Developmental and Comparative Immunology 35 (2011) 1388–1399

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Structural and functional diversity of the lectin repertoire in teleost fish: Relevance to innate and adaptive immunity

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ARTICLE INFO

Article history:
Available online 30 August 2011

Keywords:
Lectins
Innate immunity
Fish
Self/non-self recognition
Effector
Regulatory functions
Complement activation

ABSTRACT

Protein–carbohydrate interactions mediated by lectins have been recognized as key components of innate immunity in vertebrates and invertebrates, not only for recognition of potential pathogens, but also for participating in downstream effector functions, such as their agglutination, immobilization, and complement-mediated opsonization and killing. More recently, lectins have been identified as critical regulators of mammalian adaptive immune responses. Fish are endowed with virtually all components of the mammalian adaptive immunity, and are equipped with a complex lectin repertoire. In this review, we discuss evidence suggesting that: (a) lectin repertoires in teleost fish are highly diversified, and include not only representatives of the lectin families described in mammals, but also members of lectin families described for the first time in fish species; (b) the tissue-specific expression and localization of the diverse lectin repertoires and their molecular partners is consistent with their distinct biological roles in innate and adaptive immunity; (c) although some lectins may bind endogenous ligands, others bind sugars on the surface of potential pathogens; (d) in addition to pathogen recognition and opsonization, some lectins display additional effector roles, such as complement activation and regulation of immune functions; (e) some lectins that recognize exogenous ligands mediate processes unrelated to immunity: they may act as anti-freeze proteins or prevent polyspermia during fertilization.

1. Protein–carbohydrate interactions in animal–microbe consortia

Protein–carbohydrate interactions are critical in the establishment and homeostasis of highly specific mutualistic associations in complex animal–microbe consortia. The success of individual consortium components depends on either the maintenance of a tightly regulated balance for mutual benefit (symbiosis or commensalism), or host colonization beneficial to the microbe while leading to loss of fitness in the host (pathogenic), the latter usually associated with host defense responses aimed at rejecting the microbe (Casadevall and Pirofski, 2000).

The term "lectin" is commonly used *sensu lato* to encompass a wide variety of carbohydrate-binding proteins and glycoproteins from virus, bacteria, fungi, protista, plants, and animals (Mirelman, 1986; Vasta and Ahmed, 2008). A wide variety of microbial pathogens, ranging from virus to protistan parasites, use lectins that recognize the host cell surface glycans as colonization factors (Mandlik et al., 2008). Among viral pathogens, the best characterized of these are probably the influenza A hemagglutinins which bind to sialic acids on bird and mammalian cells surface glycans in a species- and tissue-specific manner. Similarly, specific adhesins that form part of heteropolymeric fibers such as pili and fimbriae on the bacterial surface, mediate the interactions between the bacterial pathogen and a cell-surface ligands on specific host tissues. Furthermore, lectins function as host colonization and virulence factors for several protistan parasites (Frederick and Petri, 2005; von Itzstein et al., 2008). In turn, humoral and membrane-associated lectins from the host are critical recognition molecules that may facilitate the establishment of favorable mutualistic interactions with the colonizing microbes, or initiate

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0145-305X/$ – see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.dci.2011.08.011

innate and adaptive responses against the potentially pathogenic ones. In addition, host lectins also mediate downstream effector functions, such as agglutination, immobilization, and complement-mediated opsonization and killing of potential pathogens (Vasta et al., 2004a). Further, in vertebrates, lectins also function as homeostatic regulators of adaptive immune responses, significantly modulating embryonic development, cell differentiation, and activation of dendritic, B, and T cells (Rabinovich and Toscano, 2009). This review will focus on the current knowledge of lectin repertoires in fish and their roles in the immune responses to infectious challenge.

2. Structural and functional aspects of animal lectins

2.1. Current classification of animal lectins

Animal lectins are usually covalently or non-covalently bound oligomeric assemblages of peptide subunits characterized by the presence of a carbohydrate recognition domain (CRD) (Taylor and Drickamer, 2003) (Fig. 1). The presence of conserved amino acid sequence motifs within the CRD, unique structural domains, and distinct properties such as requirement of divalent cations or a reducing environment for ligand binding, has lead to their classification in several major families (Table 1). More recently, the structural characterization of selected family members has enabled the identification of their structural folds (Fig. 2). It is noteworthy that in some lectin families, a lectin polypeptide subunit can include multiple tandemly arrayed CRDs, or a CRD can be linked to distinct functional domains, as in C-type lectins (CTLs) and F-type lectins (FTLs) (Fig. 3). Through this structural diversity, a lectin subunit whether soluble or membrane-associated, can display multiple biological activities (Zelensky and Gready, 2005; Odom and Vasta, 2006).

2.2. Structural and functional diversity of the lectin repertoire in invertebrates and vertebrates

In most animal taxa, highly diversified lectin repertoires that comprise representatives of multiple lectin families have the potential to greatly expand the range of carbohydrate ligands that

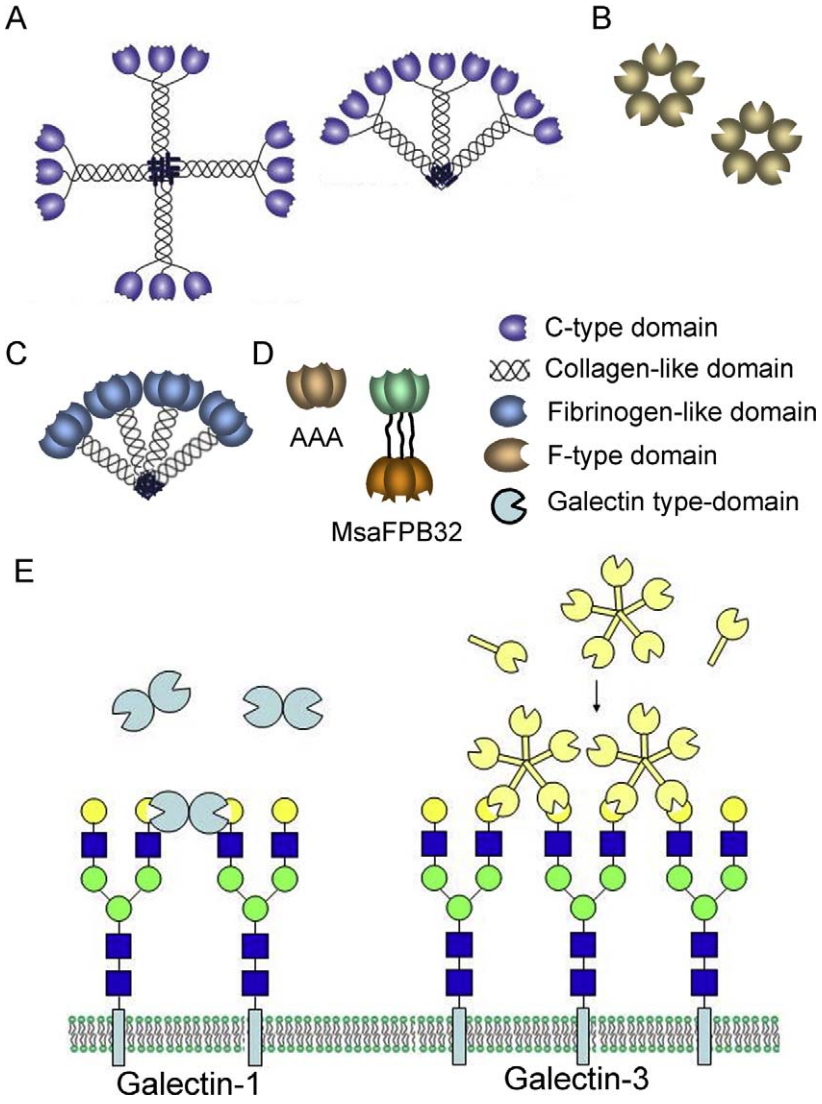

Fig. 1. Quaternary structure arrangements of animal lectins. Examples of oligomeric organization of C-type lectins: (A) cruciform and bouquet collectins, (B) pentraxins, (C) ficolins. (D) Examples of oligomeric fish F-lectins: AAA trimer, with 1 CRD per monomer, and MsaFBP32 trimer, with 2 tandemly arrayed related CRDs per molecule. (E) Proto-type galectins (galectin 1, in blue) associate as non-covalently bound dimers through a hydrophobic interphase, whereas chimera galectins (galectin 3, in yellow) associate through their amino–terminal domain to form oligomers that, in the presence of multivalent oligosaccharides, display binding cooperativity.

Table 1
Animal lectin families.

Lectin families	Cation	Specificity	Defining features
C-type	Yes	Man, Gal, Fuc (Variable)	C-type sequence motif
Galectins	No	β-Galactosides	S-type sequence motif
P-type (Man-6-P)	Variable	Man-6-P	P-type sequence motif
I-type (siglecs)	No	Variable	Ig-like domains
F-type	Variable	L-fucose	F-type sequence motif
Calnexin	Yes	Glc_1Man_9	Calnexin sequence motif
M-type	Yes	Man_8	M-type sequence motif
L-type	Yes	Variable	L-type sequence motif
R-type	No	Variable	R-type sequence motif
F-box	No	$GlcNAc_2$	F-box sequence motif
Ficolin	Yes	GlcNAc, GalNAc	Ficolin sequence motif
Chitinase-like (chilectins)	No	Chito-oligosaccharide	Triose-phosphate isomerase (TIM) barrel-like structure
Pentraxins	Most	PC/galactosides	Multimeric binding motif
Heparin-binding type	No	Heparin/heparin-$(SO_4)^{2-}$	Basic amino acid clusters
Intelectins (X-type)	Yes	Gal, galactofuranose, pentoses	Intelectin sequence motif

can be recognized on endogenous and exogenous glycans. The association of multiple lectin and effector domains in the same polypeptide subunit described above increases the functional efficiency of the system, and the presence of isoforms with subtle differences in specificity within each lectin type further enhances their diversity in ligand recognition. However, unlike immunoglobulins and the recently identified variable lymphocyte receptors (VLRs) of agnathans (Pancer and Cooper, 2006), lectins do not generate diversity in recognition by genetic recombination and are "hardwired" in the germline. Therefore, the genetic basis of diversification of lectin repertoires, including tandem gene duplications and development of complex multigene families, their allelic variation, formation of chimeric structures by exon shuffling, additional variability by alternative splicing, and the structural basis for the potential "plasticity" of their carbohydrate binding sites is currently an area of great interest (Vasta and Ahmed, 2008).

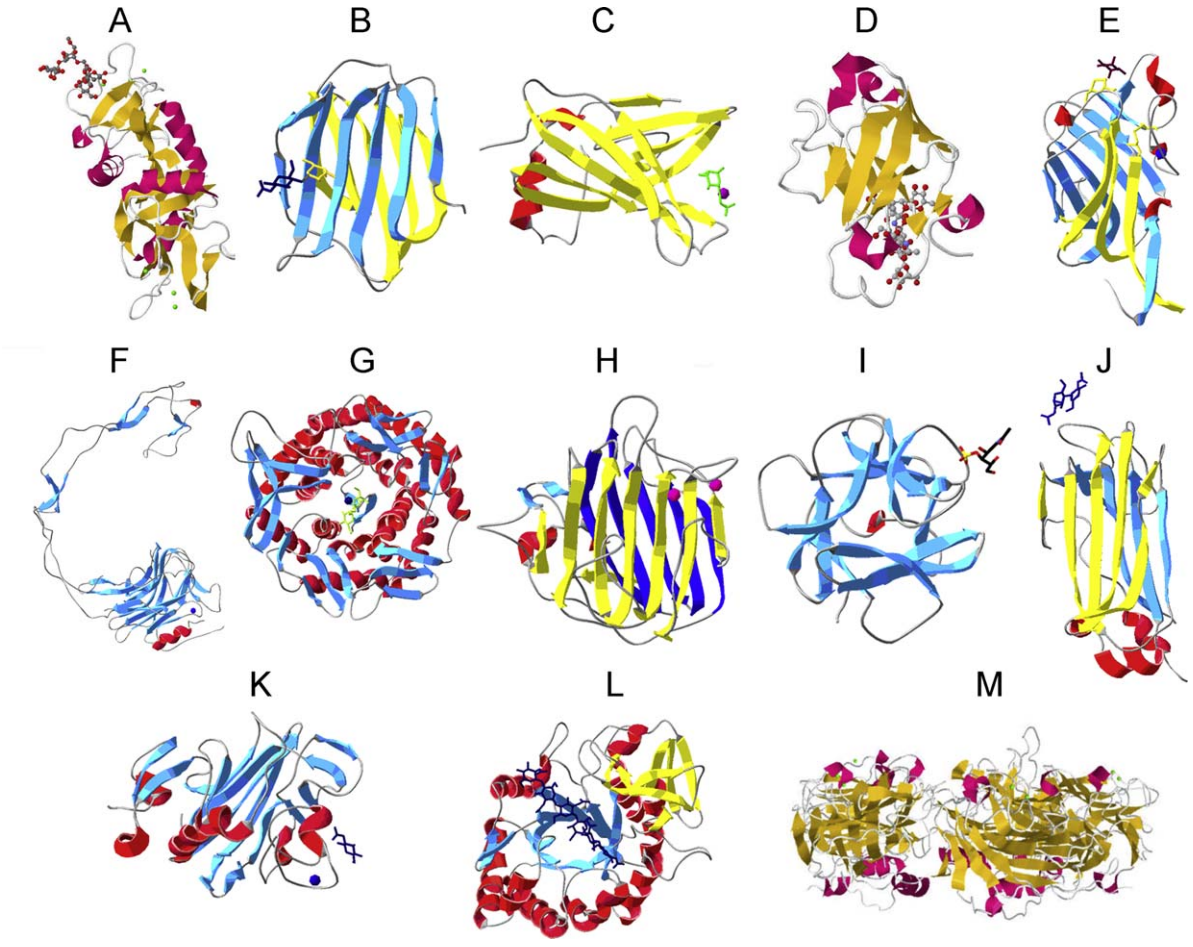

Fig. 2. Structure of representatives from selected lectin families: PDB IDs are in square brackets. (A) C-type lectin: mannose-binding protein A PDB [2MSB]; (B) galectin: Human galectin-3 [1KJL]; (C) P-type lectin: extracytoplasmic domain of the mannose 6-phosphate receptor [1M6P]; (D) I-type lectin: Siglec-7 [1O7V]; (E) F-type lectin: *Anguilla anguilla* agglutinin [1K12]; (F) calnexin: Luminal domain of canine calnexin [1JHN]; (G) M-type lectin: Catalytic domain of human ER mannosidase [1X9D]; (H) L-type lectin: rat ERGIC-53 [1R1Z]; (I) R-type lectin: murine mannose receptor [1DQ0]; (J) F-box lectin: murine Fbs1 [1UMI]; (K) ficolin: TL-5A CRD of Japanese horseshoe crab [1JC9]; (L) chitinase-like lectin: human cartilage glycoprotein (HCGP39) [1HJW]; (M) pentraxin: human C-reactive protein [1GNH].

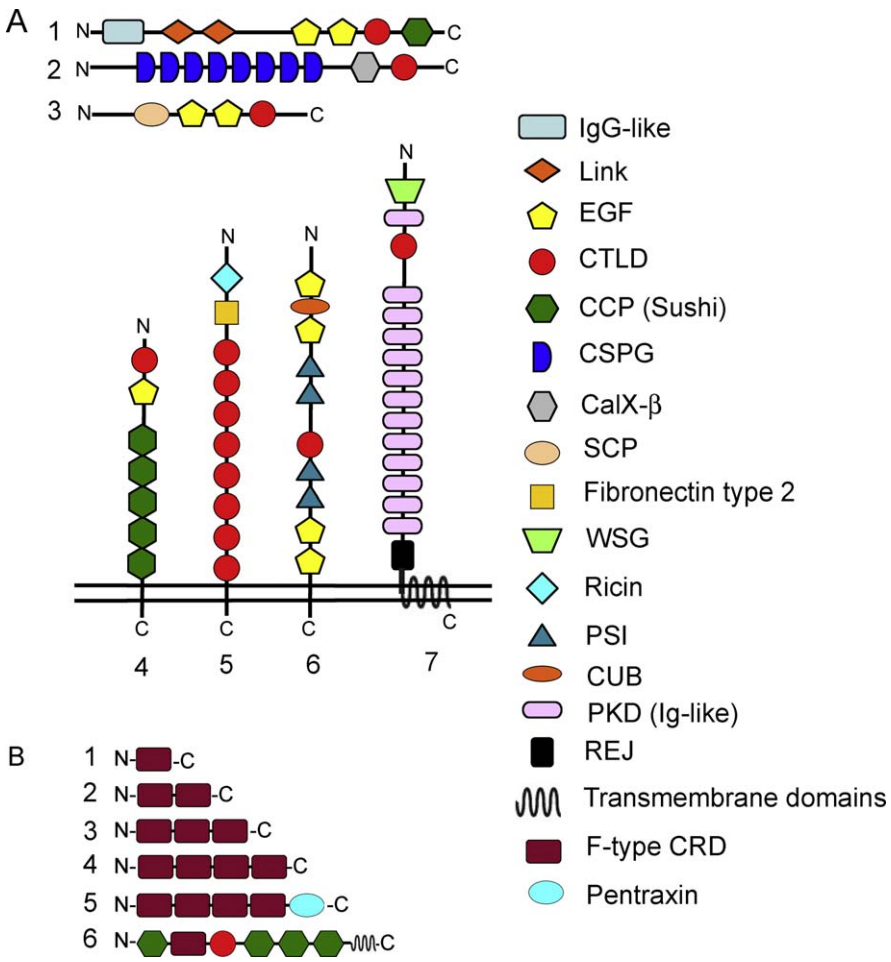

Fig. 3. Domain architecture of C-type and F-type lectins. (A) Examples of chimeric C-type lectins (adapted from (Zelensky and Gready, 2005)): (1) lecticans, (2) Calx-β and CTLD containing Protein (CBCP), (3) soluble protein containing SCP, EGF, EGF and CTLD domains (SEEC), (4) selectins, (5) the macrophage mannose receptor group, (6) the tetranectin group, (7) polycistin; (B) examples of different arrangements of F-type lectins (adapted from (Odom and Vasta, 2006)): (1) tachylectin-4, eel agglutinin; (2) *Morone saxatilis* FBP32 and *Danio rerio* FBP38; (3) *Xenopus tropicalis* XtrIII-FBPL; (4) *Xenopus laevis Xl* -FBL22; (5) *Xl* PXN120; (6) *Drosophila* "furrowed"-like receptor.

2.3. Current approaches for assessing the structural and functional diversity of lectin repertoires

The dramatic increase in availability of genomic and EST public databases for many organisms during the past two decades, has greatly facilitated the search for lectin canonical sequence motifs, and therefore have greatly facilitated the assessment of the structural diversity of their lectin repertoires. Moreover, the use of forward and reverse genetic approaches in model organisms amenable to genetic manipulation has provided further insight into their functional aspects. A continuously expanding variety of bioinformatic tools (BLASTP, ProfileScan, SwissProt and PfamA) have enabled not only the identification of lectins, but also lectin domains present in complex mosaic proteins. For example, mining of the zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes*) genomes has revealed the presence of greatly diversified lectin repertories, with multiple members of the galectin, CTL, FTL, and X-type lectins (XTL) (Vasta et al., 2004b). The identification of complex and diverse lectin repertoires in a single organism has led to the development of high throughput, in some cases automated, novel technologies for analyzing in detail the carbohydrate specificities of the proteins of interest. Although techniques such as agglutination-inhibition or binding-inhibition in solid phase are simple, inexpensive, and reliable, the use of glycan microarrays or "glycochips" for comparative analysis of lectin specificity has enabled the simultaneous testing of large numbers of potential carbohydrate ligands (Comelli et al., 2006). In addition, frontal affinity chromatography (FAC) (Nakamura-Tsuruta et al., 2006) and methods based on surface plasmon resonance (Biacore) (Smith et al., 2003) have allowed the rigorous quantitative kinetic analysis of protein-carbohydrate interactions with a relatively high throughput.

2.4. Lectins as recognition and effector factors in innate immunity and modulators of adaptive immune responses

The various lectin types described above can be present in the intracellular compartments mediating critical processes from RNA splicing to protein folding and glycoprotein trafficking, or can be released to the extracellular compartment and remain at the cell surface or as soluble components in biological fluids. Lectins function as recognition and effector factors, as agglutinins, opsonins, complement activating factors, LPS-binding molecules, signaling receptors, and as key immune regulators of immune homeostasis (Vasta and Ahmed, 2008). By recognizing endogenous or exogenous ligands, lectins mediate multiple biological processes encompassing cell–cell or cell–extracellular matrix interactions, leading to lattice formation at the cell surface and activation of signaling pathways that are key to innate and adaptive immune defense processes (Vasta et al., 1999; Rabinovich et al., 2007). The instructive roles of innate immunity on adaptive immunity have been widely

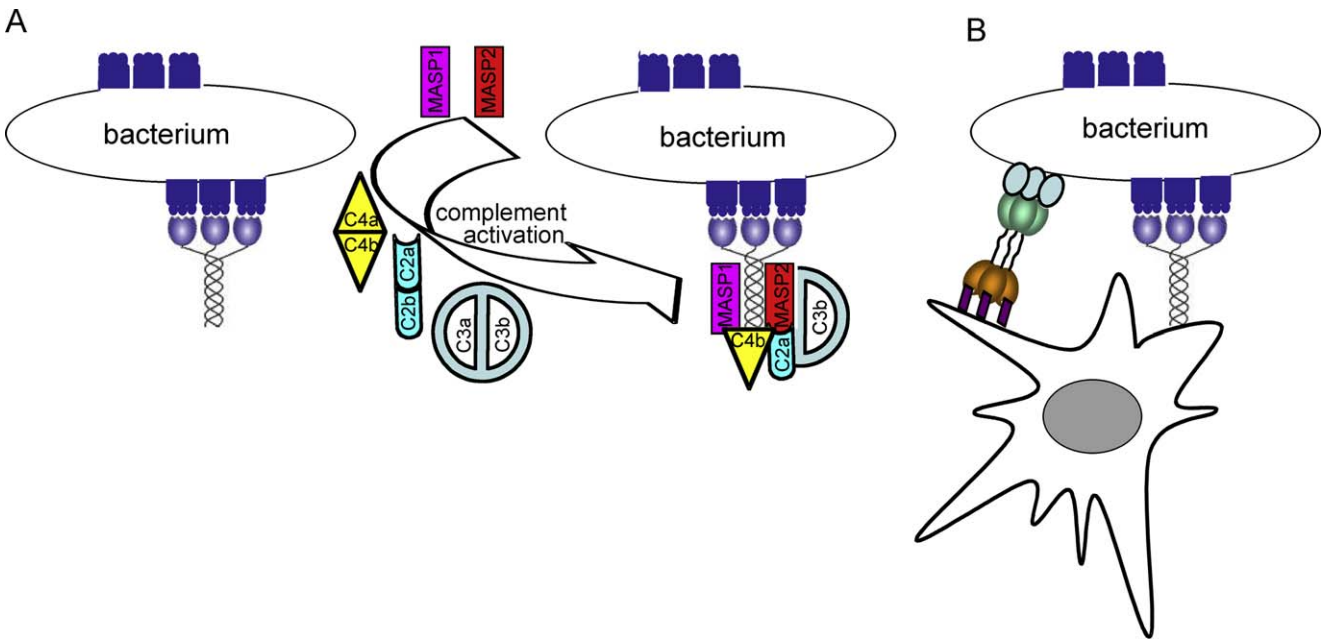

Fig. 4. Effector functions of C-type and F-type lectins. (A) Complement activation by binding of a collectin to a microbial surface; (B) lectin binding and opsonization of a microbe by an F-type lectin (left) and a collectin (right) to a macrophage.

recognized, and in recent years the functional interplay between lectins and their receptors has been characterized in considerable detail (van Kooyk and Rabinovich, 2008). Upon recognition of potential pathogens, some humoral lectins, such as collectins, ficolins and pentraxins, function as effector factors by promoting their phagocytosis and activating the complement system (Fujita, 2002) (Fig. 4). This pathway can be initiated by the binding of a lectin, such as the mannose-binding lectin (MBL) or ficolin, to microbial surfaces bearing non-self carbohydrate determinants, and subsequent association with a serine protease [MBL-associated serine protease (MASP)] which, by activating C3, can lead to either phagocytosis of the opsonized target *via* the complement receptor, or its lysis *via* assembly of the membrane attack complex. Although initially described as mediators of developmental processes, galectins have been recently demonstrated to participate directly or indirectly in immune functions (Vasta et al., 1999). Like CTLs, galectins can function as recognition factors of microbial glycans (Vasta, 2009; Stowell et al., 2010), or as regulators of multiple adaptive immune functions by modulating B cell development, inducing or preventing T cell apoptosis, and modulating T cell responses and the activity of macrophages, eosinophils, and neutrophils (Vasta et al., 1999; Rabinovich et al., 2007; van Kooyk and Rabinovich, 2008; Jayaraman et al., 2010; Sehrawat et al., 2010; Liu and Rabinovich, 2010). Other lectin-like proteins, such as calnexin and calreticulin, participate in the processing of antigens by dendritic cells for presentation in the context of MHC to the naïve T cells (Vasta and Ahmed, 2008). Lectins and other pattern-recognition molecules also play critical roles in qualitatively and quantitatively modulating other innate immune responses such as NK cell function, and adaptive immune responses through the requirement of co-stimulatory signals from cell surface proteins on antigen presenting cells to naïve T lymphocytes, via CD28 receptors (van Kooyk and Rabinovich, 2008). In summary, the functional interplay of various humoral and cell-associated lectin types in the immune response contributes not only to quickly recognize and neutralize the microbial challenge, but lead to an effective long term adaptive immunity. The increasing environmental and economic relevance of viral, bacterial, fungal and parasitic infections in fisheries and aquaculture (Harvell et al., 1999) has greatly enhanced the interest in the role of lectins in innate immune responses as potential targets for intervention.

3. The lectin repertoire in fish: genomic and structural diversity

Virtually all components of the mammalian adaptive immune response are present in elasmobranchs and teleost fish. However, it is currently accepted that their innate immune responses carry a substantial burden of the defense functions against infections disease. Therefore, great interest has been generated in the past few years on the structural-functional aspects of innate immune components, particularly lectins, toll-like receptors, and complement. The recent identification of dendritic cells (DCs) in teleost fish (Lugo-Villarino et al., 2010) opens the possibility that similar lectin endocytic receptors, antigen presentation mechanisms, and instructional activity of DCs on T and B cells are operative in this taxon. Early evidence about the presence of lectins of teleost fish, particularly in plasma and eggs, was obtained by the application of serological approaches. More recently, implementation of biochemical and molecular approaches has contributed with a more accurate appreciation of the complexity of the lectin repertoires, not only in plasma and eggs, but also in skin mucus (Anderson and Ingham, 2003) and gastrointestinal tract (Wittbrodt et al., 2002), and their roles in innate immunity. Finally, structural approaches either by determination of the structures of fish lectins or by homology modeling over structures determined in other taxa have provided detailed information about the critical amino acid residues that interact with the carbohydrate ligands and their true recognition spectrum. Current evidence suggests that among the highly diversified teleost fish taxa, the lectin repertoires are ample and complex, with representatives from most lectin families described so far. Further, recent studies on teleost fish have identified novel families of lectins (Fig. 5), some of them with members present in other vertebrate and invertebrate taxa. In addition, this lectin diversity is greatly amplified by the presence of isoforms that exhibit subtle differences in sugar specificity, and presumably, pathogen recognition (Suzuki et al., 2003; Bianchet et al., 2002). The available genomes of tetraodontid pufferfish (*Takifugu rubripes*

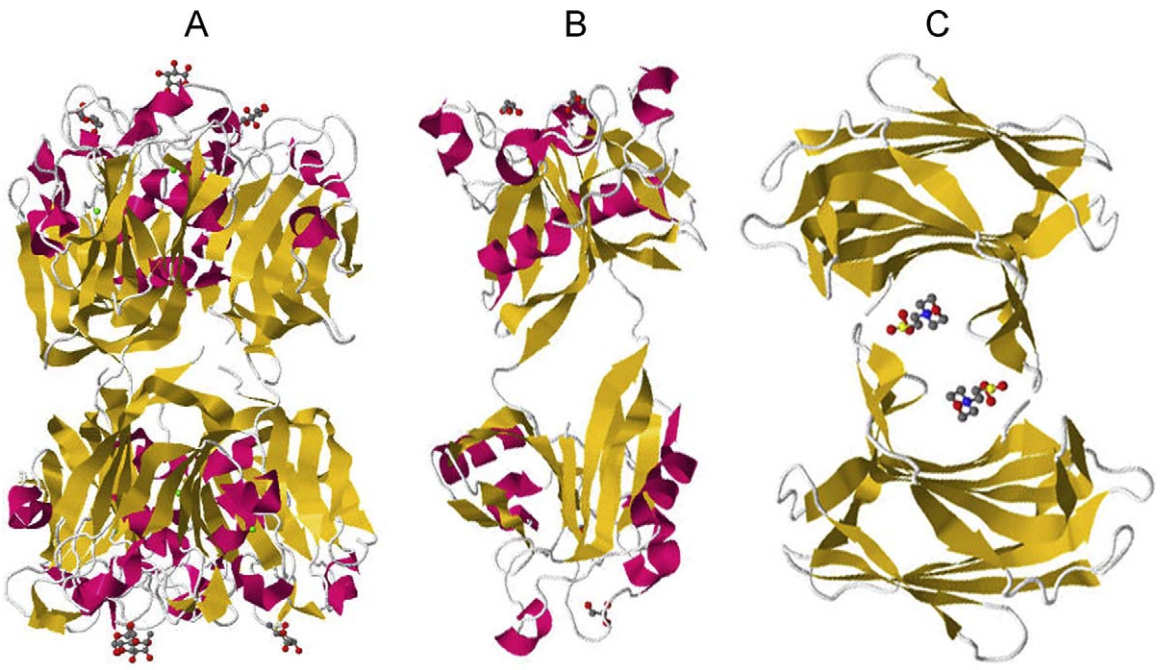

Fig. 5. Structural folds of lectins unique to fish. (A) F-type lectin MsaFBP32 (trimer) striped bass; (B) rhamnose-binding lectin CSL3 of keta salmon (*Oncorhynchus keta*), (PDB ID: 2ZX4); (C) Congerin II of conger eel.

and *T. nigroviridis*) (Aparicio et al., 2002) (www.genoscope.com), zebrafish (*Danio rerio*)(Anderson and Ingham, 2003), and medaka (*Oryzias latipes*) (Wittbrodt et al., 2002) are expanding this view even further. In summary, the implementation of serological, biochemical, structural, and genomic approaches has resulted in not only representatives of most lectin classes but also new lectin families having been identified in teleost fish. It must be noted that a large number of reports describing fish lectins lack information about their primary structure and thus, the requisite evidence for their classification in any of the currently established lectin classes. A description of the lectin families identified in fish follows below.

3.1. C-type lectins

CTLs (Fig. 1) are characterized by Ca^{2+} requirement, diverse carbohydrate specificity, and multiple structural domains sometimes forming chimeric structures (Fig. 3A). CTLs have been classified in several groups comprising the collectins, proteoglycan core proteins, selectins, endocytic receptors, and the mannose-macrophage receptor, some directly or indirectly involved in immune function (Vasta and Ahmed, 2008). The collectins include the MBLs and conglutinin from serum and saliva, and the pulmonary surfactant, with critical roles in innate immunity against viruses and bacteria. Some NK cell and macrophage receptors, and selectins are also CTL-related. Various types of CTLs of diverse carbohydrate specificities have been identified in various tissues, plasma, and skin mucus of several teleost fish species, including rainbow trout (*Oncorhynchus mykiss*), catfish (*Silurus asetus*), carp (*Cyprinus carpio*), eel (*Anguilla japonica*), fugu (*Takifugu rubripes*) and zebrafish (Zhang et al., 2000; Vitved et al., 2000; Tasumi et al., 2002; Singha et al., 2008). Recent studies in rainbow trout revealed CTL domains associated with two extracellular immunoglobulin domains and cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the "novel immune-type receptors" (NITRs) (van den Berg et al., 2004). In addition, selectin and macrophage mannose receptor gene sequences are present in the whole-shotgun genome assembly of zebrafish and of fugu indicating that this receptor appeared early in vertebrate evolution.

3.2. Galectins

Galectins are β-galactosyl-binding lectins that require a reducing environment for binding activity. Based on the primary structure and polypeptide architecture of the subunits, galectins are classified as "proto", "chimera", and "tandem-repeat" types (Vasta and Ahmed, 2008). Although the biological roles of galectins are not fully understood, they mediate cell–cell or cell–intercellular matrix interactions in developmental processes and immunity. Electrolectin, the first galectin identified and characterized, was isolated from electric organs of the electric eel *Electrophorus electricus* (Teichberg et al., 1975). Since then, a substantial number of galectins from the three structural types have been identified and characterized in various tissues, plasma and mucus of elasmobranch and teleost fish (Vasta et al., 2004b; Ahmed et al., 1994; Muramoto et al., 1999; Ahmed et al., 2004; Inagawa et al., 2001), which show remarkable structural and binding specificity conservation with the mammalian homologues (Ahmed et al., 2004). In addition, a substantial number of galectin sequences from teleost fish are available from GenBank.

3.3. F-type lectins

The FTLs, also known as fucolectins, constitute the most recent lectin family to be identified and structurally characterized in teleosts (Odom and Vasta, 2006; Bianchet et al., 2002, 2010). The FTL family is constituted by a large number of proteins exhibiting greater multiples of the F-type motif, either tandemly arrayed or in mosaic combinations with other domains, including a putative transmembrane receptor, that suggests an extensive functional diversification of this lectin family from prokaryotes to amphibians (Odom and Vasta, 2006; Bianchet et al., 2002) (Fig. 3). Although the European eel (*Anguilla anguilla*) agglutinin (AAA) possesses a single F-type CRD, in most teleosts FTLs contain either duplicate (Zebrafish, striped bass (*Morone saxatilis*), and fugu) or quadruplicate (steelhead trout) tandemly arrayed domains yielding subunits of variable sizes, even within a single fish species. It is noteworthy that the multiple duplicate tandem homologues present in

modern teleost orders appear to be the product of independent duplications (Odom and Vasta, 2006). Further, these tandem arrays may yield mosaic proteins by including pentraxin (*X. laevis*) or C-type domains (*Drosophila melanogaster* CG9095). In *Xenopus* spp., however, these lectins are composed of either triplicate or quintuple tandem F-type domains (Odom and Vasta, 2006). The native structures of the European eel and striped bass agglutinins are homotrimers (Bianchet et al., 2002, 2010), which suggests that ligand binding is enhanced through avidity. The striped bass FTL (MsaFBP32) consists of two non-identical F-type CRDs in tandem. The structure of MsaFBP32 revealed a cylindrical trimer divided into two globular halves: one containing N-terminal CRDs (N-CRDs) and the other containing C-terminal CRDs (C-CRDs) (Bianchet et al., 2010). The resulting binding surfaces at the opposite ends of the cylindrical trimer have the potential to cross-link fucosylated glycans that are widely distributed in prokaryotes and eukaryotes supporting the results that binary tandem CRD FTLs function as opsonins (Salerno et al., 2009) by cross-linking sugar structures displayed by microbial pathogens and glycans on the surface of phagocytic cells from the host (Bianchet et al., 2010). Variability of critical residues in the binding pocket and surrounding loops in the multiple isoforms, as expressed in the Japanese eel FTL (Honda et al., 2000), suggests that alternative interactions with terminal and subterminal sugar units may expand the range of diverse oligosaccharides recognized by the lectin isoform repertoire (Odom and Vasta, 2006). Closely related FTLs have been identified and characterized in other teleost species (Cammarata et al., 2007). Other fucose-binding lectins have been identified in bighead carp (*Aristichthys nobilis*) (Pan et al., 2010) and Nile tilapia (*Oreochromis niloticus L.*) (Argayosa and Lee, 2009) (*Oreochromis niloticus*), although the lack of full sequence information prevents their identification as members of the FTL family.

3.4. Rhamnose-binding lectins

A novel family of rhamnose-binding lectins (RBLs) was identified in eggs of steelhead trout (*Oncorhynchus mykiss*), catfish (*Silurus asetus*), white-spotted char (*Salvelinus leucomaenis*), and Spanish mackerel (*Scomberomorous niphonius*) (Tateno et al., 2002; Terada et al., 2007). These lectins, that bind L-rhamnose and sugars possessing hydroxyls in the same configuration at C2 and C4, such as L-arabinose and D-galactose, are homologous to members of the low-density lipoprotein receptor superfamily. Multiple RBLs, synthesized in liver and oocytes, are widely distributed in the adult tissues including spleen, thrombocytes, and blood leukocytes. The crystal structure of the RBL CSL3 revealed that it is a homodimer of two 20 kDa subunits with a dumbbell-like overall shape, and forms a pseudo-tetrameric structure (Shirai et al., 2009). The gene organization of the RBL from snakehead *Channa argus* (SHL) suggests that the RBL ancestral gene may have diverged and evolved by exon shuffling and gene duplication, producing functionally diversified forms in different organisms. The structure of the SHL promoter suggests that expression may be induced in response to inflammatory stimuli, such as LPS, IL-6, and IFN-gamma (Jia et al., 2010).

3.5. Pufflectins

Mannose-binding lectins (pufflectins) isolated from the skin mucus and intestine of pufferfish, *Fugu rubripes*, are homodimers of non-covalently associated 13 kDa subunits (Zhang et al., 2010a). They show intriguing homology (about 30% identity) to mannose-specific lectins from monocotyledoneous plants (Zhang et al., 2010a). Pufflectin genes are expressed in gills, oral cavity wall, esophagus, and skin, as highly similar isoforms, with an isoform exclusively expressed in the intestine. Pufflectins differ from mannose-binding lectins purified from pufferfish plasma, and recent work has revealed that they are widely distributed among teleost fish families.

3.6. X-type lectins/intelectins

The X-lectin (XL) lectin family [reviewed in Vasta and Ahmed, 2008] was initially described as the *Xenopus laevis* oocyte cortical granule lectin XL35. Since then, homologues have been identified (designated "intelectins") in protochordates, agnathans, fish, other amphibian species, mouse (intelectins-1 and -2) and man (HL-1 and HL-2 intelectins, omentin, and lactoferrin receptor). XL/intelectins are Ca^{2+}-dependent, soluble oligomers of 34–45 kDa glycosylated subunits with a fibrinogen-like domain that functions as the CRD with specificity for alpha-galactosides. Several XL35 homologs have been identified in fish, including the rainbow trout (about 46% identical to the XL35 C-terminal region) expressed in liver, and the grass carp (*Ctenopharyngodon idella*) intelectin gcIntL, about 55% identical to HL-1 and expressed in multiple tissues including head kidney, spleen, heart, gill, intestine, and brain [reviewed in Vasta and Ahmed, 2008].

3.7. Pentraxins

Pentraxins, which includes C-reactive protein (CRP) and serum amyloid P (SAP), are disc-shaped cyclic pentamers of identical subunits of Mr ranging from 20,000 to 30,000, non-covalently bound in most examples (Bottazzi et al., 2010). CRP is a prototypical pentraxin that distinguishes self from non-self, opsonizes bacteria, and activates complement. In addition to binding phosphocholine, it exhibits lectin-like properties including binding to carbohydrates and related structures, divalent cation requirements, overall molecular structure and biological functions (Bottazzi et al., 2010). Some pentraxins behave as acute phase reactants, rapidly increasing their plasma concentration up to 1000-fold or more in response to stress, injury or infection and modulating immune responses (Bottazzi et al., 2010). Pentraxins constitute a highly conserved family and distant species that exhibit a considerable level of structural similarity, have been identified in several fish species including carp, snapper (*Pagrus auratus*), common wolffish (*Anarhichas lupus*), cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*), and rainbow trout (Szalai et al., 1992; Jensen et al., 1995; Lund and Olafsen, 1999; Cartwright et al., 2004; Cook et al., 2005; Gerwick et al., 2007). However, the architecture of the native CRPs from the channel catfish (*Ictalurus punctatus*) is different from the human prototype (Szalai et al., 1992). SAP-like proteins have been isolated from serum of rainbow trout (Jensen et al., 1995) and Atlantic salmon (*Salmo salar L.*) (Lund and Olafsen, 1999).

3.8. Calnexin and calreticulin

Calnexin and calreticulin are ER lectins that function as molecular sensors [reviewed in Vasta and Ahmed, 2008], recognizing a non-reducing-end glucose residue in an N-linked oligosaccharide precursor, Glc1Man9GlcNAc2, which ensure the proper folding of newly synthesized glycoproteins. The N-terminus domain of calnexin and calreticulin interacts with misfolded proteins and glycoproteins, and binds ATP, Zn^{2+} and Ca^{2+}. Further, they have critical roles in MHC class I-related immune functions. In spite of similar sugar-binding specificity, calnexin and calreticulin bind to a variety of distinct target proteins. Null mice for either calnexin or calreticulin are lethal, indicating that these proteins are not functionally compensatory in development. These also suggest that calnexin and calreticulin play distinct biological roles in the cell. Calreticulin has been recently isolated in rainbow trout (Kales et al., 2004),

whereas calnexin was identified in the channel catfish (*Ictalurus punctatus*) (Fuller et al., 2004).

3.9. Other proteins with lectin-like properties

Recently, lectin-like activity was identified in a TNF-alpha homolog from carp (*Cyprinus carpio*), suggesting that these proteins might promote macrophage activation (Forlenza et al., 2009). Similarly, lectin-like fucose-binding activity was identified in a C1q-like protein from the surfperch (*Neoditrema ransonnetii*); it is expressed in liver, stomach and intestine, and is upregulated by bacterial challenge (Nakamura et al., 2009). Like the pufflectins, an intriguing homotetrameric protein (plumieribetin) from the fin stings and skin mucus of the scorpionfish (*Scorpaena plumieri*) reveals structural characteristics shared with the plant mannose-binding B-lectins, known for the roles in defense against pathogens. However, plumieribetin inhibits binding of $\alpha1\beta1$ integrin to basement membrane collagen IV in a Ca^{2+}-independent, protein–protein interaction, weakening the cell–collagen contacts, and reducing cell spreading (de Santana Evangelista et al., 2009).

4. Functional aspects of lectins in fish immunity

A. Innate and adaptive immunity in teleost fish Given that extant elasmobranch and teleost species display most hallmarks of the antibody-based immune system, as most commonly described in mammals, it has been proposed that the adaptive immune system evolved very rapidly in the early stages of vertebrate evolution (Litman et al., 1999). Newly described immunoreceptor types (Criscitiello et al., 2006; Yoder et al., 2010), immunoglobulin classes (Zhang et al., 2010a), and immune cell functions (Li et al., 2006) also reflect the unique or specific adaptations of immune responses elasmobranchs and teleosts, some of which have been conserved along the mammalian lineages. Like all jawed vertebrates, teleosts are endowed with the capacity for randomly generating an enormous diversity of binding sites on immunoglobulin receptors through somatic gene recombination during development of humoral lymphoid cells. However, in contrast to mammals and birds, teleosts lack class switching between antibody isotypes that is associated with specific effector responses, and IgM remains the dominant isotype.

Unlike vertebrates, protostome and deuterostome invertebrates lack a defense system based on immunoglobulin receptor adaptability for pathogen recognition, but instead rely on “hard-wired” responses based on innate receptors such as Toll receptors, complement components, lectins, and peptidoglycan receptor proteins (PGRPs). Evidence of the effectiveness of these receptors, however, is illustrated by their conservation in mammals, even in the presence of an optimized adaptive immune system. The targets of these receptors are molecules essential to the microbial surface or metabolism, and ubiquitous among microbes. Extensive research on humoral and cell-associated lectins from invertebrates has been carried out through the past few years, but much of the current knowledge on the specific roles of animal lectins in immunity has been obtained from mammalian models [reviewed in Vasta and Ahmed, 2008]. Although immunoglobulin-mediated immune responses endowed with affinity maturation and memory, probably constitute the most sophisticated internal defense system, the fundamental role played by lectins in the mammalian internal defense mechanisms against microbial pathogens, becomes evident in the early childhood immunodeficiencies caused by defective mutations in the collagenous tail of the MBL (Super et al., 1989). Although this critical role in immunity becomes apparent at a developmental stage of human development when adaptive immunity is not yet mature, few studies have focused on the function of lectins in lower vertebrates such as teleosts. The aim of the following sections is to discuss current evidence about the role(s) of lectins in innate and adaptive immunity of teleost fish.

4.1. Roles of lectins in fish innate and adaptive immunity

Although defense responses mediated by lectins lack advantages in adaptability typical of immunoglobulins, the swiftness of the acute phase response and the wide spectrum of microbial species recognized, clearly represents a compensatory advantage that provides an immediate opportunity to eliminate the pathogen, or at least limit the infection, prior to activation of the full immune repertoire. When mammalian neutrophils and macrophages respond to a local site of infection, they are induced to secrete cytokines that regulate expression of multiple acute-phase proteins and induce a systemic response in which collectins, ficolins, pentraxins, and other lectins are released to body fluids including plasma, saliva, alveolar fluid, and mucus. Lectins and lectin-like receptors are expressed in a variety of tissues and cell types, including NK cells (Lopez-Botet et al., 2001) but together with several acute phase reactants, upon immune challenge the mammalian MBL is expressed in the liver and released to the blood stream. By binding to the microbial surface, lectins target potential pathogens for immobilization, opsonization, and killing by complement components, and mediate downstream regulatory immune functions. In the Japanese flounder (*Paralichthys olivaceus*) CTLs are expressed in liver (Kondo et al., 2007) whereas in other fish species the presence of lectins in skin and gut mucus has been particularly intriguing with regards to their potential biological role(s) in external defense against microbial pathogens. CTLs are present in skin mucus of the Japanese eel (*A. japonica*) (Tasumi et al., 2002), and in the conger eel (*Conger myriaster*) (Tsutsui et al., 2007). In the latter, conCL-s, a CTL is expressed in club cells of external and internal mucosal tissues (Tsutsui et al., 2007). In the same species, also proto type galectins (congerins) are present in the mucus and epidermis of the skin (Kamiya et al., 1988). In the striped bass *Morone saxatilis* a prototype galectin is present in skin mucus and most tissues, with the exception of the plasma, and agglutinates several bacterial species, including fish pathogens (Henrikson, Ahmed, and Vasta, unpublished). The rainbow trout ladderlectins RTLL1 and RTLL 2 are expressed in kidney, intestine, gills, and skin (Russell et al., 2008). An FTL from sea bass (*Dicentrarchus labrax*) is expressed in hepatocytes and intestinal cells, and the protein localizes in plasma and the gut tissues (Salerno et al., 2009). In the sea bass hatchlings the FTL (DlFBL) is expressed in columnar and goblet cells of the intestinal epithelium. A similar FTL localizes in the embryo’s residual yolk sack, while related transcripts are present in eggs and embryos (Parisi et al., 2010).

Like members of the collectin family, some pentraxins behave as acute phase reactants in teleosts, rapidly increasing their plasma concentration in response to stress, injury or infection whereas in others, pentraxins are constitutive proteins present at high concentrations in the normal plasma (Lund and Olafsen, 1999). Similarly, FTLs from striped bass appear to be present at substantial levels in plasma of unchallenged individuals, with relatively moderate increases upon Lipopolysaccharides (LPS) exposure (Odom and Vasta, unpublished), while in hepatocytes of the Japanese eel (*A. japonica*), FTLs are rapidly upregulated by infectious challenge, thus behaving as true acute phase reactants (Tasumi et al., 2002). In this species, expression of a mucus galectin was higher in individuals resistant to infection (Tasumi et al., 2004). In LPS-challenged grass carp, intelectin (gcIntL) transcripts are induced in head kidney, spleen, and intestine, and the protein can be detected in various organs and tissues [reviewed in Vasta and Ahmed, 2008]. In contrast, the levels of the rainbow trout plasma ladderlectin remain unchanged or undergo a moderate decrease upon experimental

challenge with live *Aeromonas salmonicida* or LPS (Young et al., 2009).

From the above, the diversity in spatiotemporal expression and tissue distribution of lectins in teleosts suggests that they carry distinct functions in innate immunity, from the time when oocytes are released and fertilized, the larval and juvenile stages, to the adult. Complex lectin repertoires and their molecular partners are present in various body fluids of teleost fish, including plasma and mucus that covers surfaces from skin and the gastrointestinal tract, suggesting roles in both systemic humoral and mucosal immune functions.

Lectins from diverse families identified in fish recognize and agglutinate virus, bacteria, fungi, and parasites. The diversity of the lectin repertoire is further enhanced by the presence of isoforms. FTLs in the Japanese eel (*A. japonica*) are heterogeneous proteins -at least seven isoforms can be identified- synthesized in the liver, exocrine mucus cells in the gill and intestine (Honda et al., 2000). The structure of the eel agglutinin revealed that the highest sequence variability among isoforms resides in the areas that interact with the carbohydrate ligand, suggesting subtle differences in sugar specificity that would expand the spectrum of potential surface ligands and thus, potential pathogens that could be recognized (Bianchet et al., 2002).

Among the fish CTLs, binding and agglutination of various virus, bacteria, yeast, and parasites, some of these specific fish pathogens, has been well documented (Russell et al., 2008; Wei et al., 2010; Ourth et al., 2007; Zhang et al., 2010b). Downstream effector functions have also been reported for fish lectins. The mannose-specific CTL conCL-s from skin mucus of the conger eel (*Conger myriaster*) opsonizes yeast and *Escherichia coli*, possibly representing a skin defense mechanism (Tsutsui et al., 2007). In the same species, the galectin congerin expressed in skin and epithelia of the oral cavity and esophagus, agglutinates and opsonizes enteric bacteria, and it has been proposed that it participates in innate immune responses in skin and the gut mucosa (Nakamura et al., 2007). Similar observations have been reported for FTLs and other fucose-binding lectins, confirming their role in immune recognition (Salerno et al., 2009; Pan et al., 2010; Argayosa and Lee, 2009). An FTL from serum of the gilt head bream (*Sparus aurata*, SauFBP) binds to formalin-killed *Escherichia coli* and enhances their phagocytosis by peritoneal macrophages (Cammarata et al., unpublished). Although RBLs bind egg yolk proteins and are developmentally regulated, they also recognize Gram-negative and Gram-positive bacteria and parasites in a rhamnose-inhibitable manner, suggesting a role in recognition of potential infectious agents that may compromise egg viability during development, or survival of the hatchlings (Tateno et al., 2002; Watanabe et al., 2008; Tsutsui et al., 2003, 2006a,b).

The specific binding, agglutination and opsonization of potential microbial pathogens, together with their capacity to activate complement indicates that lectins are endowed not only of recognition, but effector functions as well. The lectin pathway for complement activation characterized by MBLs and MBL-associated serine proteases (MASPs) is well developed in protochordates and agnathans (Endo et al., 2006; Ourth et al., 2008), and clearly, teleosts inherited and expanded this key component of innate immunity (Nonaka and Smith, 2000). Because the alternative pathway is characterized by the participation of factor D, which is only found in teleosts and higher species, it should be assumed that like the classical pathway that is triggered by antibody/antigen complexes, the alternative pathway represents an acquisition unique to the emergence of jawed vertebrates (Dodds, 2002). The intriguing diversification of C3 in teleosts (Sunyer et al., 1997), begs the question about the existence of an equivalent diversification of its lectin-mediated activation pathways, consistent with the complex lectin repertoires present in this taxon. Thus, the diversification of the lectin repertoire would not only be reflected in a wide recognition spectrum of non-self ligands on potential pathogens, but would extend to their effector functions aimed at their destruction and clearance. Multiple MBL isoforms have been identified in teleost fish including salmonids, carp, and zebrafish as Ca^{2+}-dependent opsonins as well as activators of the complement system, and are expressed exclusively in liver and spleen (Nikolakopoulou and Zarkadis, 2006). MASPs and complement components have been well characterized in carp (Nakao et al., 2006) and rainbow trout (Kania et al., 2010).

Some lectins show biological activities that suggest their involvement in immune regulation and homeostasis. The turbot CTL SmLec1 stimulates kidney lymphocyte proliferation and enhance the killing of bacterial pathogen by macrophages, thereby suggesting that SmLec1 has immunomodulatory activity (Zhang et al., 2010b). In ayu, the interaction between the CTL receptor aCLR, which is expressed in head kidney and peripheral blood leukocytes, and the leukocyte cell-derived chemotaxin 2 (LECT2) has been proposed as responsible for the chemotactic activity (Chen et al., 2010). In the sea bass, nodavirus infection induces expression of a prototype galectin with anti-inflammatory activity (Poisa-Beiro et al., 2009). The RBLs CSL1, 2 and 3, from chum salmon (*Oncorhynchus keta*) bind globotriaosylceramide (Gb3) on the surface of peritoneal macrophage (RTM5) and fibroblastic-like (RTG-2) rainbow trout cell lines and induce proinflammatory cytokines, including IL-1beta1, IL-1beta2, TNF-alpha1, TNF-alpha2 and IL-8. In addition, CSLs had an opsonic effect on RTM5 cells and this effect is significantly inhibited by L-rhamnose, indicating that CSLs enhances their phagocytosis by binding to Gb3 on cell surfaces (Watanabe et al., 2009).

Most lectins appear to carry out multiple functions depending on their spatiotemporal tissue/organ expression and localization throughout the various developmental stages of the organism. The RBLs in fish eggs have been proposed to participate in various biological functions, ranging from recognition, opsonization, and killing of bacterial pathogens to blocking polyspermy, formation of the fertilization envelope, and apoptosis (Tateno et al., 2002). Similarly, the XLs are functionally diverse [reviewed in Vasta and Ahmed, 2008]. In the oocyte, XL-35 is mostly contained within the cortical granules and released upon fertilization, participating in the formation of the fertilization envelope that blocks polyspermy while in the embryo it mediates cell adhesion. In fish, mouse, and human, however, XLs have been proposed to function in innate immune responses by binding to microbial pathogens [reviewed in Vasta and Ahmed, 2008]. Further, the RBL from Asian catfish (*Silurus asotus*) egg SAL induces early apoptosis via binding to surface Gb3 (Nitta et al., 2007; Kawano et al., 2009). SAL also induces the expression of HSP70, and by binding to Gb3 modulates the half-life of membrane-bound HSP70 (Sugawara et al., 2009). Further, the human HL-1/omentin functions as an adipocytokine and enhances glucose transport. The galactose-specific egg lectin SEL24K of Chinook salmon (*Oncorhynchus tshawytscha*) is released during the cortical reaction and blocks polyspermy during fertilization (Yu et al., 2007; Murata et al., 2007).

Taken together, these observations indicate that lectins are clearly pleiotropic molecules that may bind both self and non-self ligands and mediate diverse functions from fertilization and embryogenesis to immune recognition. Given the wide distribution of complex glycans at the surface of pro-and eukaryotic cells, with some oligosaccharide structures common to both, the ability of these lectins to discriminate self-vs non-self probably resides not only on the presence or absence of a particular carbohydrate moiety in either surface but also in the architecture of the displayed oligosaccharides, such as their density and particular topology.

Finally, some lectins may have been coopted in evolution to carry out divergent functions. The type II antifreeze proteins (AFPs) present in plasma of arctic teleosts, such as herring (Atlantic herring (*Clupea harengus*), Pacific herring (*Clupea pallasii*)) and sea raven

(*Hemitripterus americanus*), are CTLs that have lost the capacity to bind sugars, instead disrupting the growth of ice crystals (Loewen et al., 1998). It has been recently proposed that the presence of AFPs in distantly related species such as herring, smelt (*Osmerus mordax*) and sea raven is the result of lateral gene transfer (Graham et al., 2008). Although FTLs have not been identified beyond the amphibians, the C1 and C2 domains of the human coagulation factor V (hCFV) not only share the FTL fold with the fish lectins, but also some of the key primary structure determinants that characterize this lectin family (Odom and Vasta, 2006; Bianchet et al., 2002). However, none of the three critical basic amino acid residues that bind carbohydrate is present in hCFV, which instead of binding sugars binds membrane phospholipids (Zwaal et al., 1998). Thus, it is tempting to speculate that beyond ectothermic vertebrates, FTLs may have evolved to mediate different functions.

From the above it is clear that very little is known about functional aspects of lectins in fish immunity, except that they are expressed in cells, tissues, or organs relevant to immune functions, are up- or down-regulated by infectious challenge, bind potential pathogens, and participate in effector and regulatory immune functions. However, information about mechanistic and regulatory aspects of immune homeostasis lags far behind our current knowledge in mammals. In this regard, genetically tractable fish models systems such as the zebrafish hold great promise for unraveling the contributions of the complex lectin repertoires to immune function and homeostasis, relevant not only to fish but also to mammals, including man.

5. The zebrafish (*D. rerio*) as a model for glycoimmunology

A small number of fish species such as rainbow trout, channel catfish, and nurse shark (*Ginglymostoma cirratum*) have been used to examine specific aspects of their immune response, although they have serious limitations as model organisms. Recent advances in the cell biology, and technical manipulations of zebrafish (*D. rerio*) make this model increasingly attractive for studies on immunity, development, and oncology (Vasta et al., 2004b). As a model system, zebrafish offers many advantages over mammalian models. For example, its external fertilization, transparent embryos, and rapid development allow visualization the effects of genes involved in developmental processes, such as cell migration, organogenesis, etc. Moreover, gene expression can be disrupted in zebrafish embryos and the effect(s) can be analyzed easily. However, because of the genome duplication that took place after the divergence of teleost fish from the mammalian lineage, identification of zebrafish orthologues of mammalian genes may require caution. Various somatic cell lines (ZEM2S, ZF4, ZFL, SJD.1, AB.9) from zebrafish have been established (American Type Culture Collection (ATCC), Manassas, VA) and can be used for studying the expression of gene of interest and protein export at a cellular level. Further, germ-line chimeras can be produced when rainbow trout spleen RTS34st cells are introduced into a host zebrafish embryo. Several unique experimental approaches have been established in the zebrafish model to address the role(s) of genes of interest in immune responses, and include morpholino-modified antisense methodology, expression of dominant negative or specific tissue/organ over expression of an active protein, and the use of established mutants, combined with experimental infectious challenges. Therefore, the zebrafish model should constitute an ideal model to dissect both the ontogenic and mechanistic aspects of lectin functions in immune responses: mutagenesis screens and tilling can be implemented to examine the genetic aspects of lectin-mediated disease resistance and susceptibility.

By using various approaches (protein purification and characterization, cloning, and *in silico* data mining), we identified and characterized the zebrafish galectin repertoire: six prototype galectins (Drgal1-L1, Drgal1-L2, Drgal1-L3, Drgal1-L4, Drgal5 and DrGRIFIN), two chimera type galectin (Drgal3-L1, Drgal3-L2), and at least five tandem-repeat type galectins (Drgal4, Drgal8, Drgal9–L1, Drgal9-L2 and Drgal9-L3 (Ahmed et al., 2004). All zebrafish galectins characterized showed remarkable sequence similarities with mammalian galectins, and this enabled their unambiguous classification within the three well-established galectin groups. The proto-type galectins expressed in the zebrafish notochord play a key role in somatic cell differentiation and development, and disruption of their expression causes defects in skeletal muscle architecture (Ahmed et al., 2009), whereas the DrGRIFIN may function as a crystalline in the fish eye (Ahmed and Vasta, 2008). Further, the prototype galectin Drgal1-L2 is induced by photoreceptor cell death and secreted by stem cells and neuronal progenitors, and regulates the regeneration of rod photoreceptors (Craig et al., 2010).

Expression patterns of seven zebrafish intelectins (zINTLs) in various development stages, normal adults, and *Aeromonas salmonicida* infected adults, have provided clues to the function of this gene family. Interestingly, zINTL3 was expressed predominantly in the liver and highly upregulated upon infection, suggesting its important roles in immunity. Further, a comparative analysis with intelectins from various species revealed that the XL/intelectin family may be unique to deuterostomes, and in spite of their sequence conservation, expression patterns, quaternary structures and glycosylations are diverse among species (Lin et al., 2009).

The zebrafish model has been useful to examine the spatiotemporal expression patterns of complement components (C3, C1r/s, C4, C6, Bf, MBL and MASP) in fish exposed to infectious challenge, and has revealed that the system is well developed in the hatched larvae (Wang et al., 2008). Mining the zebrafish genome database has revealed a multi-gene family of Group II immune-related, lectin-like receptors (illrs) whose members possess inhibiting and/or activating signaling motifs typical of Group V NK receptors. Illr genes are differentially expressed in the myeloid and lymphoid lineages, suggesting that they may play important roles in the immune functions of multiple hematopoietic cell lineages (Panagos et al., 2006). By adapting the Genomic Matching Technique to define haplotypes of the MBL region in zebrafish, four ancestral haplotypes were identified, with at least one of these demonstrating a significant increase in resistance to *L. anguillarum*. Genomic analysis of an MBL gene cluster suggests that duplication, retroviral insertion, and possibly gene mutation and/or deletion have been key factors in MBL evolution. These findings, together with the extensive MBL polymorphism identified supports the notion that unique MBL variants are likely to define the zebrafish susceptibility/resistance to infection (Jackson et al., 2007).

Taken together, the selected studies above illustrate the great potential of the zebrafish as a model system to address in great detail the functional, structural, and evolutionary aspects of lectins that are relevant to fish and mammalian innate and adaptive immune responses.

6. Conclusions

Unlike immunoglobulins and the recently identified variable lymphocyte receptors (VLRs) of agnathans, lectins do not generate diversity in recognition by genetic recombination and therefore, additional interest has arisen on the germline-encoded diversity of the lectin repertoires, including their allelic variation or polymorphisms, presence of tandem gene duplications and multigene families, formation of chimeric structures by exon shuffling, additional mechanisms for expanding the ligand recognition spectrum by alternative splicing, and the structural basis for the potential

"plasticity" of their carbohydrate binding sites. Complex lectin repertoires, including C-type, galectins, pentraxins and the newly discovered families of rhamnose-binding, pufflectins and FTLs, appear to reach a high level of diversification in teleost fish, thereby enhancing the spectrum of ligands that can be detected on potential pathogens by this carbohydrate-based recognition system. The association of multiple lectin and effector domains in the same polypeptide subunit increases the functional efficiency of the system, and the presence of isoforms with subtle differences in specificity within each lectin type further enhances its diversity in ligand recognition. The multiple C3 forms present in teleost fish also suggest that a diverse repertoire of complement components may reflect a highly diversified lectin-mediated complement activation pathway. It is expected that in the near future the ongoing genome and transcriptome projects on representative fish models will reveal not only the extent of the full lectin repertoire in teleost fish but a comprehensive view of their involvement in defense functions, directly as recognition and effector factors in innate immunity, but indirectly as regulators of adaptive immune responses. Yet, the rigorous demonstration of the mechanisms by which each lectin or its isoforms accomplish their role(s) will only emerge from carefully designed experimental studies.

Acknowledgements

Supported by grant MCB-00-77928 from the National Science Foundation, and grants 1R01GM070589-01 and 5R01GM070589-06 from the National Institutes of Health to G.R.V.

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