

Ficolins: innate immune recognition proteins for danger sensing

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FICOLINAS: PROTEÍNAS DE RECONOCIMIENTO DE LA INMUNIDAD INNATA COMO SENSORES DE PELIGRO

Recibido: 13 Julio 2007

Aceptado: 31 Julio 2007

RESUMEN

Las ficolinas son proteínas de defensa que forman oligómeros a partir de tallos homólogos al colágeno y dominios semejantes a fibrinógeno. Son capaces de sentir señales de peligro tales como patrones moleculares asociados patógenos o a células apoptóticas. En el hombre, las ficolinas L y H se han caracterizado en el suero, mientras que la ficolina M es secretada por células monocíticas. Al igual que la lectina de unión a manano ("mannan-binding lectin", MBL), pueden asociarse a las serina-proteasas asociadas a MBL e iniciar la vía de activación de complemento de las lectinas, un importante sistema efector de la inmunidad humoral. También pueden actuar como opsoninas, incrementando la eliminación de sus dianas por fagocitosis. Estudios estructurales recientes muestran que la ficolina L es una proteína de reconocimiento versátil, capaz de unir moléculas acetiladas y carbohidratos neutros por medio de sitios de unión diferentes, mientras que la ficolina H posee un único sitio de unión con una especificidad más restringida hacia los carbohidratos neutros. Los estudios filogenéticos revelan que las ficolinas han sido conservadas en el proceso evolutivo, apoyando la hipótesis de que el sistema de complemento primitivo era un sistema de opsonización basado en lectinas, y ponen de relieve la importancia de las proteínas de reconocimiento de carbohidratos en la inmunidad innata.

PALABRAS CLAVE: Ficolina / Inmunidad Innata / Receptores de reconocimiento de modelos / Activación del Complemento / MASP.

ABSTRACT

Ficolins are oligomeric defence proteins assembled from collagen-like stalks and fibrinogen-like domains that are able to sense danger signals such as pathogen- or apoptotic cell-associated molecular patterns. In humans, L- and H-ficolins have been characterized in serum whereas M-ficolin is secreted by monocytic cells. Like mannan-binding lectin (MBL), they are able to associate with MBL-associated serine proteases and to trigger activation of the lectin pathway of complement, a major effector system of humoral innate immunity. They can also act as opsonins to enhance clearance of their targets by phagocytosis. Recent structural studies have shown that L-ficolin is a versatile recognition protein able to bind acetylated molecules and neutral carbohydrates through different binding sites, whereas H-ficolin has a single binding site with a more restricted specificity for neutral carbohydrates. Phylogenetic studies reveal that ficolins have been conserved through evolution, supporting the hypothesis that the primitive complement system was a lectin-based opsonic system, and emphasizing the essential role of carbohydrate recognition proteins in innate immunity.

KEY WORDS: Ficolin / Innate immunity / Pattern recognition receptor / Complement activation / MASP.

INTRODUCTION

Protection against infection lies on the host's ability to identify and eliminate pathogens while preserving its own integrity. To fulfill this challenge, vertebrates have evolved two complementary systems, innate immunity and adaptive immunity. The innate system not only represents the first line of defence against pathogens, but also stimulates and orientates the adaptive response that then provides a delayed but memorized response to infection⁽¹⁾. Moreover, the innate immune system is also involved in the clearance of potentially dangerous substances from self such as cellular debris and damaged cells⁽²⁾. Innate immunity involves a set of constitutive recognition molecules able to identify invariant structures exposed at the surface of large groups of pathogens, referred to as pathogen-associated molecular patterns (PAMPs)⁽³⁾, and to distinguish them from self-antigens. In a similar way, innate immune molecules recognize apoptotic cell-associated molecular patterns (ACAMPs)⁽⁴⁾ that are not exposed on healthy cells. Binding of these molecules (also called pattern recognition receptors or PRRs) to the PAMPs triggers effector mechanisms aimed at limiting early infection and inducing an appropriate adaptive response. In contrast, binding to ACAMPs normally leads to removal of dying cells without induction of an adaptive response in order to prevent auto-immune disorders. Pattern recognition receptors include membrane-bound proteins such as Toll-like and phagocytic receptors as well as humoral recognition molecules. One group of these soluble PRRs, called "defence collagens", encompasses oligomeric proteins with a globular recognition domain and a collagen-like region providing the link with effectors of the innate immune system. These proteins include complement component C1q, mannan-binding lectin (MBL), and the recently identified lectin-like proteins ficolins, that are able to trigger complement activation through their association with serum proteases (Figure 1). The classical complement pathway is initiated by binding of C1q to the antibody moiety of antigen-antibody complexes or directly to certain pathogens and elements of altered self, which induces sequential activation of two proteases, C1r and C1s⁽⁵⁾. The lectin pathway is triggered through activation of MBL-associated protease (MASP-2) in response to recognition of neutral carbohydrate patterns and other microbial surface motifs by MBL and the ficolins^(6,7). Once activated, C1s and MASP-2 are both able to cleave C4 and C2, thus generating a C3-cleaving enzymatic complex called C3-convertase. Opsonization of the target by C3-derived fragments enhances its clearance by C3 receptors-bearing phagocytes and triggers assembly of a cytolytic membrane attack complex involving components C5b to C9 (Figure 1). C3- and C5-derived fragments are also involved in activation of the inflammatory

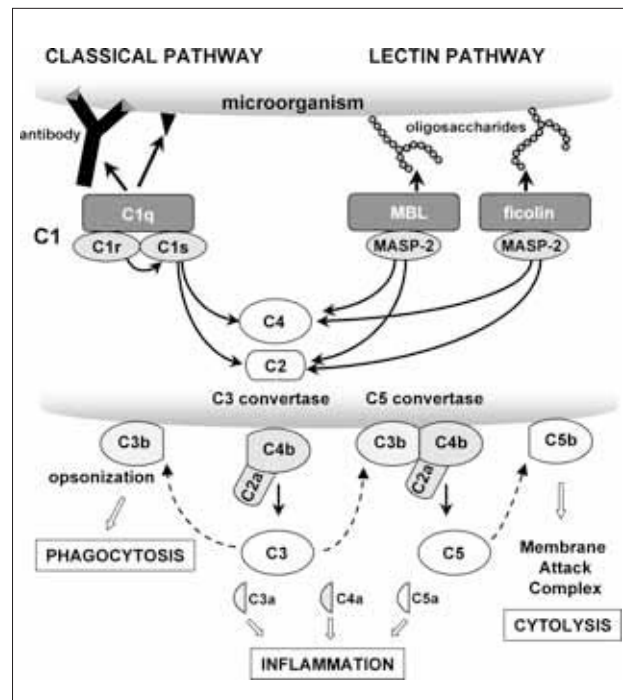


Figure 1. Schematic representation of the classical and lectin pathways of complement activation. The C1 complex and the MBL/MASP-2 or ficolin/MASP-2 complexes recognize microbes through different mechanisms and trigger the same enzymatic cascade. Released proteolytic fragments mediate various biological activities contributing to the elimination of the pathogen.

response and stimulation of the adaptive immune response through interaction with specific cellular receptors⁽⁸⁾.

Whereas the structure, function, genetics and disease association of MBL have been extensively studied since the discovery of the lectin pathway about 15 years ago, this is not the case for ficolins as their role in innate immunity was only discovered at the beginning of the 2000s. The purpose of this review is to summarize our current knowledge on ficolins, including recent data from our laboratory on the structure of their recognition domain that provide insights into their innate immune recognition specificities.

GENERAL FEATURES OF HUMAN FICOLINS

Ficolins were initially isolated from porcine uterus membranes as transforming growth factor- β 1 (TGF- β 1)-binding proteins⁽⁹⁾ and further identified as multimeric proteins with fibrinogen- and collagen-like domains⁽¹⁰⁾. In human, three types of ficolins have been described, called L (synthesized in the liver), M (of monocyte origin)⁽¹¹⁾ and H (Hakata antigen).

L-ficolin or ficolin-2 (also called hucolin⁽¹²⁾, elastin-binding protein (EBP)-37⁽¹³⁾ and P35⁽¹⁴⁾) is a serum protein that is

essentially synthesized in the liver. Serum concentrations varying from 1.0 to 12.8 µg/ml with median values of 3.0–5.4 µg/ml have been estimated in Caucasian, Indian and Singapore healthy individuals^(15–20), whereas a higher mean value of 13.7 µg/ml has been reported in a Japanese population⁽²¹⁾. The gene for human L-ficolin (*FCN2*) is located on chromosome 9q34 and contains 8 exons⁽²²⁾.

H-ficolin, also termed Hakata Antigen or ficolin-3, was first identified as a serum protein found to react with an autoantibody from patients with systemic lupus erythematosus (SLE)⁽²³⁾. It was shown subsequently that Hakata antigen is a thermolabile β2-macroglycoprotein (TMG) present in normal human serum⁽²⁴⁾ and a new member of the ficolin family⁽²⁵⁾. Its concentration in healthy blood donors was initially reported to range from 7 to 23 µg/ml⁽²⁴⁾ and two different mean values of 5.0⁽²⁶⁾ and 18.4 µg/ml⁽¹⁷⁾ have been estimated recently. H-ficolin is synthesized in the liver by hepatocytes and bile duct epithelial cells as well as in the lung by ciliated bronchial and type II alveolar epithelial cells⁽²⁷⁾, and it is secreted into blood, bile and bronchus/alveolus. It has been shown recently that H-ficolin is expressed by the human glioma cell line T98G, suggesting that it is produced in the brain⁽²⁸⁾. The gene for human H-ficolin (*FCN3*) is located on chromosome 1 (1p35.3) and its exon organization is similar to that of *FCN2*⁽²⁹⁾.

M-ficolin (also known as ficolin-1^(30,31) or P35-related protein⁽²²⁾) is primarily synthesized in monocytes, the bone marrow, the lung and the spleen. It is detected on the surface of peripheral blood monocytes and promonocytic U937 cells and is not present in serum^(32,33). Recent studies have shown that M-ficolin is localized mainly in secretory granules in the cytoplasm of neutrophils, monocytes, and type II alveolar epithelial cells in the lung⁽³⁴⁾. This location is consistent with the fact that the protein sequence contains an N-terminal signal peptide, but no membrane anchor motif. The gene of human M-ficolin (*FCN1*) is located close to the *FCN2* gene on chromosome 9q34 and again has a similar exon organization, except that it contains a ninth exon encoding four additional Gly-X-Y repeats in the collagen-like region⁽²²⁾.

At the amino acid level, the identities between L- and H-ficolins, L- and M-ficolins, and H- and M-ficolins are 48, 80 and 48%, respectively.

MOLECULAR ORGANIZATION OF FICOLINS

The oligomeric nature of L- and H-ficolins was revealed from their mobility upon SDS-PAGE analysis, as they migrate as high molecular weight species under non-reducing conditions and yield a single band at 34–35 kDa under reducing conditions^(14,25). The polypeptide chain of ficolins

consists of an N-terminal region containing two cysteine residues, a collagenous region comprising 11–19 repeating Gly-X-Y triplets, and a C-terminal fibrinogen-like (FBG) domain of about 210 residues preceded by a short linker segment (Figure 2, Table I). Three polypeptide chains associate to form a homotrimeric structural subunit comprising a collagen-like triple helix and a globular recognition domain composed of three FBG domains. Contrary to the proteins of the collectin family, ficolins have no α-helical "neck" region between the collagen-like region and the recognition domain. The interruption in the collagen-like sequence that gives rise to a kink in the triple helix of MBL and C1q is also missing in the ficolins. The cysteines in the N-terminal region form interchain disulfide bonds that mediate oligomerisation to higher structures^(35,36). Based on electron microscopy pictures and gel filtration experiments, it has been proposed that L- and H-ficolins are tetramers and hexamers of the structural units, respectively, with an overall parachute-like structure^(25,37). It has been shown recently that recombinant M-ficolin is also an oligomeric protein with an estimated molecular mass ranging from 440⁽³³⁾ to 610 kDa⁽³⁴⁾, consistent with tetrameric and hexameric species, respectively.

PATHOGEN RECOGNITION AND LIGAND SPECIFICITY

It was first proposed that L-ficolin is a Ca²⁺-dependent lectin specific for clustered N-acetyl-glucosamine (GlcNAc) residues as found in GlcNAc-agarose, the neoglycoprotein GlcNAc-BSA or asialofetuin, but not for mannose residues⁽¹⁴⁾. In accordance with this preference, L-ficolin binds to a Ra chemotype strain (TV119) of *Salmonella typhimurium*, a microorganism with a large number of surface-exposed non-reducing GlcNAc residues. It was shown later that Ca²⁺ is not essential for GlcNAc binding⁽¹¹⁾ and that L-ficolin also binds to GalNAc residues, a property that could be assigned to its FBG domain⁽¹⁵⁾. Further reports indicated that L-ficolin specifically binds to lipoteichoic acid (LTA), a cell wall constituent of gram-positive bacteria, including the clinically important strains *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus agalactiae*⁽³⁸⁾, and to the fungal cell wall component 1,3-β-D-glucan, consisting of a linear polymer of β-1,3-linked glucose residues⁽³⁹⁾. On the other hand, Krarup et al.⁽⁴⁰⁾ reported specificity for N-acetylated carbohydrates and other acetylated compounds such as acetylcholine, as determined from inhibition of L-ficolin binding to *Streptococcus pneumoniae*. Further investigations revealed binding to opportunistic pathogenic bacteria such as some capsulated *S. aureus* and *S. pneumoniae* serotypes, but not to non-

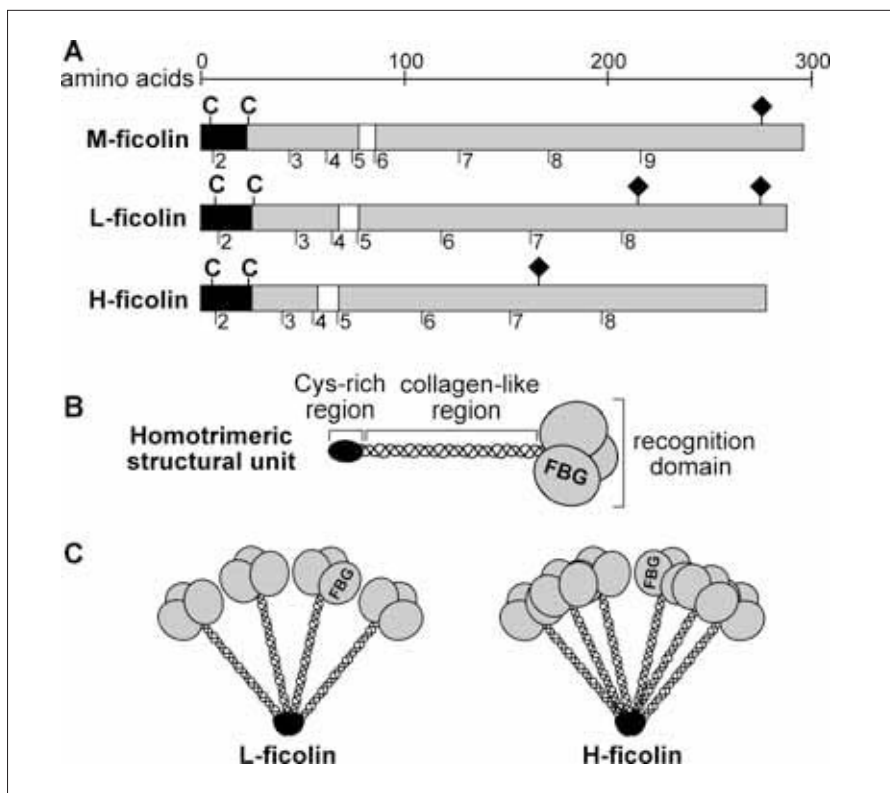


Figure 2. Domain structure and oligomeric organization of human ficolins. (A) Each mature polypeptide chain comprises a cysteine-rich N-terminal sequence (black), a collagen-like sequence (hatched), a linker segment (white) and a fibrinogen-like sequence (grey). The exon boundaries, N-linked oligosaccharides (◆) and the two N-terminal cysteine residues are indicated. (B) Three polypeptide chains assemble to form a structural subunit. (C) Several subunits assemble to form tetramers (L-ficolin) or hexamers (H-ficolin).

TABLE I. General features of human ficolins

	Gene			Protein			
	Chromosomal locus	Tissue expression	Exons	Monomer (kDa)	Polypeptide chains	Collagen triplets	Location
M-ficolin (FCN1)	9q34	Monocytes, lung	9	35	12 or 18	19	Monocytes
L-ficolin (FCN2)	9q34	Liver	8	35	12	15	Serum
H-ficolin (FCN3)	1p35.3	Liver, lung	8	34	18	11	Serum, bronchus, bile

capsulated strains⁽¹⁷⁾, as well as to the capsular polysaccharide of serotype III group B streptococci (*S. agalactiae*)⁽⁴¹⁾.

H-ficolin was initially reported to agglutinate human erythrocytes coated with lipopolysaccharides derived from *Salmonella minnesota*, *Escherichia coli* (0111), and *Salmonella typhimurium*⁽²⁵⁾. In the latter case agglutination could be inhibited by GlcNAc, GalNAc and fucose. However, H-ficolin did not bind to GlcNAc-Sepharose⁽⁴²⁾ and its binding to polysaccharide capsule constituents derived from *Aerococcus viridans* could not be prevented by carbohydrates⁽⁴³⁾. In the same way, the binding of H-ficolin to *A. viridans* could not be inhibited by acetylated sugars or non-carbohydrate compounds⁽⁴⁰⁾, and no binding to different *S. pneumoniae* and *S. aureus* serotypes could be detected⁽¹⁷⁾.

Recombinant M-ficolin shows a marked preference for acetylated compounds, as observed for L-ficolin⁽³³⁾ and binds to GlcNAc, GalNAc and sialic acid⁽³⁴⁾, consistent with the ability of its recombinant FBG domain to bind GlcNAc⁽³²⁾. Binding to the smooth type LT2 strain of *S. typhimurium* and to *S. aureus* has been reported, but only binding to the latter could be inhibited by GlcNAc⁽³²⁾.

Recent studies have shown that, in addition to pathogenic microorganisms, L-ficolin binds specifically to apoptotic HL60, U937 and Jurkat T cells, whereas binding of H-ficolin is restricted to apoptotic Jurkat T cells^(44,45). L-ficolin recognizes DNA and its binding to late apoptotic and necrotic Jurkat T cells is inhibited by DNA, which is not the case for H-ficolin^(45,46).

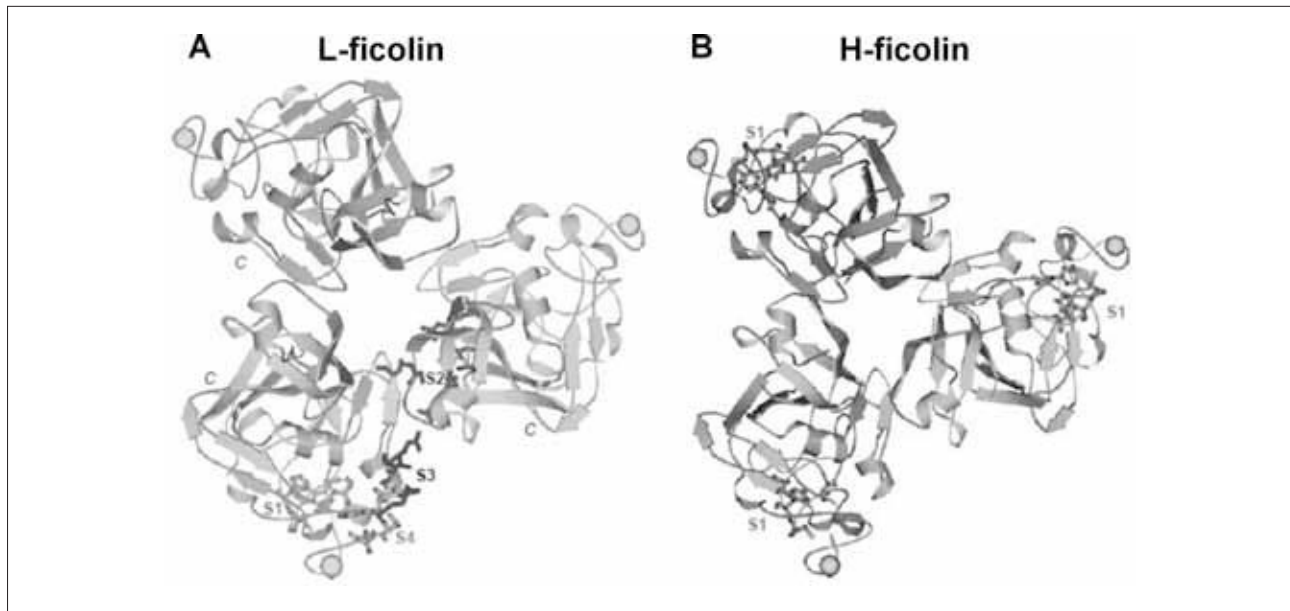


Figure 3. X-ray structures of the recognition domains of human L- and H-ficolins. Bottom views of L-ficolin (A) and H-ficolin (B) are shown. The side chains of the binding site residues are indicated. Only one of each representative binding site (S1-S4) is shown in L-ficolin. Ca^{2+} ions are represented as grey spheres (from 47).

STRUCTURAL BASIS FOR RECOGNITION

With a view to decipher the structural determinants for the binding specificity of L- and H-ficolins, we have solved recently the X-ray crystal structures of their recombinant recognition domains, alone or in complex with different ligands⁽⁴⁷⁾. The fibrinogen-like domains associate in both cases as homotrimers of protomers homologous to tachylectin 5A (TL5A), a fibrinogen-like lectin from the horseshoe crab *Tachypleus tridentatus*⁽⁴⁸⁾. Both ficolin trimeric domains have three-lobed structures with clefts separating the distal parts of the protomers and a small central cavity (Figure 3). The L-ficolin trimer exhibits variable angles between the protomers, revealing a significant plasticity at the inter-subunit interfaces. A single binding site S1 was found in H-ficolin, located on the outer side of the trimer close to the Ca^{2+} -binding site, and homologous to the GlcNAc binding pocket of TL5A⁽⁴⁸⁾. However, ligand binding was observed only with D-fucose and galactose. Unexpectedly, three additional binding sites S2, S3 and S4 were identified in L-ficolin, located in an area surrounding the cleft between the protomers (Figure 3). This arrangement was shown to allow accommodation of a four-residue linear 1,3-,D-glucan molecule, a molecular marker of yeast and fungal cell walls, which bound through a network of interactions spanning the S3 edge and site S4. Interestingly, galactose bound specifically to the inner site S2, in a way that would be compatible with interaction to the terminal galactose residue of a linear oligosaccharide

chain. In contrast to the highly specific GlcNAc binding observed in TL5A⁽⁴⁸⁾, small acetylated ligands bound to site S2 and mostly to site S3 in diverse orientations, reflecting different modes of interaction with minimal structural requirements for the acetyl group. These new sites thus define a continuous recognition surface allowing L-ficolin to bind elongated carbohydrates featuring acetylated and neutral residues, as found at the surface of microbes and apoptotic cells. This differs significantly from the highly specific recognition mechanism of MBL, which only binds to terminal carbohydrate residues with equatorial C3- and C4-OH groups, such as those present in mannose, GlcNAc, but not in galactose⁽⁴⁹⁾.

The crystal structure of the trimeric recognition domain of M-ficolin has been recently solved by Tanio et al.⁽⁵⁰⁾ revealing an overall structure comparable to that of L- and H-ficolins. However, no ligand binding was observed in this study and current investigation is underway in our laboratory to determine the ligand binding specificity of M-ficolin.

Our structural studies indicate that, besides C1q and collectins, L- and H-ficolins represent novel types of multivalent pattern recognition molecules with trimeric ligand binding domains. In addition to major differences in the structural determinants involved in their ligand specificity, comparison of the overall structures of ficolins, C1q and MBL reveal distinguishing features in trimer assembly. Indeed, the semi-

open ficolin structure appears intermediate between the compact subunit assembly seen in the gC1q domain⁽⁵¹⁾ and the widely open shape of the MBL carbohydrate recognition domain, where the three lectin domains are connected through a neck region^(52,53) (Figure 4).

MBL/FICOLIN-ASSOCIATED PROTEASES AND COMPLEMENT ACTIVATION

Binding of ficolins to danger signals on pathogens and damaged cells leads to their elimination through activation of the lectin pathway of complement. The discovery of this pathway dates back to studies showing that MBL is able to activate complement in a C4-dependent and C1-independent manner^(54,55), through a novel C1r/C1s-like protease termed MBL-associated serine protease (MASP)^(56,57). Three additional MBL-associated proteins were discovered later, the MASP-2 and MASP-3 proteases^(58,59) and a non-enzymic protein called MAP19 (MBL-associated protein of 19 kDa) or sMAP (small MBL associated protein)^(60,61). It was also shown that complexes containing MBL and MASP-2 alone are sufficient to elicit C4 and C2 cleavage⁽⁶²⁾, thus raising the question of the role of the MASP (MASP-1) initially identified, and of MASP-3 and MAP19. It was shown more recently that, in addition to MBL, serum L- and H-ficolins also associate with the three MASPs and MAP19 and trigger the lectin pathway of complement^(42,63). In the same way, recombinant M-ficolin forms complexes with MASP-1 and MASP-2 and activates complement^(33,34).

The MASPs have a modular organization homologous to that of the C1r and C1s proteases, with an N-terminal CUB module, a Ca²⁺-binding epidermal growth factor (EGF) module, a second CUB module, two complement control protein (CCP) modules, a short linker segment and a C-terminal chymotrypsin-like serine protease (SP) domain. MASP-1 and MASP-3 are generated through alternative splicing of the *MASP1/3* gene and differ only through their linker segment and SP domain⁽⁵⁹⁾. MAP19 is an alternative splicing product of the *MASP-2* gene that comprises the N-terminal CUB1 and EGF modules of MASP-2 prolonged by four specific residues at the C-terminal end^(60,61).

Assembly of the ficolin/MASP complexes

Using purified recombinant rat and human proteins, it was shown that MASP-1, MASP-2, MASP-3, their N-terminal CUB1-EGF-CUB2 and CUB1-EGF segments, and MAP19 each associate as homodimers that interact individually with MBL and L-ficolin in the presence of Ca²⁺ ions^(36,64-67). The full-length proteases and their CUB1-EGF-CUB2 segments bind to MBL and L- and H-ficolins

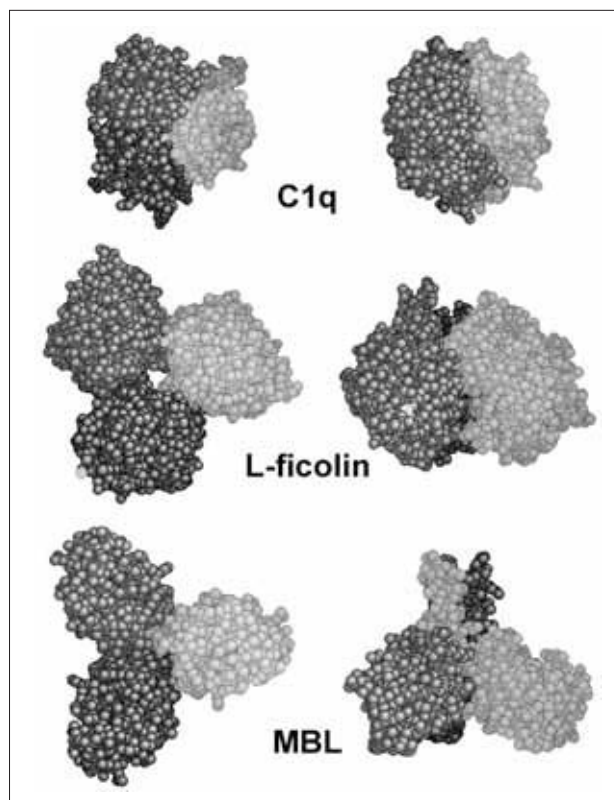


Figure 4. The trimeric recognition domains of C1q, L-ficolin and MBL. Space filling bottom views (left) and side views (right) are shown (adapted from 47).

with comparable high affinities (KD in the nanomolar range), whereas the shorter CUB1-EGF segments and MAP19 interact with lower affinities (66; M Lacroix and NM Thielens, unpublished data). These data indicate that the interaction involves a major contribution of the CUB1-EGF module pair of each protease, but is reinforced by the following CUB2 module. Preincubation of the MASPs and MAP19 with soluble MBL was shown to inhibit their subsequent binding to immobilized L-ficolin, and conversely, indicating that these recognition proteins compete with each other for binding to the MASPs⁽⁶⁶⁾.

Resolution of the X-ray structure of human MAP19 and site-directed mutagenesis allowed us to locate its interaction site with MBL and L-ficolin⁽⁶⁸⁾. The structure reveals a head-to-tail homodimer held together by interactions between the CUB1 module of a monomer and the EGF module of its counterpart. The dimer contains 4 calcium ions, one bound to each EGF module at the CUB1-EGF interface and one bound to the distal end of each CUB module. To identify the residues of MAP19 involved in the interaction with MBL and L- and H-ficolins, point mutants were produced and their binding capacity was assessed using surface plasmon

resonance (SPR) spectroscopy. Mutations that strongly decreased or abolished interaction with both MBL and the ficolins were found to define a common binding site for these proteins, located at the distal end of each CUB1 module and stabilized by a Ca^{2+} ion (68; NM Thielens, unpublished results).

The X-ray structure of the CUB1-EGF-CUB2 interaction region of human MASP-1/3 has been solved recently and provides further insights into the assembly of the MBL/MASP and ficolin/MASP complexes. The structure reveals that each CUB2 module is aligned with the preceding head-to-tail CUB1-EGF dimer, and that three Ca^{2+} ions are bound per monomer, one in the EGF module and one in each of the CUB modules. Site-directed mutagenesis and SPR spectroscopy allowed us to map two binding sites for MBL and L- and H-ficolins in each MASP-3 monomer, each stabilized by Ca^{2+} ions. The fact that these sites are structurally homologous and located within the same plane strongly suggests that the MASPs interact with homologous sites on four collagen triple helices of MBL and the ficolins⁽⁶⁹⁾.

Recent studies have led to the identification of the site of human MBL involved in the interaction with its partner proteases, providing evidence for an essential role of residue Lys55 of the collagenous region⁽⁷⁰⁾, in accordance with previous studies on rat MBL⁽⁷¹⁾. Sequence alignment of the collagen-like regions of MBL and ficolins reveals that Lys55 is conserved in all ficolins of known sequence, except pig ficolin α . To test the possible involvement of the homologous residues Lys57 of L-ficolin and Lys47 of H-ficolin, point-mutants of both proteins have been produced, in which these residues were mutated to Ala, Glu and Arg, and their ability to bind the MASPs was determined by SPR spectroscopy. All three mutations had a strong inhibitory effect on the interaction of L- and H-ficolins with MASP-1 and MASP-2. In the case of MASP-1 and MASP-3, replacement of the target Lys residues by Ala or Glu abolished interaction, whereas the Lys to Arg mutation had only slight inhibitory effects (M Lacroix and NM Thielens, unpublished results). These results reveal that residues Lys57 of L-ficolin and Lys47 of H-ficolin are key components of the interaction with the MASPs and MASP-1, thus providing strong indication that MBL and ficolins share homologous binding sites for their partners. However, the binding sites are not identical since a Lys to Arg mutation prevents interaction of the ficolins with MASP-1/MASP-2 but allows binding to MASP-3, as observed previously in the case of MBL⁽⁷⁰⁾.

The stoichiometry of circulating ficolin-MASPs complexes is unknown, but it is likely that L-ficolin tetramers or H-ficolin hexamers can accommodate one MASP or MASP-1 dimer, as previously determined in the case of MBL tetramers⁽⁷²⁾.

Activation of the ficolin/MASP complexes

Activation of the proenzyme MASPs proceeds through cleavage of a conserved arginine-isoleucine bond, thus generating an active protease domain that remains disulfide-linked to the N-terminal part of the protease. MASP-1 and MASP-2 are each able to autoactivate, in contrast to MASP-3 that is activated through a yet unknown mechanism⁽⁶⁷⁾.

Activation of the lectin pathway is thought to involve transmission of a conformational signal from the recognition domain of the ficolins to the catalytic domain of the associated proteases, likely through the interface between the ficolin collagen-like region and the MASP interaction region. Recent studies on rat MBL have proposed that this protein modulates MASP-2 enzymatic activity and substrate recognition through two mechanisms. First, MBL increases the rate of MASP-2 autocatalysis upon binding of the MBL-MASP-2 complex to a target. Second, conformational changes of MASP-2 within the bound complex expose accessory C4 binding sites while separate structural changes yield a functional catalytic site able to cleave C4⁽⁷³⁾. C2 would then associate with the immobilized C4b fragment before cleavage by activated MASP-2. Whether such a mechanism also applies to the ficolin/MASP-2 complexes remains to be demonstrated, but this would imply the existence of interdomain flexibility in both the recognition protein and the associated protease.

ENHANCEMENT OF PHAGOCYTOSIS

Complement activation by the ficolins results in opsonization of microorganisms and apoptotic cells with C3b and C4b, thus promoting their clearance through interaction with C3b and C4b receptors on phagocytes^(41,44). Alternatively, ficolins may themselves function as opsonins, as suggested by the ability of L-ficolin to enhance phagocytosis of *Salmonella typhimurium* by neutrophils⁽¹⁴⁾ and of an antibody directed against M-ficolin to inhibit phagocytosis of *Escherichia coli* K-12 by promonocytic U937 cells⁽³²⁾. It has also been shown recently that binding of H-ficolin to late apoptotic cells results in enhanced adhesion/uptake by macrophages⁽⁴⁵⁾. These opsonizing effects of ficolins are likely exerted through receptors present on phagocytic cells, which may be shared with C1q and the collectins. Several candidates can be proposed, among which cC1qR (receptor for the collagenous domain of C1q) or calreticulin (CRT)⁽⁷⁴⁾ that functions in complex with the cell surface receptor CD91^(75,76). L- and H-ficolins were recently found to bind to cC1qR/CRT⁽⁴⁴⁾ and H-ficolin binding to CRT could be inhibited by MBL, suggesting that both proteins have a common binding site on CRT⁽⁴⁵⁾. However the precise CRT binding site on the collagen-like region of C1q, collectins and ficolins remains to be determined.

It has been proposed that the GEK/Q/RGEP sequence of the collagen-like region of C1q and collectins forms a consensus motif critical for enhancement of phagocytosis through another receptor called C1qRp (C1q receptor for phagocytosis enhancement)⁽⁷⁷⁾. However this motif is absent in all ficolins of known sequence, except pig ficolin α , and recent studies have identified C1qRp as CD93, casting doubt about its responsibility for *in vivo* enhancement of phagocytic activity by C1q and the collectins^(78,79).

Other studies have shown that complement receptor 1 (CR1/CD35), a phagocytic receptor for C3b and C4b, also binds to the collagen-like region of C1q and MBL^(80,81), but it is not known whether ficolins also interact with CR1. Lastly, Edelson et al.⁽⁸²⁾ have recently identified new ligands for the $\alpha 2\beta 1$ integrin, including C1q and the collectins. They suggested that binding occurs through the collagen-like region of C1q and collectins and that this interaction may stimulate mast cell activation and cytokine secretion in response to *Listeria*. The possibility that ficolins bind to this integrin and play a similar role remains to be investigated.

GENETIC POLYMORPHISM AND CLINICAL IMPLICATIONS

Two recent studies involving Dutch and Danish adult blood donors have provided evidence for a high polymorphism of the *FCN2* gene^(18,83). Fourteen single nucleotide polymorphisms (SNPs) were found in different regions of the gene, with allele frequencies ranging from 0.01 to 0.45. Five polymorphisms were identified in the promoter region and nine in the coding region, of which two result in amino acid changes, namely substitutions of threonine 211 and alanine 233 of the fibrinogen-like domain by methionine and serine, respectively. These two structural mutations were reported to be associated with decreased and increased ficolin binding to GlcNAc coupled to agarose beads, respectively⁽¹⁸⁾. The promoter SNPs at positions -986, -602 and -4 and the Ala233Ser mutation were associated with marked changes in the serum concentration of ficolin L. Whether certain alleles may be associated with disease susceptibility, as observed in the case of MBL, remains to be further investigated. In this respect, it should be mentioned that low L-ficolin concentrations have been associated with recurrent respiratory infections in children⁽⁸⁴⁾. It has also been reported that L-ficolin may contribute to the progression of renal disease in IgA nephropathy⁽⁸⁵⁾ and that *FCN2* gene promoter polymorphisms may influence the pathogenesis of the recurrent inflammatory Behçet's disease among HLA-B51 positive patients⁽⁸⁶⁾. On the other hand, no strong relationship could be established between low levels of

MBL, L- and H-ficolins and chemotherapy-related infections⁽²⁶⁾, as well as between functional polymorphisms of the L-ficolin gene and susceptibility to invasive pneumococcal disease⁽⁸⁷⁾.

Although no polymorphism was observed upon sequencing of the *FCN3* gene⁽¹⁸⁾, the serum concentration of H-ficolin has been reported to be elevated in systemic lupus erythematosus (SLE)⁽⁸⁸⁾ and to decrease with increasing severity of cirrhosis⁽⁸⁹⁾. However it should be stressed that the H-ficolin (Hakata antigen) concentration in SLE patients is likely influenced by the presence of autoantibodies. It has been reported recently that variations in H-ficolin serum levels are not associated with the pathogenesis of Crohn's disease⁽⁹⁰⁾.

The *FCN1* gene encoding M-ficolin is highly polymorphic as 12 SNPs have been identified in the promoter and in structural parts of the gene with frequencies varying from 0.05 to 0.40, but none resulted in amino acid substitution⁽¹⁸⁾. The influence of the promoter polymorphisms on the expression level of M-ficolin in peripheral monocytes awaits further characterization.

NON-HUMAN FICOLINS AND PHYLOGENY

Apart from humans, ficolins have been identified in different mammalian species including pig⁽¹⁰⁾, cow⁽⁹¹⁾, rodents⁽⁹²⁾ and hedgehog⁽⁹³⁾, in chicken⁽⁹⁴⁾, frog⁽⁹⁵⁾ and the invertebrate ascidian *Halocynthia roretzi*⁽⁹⁶⁾. Four ficolin cDNAs have been isolated from *H. roretzi* (AsFCN1, 2, 3 and 4) and from *Xenopus laevis* (XeFCN1, 2, 3 and 4). The ascidian ficolins and XeFCN3 have shorter collagen-like regions (5 and 7 Gly-X-Y repeats, respectively) than those of their mammalian counterparts (11-19 repeats). Rodents and pigs have two related ficolin genes, called A, B and α , β , respectively, that are orthologous to human L- and M-ficolin genes, respectively, and arose by separate gene duplication in each species⁽²⁹⁾. Chickens have a single ficolin gene that could represent an undiversified ancestor of the L- and M-ficolin genes⁽⁹⁴⁾. Interestingly, XeFCN1, rodent ficolin A, pig ficolin α and human L-ficolin are serum proteins, whereas XeFCN2/4, rodent ficolin B, pig ficolin β and human M-ficolin are mainly expressed in cells of monocytic origin, thus defining two ficolin types able to mediate innate immune recognition either in the circulation and locally. H-ficolin is a serum protein that up to now has only been identified in humans, despite its ancient origin by gene duplication prior to the emergence of amphibians. The mouse and rat homologues of the H-ficolin gene have been recently identified as pseudogenes, which accounts for the absence of the corresponding protein in rodents⁽²⁹⁾. It has been reported that erinacin, an antihemorrhagic factor

from muscle extracts of the hedgehog *Erinaceus europaeus*, is a multimeric protein consisting of two homologous α and β subunits in a 1:2 ratio, each containing a collagen-like region and a fibrinogen-like domain⁽⁹³⁾. Electron microscopy pictures revealed the parachute-like structure characteristic of ficolins, but the corresponding genes have not been identified and the mechanism underlying metalloprotease inhibition is unknown.

Phylogenetic analyses have provided evidence for a close relationship between the fibrinogen-like domains of ficolins and of non-collagenous proteins such as tenascins and horseshoe crab tachylectins, suggesting that the FBG domains of each of these families evolved from a common ancestor derived from fibrinogen⁽²⁹⁾. Interestingly, tachylectins 5A and 5B are innate immune recognition proteins with a propeller-like oligomeric arrangement that bind through their FBG domain to acetylated carbohydrates present at the microbial surface⁽⁹⁷⁾. However, recent structural studies have shown that the highly specific GlcNAc binding site observed in TL5A is not conserved in L-ficolin, which evolved to recognize a variety of acetylated and carbohydrate compounds through three different sites⁽⁴⁷⁾.

Based on evolutionary studies of the complement system, a minimal primitive system was proposed to involve a lectin (MBL/ficolin)-protease (MASP) complex, C3, and a C3 receptor. This ancestor of complement functioned through opsonophagocytosis and appeared in the ascidian lineage, at an intermediary stage between vertebrates and invertebrates^(98,99). However, recent studies have revealed the presence of a similar lectin-based opsonic system in the horseshoe crab, pointing out an earlier origin of these critical complement components and their essential role in innate immunity⁽¹⁰⁰⁾.

CONCLUDING REMARKS

Ficolins have emerged over the past few years as novel types of carbohydrate pattern recognition molecules, thus providing a further illustration of the diversity of the mechanisms used by innate immunity to sense danger signals. Structural studies on L-ficolin have led to identification of an extended recognition surface for both acetylated compounds and neutral carbohydrates that could accommodate elongated polysaccharides exposed at the surface of microbes or apoptotic cells. However this hypothesis will need to be verified in more physiological contexts and further microbiological studies are clearly necessary to define the pathogen recognition spectrum of human ficolins. Intriguing features of M-ficolin lie in its ability to bind sialic acid and in its cellular localization, thus raising the possibility that it may function in local host defense.

The effector mechanisms triggered by ligand-bound ficolins remain to be fully characterized at the molecular level. Ficolins may either act as direct opsonins able to present the bound pathogens to phagocytes or indirectly through further opsonization with complement activation fragments. The nature of the ficolin/collectin receptors on phagocytes and the precise intracellular signalling pathways triggered upon this interaction remain to be fully elucidated. Further investigation will also be needed to analyse the structural changes underlying the activation mechanism of the ficolin-MASPs complexes upon binding to a target.

Besides these fundamental aspects, a future challenge is to understand the precise function of ficolins in innate immunity by producing gene-targeted animal models. In this respect, single and double knock-out mice would be necessary to elucidate the role of each ficolin type, the serum type (mouse ficolin A/human L-ficolin) and the locally secreted one (mouse ficolin B/human M-ficolin). However this strategy will not apply to H-ficolin as its gene is dysfunctional in rodents. The role of polymorphisms of the ficolin genes in the predisposition to infectious or autoimmune pathologies will also need investigation, both through animal models and human studies. This may lead to the identification of subgroups of patients at particular risk for certain infections or for increased disease severity and possibly to the elaboration of novel therapeutic strategies.

ACKNOWLEDGEMENTS

Work in the authors' laboratories has been funded by the "Agence Nationale pour la Recherche" (ANR) (MIME programme, 2006-2008).

DISCLOSURES

The authors declare no financial conflict of interest.

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