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MINIREVIEW

The immune role of C-type lectins in molluscs

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Abstract

The phylum Mollusca is one of the largest and most important group in the animal kingdom. Recently, interest in molluscan immunity has increased due to their importance in worldwide aquaculture, their role in aquatic environmental science and their phylogenetic position, and a great number of immune molecules have been identified and characterized from molluscs. C-type lectins are a superfamily of diverse proteins with one or more carbohydrate recognition domains (CRDs) of ~130 amino acid residues. They recognize and bind to terminal sugars on glycoproteins and glycolipids and function in non-self recognition and clearance of invaders. This chapter provides a short review of C-type lectins in molluscs, including their structure, function and possible use in science and technology.

Key Words: molluscs; C-type lectin; carbohydrate-recognition domain; non-self recognition; agglutination; phagocytosis; encapsulation

Introduction

Lectins are carbohydrate-recognition proteins that bind to specific carbohydrate structures endogenous to the host or presented by microbial invaders (Drickamer *et al*., 1993; Barondes *et al.*, 1994), which make them the mediators of non-self recognition in the innate immune response (Epstein *et al.*, 1996). Although they were first discovered more than 100 years ago in plants, they are now known to be present throughout nature (including the microbial world, wherein they tend to be called by other names, such as hemagglutinins, adhesins, and toxins). The first of the animal lectins shown to be specific for a sugar (L-fucose) was from the eel (Watkins and Morgan, 1952), and lectins in animals were further known since the purification of an agglutinin in the hemolymph of horseshoe crab (Finstad *et al.*, 1974). Based on the structure, animal lectins have been classified into at least 13 lectin families, including C-type lectins and galectins, which are classic major families (Kilpatrick, 2002). The C-type lectins, structurally characterized by double-loop composed of two highly conserved disulfide bridges located at the bases of the loops, are believed to mediate pathogen recognition and play important roles in the innate immunity of both vertebrates and invertebrates due to their ability to bind specific carbohydrate in a Ca^{2+}-dependent manner (Devi *et al.*, 2010).

The phylum Mollusca is one of the largest and most important group in the animal kingdom, and there are about 200,000 living species distributed in terrestrial, freshwater and marine environments (Zuschin, 2009). As invertebrates, molluscs lack adaptive immune system, but have evolved sophisticated strategies and rely exclusively on their innate immunity to defend themselves against a variety of pathogens (Loker *et al.*, 2004). Since a sialic acid-specific lectin was first found in the slug *Limax flavus* (Miller, 1982), a number of lectins have been purified and characterized from molluscs.

Searching in the database of NCBI has revealed that C-type lectins attract much more attention, and totally 246 nucleotide sequences of molluscan C-type lectin, such as 119 from *Mytilus galloprovincialis*, 8 from *Haliotis discus discus*, 7 from *Chlamys farreri*, and 3 from *Crassostrea gigas* are identified. Accumulating evidences have favored that these molecules differ significantly in the amino acid sequences and geometrical arrangement of carbohydrate-recognition domain (CRD), and participate in many aspects of fundamental biological events, such as recognition of self and non-self, cell to cell interaction, serum glycoprotein turnover and so forth. This chapter reviews the

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interest arose around C-type lectins in molluscan animals, mainly in scallops, clams, oysters, mussels and snails, and especially highlights the diversity of their structure and functions.

The CRD structure of molluscan C-type lectin

The ability of C-type lectins to discriminate self and non-self is determined by their broad selectivity of carbohydrate-binding site and the geometrical arrangement of CRD (Weis *et al.*, 1998). The CRD is a typical structure of most C-type lectins, which consists of 115-130 amino acid residues with several conserved motifs (Drickamer *et al.*, 1993; Weis and Drickamer, 1996). The CRD always contains a double-loop structure, and the second loop also called long loop region is involved in Ca^{2+}-dependent carbohydrate binding. There are four Ca^{2+}-binding sites in this structure, among which the site 2 is known to be involved in the carbohydrate binding, and it is a useful simplification to predict the binding specificity of C-type lectins (Drickamer *et al.*, 1993; Zelensky and Gready, 2005). In this site, there are two conserved motifs determining the CRD binding ability, and the first one is always Glu-Pro-Asn (EPN) or Gln-Pro-Asp (QPD) in vertebrates (Zelensky and Gready, 2005). This motif seems to be more various in molluscan C-type lectins, and up to now seven types of motifs have been identified in molluscs, including EPN, Glu-Pro-Asp (EPD), QPD, Gln-Pro-Gly (QPG), Gln-Pro-Ser (QPS), Tyr-Pro-Gly (YPG) and Tyr-Pro-Thr (YPT). EPN motif has been considered to determine the CRD binding ability to mannose or similar sugar with the 3- and 4-OH (Weis *et al.*, 1998). This motif is widely spread in molluscan C-type lectins, such as Codakine from the tropical clam *Codakia orbicularis* (Gourdine *et al.*, 2008), Cflec-3, Cflec-4 and Cflec-5 from Zhikong scallop *C. farreri* (Zhang *et al.*, 2009a, b, 2010) and AiCTL-9 from bay scallop *Argopecten irradians* (Wang *et al.*, in press). EPD motif is a conserved motif in C-type lectins of invertebrates, which has also been identified in molluscs, such as Cflec-1, Cflec-2, Cflec-3, Cflec-4 from *C. farreri* (Wang *et al.*, 2007; Zheng *et al.*, 2008; Zhang *et al.*, 2009a, b), as well as AiCTL-2, AiCTL-6, AiCTL-7 from *A. irradians* (Zhu *et al.*, 2009; Kong *et al.*, 2011; Zhang *et al.*, 2011). Although the hydrogen bond donor Asn in EPN motif is replaced by an acceptor Asp in EPD motif, this replacement has no effect on agglutination towards microbes in D-mannose manner and specificity of carbohydrate binding in scallops (Zhang *et al.*, 2011). The motif QPD is only found in MCL-3 from Manila clams *Ruditapes philippinarum* (Kang *et al.*, 2006) and AiCTL-1 from *A. irradians* (Zhu *et al.*, 2008) and endows the ability to bind galactose similarly to vertebrate QPD motif in C-type lectins. YPT motif, which is quite different from motifs in vertebrates, is found in CfLec-3 from *C. farreri* as well as AiCTL-9 from *A. irradians* and has wider binding spectrum including lipopolysaccharides (LPS), peptidoglycan (PGN), yeast glucan, and even CpG oligodeoxynucleotide (data not published). The motifs QPG, QPS, and YPD are unusual motifs only found in CLHd from abalone *H. discus discu*s (Wang *et al.*, 2008), MeML from *Mytilus edulis* (Espinosa *et al.*, 2010), CvML from *Crassostrea virginica* (Jing *et al.*, 2011). QPG motif in CLHd offered galactose binding ability which is similar to that of QPD motif (Wang *et al.*, 2008). The carbohydrate specificity of CRDs with QPS and YPD motifs is still not well understood and requires further investigations. The second motif in Ca^{2+}-binding site 2, always Trp-Asn-Asp (WND) in vertebrates, has also been reported in invertebrate C-type lectins. The diversity of this motif in molluscs is even greater than that of the first one, and more than 10 motifs have been reported, such as WND, Trp-Ile-Asp (WID), Trp-Ser-Asp (WSD), Trp-His-Asp (WHD), Phe-Ser-Asp (FSD), and Leu-Ser- Asp (LSD). The first motif is believed to be the key switch in the specificity of binding with carbohydrate, and the second one can increase the affinity and specificity of this binding (Drickamer, 1992; Iobst and Drickamer, 1994). However, the function of the second motif in invertebrate has not been studied thoroughly, which is very important for us to understand the mechanism of C-type lectin functioning as a pattern recognition receptor (PRR).

Studies in other invertebrates implied that the clustering of multiple CRDs in one molecule endowed C-type lectin with broader spectrum and higher affinity of binding pathogen-associated molecular patterns (PAMPs) (Watanabe *et al.*, 2006; Zhang *et al.*, 2009c). To our knowledge, most of known molluscan lectins have single CRD, but there are also multi-CRD ones, such as Cflec-3 with three CRDs (Zhang *et al.*, 2009b), Cflec-4 and AiCTL-9 with four CRDs (Zhang *et al.*, 2009a; Wang *et al.*, in press). Clustering of multiple CRDs may also result in wider specificity of carbohydrate binding in molluscs. For instance, Cflec-3 with three CRDs can bind more PAMPs than Cflec-1 and Cflec-2 with single CRD (data not published). However, the influence of potential cooperation of multi-CRDs for binding affinity in molluscan C-type lectins still remains of interest.

Considerable information has become available of the chemical groups on the lectin and on the carbohydrates that interact with each other and of the types of bond formed, primarily hydrogen bonds and hydrophobic interactions (Sharon, 1993). Moreover, during the past few years, the number of lectin primary and 3D structures has increased dramatically. Interestingly, remarkable similarities have been noticed between the tertiary structures of lectins from diverse sources, in spite of the lack of primary sequence similarities (Sharon and Halina, 2004). Further knowledge of lectin structure will deduct these ubiquitous recognition molecules with myriad exciting functions and applications.

Immunological functions of C-type lectins

Accumulating evidences have demonstrated the diversity of sequence and function of C-type lectin in molluscs. Their functions in defense processes such as non-self recognition, microbe agglutination, induction of phagocytosis and encapsulation, anti-bacterial properties, will be discussed in detail below.

PAMPs binding and non-self recognition

The ability to distinguish self from non-self is

one of the fundamental functions of immune system. Due to the lack of adaptive immunity, invertebrate lectins play a major role in non-self recognition (Janeway and Medzhitov, 2002). C-type lectins specifically bind PAMPs on the surfaces of many pathogens, which provides them with the ability to recognize a wide variety of pathogens (Kilpatrick, 2002; Devi *et al.*, 2010). There are increasing evidences that the senescent (*i.e.*, apoptotic) cells are also recognized by lectins for their subsequent clearance by phagocytes. In recent years, many C-type lectins have been identified in molluscs, and their transcription levels increase after stimulation with pathogens or PAMPs, implying that they are involved in innate immune response (Wang *et al.*, 2007; Zheng *et al.*, 2008; Yang *et al.*, 2011). Moreover, molluscan C-type lectins displayed high affinity to various PAMPs on the surface of pathogens, such as LPS from Gram-negative bacteria, PGN from Gram-positive bacteria, glucan and mannan from fungi, and so on. For instance, a multi-CRD lectin from scallop *A. irradians* (AiCTL-9) can bind LPS, PGN, glucan and mannan (Wang *et al.*, in press). Manila clam lectin from *R. philippinarum* can bind N-acetyl-D-galactosamine and mannan (Bulgakov *et al.*, 2004). It is noteworthy that scallop C-type lectins with the same first motif of Ca^{2+}-binding site 2 had different PAMPs binding spectrums. Cflec-1 containing the motif EPD could bind LPS, PGN and mannan *in vitro*, while Cflec-2 with the same motif could also bind zymosan besides these three PAMPs (Yang *et al.*, 2010, 2011). These special binding patterns may represent a ligand-receptor interaction that is involved in the recognition of various pathogens through the limited germline-encoded PRRs and play key role in immune defense process.

Agglutination (microbes and erythrocytes)

Besides non-self recognition, molluscan lectins participate in innate immune responses, including agglutination, hemocyte phagocytosis as well as encapsulation, and even bactericidal effect (Wang *et al.*, 2007; Zheng *et al.*, 2008; Yang *et al.*, 2010, 2011). Like other invertebrate lectins, molluscan C-type lectins have the property of agglutinating various microbes as well as vertebrate erythrocytes. For instance, most of scallop C-type lectins exhibited agglutinating activity towards various bacteria and fungi (Wang *et al.*, 2007; Zheng *et al.*, 2008; Zhang *et al.*, 2009b, 2010, 2011; Kong *et al.*, 2011). Manila clam lectin from *R. philippinarum* agglutinated erythrocytes from sheep and rabbit (Takahashi *et al.*, 2008). Moreover, purified lectins from the giant African snail *Achatina fulica* can agglutinate not only bacteria, but also rabbit red blood cells (Ito *et al.*, 2011). Interestingly, scallop C-type lectins with similar carbohydrate-binding specificity may distinguish different invading microbes in humoral immune system. For example, Cflec-1, Cflec-2, Cflec-3 and Cflec-5 from *C. farreri*, agglutinated *E. coli*, *Staphylococcus haemolyticus*, *Pseudomonas stutzeri* and *Pichia pastoris*, respectively, though they all possessed mannose-binding specificity (Wang *et al.*, 2007; Zheng *et al.*, 2008; Zhang *et al.*, 2009b, 2010). Additionally, PAMPs, with the same YPT, EPD and EPN motifs, multi-CRD lectin AiCTL-9 agglutinated not only Gram-negative bacteria *E. coli and Vibrio anguillarum*, but also Gram-positive bacteria *Bacillus subtilis* (Wang *et al.*, in press), while another multi-CRD lectin Cflec-3 aggregated only the Gram-negative bacteria *Pseudomonas stutzeri*, although remarkably (Zhang *et al.*, 2009b). The agglutination of foreign particles by C-type lectins have been considered to enable phagocytic cells to recognize invading cells as non-self and therefore initiate the clearing (phagocytosis, encapsulation) process (Devi *et al.*, 2010).

Induction of phagocytosis and encapsulation

Even there is a great difference between vertebrate and invertebrate immunity, invertebrates share some similar innate immune defense mechanisms with vertebrates, such as encapsulation and phagocytosis (Medzhitov and Janeway, 2000; Plows *et al.*, 2005). Molluscan C-type lectins have been reported to play significant roles in hemocyte phagocytosis and encapsulation. For example, Manila clam lectins can significantly enhance the hemocyte phagocytic ability toward the bacteria and fluorescent beads (Kim *et al.*, 2006; Takahashi *et al.*, 2008). Cflec-1 and Cflec-2 from *C. farreri* can bind to the surface of scallop hemocytes and recruit them to enhance their *in vitro* encapsulation (Yang *et al.*, 2010, 2011). Meanwhile, Cflec-1 could also enhance the phagocytic activity of scallop hemocytes against *E. coli* (Yang *et al.*, 2011). The C-type lectin-enhanced hemocytes activity towards microorganisms suggested that these molecules could function as receptors to transduce extracellular signals into the cell, which was similar as the lectins in vertebrates (Zelensky and Gready, 2005).

Anti-bacterial properties

The mechanism of humoral immune defenses in invertebrate mainly refers to a class of significant effector molecules, such as inducible antimicrobial peptides (AMPs), to be involved in a direct attack on infectious agents (Hoffmann *et al.*, 1999; Roch, 1999). Some identified molluscan C-type lectins can function in directly suppressing and clearing the microbes, although the underlying mechanism is not exactly known. Purified MCL-4 from the plasma of Manila clam *R. philippinarum* could markedly suppress the growth of *Alteromonas haloplanktis* (Takahashi *et al.*, 2008). In addition, The recombinant C-type lectins from scallop *C. farreri* also inhibited the growth of bacteria, such as rCflec-1 inhibiting the growth *E. coli* and *Micrococcus luteus* (Wang *et al.*, 2007), rCflec-2 suppressing the growth of *E. coli* (Zheng *et al.*, 2008). All these studies indicated the molluscan C-type lectins could also contribute to the host defense mechanisms as an effector molecule.

The possible use of molluscan lectins in science and technology

The activities of lectin are of advantageous within the immune system, both for self/non-self discrimination and interactions between components of the immune system. Considering the high

abundance of C-type lectins discovered in molluscs as well as their functions in immune system, it is likely that many molluscan C-type lectins are of great significance.

Use in immunological research and disease control

Since there is no antibody-mediated immunity in the relatively simple invertebrates, abundant lectins with diverse expression profiles and bioactivities might function as effectors in the immune system. Some molluscan C-type lectins, such as Cflec-1 (Wang *et al.*, 2007; Yang *et al.*, 2011) and Cflec-2 (Zheng *et al.*, 2008; Yang *et al.*, 2010), not only function to suppress the growth of microbes and clear the pathogen, but also play significant role in hemocyte phagocytosis and encapsulation.

It may be that carbohydrate binding has evolved as a useful additional property amongst unrelated proteins fulfilling a variety of principal functions. Future progress will elucidate the contribution of those lectins in mounting protective immune responses for molluscs against infection, which may promote the cognition of invertebrate immune system as well as the development of comparative immunology. Furthermore, the abilities of those molluscan lectins to confer resistance to certain bacterial species have opened a new scope in the field of application in disease control for aquaculture animals.

Use in studying molecule interactions and developing chemical tools

Lectins are multivalent carbohydrate-binding proteins with the ability to agglutinate erythrocytes, bacteria and other normal and malignant cells displaying more than one saccharide of sufficient complementarity (Barondes, 1981). C-type lectins were implicated as the indispensable players in carbohydrate recognition, suggesting the possible application in discrimination of various correlative microbes, and developing biochemical tools.

Molluscan C-type lectins has the property of agglutinating various microbes as well as vertebrate erythrocytes like other invertebrate lectins. Lectins from the giant African snail and Manila clam could agglutinate rabbit red blood cells and erythrocytes from sheep and rabbit (Takahashi *et al.*, 2008; Ito *et al.*, 2011), respectively. C-type lectins from scallops exhibited agglutinating activity of various bacteria and fungi (Wang *et al.*, 2007; Zheng *et al.*, 2008; Zhang *et al.*, 2009b, 2010, 2011; Kong *et al.*, 2011). Some of lectins in other invertebrates are found specific in their cognition reactions, such as with human blood groups and somewhat bacteria, for instance, crude *Limulus polyphemus* lectin agglutinated 96 % of coagulase-negative strains of *Staphylococci* and none of the human strains of *Staphylococcus aureus* (Boyd, 1963; Davidson *et al.*, 1982). The special agglutination of lectins may lead to the future development of methodology for the differentiation of certain bacteria and erythrocytes.

C-type lectins from molluscs have attracted much attention for their great diversity in structure and activity, and they also provide a model system to understand the molecular basis of how proteins recognize carbohydrates. Because of their wide variety of sugar specificities, the molluscan C-type lectins are becoming attractive candidates for the development of biochemical tools in chromatography, blotting, and electrophoresis to purify and characterize the relative molecules and cellular structures. Regarding the great diversity, the specific carbohydrate binding and recognition for molluscan C-type lectins still need further investigation, and the search for lectins in such diverse molluscan animals including their identification and characterization may provide the potential to uncover unique lectins, which may enrich the library of future biomedical tools.

Conclusions

A variety of molluscan lectins have enabled greater insight into the diversity and complexity of lectin repertoires in invertebrates. The recent knowledge on the structure and functions of molluscan C-type lectins is underlined in this review. These identified molluscan C-type lectins differ in CRD number and motif characterization, which endow them with different property fulfilling a variety of vital functions, and thus they are expected to be applied in several biological and biomedical aspects. The nature of the protein-carbohydrate interaction as well as the potential mechanism of different function for those molluscan lectins still remain of intense interest. Future progress will elucidate the contribution of those lectins and their crosstalk with each other or with other molecules with respect to mounting protective immune responses in molluscs.

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