Human L-ficolin: plasma levels, sugar specificity, and assignment of its lectin activity to the fibrinogen-like (FBG) domain

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Received 16 January 1998; revised version received 24 February 1998

Abstract Ficolins are characterised by the presence of collagen-like and fibrinogen-like (FBG) sequences. Human L-ficolin is synthesised in the liver and secreted into blood circulation. In previous studies, it was shown to bind to *N*-acetyl-D-glucosamine (GlcNAc). In the present study, its detailed sugar specificity and binding site have been investigated. It was found to bind to GlcNAc and GalNAc (*N*-acetyl-D-galactosamine) while showing no significant affinity for the precursor sugars. The structure in these molecules which is recognised by L-ficolin has been deduced to include an amide (-CO-NH-) or similar group. L-Ficolin was digested with collagenase and the collagenase resistant FBG domain was shown to bind to GlcNAc. Its levels in adult and cord blood-derived human plasma were also determined and showed that adult plasma contains approximately three times more L-ficolin than that of newborn babies.

Key words: Ficolin; Fibrinogen-like domain; Collagen-like; Lectin; Collectin; C1q

1. Introduction

Ficolins, the collectins and C1q comprise a family of proteins that contain collagen-like sequences and have similar domain organisations [1]. As a result, all these proteins are assembled into a 'bundle-of-tulips' or 'cruciform' overall structure [1,2]. L-Ficolin is approximately 400 kDa and electron microscopy reveals that it is probably composed of 12 polypeptide chains assembled into four trimeric 'structural units' that are disulphide-linked via the N-terminal region [2]. The tetrameric L-ficolin molecules can also form non-covalent, 'spindle-like' dimers [2]. However, the three types of proteins have distinct independently folded C-terminal globular domains [1,2] which form the basis for assigning the three types of proteins to distinct protein families [1,3,4]. C1q has C-terminal sequences which are related to globular sequences in certain collagens and bind to immune complexes leading to complement activation [3,5]. The collectins have C-terminal Ca^{2+}-dependent carbohydrate recognition domains (C-type CRDs) which bind to sugar residues on the surface of microorganisms leading to killing of bound microbes through complement activation and/or enhanced phagocytosis [1,6–8]. The corresponding regions in the ficolin polypeptides are related to the C-terminal halves of fibrinogen β and γ chains [1,9–11]. Similar sequences have also been found in a number of other proteins and have been termed the fibrinogen-like or FBG domains [12].

Although the FBG domain is unrelated to CRD [1,9–11,13], L-ficolin has been shown, like the collectins, to bind to sugar structures [2,11,14]. However, unlike the collectins, human L-ficolin could bind to GlcNAc in the absence of Ca^{2+} [2,14]. L-Ficolin has also been identified as an elastin- and corticosteroid-binding protein [15,16]. Together with the initial isolation of porcine ficolin as a transforming growth factor-β1-binding protein [9], ficolins apparently have affinity for various unrelated structures. It has also been shown recently that L-ficolin simply bound to CNBr-activated and Tris-reacted Sepharose at its GlcNAc-binding site(s) [14]. The binding of L-ficolin to GlcNAc on *Salmonella typhimurium* has been shown to enhance the phagocytosis of bound bacteria by polymorphonuclear leucocytes [11].

In the present study, the sugar specificity of L-ficolin has been further investigated. The structures in the sugars which are recognised by L-ficolin have also been suggested and the sugar binding site in L-ficolin has been assigned to the FBG domain. The level of L-ficolin in adult and cord blood-derived human plasma was also determined.

2. Materials and methods

CNBr-activated Sepharose, glutaraldehyde, biotinamidocaproate *N*-hydroxysuccinimide ester, streptavidin-alkaline phosphatase, galactose, galactosamine, GalNAc, glucose, glucosamine and GlcNAc were purchased from Sigma Chemical Co. (St. Louis, MO, USA). GlcNAc-Sepharose was prepared following the method described by Fornstedt and Porath [17]. Expired human plasma and fresh human plasma samples were obtained from apparently healthy adult blood donors and cord blood samples were collected in the National University Hospital, Singapore.

2.1. Purification of L-ficolin

Human L-ficolin was purified from plasma essentially as previously reported [14] with slight modifications. Human plasma was, after removal of debris by centrifugation, passed through a Sepharose 4B column (50 ml packed volume), which had been equilibrated with a TBS-TEDTA buffer (50 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween 20 and 2 mM EDTA, pH 7.8), and then applied onto a GlcNAc-Sepharose column (50 ml) similarly equilibrated. After washing, the column was eluted with 200 mM GlcNAc in TBS-TEDTA. The eluate was diluted with 2 volumes of buffer D (50 mM Tris, 0.05% (v/v) Tween 20 and 2 mM EDTA, pH 7.8) and then subjected to FPLC ion-exchange on a mono-Q column. The FPLC step was repeated with the flow-through from the mono-Q column until L-ficolin was not detectable in the subsequent eluate of a NaCl gradient. Fractions containing L-ficolin were pooled and applied onto a CNBr-activated and Tris-reacted Sepharose column (5 ml). After washing with TBS-TEDTA, L-ficolin was eluted from the column with 200 mM GlcNAc in TBS-TEDTA.

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Abbreviations: FBG, fibrinogen β/γ C-terminal; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; CRD, carbohydrate recognition domain

PII S0014-5793(98)00267-1

2.2. Assay for sugar specificities of purified L-ficolin

Purified human L-ficolin (30 μg) was re-bound to CNBr-activated and Tris-reacted Sepharose columns (1 ml). The columns were eluted sequentially either with (100 mM) glucose, glucosamine and GlcNAc, or with the same concentration of galactose, galactosamine and GalNAc. The re-bound L-ficolin was also eluted sequentially with 10 mM glutathione, 50 mM glutathione and 100 mM GlcNAc. Fractions of each elution were examined by SDS-PAGE, under reducing conditions, on 10% (w/v) gels.

2.3. Collagenase digestion and identification of GlcNAc-binding site in L-ficolin

Purified L-ficolin (20 μg) was subjected to collagenase digestion as described previously [14]. The digest was applied onto a GlcNAc-Sepharose column (2 ml) and, after washing of the column with TBS-TEDTA (10 ml), the column was eluted with 200 mM GlcNAc in TBS-TEDTA collecting 1 ml fractions. The digest, the flow-through from the GlcNAc-Sepharose column, the washing, and the GlcNAc eluate were subjected to SDS-PAGE under reducing conditions on a 10% (w/v) gel.

2.4. Assay for plasma levels of human L-ficolin(s)

Purified L-ficolin was used to immunise New Zealand White rabbits and IgG was isolated from the antiserum by sodium sulphate precipitation. A fraction of the purified IgG (5 mg) was labeled with biotin following the method described by Guesdon et al. [18].

The level of L-ficolin in plasma samples was measured by a sandwich antigen capture assay. Briefly, microtitre plates were coated with rabbit anti-L-ficolin IgG at 1 μg/well. After washing with TBS-TEDTA-BSA (BSA 1 mg/ml) and blocking with the same buffer for 1 h at room temperature, the plates were incubated with plasma samples diluted in TBS-TEDTA. The plates were washed with PBS and then incubated for 15 min with 0.125% (v/v) glutaraldehyde dissolved in PBS. Free binding sites were blocked for 1 h with 0.5 M ethanolamine. After washing with TBS-TEDTA-BSA, the plates were incubated for 2 h with biotinylated anti-L-ficolin IgG (0.1 μg/well) and, after washing, incubated for 2 h with streptavidin-alkaline phosphatase diluted 3000-fold in TBS-TEDTA-BSA. The plates were washed and developed by incubation with a *p*-nitrophenyl phosphate disodium solution (1 mg/ml). The L-ficolin concentration in each plasma sample was determined by comparison with the standard curve of a plasma pool which was assayed in the same plates and whose L-ficolin concentration had been determined using purified L-ficolin as a reference.

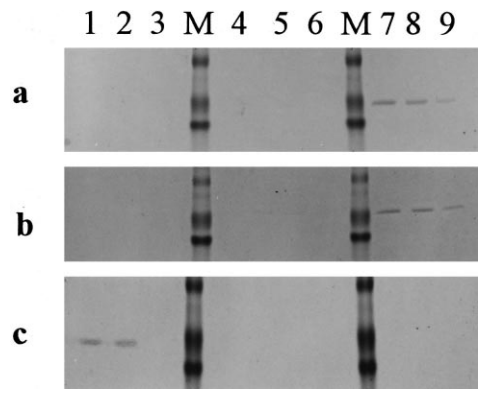

Fig. 1. Sugar specificity of L-ficolin. L-Ficolin was applied to a CNBr-activated and Tris-reacted Sepharose column (1 ml). After washing with TBS-TEDTA, the column was eluted sequentially with (a) 100 mM D-glucose (lanes 1–3), D-glucosamine (lanes 4–6), and then GlcNAc (lanes 7–9), (b) 100 mM D-galactose (lanes 1–3), D-galactosamine (lanes 4–6), and then GalNAc (lanes 7–9), or (c) 10 mM glutathione (lanes 1–3), 50 mM glutathione (lanes 4–6), and then 100 mM GlcNAc (lanes 7–9). Molecular weight standards are included between each elution and are 58 kDa, 40 kDa, and 32 kDa, respectively, from the top.

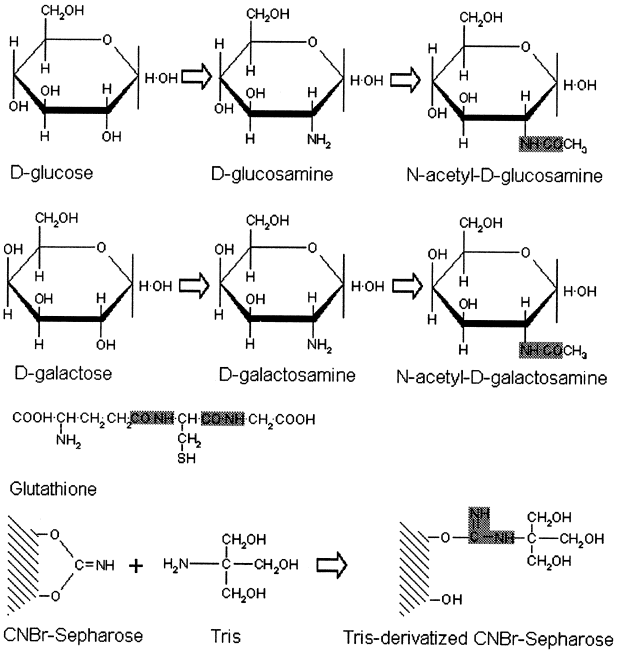

Fig. 2. Molecular structures of GlcNAc, GalNAc, glutathione and CNBr-activated/Tris-derivatised Sepharose. Chemical reactions which generate these structures from their precursors are also illustrated. The amide groups (-CO-NH-) in GlcNAc, GalNAc and glutathione and a similar group (-CNH-NH-) in Tris-derivatised Sepharose, potentially involved in L-ficolin binding, are shaded.

3. Results

3.1. Sugar specificity of L-ficolin

L-Ficolin has previously been shown to bind to a number of unrelated structures including elastin, corticosteroid and GlcNAc. Unlike the collectins, it binds to GlcNAc in the absence of Ca^{2+} [2,14]. L-Ficolin has also been shown to bind to CNBr-activated Sepharose from which it could be eluted with GlcNAc [14]. In the present study, the sugar specificity of L-ficolin has been further investigated. Purified L-ficolin was re-bound to CNBr-activated and Tris-reacted Sepharose and was then sequentially eluted with two sets of sugars. The bound L-ficolin was not eluted from the column with glucose and glucosamine, but was eluted with GlcNAc (Fig. 1a). Similarly, galactose, galactosamine and GalNAc were used sequentially to elute bound L-ficolin. However, L-ficolin was only eluted with GalNAc (Fig. 1b). Both GlcNAc and GalNAc are different from their amino sugar precursors by modification of the amino group into an acetamide (CH_3-CO-NH-) group (Fig. 2). Interestingly, L-ficolin was also eluted with glutathione, which has no structural similarity to GlcNAc and GalNAc apart from the presence of two similar amide groups (Fig. 2). L-Ficolin binds to CNBr-activated and Tris-reacted Sepharose but not to underivatised Sepharose [14]. It is interesting that a similar structure (-CNH-NH-) is also generated in CNBr-activated Sepharose when the resin is reacted with Tris (Fig. 2). Therefore, the amide group in these molecules/structures is likely to be, at least part of, the L-ficolin-binding site(s).

3.2. Sugar binding site in L-ficolin

L-Ficolin was digested with collagenase in order to isolate the FBG domain. The digestion yielded a collagenase-resistant

fragment of approximately 32 kDa under reducing conditions (Fig. 3, lanes a and b). The reduction of 8 kDa when compared with the undigested 40 kDa L-ficolin polypeptide is consistent with the loss of the N-terminal segment and the collagen-like sequence (total of 71 residues) after collagenase digestion [11]. Therefore, the 32 kDa fragment is most likely to correspond to the FBG domain. Despite the presence of two additional cysteine residues in the N-terminal region of the L-ficolin FBG domain, corresponding to the neck regions of the collectins [7,10,11], no covalent FBG trimer (predicted molecular weight of approximately 90 kDa) was detected after collagenase digestion of L-ficolin, as judged by SDS-PAGE under non-reducing conditions and by Western blotting (data not shown). By affinity chromatography analysis of the collagenase digest, it was shown that the 32 kDa fragment could re-bind to GlcNAc-Sepharose as it was not detected in the flow-through (data not shown). The bound 32 kDa fragment was subsequently, after washing of the column with 5 volumes of TBS-TEDTA, eluted from the column with GlcNAc in the second and third fractions (Fig. 3, lanes d and e). Therefore, like the collectins, L-ficolin probably also binds to sugars via its C-terminal globular FBG domains.

3.3. Plasma levels of L-ficolin

Eighty adult human plasma and 24 cord blood plasma samples were assayed for levels of L-ficolin (Fig. 4). The concentration of L-ficolin in a pooled plasma sample was determined using purified L-ficolin of known concentration as a reference. The level of L-ficolin in the 80 adult human plasma samples showed significant variation ranging from 1 to 7 μg/ml with an average of 4.13 μg/ml. However, most samples (>90%) contained L-ficolin at concentrations of 2.5–5.5 μg/ml. Cord blood plasma samples were similarly assayed for the level of L-ficolin and were shown to contain 0–2.25 mg/ml L-ficolin, i.e. much lower than in adult human plasma. The serum level of collectin MBL increases sharply after birth and it has been demonstrated to play an important role in the first line of immune defence in young children [19,20]. The increase of the plasma L-ficolin level after birth may play similar roles.

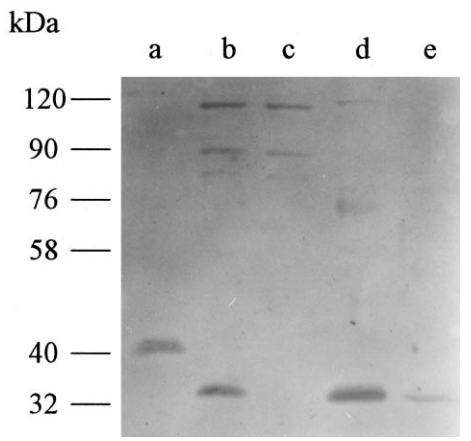

Fig. 3. Identification of GlcNAc binding site on L-ficolin. L-Ficolin was incubated at 37°C for 24 h in the absence (lane a) or presence (lane b) of collagenase. Collagenase alone (lane c) was similarly incubated. Collagenase-digested L-ficolin was applied onto a GlcNAc-Sepharose column (2 ml). After washing, the column was eluted with 100 mM GlcNAc and the 2nd and 3rd fractions (lanes d and e) were examined by SDS-PAGE. The samples were examined by SDS-PAGE under reducing conditions on a 10% (w/v) gel and the gel was silver-stained.

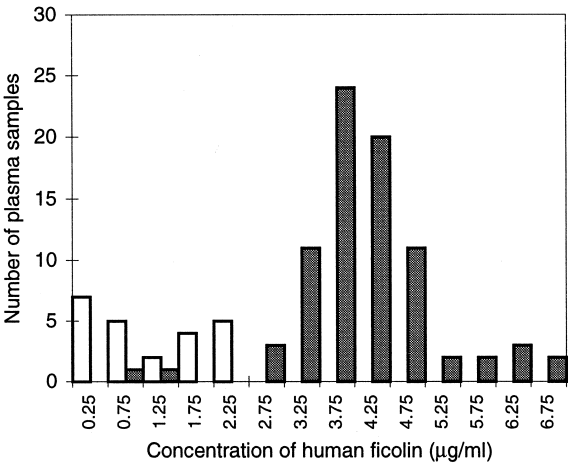

Fig. 4. Assay of human L-ficolin in the plasma of healthy adults and that derived from cord blood. Microtitre plates were coated with rabbit anti-human L-ficolin IgG. Plasma samples were diluted in TBS-TEDTA-BSA and then incubated in the plates. After washing, bound L-ficolin was detected with biotinylated anti-ficolin IgG and streptavidin-alkaline phosphatase. Solid bars represent plasma samples from healthy adult and hatched bars cord blood plasma samples.

4. Discussion

L-Ficolin was previously reported to bind to GlcNAc and similar sugar structures expressed on bacterial surfaces [11,21]. Binding of L-ficolin to *S. typhimurium* has been reported to enhance the phagocytosis of the bacteria by polymorphonuclear leucocytes [11]. The sugar-binding site in L-ficolin was not defined in these studies which, however, is important to the understanding of functional mechanism(s) of L-ficolin. Like C1q and the collectins, ficolins also have clear domain organisations and the different domains can be isolated by collagenase or pepsin digestion [14,22,23]. In this study, L-ficolin was similarly digested with collagenase and a 32 kDa fragment corresponding to the FBG domain was shown to retain the GlcNAc-binding activity of L-ficolin. This implies that, like the collectins, L-ficolin probably binds to sugar residues on microorganisms via the FBG domains and subsequently interacts with effector mechanisms, e.g. receptor(s) on phagocytes, to bring about the killing of the bound microorganisms [1,11].

Previous studies showed that L-ficolin bound to GlcNAc but not a range of other sugars including mannose, glucose, galactose, lactose and cellobiose [11]. In our previous study, it was shown that L-ficolin bound CNBr-activated and Tris-reacted Sepharose with its GlcNAc-binding site [14]. In the present study, it was found that L-ficolin could not bind to glucosamine or galactosamine unless these sugars were *N*-acetylated. The *N*-acetylation of the two amino sugars generates an acetamide structure in each molecule (Fig. 2). Glutathione, a molecule containing two similar amide groups and otherwise distinct from GlcNAc and GalNAc, was also shown to bind L-ficolin implying that the amide groups are probably essential for L-ficolin binding. This hypothesis was further strengthened by the fact that CNBr-activated and Tris-derivatised Sephar-

ose also generates a structure similar to acetamide. However, in addition to the amide group or amide-like group, another structure is clearly required in the L-ficolin FBG ligand(s) since the amide groups are found in all proteins and many other molecules, e.g. BSA, which apparently do not bind to L-ficolin (data not shown).

Acknowledgements: The authors wish to thank Jackie Lau for assistance. This project is supported by an NUS Academic Research Grant (RP940311) and Y. Le is the recipient of a NUS Scholarship.

References

[1] Lu, J. (1997) BioEssays 19, 509–518.
[2] Ohashi, T. and Erickson, H.P. (1997) J. Biol. Chem. 272, 14220–14226.
[3] Sellar, G.C., Blake, D.J. and Reid, K.B.M. (1991) Biochem. J. 274, 481–490.
[4] Drickamer, K. and Taylor, M.E. (1993) Annu. Rev. Cell. Biol. 9, 237–264.
[5] Reid, K.B.M. (1983) Biochem. Soc. Trans. 11, 1–12.
[6] Holmskov, U., Malhotra, R., Sim, R.B. and Jensenius, J.C. (1994) Immunol. Today 15, 67–74.
[7] Hoppe, H.-J. and Reid, K.B.M. (1994) Protein Sci. 3, 1143–1158.
[8] Sastry, K. and Ezekowitz, R.A.B. (1993) Curr. Opin. Immunol. 5, 59–66.
[9] Ichijo, H., Helman, U., Wernstedt, C., Gonez, L.J., Claesson-Welsh, I., Heldin, C.-H. and Miyazono, K. (1993) J. Biol. Chem. 268, 14505–14513.
[10] Lu, J., Tay, P.N., Kon, O.L. and Reid, K.B.M. (1996) Biochem. J. 313, 473–478.
[11] Matsushita, M., Endo, Y., Taira, S., Sato, Y., Fujita, T., Ichikawa, N., Nakata, M. and Mizuochi, T. (1996) J. Biol. Chem. 271, 2448–2454.
[12] Lu, J. and Le, Y. (1998) Immunobiology (in press).
[13] Yee, V.C., Pratt, K.P., Cote, H.C.F., Le Trong, I., Chung, D.W., Davie, E.W., Stenkamp, R.E. and Teller, D.C. (1997) Structure 5, 125–138.
[14] Le, Y., Tan, S.M., Lee, S.H., Kon, O.L. and Lu, J. (1997) J. Immunol. Methods 204, 43–49.
[15] Harumiya, S., Omori, A., Sugiura, T., Fukumoto, Y., Tachkawa, H. and Fujimoto, D. (1995) J. Biochem. (Tokyo) 117, 1029–1035.
[16] Edgar, P.F. (1996) FEBS Lett. 375, 159–161.
[17] Fornstedt, N. and Porath, J. (1975) FEBS Lett. 57, 187–191.
[18] Guesdon, J.L., Ternynck, T. and Avrameas, S. (1979) J. Histochem. Cytochem. 27, 1131–1137.
[19] Thiel, S., Bjerke, T., Hansen, D., Poulsen, L.K., Schiotz, P.D. and Jensenius, J.C. (1995) Pediatr. Allergy Immunol. 6, 20–23.
[20] Super, M., Thiel, S., Lu, J., Levinsky, R.J. and Turner, M.W. (1989) Lancet 2, 1236–1239.
[21] Lu, J., Le, Y., Kon, O.L., Chan, J. and Lee, S.H. (1996) Immunology 89, 289–294.
[22] Strang, C.J., Slayter, H.S., Lachmann, P.J. and Davis, A.E. (1986) Biochem. J. 234, 381–389.
[23] Reid, K.B.M. (1976) Biochem. J. 161, 239–248.