Structural Basis for Innate Immune Sensing by M-ficolin and Its Control by a pH-dependent Conformational Switch*

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Virginie Garlatti‡, Lydie Martin‡, Evelyne Gout§, Jean-Baptiste Reiser†, Teizo Fujita‡, Gérard J. Arlaud§, Nicole M. Thielens§, and Christine Gaboriaud‡

From the ‡Laboratoire de Cristallographie et Cristallogénesis des Protéines and §Laboratoire d’Enzymologie Moléculaire, Institut de Biologie Structurale Jean-Pierre Ebel, Commissariat à l’Energie Atomique-CNRS-Université Joseph Fourier, 41 Rue Jules Horowitz, 38027 Grenoble, France and the †Department of Biochemistry, Fukushima Medical University, Fukushima, Japan

Ficolins are soluble oligomeric proteins with lectin-like activity, assembled from collagen fibers prolonged by fibrinogen-like recognition domains. They act as innate immune sensors by recognizing conserved molecular markers exposed on microbial surfaces and thereby triggering effector mechanisms such as enhanced phagocytosis and inflammation. In humans, L- and H-ficolins have been characterized in plasma, whereas a third species, M-ficolin, is secreted by monocytes and macrophages. To decipher the molecular mechanisms underlying their recognition properties, we previously solved the structures of the recognition domains of L- and H-ficolins, in complex with various model ligands (Garlatti, V., Belloy, N., Martin, L., Lacroix, M., Matsushita, M., Endo, Y., Fujita, T., Fontecilla-Camps, J. C., Arlaud, G. J., Thielens, N. M., and Gaboriaud, C. (2007) *EMBO J.* 24, 623–633). We now report the ligand-bound crystal structures of the recognition domain of M-ficolin, determined at high resolution (1.75–1.8 Å), which provides the first structural insights into its binding properties. Interaction with acetylated carbohydrates differs from the one previously described for L-ficolin. This study also reveals the structural determinants for binding to sialylated compounds, a property restricted to human M-ficolin and its mouse counterpart, ficolin B. Finally, comparison between the ligand-bound structures obtained at neutral pH and nonforming conformations observed at pH 5.6 reveals how the ligand binding site is dislocated at acidic pH. This means that the binding function of M-ficolin is subject to a pH-sensitive conformational switch. Considering that the homologous ficolin B is found in the lysosomes of activated macrophages (Runza, V. L., Hehlgens, T., Echtenacher, B., Zahringer, U., Schwaeble, W. J., and Mannel, D. N. (2006) *J. Endotoxin Res.* 12, 120–126), we propose that this switch could play a physiological role in such acidic compartments.

To protect themselves against infection, multicellular organisms have acquired innate immunity systems that rely upon the ability of a restricted pool of recognition molecules to sense conserved molecular patterns exposed at the surface of microbes and to elicit effector mechanisms designed to provide a first line of defense (1, 2). Among these molecules are the ficolins, a family of proteins found in a variety of animals ranging from invertebrates to mammals (3, 4). Ficolins are oligomers of trimeric subunits, which are made of three identical polypeptide chains, comprising collagen-like triple helices prolonged by a globular recognition domain structurally related to the fibrinogen β and γ chains (5). Three ficolins have been identified in humans: L-ficolin and H-ficolin, which are both serum proteins, and M-ficolin, a secretory protein synthesized in bone marrow, lung, and spleen and by blood monocytes and neutrophils (6). L-ficolin is known to recognize various capsulated bacteria and exhibits binding specificity for diverse ligands, such as lipoteichoic acid (7), 1,3-β-d-glucan (8), and the capsular antigen of type III group B streptococci (9). H-ficolin has only been reported to bind to *Aerococcus viridans* (10). 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 (11, 12). The structures of the recognition domains of human L- and H-ficolins, alone and in complex with various ligands, have been solved by x-ray crystallography (13), revealing the structural determinants for their binding specificities. In addition to an outer S1 binding site, homologous to a site identified in the invertebrate tachylectin 5A (TL5A) (14), three additional sites, called S2, S3, and S4, were discovered in L-ficolin. Together, these new sites define a continuous recognition surface able to sense various acetylated and neutral carbohydrate markers in the context of extended polysaccharides, as found on microbial or apoptotic surfaces (13). Recombinant M-ficolin shows a marked preference for acetylated compounds, as also observed for L-ficolin (15) and binds neoglycoproteins bearing GlcNAc, GalNAc, and sialyl-N-acetyllactosamine (16). Binding to the smooth type LT2 strain of *Salmonella typhimurium* and to *Streptococcus aureus* has been reported, but only binding to the latter could be inhibited by GlcNAc (17). The structure of the recognition domain of human M-ficolin was recently reported,

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The atomic coordinates and structure factors (code 2jhm, 2jhk, 2jhi, 2jhl, and 2jjh) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† To whom correspondence should be addressed. Tel.: 33-4-38789599; Fax: 33-4-38785122; E-mail: christine.gaboriaud@ibs.fr.

‡ The abbreviations used are: TL5A, tachylectin 5A; Neu5Ac, N-neuraminic acid; r.m.s., root mean square; Mes, 4-morpholineethanesulfonic acid.
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### MATERIALS AND METHODS

#### Recombinant Protein Production and Purification

The DNA segment encoding the C-terminal residues 80–297 of mature human M-ficolin was amplified using VentR polymerase and the pMT/Bip/V5-HisA plasmid containing the full-length cDNA (16) as a template, according to established procedures. This segment starts at the first residue following the collagen-like sequence. The DNA was cloned in frame with the melittin signal peptide of the pNT-Bac baculovirus transfer vector (19), and the recombinant baculovirus was generated using the Bac-to-Bac™ system (Invitrogen Corp.) and amplified as described previously (20). High Five cells were infected with the recombinant virus for 96 h at 27 °C. The protein was purified from culture supernatants by ion exchange chromatography on a Q-Sepharose Fast Flow column (GE Healthcare) equilibrated in 50 mM triethanolamine-HCl, pH 7.6, using a linear gradient to 250 mM NaCl. Mass spectrometry analysis was performed using the matrix-assisted laser desorption ionization technique under conditions described previously (21).

**Crystallographic Data Collection Statistics**

| Protein Data Bank code | ESRF beamline | Resolution range (Å) | Space group | Unit cell dimensions | Observed reflections | Unique reflections | Red. (%) | Comp. (%) | I/σI | Rsym (%) |
|------------------------|---------------|----------------------|-------------|---------------------|---------------------|------------------|---------|----------|-----|----------|
| 2jhm                   | ID14-eh2      | 1.52–56.8 (1.52–1.60) H3 | a = b = 73.71, c = 124.61 | 98,652 (9529) | 36,615 (4482) | 2.7 (2.1) | 94.3 (94.3) | 11.3 (3.0) | 7.4 (27.7) |
| 2jkh                   | ID23-eh2      | 1.75–23.7 (1.75–1.80) H3 | a = b = 73.75, c = 124.89 | 146,501 (11,878) | 25,519 (2072) | 5.7 (5.72) | 99.3 (98.7) | 17.7 (4.2) | 6.3 (27.4) |
| 2jhi                   | ID23-eh2      | 1.80–283.1 (1.80–1.85) H3 | a = b = 73.97, c = 126.87 | 135,776 (10,669) | 23,757 (1662) | 5.7 (5.72) | 99.3 (97.7) | 10.4 (3.0) | 9 (36.4) |
| 2jhl                   | ID14-eh4      | 1.75–28.0 (1.75–1.80) H3 | a = b = 73.93, c = 124.83 | 137,933 (11,099) | 24,703 (1973) | 5.6 (5.6) | 98.9 (97.6) | 23.5 (4.9) | 4.6 (24.4) |
| 2jhh                   | ID23-eh2      | 1.70–280 (1.70–1.75) P3 | a = b = 69.40, c = 77.63 | 258,896 (20,733) | 45,425 (3642) | 5.7 (5.7) | 98.7 (95.5) | 14.9 (3.9) | 7.3 (36.9) |

* Data corresponding to the last resolution shell are indicated in parentheses.

#### Results

In order to determine the three-dimensional structure of the fibrinogen-like recognition domain of human M-ficolin, the segment corresponding to this domain (residues 80–297 of mature M-ficolin) was expressed in a baculovirus/insect cell system. As assessed by mass spectrometry, a single species with a mass of 24,549 ± 12 Da was purified, accounting for the unmodified polypeptide chain (calculated value 24,553 Da). Selection of the best diffracting crystals among several crystallization hits obtained at two different pH values allowed us to solve to the five x-ray structures presented here (Table 2).

**The Ligand-free and Ligand-bound Structures Solved at Neutral pH**

The ligand-free structure obtained at pH 7.0 was solved by molecular replacement using L-ficolin (13) as a starting model and refined to 1.5 Å resolution (Table 2). The protein is homotrimeric, with crystallographic 3-fold symmetry (Fig. 1A). As expected from the amino acid sequence conservation of the interprotomer interfaces in ficolins, this assembly is very similar to those previously described for the L- and H-ficolin recognition domains (13). Likewise, homologous Ca\(^{2+}\) binding sites are found in the most external part of the trimer, with a distance of 65 Å between the Ca\(^{2+}\) ions, as observed in L-ficolin (Fig. 1, A and D). Ca\(^{2+}\) coordination in M-ficolin involves two water molecules, both carboxylate oxygens of Asp\(^{239}\), one of the side-chain oxygens of Asp\(^{239}\), and the main-chain carbonyl oxygens of Ser\(^{237}\) and Ser\(^{239}\). M-ficolin and L-ficolin have highly similar overall protomer structures, with an r.m.s. deviation value of 0.5 Å for 211 superposed Ca atoms (Fig. 1B). Only
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TABLE 2
Information about the structures solved and their crystallographic refinement statistics

| PDB code | pH   | Ligand       | Resolution high/low | $R_{work}$/$R_{free}$ | r.m.s. deviation bond/angle | Mean B factor protein/ligand | Ramachandran FR/AAR/GAR/DR $^{ab}$ |
|----------|------|--------------|---------------------|-----------------------|-----------------------------|-----------------------------|-----------------------------------|
| 2jhm     | 7    | GlcNAc       | 1.52–56.79 (1.52–1.56) | 18.1/20.7 (21.9/25.6) | 0.009/1.19                  | 11.7                        | 84.5/15.5/0.0/0.0                 |
| 2jkh     | 7    | GlcNAc       | 1.75–23.71 (1.75–1.81) | 17.3/18.6 (20.4/23.9) | 0.008/1.17                  | 21.0/42.1                   | 85.0/15.0/0.0/0.0                 |
| 2jhi     | 7    | GlcNAc       | 1.80–28.35 (1.80–1.86) | 19.6/21.4 (23.8/31.7) | 0.01/1.23                   | 25.3/36.7                   | 85.0/15.0/0.0/0.0                 |
| 2jhd     | 7    | GlcNAc       | 1.75–27.98 (1.75–1.81) | 21.0/23.9 (23.2/23.7) | 0.008/1.24                   | 27.4/36.0                   | 84.5/15.5/0.0/0.0                 |
| 2jhe     | 7    | Neu5Ac       | 1.70–28.03 (1.70–1.74) | 21.9/25.1 (26.0/30.0) | 0.009/1.27                   | 22.5                        | 87.3/12.4/0.3/0.0                 |

$^{a}$ Data corresponding to the last resolution shell are indicated in parentheses.

$^{b}$ FR, favored region; AAR, additional allowed region; GAR, generously allowed region; DR, disallowed region.

An Evolutionarily Conserved N-Acetyl-binding Pocket—The detailed interactions of the three ligands with site S1 observed at pH 7.0 are depicted in Fig. 2 (A–C). A common set of three different interactions stabilizes the ligand aceamido group: (i) its methyl group is in Van der Waals contacts with the surrounding hydrophobic pocket formed by Phe245, His255, Tyr271, Ala272, and Tyr283; (ii) its carbonyl oxygen is hydrogen-bonded to the backbone NH group of Cys254 and His255; (iii) its nitrogen atom is hydrogen-bonded to the hydroxy group of Tyr271. This latter interaction is mediated by a water molecule in the case of GlcNAc, whereas the Tyr271 side chain slightly moves toward the ligand to provide a direct hydrogen bond in the case of GalNAc and Neu5Ac. Tyr271 is thus the only flexible compo-

FIGURE 1. Structure of the recognition domain of M-ficolin and location of its S1 ligand binding site. A, bottom view of the homotrimeric structure of M-ficolin solved at neutral pH. Ca$^{2+}$ ions are represented as golden spheres. The sialic acid ligand bound to site S1 is shown in a yellow ball and stick representation. B, superposition of the similar fibrinogen-like protomers of M-ficolin (magenta) and L-ficolin (green). Domains A, B, and P are labeled. C, detailed superposed views of the structures of M-ficolin (magenta) and L-ficolin (green) highlighting the common cis-conformation of their respective Asp253-Cys254 and Asn244-Cys245 peptide bonds. D, sequence alignment of the P domains of human ficolins M, L, and H; mouse ficolins FCN B and FCN A; and TL5A. The residue numbering and the secondary structure elements apply to M-ficolin. Residues involved in the S1 binding site are colored green, and those involved in Ca$^{2+}$ binding are colored red. Small residues allowing accommodation of sialic acid in site S1 are colored blue.
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As illustrated in Fig. 2E, S1 is highly homologous to the GlcNAc binding site of the distantly related invertebrate lectin TL5A (14). Both the hydrophobic pocket and the unusual cis-conformation of the Asp<sup>253</sup>-Cys<sup>254</sup> peptide bond (Arg<sup>218</sup>-Cys<sup>219</sup> in TL5A) are conserved, the latter being essential to correctly position the two consecutive backbone NH groups for appropriate interaction with the acetamido oxygen. These characteristics are also highly conserved in mammalian ficolins, except for a slightly different hydrophobic pocket in human H-ficolin (Fig. 1D). Interestingly, the replacement of Tyr<sup>271</sup> by a phenylalanine in L-ficolin (Fig. 2F) could explain the lack of binding of N-acetylated ligands in its S1 site, where an acetate molecule is often bound instead (13).

Recognition of the three N-acetylated carbohydrates by M-ficolin also involves additional hydrogen-bonding interactions with their sugar rings, but those supplemental interactions depend on the nature of the ligand. In the case of GlcNAc, the 1-OH oxygen is hydrogen-bonded to the backbone carbonyl group of His<sup>255</sup> (Fig. 2B). This differs from the recognition of GlcNAc by TL5A, where the 1-OH oxygen forms hydrogen bonds with the guanidinium nitrogen of Arg<sup>216</sup> and with the hydroxyl group of Tyr<sup>248</sup> (Fig. 1E). More distant polar interactions are observed in the case of GalNAc, with a water-mediated hydrogen bond between 4-OH and the backbone oxygen of Asp<sup>253</sup> and a direct interaction between 1-OH and the hydroxyl group of Tyr<sup>283</sup> (Fig. 2A).

Structural Basis for Sialic Acid Recognition—The structure of the M-ficolin-Neu5Ac complex reveals a more extensive network of polar interactions required to recognize this bulkier molecule (Fig. 2C). Further stabilization is achieved by direct and water-mediated hydrogen bonds between the 7-OH oxygen and the backbone oxygen and nitrogen of Asp<sup>253</sup>, respectively. Interestingly, as illustrated by the superposition of the M- and L-ficolin structures (Fig. 2F), steric hindrance may explain why most ficolins do not interact with sialic acids. Indeed, two small residues in the vicinity of the S1 site, Gly<sup>221</sup> and Ala<sup>256</sup> in M-ficolin, are replaced in L-ficolin by the bulkier residues, phenylalanine and threonine, respectively. These two residues reduce the size of the binding pocket, thereby limiting its access to large carbohydrate molecules such as NeuNAc (Fig. 2F). Sequence alignments of mammalian ficolins show that, except for mouse ficolin B and human M-ficolin, both positions are occupied by bulkier residues (Fig. 1D).

The S1 Binding Site Is Disrupted and Exhibits Increased Flexibility at Acidic pH—It was recently reported that the GlcNAc binding activity of M-ficolin is pH-sensitive, and the structure of its recognition domain, obtained at pH 5.6, was found to exhibit inactive loop conformations around the S1 binding site (18). Such differences might have been either a direct consequence of the acidic pH of the crystallization solution or a possible artifact linked to the introduction of a 23-residue-long C-terminal tag in the recombinant domain (26). To investigate this question, our own construct, corresponding solely to the fibrinogen-like recognition domain of M-ficolin, was crystallized at pH 5.6, and its structure was solved and refined to a resolution of 1.7 Å (Table 2). Although this new crystal form differs from the one reported previously (18), the resulting structure is similar (mean subunit r.m.s. deviation value of 0.7
(Å), with some differences mostly arising from changes in the ligand-binding region, as illustrated in Fig. 3B. This additional M-ficolin structure also clearly shows the Asp$^{253}$-Cys$^{254}$ peptide bond in a trans-conformation, which drastically modifies the positioning of the His$^{255}$ side chain (Fig. 3B). In addition to this cis-trans conformational change, the acidic pH induces large displacements (>10 Å) of Tyr$^{271}$ and Tyr$^{283}$, both essential for ligand binding (Fig. 3, A and B). Thus, with the exception of Phe$^{245}$, all residues making up S1 are extensively displaced at acidic pH, resulting in a conformation clearly inappropriate for ligand binding. This acidic conformation is significantly different from the structures obtained at neutral pH, with a mean subunit r.m.s. deviation of 2.14 ± 0.45 Å, a value that increases significantly to 3.36 ± 0.64 Å when only the ligand-binding region is considered. This acidic conformation will be therefore referred to as the “non-binding” state.

The conformational transition from the binding to the nonbinding state involves the concerted displacement of four surface segments or loops, namely L1 (218–224), L2 (253–258), L3 (264–274), and L4 (278–288). Except Phe$^{245}$, all of the residues defining S1 are included in loops L2–L4, and L1 includes Gly$^{221}$, which, as stated above, is probably the key determinant for the specificity toward sialic acid. The increased flexibility of these four loops at acidic pH was assessed by analyzing both the gaps in the crystallographic models, which correspond to disordered segments (Table 3), and the mean B factor in these loops according to the experimental context (Tables 4 and 5).

As can be seen in Table 3, several disordered segments are found in loops L3 and L4 in the two structures determined at pH 5.6. This indicates a high flexibility of these loops at this pH, which corroborates the observation that they display the largest displacements when compared with the neutral pH structure (Fig. 3). A detailed analysis of the ratio of the mean B factor of each loop compared with the mean B factor of the corresponding subunit is depicted in Table 4. This ratio is referred to as the “B loop ratio.” Its value is defined for loops L1–L4 in the six M-ficolin structures currently available. A positive value means that the B factor is higher in the loop compared with the averaged value of the corresponding subunit. The highest positive values (97 and 127%) are observed for L1 in the structure determined by Tanio et al. (18), indicating a significant increase of L1 flexibility under these conditions. This is in contrast with the negative values observed for L1 at neutral pH in our study, where this loop is greatly stabilized, defining a small α7 helix (Fig. 1D). To obtain a more direct assessment of the pH-dependent increase in flexibility occurring in loops L1–L4, Table 5 summarizes the B loop ratio values averaged for each
loop depending on the pH value, revealing a significant increase at pH 5.6 compared with 7.0 for all loops.

**DISCUSSION**

We have previously solved the crystal structure of the fibrinogen-like recognition domains of human L- and H-ficolins (13) and now report the structure of the corresponding domain of M-ficolin. This additional example confirms that this type of domain associates as a three-helod homotrimeric structure that is intermediate between the compact assembly seen in the globular “head” of complement protein C1q (27) and the open structure of the carbohydrate recognition domain of mannan-binding lectin (28). In contrast to mannan-binding lectin, where trimerization requires a triple-helical “neck” region, the recognition domains of ficolins solely associate through highly conserved interprotomer interfaces and are therefore self-sufficient in terms of assembly.

The structures determined at neutral pH provide precise insights, at the atomic level, into the structural determinants involved in the recognition by M-ficolin of its three known ligands, GlcNAc, GalNAc, and Neu5Ac. Each of these three N-acetylated molecules binds to the outer site S1 homologous to that originally described in TL5A, and remarkably, binding involves in each case a common set of interactions with the ligand acetyamido group, similar to that previously described for recognition of GlcNAc by TL5A (14). Thus, it appears that M-ficolin has essentially retained during evolution the binding characteristics of TL5A, possibly with a broader specificity for a wider range of N-acetylated molecules. This is in contrast with L-ficolin, which lacks the ability to recognize acetylated molecules through its S1 site but has instead acquired additional binding sites (S2 and S3) that bind these compounds in a poorly specific manner (13).

In line with previous observations (16), the structure of the complex between the M-ficolin recognition domain and Neu5Ac (Fig. 2C) demonstrates that M-ficolin specifically recognizes sialic acids. As stated above, this particular property is probably conditioned by the presence of small residues, Gly and Ala, at strategic positions in the vicinity of site S1, allowing accommodation of the relatively bulky sialic acid molecules. The fact that, among known mammalian ficolins, this structural feature is only shared by human M-ficolin and mouse ficolin B provides a plausible explanation why the ability to recognize sialylated compounds is restricted to these proteins, which are both secreted by the monocyte/macrophage cell lineages (16, 29). This restriction may be related to the fact that, whereas sialic acid is found on some pathogens, such as the surface capsular polysaccharides of group B Streptococcus (30), it is also a marker of self cells. Thus, it is tempting to hypothesize that the inability of L- and H-ficolins to recognize sialic acid is designed to prevent inappropriate recognition of self cells by these proteins in serum. In contrast, M-ficolin would retain the ability to sense certain pathogens in peripheral compartments. Since sialic acid is found at high concentrations at the surface of immune cells, with for example a concentration about 100 mM on B cells (31), M-ficolin could also play a role in mediating or modulating immune cell-cell interactions, a dual role established for other pathogen recognition receptors, such as macrophage galactose-type lectin (32) or DC-SIGN (33).

**Structural Basis of the pH-sensitive Switch**—A major lesson from this study is that the ligand-binding activity of M-ficolin is subject to a pH-dependent switch. The conformational transition from the binding to the nonbinding state involves the concerted displacement of four surface loops, namely 218–224, 253–258, 264–274, and 278–288, with Trp277 serving as an anchor (Fig. 3C). Because a $K_a$ of 6.2 was derived from the pH dependence of the GlcNAc binding activity of M-ficolin, it has been suggested that this is possibly related to changes in the charged state of some of its histidine residues (18). Indeed, comparison of the structures of the binding and nonbinding states reveals changes in the stabilizing interactions mediated by the side chains of His, His, and His (Fig. 3C). In the binding state, His is hydrogen-bonded to the main chain oxygen of Trp and to the main chain nitrogen of Ala (i.e. to the backbone of the “active loop” comprising the Asp-Cys-cis-peptide bond. In this “active loop,” His is hydrogen-bonded to the carbonyl group of Ala (27). The third histidine residue, His, also mediates two hydrogen bonds within the same loop (with Ser and Asn OD1). These histidine-mediated stabilizations are disrupted at acidic pH (Fig. 3C), with an increased flexibility in these loops; the side
chain of His\textsuperscript{223} becomes disordered, and the side chain of His\textsuperscript{255} flips toward the Asp\textsuperscript{253} side chain, where it fills the space occupied by the ligand in the structures of complexes. Finally, the long neighboring loop, including His\textsuperscript{266}, displays the largest displacement (Fig. 3C).

A pH-induced Functional Switch in Lysosomes?—Remarkably, the pH-sensitive conformational switch revealed by this study only affects the loops holding the ligand binding site residues. This strongly suggests, therefore, that this switch supports some functional role. Indeed, considering that the mouse counterpart of M-ficolin (ficolin B) was recently found in the lysosomes of activated macrophages (34), it is tempting to speculate that this mechanism is involved in ligand release. Thus, the M-ficolin secreted upon macrophage activation would bind to its target microorganism and then become internalized as a complex with its ligand. The pH drop in the lysosomes would then be expected to trigger the conformational switch, resulting in the release of the ligand. This intracellular trafficking would be similar to some extent to that described for several receptor-ligand complexes, such as the asialoglycoprotein receptor (35) or the low density lipoprotein receptor family (36, 37). Whether M-ficolin would then be recycled or not remains to be investigated. In future studies, the histidine residues that are likely to play a role in the conformational switch could be modified to further investigate their functional implication.

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