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Recognition of Acetylated Oligosaccharides by Human L-ficolin

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1. Summary

The complement system is a protein cascade capable of neutralizing invading pathogens. One of its activation pathways is the lectin pathway which is dependent on the binding of MBL or the ficolins. The specificity of L-ficolin binding has been investigated previously and it was observed that binding is dependent on acetyl groups. If this was the only requirement this would enable L-ficolin to bind to most mammalian glycosylations since they contain acetylated monosaccharides. To investigate this further L-ficolin was subjected to glycan-array analysis in which L-ficolin binding to 279 different glycans was investigated. Few of these bound L-ficolin above background level but clear structural requirements were discovered.

2. Introduction

The immune system is composed of a multitude of different recognition and effector mechanisms preventing omnipresent microorganisms from invading the nutrient-rich internal environment of multicellular organisms. The complement system is one such immune system mechanism and it comprises more than 30 soluble and cell-bound recognition, effector and control proteins. Upon activation the complement system can lead to opsonization or direct lysis of pathogens and inflammation at the site of infection. It is activated through three different pathways all leading to the formation of a C3-convertase complex. The classical pathway is dependent on C1q binding to a target and subsequent activation of the associated proteases C1r and C1s [1]. The activation of C1s leads to the cleavage of C4 and C2 and the formation of the C3 convertase complex C4b2a. The alternative pathway is dependent on the constant hydrolysis of C3 as well as the lack of complement inhibitors on pathogen surfaces. The lectin pathway is similar to the classical pathway because it relies on binding of recognition molecules which enable the cleavage of C4 and C2 through associated serine proteases. These recognition molecules are mannan-

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binding lectin (MBL) and the ficolins (L-ficolin, H-ficolin and M-ficolin). L-ficolin and H-ficolin circulate as large homomultimeric protein complexes composed of up to 18 polypeptide chains and associated with the MBL-associated serine proteases (MASP) 1, 2 and 3 [2–6]. Of these MASP2 has been identified as the protease responsible for complement activation [2] while no major substrate has yet been identified for either MASP1 or MASP3. The main difference between MBL and the ficolins is found in their globular binding domain, since MBL has a C-type carbohydrate recognition domain while the ficolins have a fibrinogen-like (fbg) domain.

MBL has since its discovery been intensely characterized in terms of ligand specificity and it has been found to bind oligosaccharides terminating in monosaccharides with 3' and 4' OH groups in the equatorial plane like mannose and glucose derivatives [7, 8]. The ficolins have not been so extensively studied in regard to ligand specificity but studies have shown that L-ficolin is a versatile recognition molecule capable of binding to a wide range of opportunistic pathogens like *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* [4, 9] as well as altered self like apoptotic and necrotic cells [10, 11]. The specificity of L-ficolin recognition remained uncertain despite studies showed that L-ficolin binding could be inhibited by various N-acetylated compounds [12, 13] but presence of such N-acetylated carbohydrates in the capsules of *S. pneumoniae* did not always lead to binding [9, 14]. The binding of all three ficolins to bacterial surface materials can be inhibited by N-acetylglucosamine (GlcNAc) [12, 15, 16]. Additionally both L-ficolin binding is inhibited by other N-acetylated monosaccharides as well as compounds like N-acetyl glycine and acetylcholine [13]. Direct binding of L-ficolin has been observed to immobilized GlcNAc monosaccharides (e.g. GlcNAc-BSA and GlcNAc-Sepharose) [12, 17] as well as to complex polymers like lipoteichoic acids and 1,3- β -D-glucans [18, 19].

Due to the complex nature of L-ficolin binding we wanted to investigate its specificity using glycan array technology. This enables us to screen multiple different oligosaccharides for their ability to bind L-ficolin and establish how L-ficolin is capable of distinguishing foreign from host cell surfaces. We found that L-ficolin binding was dependent not only on the presence of acetylated residues but also on the conformation of the oligosaccharide.

3. Materials and Methods

Fluorophore labelling of L-ficolin

Purified human L-ficolin (purified as described in [13]) was dialyzed overnight against 50 mM CHES, 140 mM NaCl, pH 9.0 at 4°C. Alexa Fluor® 488 tetrafluorophenylcarboxylate ester (Invitrogen Ltd., Paisley, UK) was dissolved in DMSO to a final concentration of 1 mg/ml. Of this 20 μ l was added to the dialyzed L-ficolin (250 μ l of a 400 μ g/ml solution) and incubated for 2 hours at room temperature. The Alexa Fluor labeled L-ficolin (labeling is via amino groups) was subsequently dialyzed into 20 mM HEPES, 140 mM NaCl, 5 mM CaCl₂ pH 7.4.

Glycan array analysis of L-ficolin

Purified L-ficolin labeled with Alexa Fluor® 488 was used for glycan array screening. Glycan array technology (Core H, The Consortium for Functional Glycomics, Emory University, Atlanta, GA) uses printed glycan microarray chips with various glycans covalently bound through amino linkers. The glycans on the chip were from a library of natural and synthetic glycans. Binding of L-ficolin to glycans was detected by measurement of Alexa Fluor® 488 fluorescence. The screening of the whole chip was performed with a total volume of 70 µl of 200 µg/ml L-ficolin in 20 mM HEPES, 140 mM NaCl, 5 mM CaCl₂ pH 7.4. For further information see the website: <http://www.functionalglycomics.org/static/index.shtml>.

4. Results

To permit investigation of the specificity of L-ficolin, purified material had to be obtained. L-ficolin was therefore purified using N-acetyl cysteine-derivatized Sepharose resin for the initial affinity step and an ion-exchange step for the secondary purification [13]. As shown in figure 1 the purified L-ficolin was analyzed for purity on SDS-PAGE. In the reduced lane the L-ficolin polypeptide chain is found as the major band at 35 kDa. In the non-reduced lane L-ficolin is found as three major bands at 35 kDa and two of more than 212 kDa. This occurs because L-ficolin is a homomultimeric protein complex where the individual chains are held together by non-covalent and covalent interactions (disulphide bridges). The disulphide bridge formation is heterogenous [17] and therefore in denaturing conditions L-ficolin will appear to consist of multiple complexes of different sizes. In both the reduced and non-reduced lanes low abundance contaminants are present but based on the staining intensity it can be estimated that the L-ficolin preparation is more than 90% pure and suitable for the glycan array analysis.

The glycan array analysis was carried out by the Consortium for Functional Glycomics and the version of the chip used contained 279 different glycan structures varying from monosaccharides to complex oligosaccharides with more than ten monosaccharide constituents. The analysis of the binding activity of L-ficolin was done by flowing the labeled L-ficolin over the chip and the amount of L-ficolin bound to the different glycans was estimated through the conjugated fluorophore. In figure 2 the fluorescence associated with each of the glycans is shown. As can be observed three glycans (nos. 49, 156 and 157) have a much higher L-ficolin binding than any of the other glycans. The structure of the five saccharides with the best L-ficolin binding potential is shown in table 1A. To allow for interpretation of the data it is assumed that each spot contains an identical number of oligosaccharides. Although glycan 156 binds L-ficolin well it also has a high standard error of measurement and therefore was not included in evaluation of the data.

Since all previous studies have shown that L-ficolin has an affinity towards N-acetylated compounds this motif most likely plays an important part. The question is how L-ficolin recognizes N-acetylated compounds incorporated in oligosaccharides. It could be that L-ficolin, like MBL, only recognizes the terminal residue on an oligosaccharide, or it may recognize any oligosaccharide containing N-acetylated compounds, or the N-acetyl groups may have to be in specific conformations to mediate L-ficolin binding. Data from

Matsushita et al. [20] indicate that binding is not dependent only on terminal N-acetylated monosaccharides. By using the binding data from the glycan we attempted to distinguish between these hypotheses.

To test if L-ficolin recognizes only terminal acetylated carbohydrates the binding potential of glycans 25 and 22 were compared to 157 and their structures and binding potential are shown in table 1B. Glycan 22 is a single GlcNAc residue and if only the terminal residue is recognized, L-ficolin should bind glycan 22 as well as it binds 25 (4 GlcNAc residues) and 157 (2 GlcNAc residues separated by a galactose). As can be seen in table 1 this is not so since glycan 22 does not bind L-ficolin over the background level (defined as the average binding potential of all the glycans of the chip i.e. a relative fluorescence of 4718) while both 25 and 157 do. This suggests that L-ficolin does not recognize only the terminal residue. However the impaired L-ficolin binding to 22 compared to 25 and 157 could also be due to steric hindrance of L-ficolin since the chip surface is in closer proximity to the terminal GlcNAc residue compared to the other two. This is unlikely since the saccharides are bound to the surface through a spacer, but the possibility cannot be eliminated. An additional effect can also be observed in that glycan 25 (4 GlcNAc) binds L-ficolin better than 22 (1 GlcNAc) but only half as well as 157 (2 GlcNAc) indicating that there are spatial conformational requirements that have to be fulfilled to maximize L-ficolin binding. To investigate the importance of the conformation of the glycan for L-ficolin binding, the binding potential of glycans 22, 153, 176 and 246 were compared to that of glycans 157 and 49. These are all shown in table 1C. Glycan 22 and 153 are truncated versions of 157 composed of a GlcNAc residue or a GlcNAc plus galactose residue, respectively. As can be observed in table 1 neither 22 or 153 binds L-ficolin above background level but 157 binds strongly indicating the importance of the terminal GlcNAc residue in 157. Glycan 176 has the same trisaccharide sequence as 157 but the linkage to the terminal GlcNAc residue is a β -bond in 176 instead of an α -bond as with 157. As can be observed the β -linkage does not support binding while the α -linkage does illustrating that L-ficolin has stringent conformational binding requirements. This is further confirmed if the binding potentials of glycans 49 and 246 are compared since these are identical apart from the presence of an additional O-acetyl group in 49. Glycan 49 is the strongest L-ficolin binder, while 246 does not bind L-ficolin at all supporting the previous assumption that the position of the acetyl groups in space is very important. The observation that L-ficolin binding is highly dependent on the presence of certain structural motifs also eliminates the steric hindrance theory presented above to explain the difference in L-ficolin binding potential between glycans with different number or residues shown in table 1B. If the varying binding of glycans was dependent on glycan length (since this increase the distance to the chip surface) the binding potential of glycans 157 and 176 as well as glycans 49 and 246 (table 1C), should be the same since they are nearly of the same size. Why it is that complex glycans rich in GlcNAc residues have an intermediate binding potential compared to 49 and 157 may arise from the high density of N-acetyl groups such that some by chance are found in the right conformation to enable L-ficolin to bind. This may also explain how glycan 247 can have the fourth highest binding potential despite its three terminal being are identical to glycan 246, which does not bind L-ficolin at all.

Matsushita et al. [20] showed, by semi-quantitative ligand blotting the binding of L-ficolin to a range of neoglycolipids, synthesized from de-sialylated glycans released from mammalian or avian glycoproteins. As noted earlier their data show that L-ficolin binding is not dependent on terminal N-acetylated monosaccharides. They showed binding to neoglycolipids containing 2 or more GlcNAc residues but not high-mannose structures containing, as acetylated species, only two adjacent core GlcNAc. Our results show best binding with two or more non-adjacent acetylated sugars (sialic acid or GlcNAc) (glycans 49, 157, 156 and 247). This suggests that two GlcNAc, separated by only one saccharide (e.g. galactose) can both interact with separate sites, presumably in a single fbg domain. Comparing glycan 157 with 176 (table 1C), the two GlcNAc in 157 are in correct orientation to interact with two sites, while in 176, they are not.

5. Discussion

The binding studies presented here show that L-ficolin has complex binding requirements since it only binds strongly to oligosaccharides containing acetylated monosaccharides but also that the actual conformation of the oligosaccharide plays an important role. Whether L-ficolin binding requires the presence of >1 GlcNAc residue has not been possible to establish but it seems likely since a single GlcNAc residue (presented at the density available on the glycan array chip) (glycan 22) is not enough to allow for L-ficolin binding indicating that L-ficolin does recognize binding motifs by multiple interactions per oligosaccharide. The crystal structure (see below) also suggests this since more than one individual site per fbg domain can coordinate N-acetyl groups.

When L-ficolin was crystallized in the presence of various potential ligands, surprisingly four different binding sites were discovered indicating a more complex recognition mechanism than that found with MBL [21]. Tachylectin 5A (TL-5A) is a protein isolated from the horseshoe crab *Tachypleus tridentatus*, which, like the ficolins, contains a fbg globular domain. It has been found to have similarity in ligand binding to L-ficolin [22] as well as a high degree of sequence similarity (49% identical) [13]. Since the crystal structure of TL-5A in complex with a GlcNAc residue had been solved [23] and since many of the amino acids in L-ficolin corresponding to the ligand coordinating sites in TL-5A were conserved, the fbg domain of TL-5A was believed to be a functional homologue to the fbg domain of L-ficolin. When L-ficolin was crystallized this model was found to be unsuitable since TL-5A only had one binding site while L-ficolin was suggested to have four [21]. Of these, two (S2 and S3) were found to interact with N-acetylated compounds while the remaining (S1 and S4), with S1 being the homologous site to that in TL-5A, bound a GlcNAc disaccharide and 1,3- β -D-glucans, respectively. Additionally three of the sites, S2-4, formed a binding groove similar to that of peptidoglycan-binding proteins and may enable L-ficolin to bind oligosaccharides through multiple interactions [21].

Since the discovery of the ability of L-ficolin to bind to acetylated compounds it has been puzzling why this does not lead to recognition of host cells since fully processed N-linked glycosylations terminate in sialic acids, which commonly have acetylations. By having complex conformational requirements L-ficolin is capable of recognizing microorganisms attempting to mask themselves with carbohydrates to which the immune system is tolerant

but if the underlying carbohydrate scaffold is foreign L-ficolin can bind, leading to complement activation.

Another possible role for L-ficolin is recognition of malignant cells since these often undergo changes in their glycosylation pattern compared to normal cells [24]. It has been shown for example that N-linked glycosylation produced by liver metastases of colon cancers is biased towards 6Gal β 1-4GlcNAc linkages while normal healthy cells predominantly produced Gal β 1-3GlcNAc [25]. The glycosylated structures found on tumor cells may be susceptible to recognition by L-ficolin, so that L-ficolin, as well as being involved in the elimination of invading pathogens and the removal of dying host cells, may also be involved in immune surveillance of altered self.

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Figure 1.

Purified L-ficolin analyzed reduced and non-reduced on SDS-PAGE and subsequently stained with Coomassie Brilliant Blue. When run reduced on SDS-PAGE L-ficolin polypeptide chains are found only in the 35 kDa band. Non-reduced three L-ficolin bands could be identified at 35 kDa and two of more than 212 kDa. The top bands are homomultimeric complexes held together by disulphide bridges while the 35 kDa band is composed of non-covalently attached L-ficolin polypeptide chains.

Figure 2.

L-ficolin binding to the different carbohydrate structures on the glycan array chip. The relative fluorescence indicates the amount of bound L-ficolin to each of the individual glycans. The error bars indicate the standard error of measurement. The numbered arrows points to the three best L-ficolin binding glycans and their exact chemical structure is shown.

Table 1

The binding potential of L-ficolin to various glycan structures. In Panel A the 5 glycan structures that have the highest L-ficolin binding potential are shown. Panel B contains the glycan structures used to investigate the terminal residue recognition hypothesis. Panel C show the glycan structures used to elucidate the conformational requirements for L-ficolin binding. The glycan number in all three tables matches with the X-axis in figure 2. The average and the standard error of measurement (STE) are based on 4 individual experiments. The Sp8 and Sp0 are linkers through which the glycans are attached to the chip. The average L-ficolin binding was estimated to 4718, i.e. only glycan nos. 49, 157, 156, 247 and 25 of the showed above average binding.

A			
Glycan No.	Glycan Name	Average[*]	STE^{**}
49	9-O-AcNeu5Acα2-6Galβ1-4GlcNAcβ-Sp8	46748	3610
157	GlcNAcα1-6Galβ1-4GlcNAcβ-Sp8	26504	2364
156	GlcNAcα1-3Galβ1-4GlcNAcβ-Sp8	22319	12469
247	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4(Fuca1-3)GlcNAcβ1-3Galβ1-4(Fuca1-3)GlcNAcβ-Sp0	16381	3647
25	GlcNAcβ1-3(GlcNAcβ1-4)(GlcNAcβ1-6)GlcNAc-Sp8	13939	1650

B			
Glycan No.	Glycan Name	Average[*]	STE^{**}
22	GlcNAcβ-Sp8	2883	1657
25	GlcNAcβ1-3(GlcNAcβ1-4)(GlcNAcβ1-6)GlcNAc-Sp8	13939	1650
157	GlcNAcα1-6Galβ1-4GlcNAcβ-Sp8	26504	2364

C			
Glycan No.	Glycan Name	Average[*]	STE^{**}
22	GlcNAcβ-Sp8	2883	1657
153	Galβ1-4GlcNAcβ-Sp8	3065	103
176	GlcNAcβ1-6Galβ1-4GlcNAcβ-Sp8	2777	546
157	GlcNAcα1-6Galβ1-4GlcNAcβ-Sp8	26504	2364
49	9-O-AcNeu5Acα2-6Galβ1-4GlcNAcβ-Sp8	46748	3610
246	Neu5Acα2-6Galβ1-4GlcNAcβ-Sp8	1707	468

* Average relative fluorescence of 4 individual experiments

** Standard error of measurement of 4 individual experiments