAGACCTCCTGACCCCT - 3' (reverse)
4. 5' - GCAGCGTACCTCCTGACCCCT - 3' (reverse)
5. 5' - GGCGGCGCCTCCTGACCCCT - 3' (reverse)
6. 5' - CCAAGCGGCGCCCTGACCCCT - 3' (reverse)
7. 5' - GGGAATCCATTTTAATTCTGACCCCTGACCCCTGACCTGC - 3' (reverse)
8. 5' - CACAGCCCTGAGGACATCGCTCCCGG - 3' (forward)
9. 5' - GAGCGGCTGCTGTTGCGGAG - 3' (reverse)
10. 5' - CTGTTAGACCTCCTGACCCCT - 3' (reverse)
11. 5' - GCGGCGGCCCTGACCCCT - 3' (reverse)
12. 5' - CACAGCCCTGAGGACATCGCTCCCGG - 3' (reverse)

Drgal1-L3 primers based on the available sequence (Unigene Dr. 15088):

13. 5' - GCAGCGTACCTCCTGACCCCT - 3' (forward)
14. 5' - CGGTTGGCTGATGGC - 3' (forward)
15. 5' - CACAGCCCTGAGGACATCGCTCCCGG - 3' (reverse)
16. 5' - GCGGCGGCCCTGACCCCT - 3' (reverse)
17. 5' - GTGTTAGACCTCCTGACCCCT - 3' (forward)
18. 5' - GCGGCGGCCCTGACCCCT - 3' (reverse)
19. 5' - CACAGCCCTGAGGACATCGCTCCCGG - 3' (reverse)

cdNA cloning and sequence analysis of galectins

cDNA cloning of galectins was carried out using a PCR-based protocol. For this purpose, total RNA was isolated from zebrafish embryos, adult whole body, or selected tissues using a standard guanidine thiocyanate, phenol/chloroform extraction (Sambrook et al., 1989). Occasionally, isolation of total RNA was performed using Rneasy Total RNA System (Qiagen, Valencia, CA). Poly (A)+ RNA was isolated from the total RNA on poly (dT)-Dynabeads using mRNA purification kit (Dynal, Oslo, Norway). Reverse transcription was done with Maloney murine leukemia virus reverse transcriptase (Life Technologies). All PCR amplifications were carried out with Taq DNA polymerase (Promega, Madison, WI) in the buffer and Mg²⁺ solution provided by the manufacturer. Annealing temperatures varied from 50°C to 65°C. PCR products were cloned into the pGEM-T vector (Promega). Plasmids for DNA sequencing were prepared using the QIAprep Miniprep Kit (Qiagen). DNA sequences were determined by the dye termination cycle sequencing method using an ABI PRISM BigDye Terminator Cycle Sequencing ready reaction Kits with AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA). All other manipulations of nucleic acids, such as ligation, transformation, gel electrophoresis, gel elution, and preparation of buffers, were carried out following standard protocols (Sambrook et al., 1989).

For the cloning and sequencing of Drgal1-L2, adult zebrafish cDNA was amplified by PCR with degenerated primer sets 1 and 2. The authenticity of the PCR product was assessed with a nested primer 3 in combination with the primer 1. Validated PCR products were cloned into pGEM-T vector and sequenced. The cDNA sequence was completed by 5'- and 3'-RACE. The 5'-end of the
full-length Drgal1-L2 was obtained from the 5′ RNA ligase-mediated RACE kit (Ambion, Austin, TX) with outer adapter primer and a gene-specific reverse primer 4 according to the manufacturer’s protocol. The PCR product generated after reamplification with the inner adapter primer and the nested gene-specific reverse primer 5 was authenticated with the nested primer 6 in combination with the primer 5. For 3′-RACE PCR, the first strand cDNA synthesis was performed using MCSDT kter VN lock-docking primer, 5′-CCGCATGCAGCCTA-GATATCGAT16VN-3′, synthesized commercially (Sigma-Genosys) based on Borson et al. (1992). The 3′-RACE PCR product, generated with primer 6 and adapter primer MCSRACE, 5′-CCGCAGATCTAGATATCGA-3′, was subcloned and sequenced.

The full-length cDNA for a Drgal1-L2 isoform (Drgal1-L2d) was obtained by PCR amplification using primers 7 and 8. The full-length cDNAs for two other protogalactins (Drgall-L1 and Drgal-L3) were obtained based on available sequences in the database using the primer sets 13–14 and 15–16, respectively. The 5′ and 3′ end of the full-length chimera and tandem-repeat galectins from D. rerio (Drgal3 and Drgal9-L1, respectively) were also performed as described. The 5′-RACE PCR product for Drgal3 was obtained with outer adapter primer and primer 18 and was authenticated with two nested primers 17 and 19. The 3′-RACE PCR product for Drgal3 was obtained with primer 17 and MCSRACE and authenticated with primer 16 in combination with the primer 17. The 3′-RACE PCR for Drgal9-L1 was performed with primer 20 and MCSRACE, and the product was confirmed with two nested primers, 21 and 22. The PCR products of expected size were subcloned into pGEM-T vector and sequenced.

Genomic structure of Drgal1-L2
Genomic DNA was prepared from adult zebrafish testis following a standard protocol (Sambrook et al., 1989). The purified genomic DNA was digested individually with seven restriction enzymes (EcoR V, Sca I, Dra I, Pvu II, Ssp I, Hpa I, Sma I). Seven libraries were constructed following Universal GenomeWalker kit (Clontech, Palo Alto, CA). For amplification of the exon I–exon III region, adapter primer AP1 and primer 4 were used to generate a PCR product, which was reamplified with nested adapter primer AP2 and 5. The sequence of the product was determined with an internal primer. 12. For amplification of exon III–exon IV region, primers 9 and 10 were used, and the product was authenticated with two nested reverse primers, 4 and 5, in combination with forward primer 9. Amplification of exon IV (in part) and the 3′ noncoding region was carried out with primers 11 and AP2. The PCR products of expected size were subcloned into pGEM-T vector and sequenced.

Chromosomal mapping of Drgal1-L2
The Drgal1-L2 gene was mapped by radiation hybrid mapping using LN54 panel developed by Hukriede et al. (1999). Briefly, 93 radiation hybrid and 3 control cell lines (gift from Dr. Marc Ekker, Loeb Health Research Institute at the Ottawa Hospital, Ottawa, ON, Canada) were tested on PCR assay in duplicate using gene-specific primers 4 and 6. The results obtained through PCR assay were scored according to Hudson et al. (1995): 1, 0, and 2 indicate a positive, negative, and ambiguous hybrid data, respectively. The mapping of Drgal1-L2 against the LN54 panel was obtained by accessing a Web tool through ZFIN (http://zfsh.uoregon.edu/zfin).

Expression of galectins in selected zebrafish developmental stages and adult organs by reverse transcriptase PCR
Total RNA was purified from several embryonic stages (1–19 h postfertilization and 1–5 days postfertilization; ~200 embryos from each stage) from adult organs of zebrafish. Poly (A) + RNA was isolated from the total RNA on poly (dT)-Dynabeads as described. For reverse transcriptase PCR analysis, first strand cDNAs were generated from the purified mRNA using the cDNA synthesis kit (Life Technologies) and used as template for PCR amplification. The presence of the Drgal1-L2 isoform (Drgal1-L2d) was determined from PCR products amplified by primers 5 and 6, following separation on a 3.5% MetaPhor agarose (FMC BioProducts, Rockland, ME).

Whole mount in situ hybridization
In situ hybridization was carried out as previously described (Du and Dienhart, 2001). Briefly, whole zebrafish embryos were fixed in 4% paraformaldehyde in azide-free PBS (pH 7.2) overnight at 4°C. After washing with PBS/0.1% Tween 20, embryos were dechorionated, soaked with acetone at –20°C for 10 min, and washed five times (5 min each) with PBS-Tween. The embryos were prehybridized with a mixture of 50% formamide, 5× sodium chloride (0.15 M)/sodium citrate (0.015 M) (SSC), 0.5 mg/ml tRNA, 50 μg/ml heparin, and 0.1% Tween 20 for at least 4 h at 70°C and hybridized with digoxigenin-labeled RNA (antisense or sense) probe for 4–12 h at the same temperature. The embryos were washed with a 1:1 mixture of prehybridization buffer (without tRNA and heparin) and 2× SSC for 15 min at 70°C followed by two washes (30 min each at 70°C) with 0.2× SSC/0.1% 3-[3-cholamidopropyl]dimethyl-ammonio]-1-propane sulfonate (CHAPS). Subsequently, the embryos were washed with maleic acid buffer (MAB) (0.1 M maleic acid/0.15 M NaCl, pH 7.5) for 5 min at RT, blocked with 10% goat serum/2% blocking reagent (Roche) in MAB for at least 4 h at RT, and incubated overnight at 4°C with a 1:2000 dilution of antigesagen antibody conjugated with alkaliine phosphatase (Roche). This was followed by six washes in PBS-Tween for 15 min each at RT and two washes with alkaline phosphate buffer (0.1 M Tris/0.05 M MgCl2/0.1 M NaCl/0.1% Tween 20, pH 9.5) for 10 min each. Color was developed with a mixture of 4.5 μl nitro blue tetrazolium (75 mg/ml) and 3.5 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/ml). For the preparation of probes, plasmid vector pGEM (Promega) containing zebrafish galectin cDNA was linearized by restriction digest with Spe I and Sac II. Antisense and sense RNA probes were synthesized by incorporating digoxigenin-UTP (Roche) using T7 and SP6 RNA polymerase (Promega) according to manufacturer’s protocol.
Examination of Drgal1-L2 protein expression by whole mount antibody staining

To study Drgal1-L2 expression in zebrafish embryos, whole mount antibody staining was performed using high-titer anti-Drgal1-L2 specific antibodies (active up to 100,000 dilution) following the protocol of Du et al. (1997). For antibody labeling, embryos were fixed with 4% paraformaldehyde for 1 h at RT, washed twice with PBS-Tween for 5 min each, and soaked in cold acetone for 10 min at −20°C. Subsequently, embryos were washed twice with PBS-Tween 5 min each, washed once with 0.1% BSA/1% dimethyl sulfoxide/PBS (BDP) for 5 min, and incubated with avidin (Vector Laboratories, Burlingame, CA) (4 drops/ml) in blocking buffer (10% of goat serum in BDP) for 30 min at RT. The embryos were then washed twice with BDP for 5 min each and incubated with avidin-biotin (Vector) (4 drops/ml) overnight at 4°C. The embryos were then washed three times for 30 min with BDP, followed by incubation with diluted (1:1000) biotin-labeled secondary antibody (goat anti-rabbit IgG) (Vector) in BDP for 1 h at RT. Embryos were then washed three times for 30 min with BDP and incubated with 1:1 diluted avidin-biotin complex solution (Vector) for 30 min at RT. Finally, embryos were washed three times for 30 min in BDP and color-developed with DAB substrate (Vector) according to the manufacturer’s protocol.

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Abbreviations

ABTS, diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate); ASF, asialofetuin; BSA, bovine serum albumin; CRD, carbohydrate recognition domain; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; MAB, maleic acid buffer; ME, 2-mercaptoethanol; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PBS, phosphate buffered saline; RACE, rapid amplification of cDNA ends; RT, room temperature; SDS, sodium dodecyl sulfate; SSC, sodium chloride (0.15 M)/sodium citrate (0.015 M).

References


