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Structural and functional studies of complement inhibitor C4b-binding protein

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Abstract

C4b-binding protein (C4BP) is a potent inhibitor of the classical pathway of the complement system. This large plasma glycoprotein consists of seven identical α-chains and a unique β-chain held together by disulphide bridges. Both types of subunits are composed almost exclusively of complement control protein domains (CCPs). Using homology-based computer modelling and mutagenesis of recombinant proteins we have localized binding sites for several ligands of C4BP: complement factor C4b, heparin and vitamin K-dependent anticoagulant protein S (PS). We found that C4b requires CCP1–3 of the α-chain for binding. The interaction is ionic in nature and mediated by a cluster of positively charged amino acids present on the interface between CCP1 and CCP2 of the α-chain. Loss of C4b-binding resulted in a loss of all inhibitory functions of C4BP within the classical pathway of complement. Binding of heparin required CCPs 1–3 of the α-chain, with CCP2 being the most important, as well as the cluster of positively charged amino acids involved in binding of C4b. The interaction between C4BP and PS is of very high affinity and conveyed by a cluster of surface exposed hydrophobic amino acids localized on CCP1 of the β-chain. Furthermore, C4BP is captured on the surface of several pathogens, which may contribute to their serum resistance and pathogenicity. We have localized interaction of C4BP with *Neisseria gonorrhoeae*, *Bordetella pertussis*, *Streptococcus pyogenes* and *Escherichia coli* to various regions of the α-chain.

Key words: C4b-binding protein, complement, heparin, protein S.
Abbreviations used: C4BP, C4b-binding protein; CCP, complement control protein domain; OmpA, outer membrane protein A; Por, porin; PS, protein S; SAP, serum amyloid P component.
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Why look into complement regulators?

The complement system was first described over a century ago and all of the major components were known by 1970, leading many to think that everything in this field was known. On the contrary, research in the last decade has revealed the amino acid sequences of all the components, the location and structure of the corresponding genes and many defects detrimental to human health. It turned out that the human complement system not only protects against invading pathogens due to its opsonic, inflammatory and lytic activities but also contributes to regulation of other biological systems, particularly adaptive immunity [1], and participates in regulation of apoptosis, although much is still to be discovered in this field [2].

Although proper activation of the complement system is pivotal for the functions mentioned above, its excessive activation is involved in pathogenesis of many diseases (e.g. arthritis, reperfusion injury, glomerulonephritis, Alzheimer's disease). This may explain why a lot of recent effort was placed into studies of complement regulators and receptors. Several complement components have

gained recognition as potential therapeutics due to their cardioprotective roles, anti-inflammatory actions or ability to assist in overcoming hyperacute rejection during xenotransplantation [3]. However, although complement regulators have therapeutic potential, the major obstacle is the fact that our knowledge of the intermolecular interactions involving complement regulatory proteins is still too basic. Therefore we have focused our studies on the structure and function of an important inhibitor of the classical pathway of complement, C4b-binding protein (C4BP).

Overall structure of C4BP

C4BP is a 500-kDa plasma glycoprotein, composed of seven identical α-chains and a unique β-chain linked together by a central core [4,5]. C-terminal parts of both chains contain two cysteine residues each and an amphipathic α-helix region, which are both required for intracellular polymerization of the molecule. The α-chains contain eight complement control protein domains (CCPs, also termed short consensus repeats) and the β-chain contains three. CCPs consist of approx. 60 amino acids forming a compact hydrophobic core surrounded by five or more β-strands organized into β-sheets [6]. Many complement regulators are composed of such domains. The whole C4BP molecule has a spider-like shape with the α-chains forming extended tentacles (Figure 1) [7].

Functions of C4BP

C4BP controls C4b-mediated reactions, thereby inhibiting the classical pathway of complement in at least three ways. Firstly, C4BP acts as a cofactor to the serine proteinase factor I in the proteolytic inactivation of C4b [4]. The mechanism by which C4BP operates as a cofactor to Factor I is not fully understood. Possibly, C4b undergoes a conformational change upon binding to C4BP and becomes susceptible to cleavage by Factor I. Secondly, C4BP prevents the assembly of the classical C3-convertase (C4bC2a) and, thirdly, it accelerates the natural decay of the complex [8]. C4BP interacts not only with C4b, but also with vitamin K-dependent anticoagulant protein S (PS) [9], heparin [10], serum amyloid P component (SAP) [11] and several bacterial proteins. The interaction with PS is of special interest since it comprises the most unequivocal link between the complement and blood coagulation systems.

Binding site for C4b

Since most, if not all, of the effects that C4BP has on the complement system are mediated by interaction with C4b it was important to localize its binding site on the α-chains. Each α-chain of C4BP is capable of binding one C4b molecule and most reports to date agree that the three most N-

Figure 1

Schematic representation of C4BP with indicated binding sites for ligands

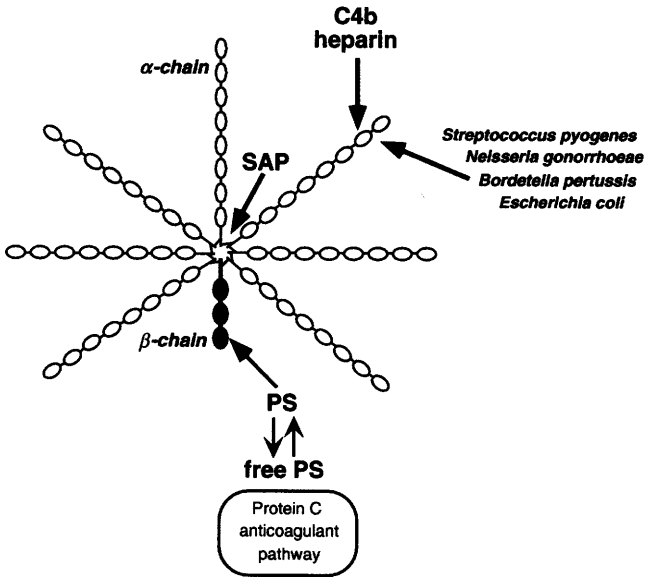

Table 1

Binding sites on C4BP for various ligands

In some cases, the major binding surface is indicated in bold and is underlined. AA, amino acid.

Ligand	Binding site	Type of interaction	References
Protein S	CCPs **1**–2 of β-chain	Hydrophobic	[20–23]
Complement factor C4b	CCPs 1–**2**–**3** of α-chain cluster of charged AAs on interface CCP1/2	Ionic	[12–16]
Heparin	CCPs 1–**2**–3 of α-chain cluster of charged AAs on interface CCP1/2	Ionic	[15,16]
S. pyogenes M-proteins	CCPs **1**–2 of α-chain	Hydrophobic	[12,27]
B. pertussis	Cluster of charged AAs on interface CCP1/2	Ionic	[30]
N. gonorrhoeae pili	CCPs 1–2 of α-chain	Ionic	[32]
N. gonorrhoeae Por1A	CCP1 of α-chain	Hydrophobic	[31]
N. gonorrhoeae Por1B	CCP1 of α-chain	Ionic	[31]
E. coli OmpA	CCP3 of α-chain	Hydrophobic	[33]

terminal CCPs are necessary and sufficient for the C4b binding (Table 1) [12–14]. We constructed a panel of eight mutants in which we removed individual CCP domains one at a time. In another series of C4BP variants, the linkers between CCPs were modified by insertion of two alanine residues. Finally, truncated monomeric forms of the α-chain were constructed by inserting stop codons after each CCP. All recombinant C4BP molecules were expressed in a eukaryotic cell line in a stable manner and purified from culture media by affinity chromatography employing monoclonal antibodies. The ability to bind C4b was severely impaired in the case of C4BP variants lacking CCP1, CCP2 (in particular) or CCP3, indicating CCPs 1–3 to be crucial for binding [15]. In addition, the spacing between the CCPs was found to be important for the integrity of the C4b-binding site, as illustrated by the disruptive consequence of alanine insertions between CCP1 and CCP2, CCP2 and CCP3, and CCP3 and CCP4 [15].

The C4BP–C4b interaction is highly sensitive to ionic strength, implying that is based on ionic interactions between amino acids. We have constructed a homology-based three-dimensional model of the α-chains and analysed its surface for potential binding sites. We found a prominent cluster of positively charged amino acids on the interface between CCP1 and CCP2 and analysed its influence on binding by site-directed mutagenesis. We found that Arg^{39}, Lys^{63}, Arg^{64} and His^{67} located in the cluster are crucial for binding of C4b [16].

Functional effects of lost C4b binding

Binding of C4b is a prerequisite for the cofactor activity of C4BP, but we wanted to investigate if additional areas of the protein may be involved in the activity. Using our mutants in addition to monomeric truncated α-chains of C4BP, we found that CCPs 1–3 of the α-chain contain all that is required for cofactor activity in the cleavage of C4b molecules [15].

Haemolytic assays were used to elucidate whether disruption of the C4b binding site in C4BP correlated with impaired ability to prevent assembly and decay of the classical pathway C3-convertase. All recombinant proteins that contained CCPs 1–3 had functional activity. Furthermore, specific deletions of CCP2 and CCP3 entirely abolished the ability of C4BP to inhibit C3-convertase. Deletion of CCP1 had a significant effect, although it did not entirely destroy the functional activity of the α-chain [15]. Mutants, in which we neutralized positively charged amino acids on the interface of CCP1 and CCP2, were also tested in several functional assays. We found that a decrease in C4b binding was directly correlated with loss of complement-inhibitory functions by C4BP [17].

Interaction of C4BP with heparin

C4BP binds heparin with relatively high affinity [10,18]. The interaction between C4BP and C4b can be inhibited by heparin, suggesting that the C4b and heparin binding sites overlap [19]. To study the binding between C4BP and heparin, we

used heparin affinity chromatography and a Biacore® technique with biotinylated heparin immobilized on a streptavidin chip. We found that the heparin-binding ability of C4BP was compromised by the removal of CCP2 and by insertion of two alanine residues between CCP1 and CCP2. In contrast with the dramatic effects on C4b binding, deletion of CCP3 and CCP1 had only minor effects on heparin binding, which suggests CCP2 is the most important domain for the interaction [15]. Furthermore, binding was nearly abolished in the C4BP mutants lacking positively charged amino acids on the interface between CCP1 and CCP2 [16].

Binding site for PS

We have previously shown that a binding site for PS is localized to CCP1 of the β-chain [20,21]. More recently, a slight contribution from CCP2 was also postulated [22]. Based on a three-dimensional model of the β-chain we have defined Ile^{16}, Val^{18}, Val^{31} and Ile^{33} as crucial for PS binding, with secondary effects from Leu^{38} and Val^{39} [23]. In addition, Lys^{41} and Lys^{42} contribute slightly to the interaction. The fact that mostly hydrophobic amino acids are involved in this interaction is in full agreement with our observation that the binding is not sensitive to ionic strength [24] and has a very high affinity (K_d = 0.2 nM). Only the molar excess of PS over C4BP is free in plasma. Interestingly, PS bound to C4BP is unable to participate in the anticoagulant protein C system. It is imperative that the balance between free and bound PS is maintained at stable levels as a lack of free PS leads to thrombosis. It is possible that PS, via its γ-carboxyglutamic acid-containing domain, is able to localize C4BP to cell surfaces on which negatively charged phospholipids are exposed. Such surfaces are present on activated platelets and apoptotic cells.

Binding of C4BP to pathogenic bacteria renders them resistant to complement attack

Infectious agents, such as viruses, bacteria and parasites, have developed many efficient strategies to avoid clearance and destruction by complement. Some pathogens hijack host complement regulators and subsequently down-regulate complement activation. Others produce their own regulators that bear remarkable similarity to the host's own proteins (reviewed in [25]). Understanding of these strategies is very important for the design of therapeutic agents to be used not only to battle infections but also to regulate complement activities in unrelated diseases.

C4BP can be captured by several bacterial pathogens and, in some cases, it is possible to correlate directly the binding with bacterial resistance to complement-mediated killing. C4BP binds with high affinity to M-proteins of *Streptococcus pyogenes* [26], an interaction based to a large extent on non-ionic or hydrophobic interactions. M-proteins interact with CCP1 and CCP2 of the α-chain and their binding site overlaps to some extent with the binding site for C4b [27]. Recently, the ability to bind C4BP was correlated to phagocytosis resistance of these bacteria [28].

Filamentous haemagglutinin from *Bordetella pertussis* is another surface protein known to interact with C4BP [29]. In this case, the binding is very similar to that with C4b. It is based on ionic interactions and requires a cluster of charged amino acids on the interface of CCP1 and CCP2 of α-chains [30].

Neisseria gonorrhoeae has evolved several ways of protection from complement attack. Apart from binding Factor H to sialylated lipo-oligosaccharide, they also employ porin molecules (Por1A and Por1B) to bind C4BP [31]. C4BP–Por1B interaction is ionic in nature (inhibited by high salt as well as by heparin), while the C4BP–Por1A bond is hydrophobic. Only recombinant C4BP mutant molecules that contain α-chain CCP1 bind both Por1A and Por1B gonococci, which implies that CCP1 contains porin-binding sites. Furthermore, isolated type IV pili (pilC subunit) from *N. gonorrhoeae* bind human C4BP [32]. From the results of an inhibition assay with C4b and a competition assay in which we tested mutants of C4BP lacking individual CCPs, we concluded that the binding area for pili is localized to CCP1 and CCP2 of the α-chain and is based primarily on ionic interactions. Inhibition of C4BP binding to serum-resistant Por1A and Por1B strains in a serum bactericidal assay using Fab fragments against C4BP CCP1 resulted in complete killing of otherwise fully serum-resistant strains, underscoring the role of C4BP in mediating gonococcal serum resistance [31].

Finally, *Escherichia coli* K1, responsible for meningitis in neonates, binds C4BP. We found that CCP3 interacts hydrophobically with the N-terminal of outer membrane protein A (OmpA) molecule (N. V. Prasadarao, A. H. Blom, B. O. Villoutreix, L. C. Limsangan, unpublished work). A compelling observation in this study is that the

synthetic peptides corresponding to CCP3 sequences block the binding of C4BP to OmpA and also significantly enhance the bactericidal activity of serum.

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