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Functions of human complement inhibitor C4b-binding protein in relation to its structure

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Summary

Considering the destructive potential of the complement cascade, it is no surprise that there are several complement inhibitors present in blood and expressed on virtually all cells of the body to protect self tissue. C4b-binding protein (C4BP) is a potent soluble inhibitor of the classical and lectin pathways of complement. This large (500 kDa) plasma glycoprotein consists of seven identical 75 kDa α -chains and a unique 40 kDa β -chain that are held together by disulphide bridges. Both types of subunit are almost exclusively composed of complement control protein (CCP) domains. In recent years, detailed studies of structure-function relationships have yielded new understanding of the interactions between C4BP and the activated complement factors C4b and C3b, heparin, and vitamin K-dependent anticoagulant protein S. This review describes the localization of binding sites for a number of C4BP ligands in relation to well-established and novel functions of C4BP such as complement inhibition, protection of apoptotic cells from complement, CD40-dependent stimulation of B cells, and the contribution of a number of human pathogens to pathogenesis.

Key words:

complement • C4b-binding protein • protein S • coagulation • heparin • B cells • CD40 • apoptosis • C3b • C4b

Abbreviations:

C3b – activated complement factor 3, **C4BP** – C4b-binding protein, **CCP** – complement control protein, **CR1** – complement receptor 1, **DAF** – decay accelerating factor, **Gla** – γ -carboxyglutamic acid, **FH** – factor H, **FI** – factor I, **LG** – laminin G-type, **LRP** – low-density lipoprotein receptor-related protein, **PS** – protein S, **SAP** – serum amyloid P component, **MBL** – mannose-binding lectin, **SLE** – systemic lupus erythematosus, **SHBG** – sex hormone-binding globulin, **RCA** – regulators of complement activation.

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THE COMPLEMENT SYSTEM

The human complement system was first described over a century ago as an aid to antibodies, and all major components of the system have been known for over 30 years. This has allowed thorough investigation of the activation pathways of complement and of various physiological functions that complement fulfils. The complement system forms the core of the innate immune system, which is able to remove pathogens without previous exposure to them. Human complement not only protects against invading pathogens due to its opsonic, inflammatory, and lytic activities, but also contributes to the regulation of other biological systems, particularly adaptive immunity^{42, 43, 61}. Moreover, it participates in the regulation of apoptosis, although much is still to be discovered in this field⁴⁷.

The complement cascade can proceed by the classical, the alternative, and the lectin pathways. The classical pathway is initiated by clustered antibodies bound to their targets, the mannose-binding lectin (MBL) pathway begins due to the recognition of certain saccharides⁸⁴, and the alternative pathway is started by the autoactivation of unstable complement factor C3 and its subsequent binding to activating pathogen surfaces (Fig. 1). Central to complement activation is the formation of enzymatic complexes such as C3- and C5-convertases following proenzyme cleavages and release of smaller chemoattractant and anaphylatoxin fragments. The end result of all complement pathways is generation of C5-convertase and the formation of the membrane-attack complex that is able to induce lysis of cells.

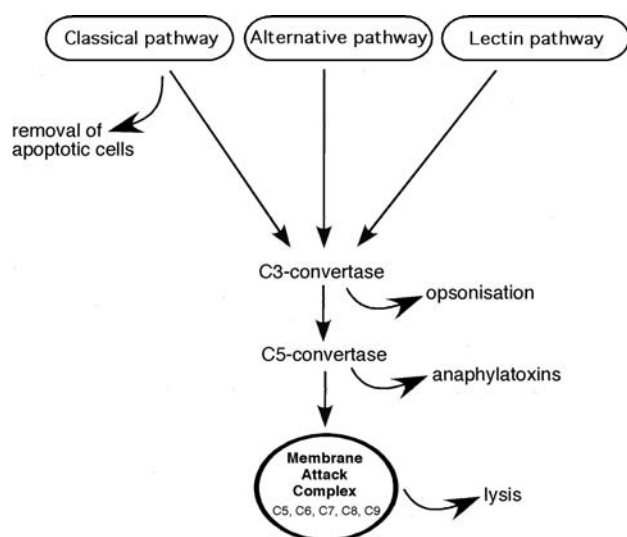


Figure 1. Three pathways of the complement system activation and their physiological effects.

A lot has been learned from the pathology of deficiency states of various components of complement cascades. They are often associated with recurrent infections with encapsulated bacteria, immune complex diseases such as systemic lupus erythematosus (SLE), and glomerulonephritis^{46, 89}. Deficiencies of complement inhibitors also cause disease. However, complement is a double-edged sword, as it is the major reason for the rejection of xenografts³⁶. Unwanted complement activation contributes also to the pathogenesis of glomerulonephritis, ischemia/reperfusion injury, rheumatoid arthritis, multiple sclerosis, and many more diseases⁸⁸. Due to the ability of the C1 molecule to recognize abnormal protein structures, complement is involved in the pathology of Alzheimer's disease^{102, 115} and prion diseases^{72, 77}.

COMPLEMENT INHIBITORS

Considering the explosive and potentially destructive character of complement, there is an obvious need for inhibitors that protect the host's own tissues. Complement inhibitors are either soluble or membrane bound and act on various levels of the complement cascades. There are four principal kinds of complement inhibitors. The first is a proteinase inhibitor, a C1 inhibitor that belongs to the serpin family and directly inhibits the proteolytic potential of C1²⁰. The second is serum carboxypeptidase N, which proteolytically inhibits anaphylatoxins generated from C5 and C3 during complement activation¹⁹. The third is CD59 (protectin), a GPI membrane-anchored inhibitor of the membrane attack complex⁷⁸. Finally, a number of inhibitors act on C3- and C5-convertases, either by increased dissociation of enzyme complexes (acceleration of decay) or by promoting enzymatic degradation of C3b or C4b mediated by the serine proteinase factor I (FI). The latter inhibitors are composed almost exclusively of 4–35 complement control protein (CCP) domains⁷¹. CCP domains consist of approximately 60 amino acids forming a compact hydrophobic core surrounded by five or more β -strands organized into β -sheets and stabilized by two disulphide bonds⁶. CCP domains are also present in a number of proteins unrelated to complement, such as selectins, haptoglobin, and coagulation factor XIII (for an up-to-date list of all CCP-containing proteins see: <http://smart.embl-heidelberg.de/>). C4b-binding protein (C4BP) is the major soluble inhibitor of the classical and lectin pathways, whereas factor H (FH) inhibits the alternative route. In recent years, a whole family of FH-like and related molecules has been identified, but it is still unclear if the major role of these molecules is to inhibit complement. Membrane-bound complement inhibitors include complement receptor 1

(CR1), membrane cofactor protein (MCP), and decay accelerating factor (DAF). All these proteins are encoded by genes on chromosome 1, the gene cluster being referred to as the regulators of complement activation (RCA) cluster⁶³. Several complement inhibitors have already gained recognition as potential therapeutics due to their cardioprotective roles, anti-inflammatory actions, and their potential to counteract hyper-acute rejection during xenotransplantation⁷⁹. However, although complement regulators have therapeutic potential, a major obstacle is that our knowledge of the inter-molecular interactions involving complement regulatory proteins is incomplete. Therefore, a number of research groups are focusing their investigations on complement inhibitors such as C4BP, which is the subject of this review. In recent years, several novel functions have been ascribed to C4BP, such as a role in the removal of apoptotic cells and protection of B cells from apoptosis. So far there is no known case of C4BP deficiency that could otherwise shed more light on the functions of C4BP. The only patient described with low levels of C4BP presented symptoms of Behcet's disease¹¹⁷.

OVERALL STRUCTURE OF C4BP

C4BP is a glycoprotein of approximately 500 kDa (over 4000 amino acid residues) with an estimated plasma concentration of 200 mg/l⁸³. The major isoform of C4BP in plasma (75–80%) is composed of seven identical α -chains and one β -chain linked by their C-terminal parts in a central core (Fig. 2)^{58, 106}. Other, less abundant forms are composed of six α -chains and one β -chain or seven exclusively α -chains^{60, 105}. All β chain-containing C4BP molecules circulate in plasma in a high-affinity, calcium-dependent complex with vitamin K-dependent, anticoagulant protein S (PS)^{32, 59, 64}. The C-terminal parts (60

amino acids each) of both the α - and β -chains contain two cysteine residues each and an amphipathic α -helix region, which are both required for the assembly of C4BP⁷⁰. The α - and β -chains contain 8 and 3 CCP domains, respectively. When analyzed by electron microscopy, C4BP has a spider-like shape with the α -chains forming extended tentacles³². Synchrotron X-ray scattering and hydrodynamic analysis suggested C4BP in solution to be rather a bundle of 7 extended arms held together at their C-termini with an average arm-axis angle of 10° than a spider-like molecule⁹⁷. Both the α - and β -chains have several N-linked carbohydrate side chains^{58, 65}. So far, the 3D structure of C4BP has not been determined experimentally by nuclear magnetic resonance or X-ray crystallography. However, several CCP domains from other proteins have been studied by these methods^{5, 6, 90, 113, 127}. The overall 3D structures of all CCP domains are highly conserved, allowing the creation of useful homology-based 3D models of all CCP domains from C4BP¹²⁰.

BIOSYNTHESIS AND REGULATION OF C4BP

The genes encoding the C4BP α - and β -chains are located in the RCA gene cluster on the long arm of chromosome 1². The two genes, which are only 4 kb apart, are arranged head to tail, supporting the hypothesis that the two genes are the result of a gene duplication event. Like most complement and plasma proteins, C4BP is synthesized in the liver²⁵, but there are secondary sites of synthesis, such as monocytes, in which mRNA was detected and shown to increase upon stimulation with interferon α and γ ⁷⁴. In addition, mRNA for the β -chain, but not for the α -chain, has been found in human ovary, the functional significance of which is unknown²⁶. Pulse-chase analysis of cells transfected with cDNA encoding the α -chain showed that polymeric C4BP is synthesized quickly and appears in the medium already after 40 min. The protein was polymerized already after 5 min of chase, and the process was inhibited by the presence of brefeldin and low (10°C) temperature, suggesting C4BP to be fully assembled in the endoplasmic reticulum⁷⁰. These experiments also demonstrated that the β -chain subunit of C4BP is not required for the polymerization process, similar to what has been shown for the J-subunit of hexameric IgM⁹⁴. The β -chain when expressed alone (without α -chain) is retained in cells and degraded (unpublished observation). Plasma levels of C4BP appear to be hormonally regulated, as they increase during pregnancy and in women using oral contraceptives⁸². Fetal and newborn levels are 5 and 20%, respectively, of adult levels^{80, 85, 86}. C4BP is classified as an acute-phase reactant because its level is elevated (up to 400%) in plas-

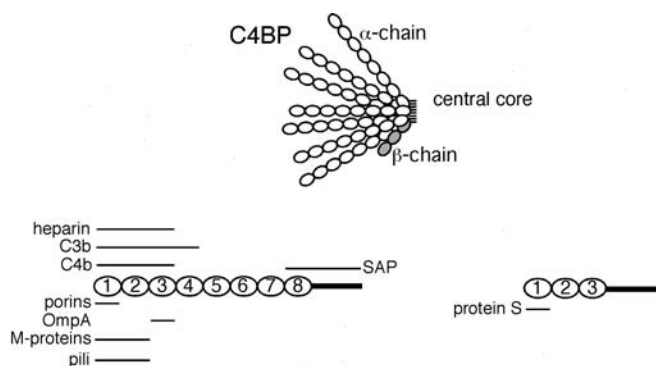


Figure 2. Schematic representation of C4BP with indicated binding sites for various ligands. The major form of C4BP is composed of seven identical α -chains and one β -chain held together by disulphide bridges and hydrophobic interactions in the central core.

ma during inflammation and after broadly defined trauma^{7, 18, 103}. The expression of α - and β -chains is regulated in different ways by cytokines in the liver-derived cell line Hep3B²⁷. Furthermore, plasma levels of the isoforms of C4BP lacking the β -chain increase in acute-phase response more than the levels of β -chain-containing C4BP⁵¹. However, a number of questions concerning the regulation of C4BP biosynthesis both in health and disease still remain unanswered.

FUNCTIONS OF C4BP IN RELATION TO ITS STRUCTURE – LOCALIZATION OF BINDING SITES FOR C4b AND C3b

C4BP is an important inhibitor of both the classical and the lectin pathways of complement¹¹⁴. It exerts its inhibitory function by controlling C4b-mediated reactions in several ways (Fig. 3). First, C4BP acts as a cofactor to FI in the proteolytic inactivation of both soluble and cell-bound C4b, thus preventing the formation and reconstitution of the classical C3-conver-

tase (complex of C4bC2a)^{48, 91, 106}. Secondly, C4BP prevents the assembly of the classical C3-convertase by binding nascent C4b, and thirdly, it accelerates the natural decay of the C4bC2a complex^{28, 54}. In addition, C4BP acts as an FI cofactor in the cleavage of C3b in the fluid phase, thereby inhibiting the alternative pathway of complement^{12, 111}. However, C4BP seems unable to inhibit the assembled alternative C3-convertase¹¹¹ and it does not reduce the hemolytic activity of cell-bound C3b unless present at very high concentration. Therefore, C4BP is not able to fully replace FH, which is the major fluid-phase inhibitor of the alternative pathway.

Although each α -chain of C4BP is capable of binding one C4b molecule, only four C4b can bind simultaneously to one C4BP due to steric hindrances¹²⁸. The affinity of C4BP for C4b is in the low micromolar range^{14, 128}, and as the physiological concentration of C4BP exceeds the observed K_D , it is expected that any C4b molecule present in serum will bind to and

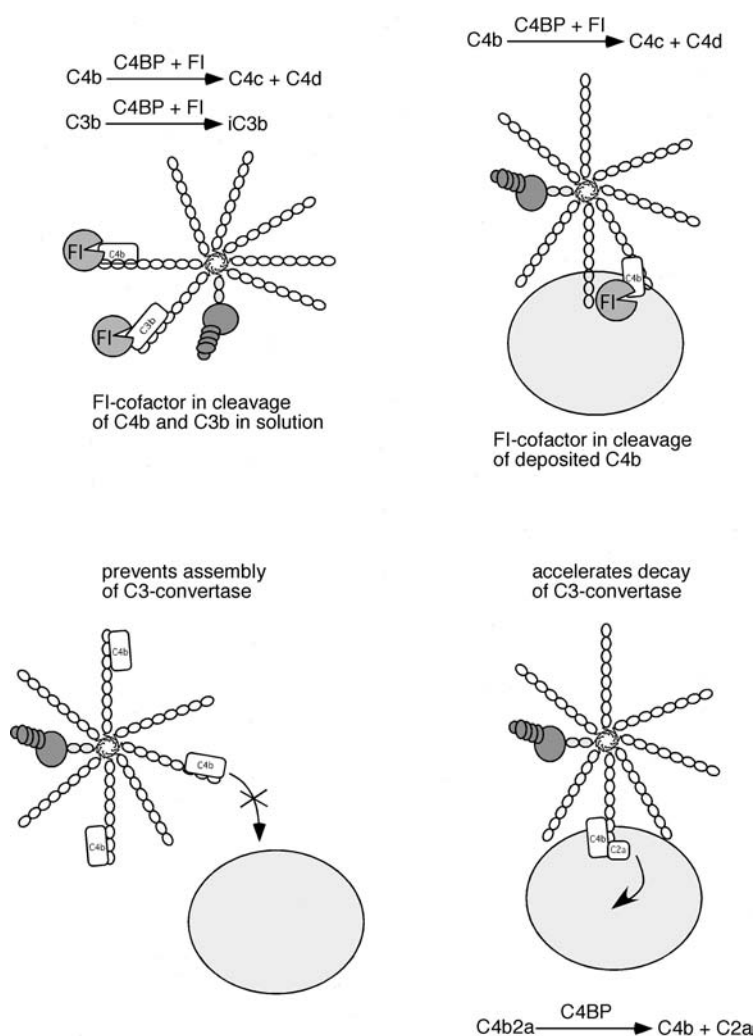


Figure 3. Mechanisms of complement system inhibition by C4BP molecule.

become inactivated by C4BP. Most reports to date agree with the concept that the three most N-terminal CCPs are necessary and sufficient for binding of C4b^{1, 13, 50, 66, 95}. C4BP is the only polymeric complement inhibitor with multiple multiple-ligand binding sites. The polymeric nature of C4BP makes the molecule particularly efficient in degrading C4b deposited on cell surfaces, as there is a co-operativity between the subunits in binding the immobilized C4b¹³. A monomeric molecule composed of the single subunits of C4BP has been shown to be less efficient in regulating surface-bound C4b, proving the concept of the polymer having unique biological properties¹³. Recent studies using a panel of C4BP mutants with systematically removed individual CCP domains as well as monomeric α -chains of various lengths showed that CCP1-3 contains the full binding site for C4b (Fig. 2)^{13, 50}. The spacing between the CCP domains was found to be important for the integrity of the C4b-binding site, since alanine insertions between CCP1/2, CCP2/3 and CCP3/4 disrupted the binding site¹³. The C4BP-C4b interaction is highly sensitive to ionic strength, implying that it is based on ionic/electrostatic interactions between amino acids from both molecules⁹. Analysis of a homology-based 3D model of the α -chain identified a prominent cluster of positively charged amino acids on the interface between CCP1 and CCP2. When the influence on binding of this electropositive cluster was analyzed by site directed mutagenesis, Arg³⁹, Lys⁶³, Arg⁶⁴ and His⁶⁷ were identified to be crucial for interaction with C4b¹⁷.

The CCP1-3 region of the α -chains contains all the features required for the FI-cofactor activity in the cleavage of C4b¹³. Hemolytic assays were used to show that disruption of the C4b-binding site in recombinant C4BP variants directly correlated with an impaired ability of the C4BP variants to prevent assembly and decay of the classical pathway C3-convertase^{11, 13}.

C4BP is also able to bind C3b, but the molecular surface on C4BP required for this interaction is larger than the one involved in C4b-binding. C3b-binding requires all four N-terminal CCPs of the α -chain, with CCP2 and CCP3 being the most important for the FI-cofactor function in the cleavage of C3b (Fig. 2)^{12, 50}. Like the binding of C4b, the cluster of positively charged amino acids at the CCP1/CCP2 interface is involved in C3b-binding, and the interaction is also sensitive to the salt concentration¹².

The mechanism by which C4BP operates as a cofactor to FI is not fully understood. It appears that the conformation of C4b changes upon binding to C4BP,

which makes C4b susceptible to proteolytic cleavage by FI. A similar situation has been observed for the degradation of C3b by FI and FH⁶⁸. Recently, we described two CCP3 mutations of C4BP, K126Q/K128Q and F144S/F149S, which selectively resulted in the loss of ability of the C4BP variants to act as FI cofactors in the cleavage of both C4b and C3b¹⁶. Both mutants showed intact binding of C4b/C3b and effected the formation and decay of the classical pathway C3-convertase in a manner similar to wild-type C4BP. It appears that C4b and C3b do not undergo the required conformational changes upon binding to these C4BP mutants, which could explain the observed loss of the cofactor activity.

INTERACTION BETWEEN C4BP AND VITAMIN K-DEPENDENT PROTEIN S, A LINK BETWEEN THE COMPLEMENT AND COAGULATION SYSTEMS

In human plasma, all β -chain-containing C4BP (C4BP β^+) circulates in a high-affinity complex with PS, whereas PS exists in both free (approximately 30% of total PS) and bound form. This is explained by the molar excess of PS in plasma^{30, 33}. One PS is bound per β -chain of C4BP, and the interaction is stronger in the presence of calcium (K_D approximately 0.2 nM) than in its absence⁵⁶. The ability of C4BP to regulate the classical C3-convertase is not affected by the bound PS³¹, whereas PS when bound to C4BP has lost its anticoagulant activity, as it can no longer act as a cofactor to activated protein C in the degradation of coagulation factor Va and factor VIIIa²⁹. It is imperative that the balance between free and C4BP-bound PS is maintained at stable levels, as a decrease in free PS leads to thrombosis^{112, 129}. It has been suggested that C4BP, by affecting the level of free PS, may have a regulatory role in coagulation. However, when C4BP levels rise in plasma due to an acute-phase reaction, the rise is mainly due to an increase in α -chain synthesis resulting in C4BP lacking the β -chain⁵¹. Thus the levels of free PS remain stable in plasma from patients with acute-phase response compared with normal controls, whereas C4BP levels are elevated. This argues against a role of C4BP in the regulation of coagulation.

PS is a 75 kDa glycoprotein synthesized mainly by hepatocytes, but also endothelial cells and testicular Leydig cells^{40, 41, 81}. PS is a multidomain protein, composed of an N-terminus containing a Gla domain (containing γ -carboxyglutamic acid), a thrombin sensitive region, four epidermal growth factor domains, and a C-terminal sex hormone-binding globule (SHBG)-like region, which is subdivided into two

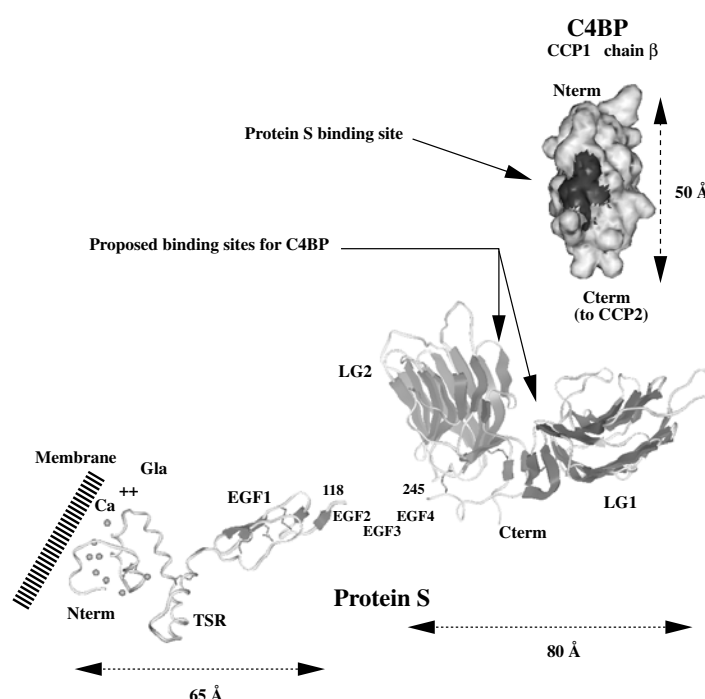


Figure 4. Interaction of C4BP with anticoagulant protein S (PS). CCP1 of the C4BP β -chain binds in a calcium-dependent manner and with very high affinity to both laminin G-type domains (LG1 and LG2) of PS. The LG domains of PS are followed by four epidermal growth factor (EGF)-type domains, thrombin sensitive regions (TSR), and a γ -carboxyglutamic acid-rich domain (Gla), which interacts with negatively charged phospholipids.

laminin G (LG)-type domains (Fig. 4). The Gla domain binds calcium ions and interacts with negatively charged phospholipids, such as phosphatidylserine, while the SHBG-domain interacts with C4BP^{56, 119}. A number of regions in the SHBG-domain have been proposed as important for C4BP-binding, but the full binding site is still to be defined. Synthetic peptides comprising residues 605–614 have been reported to inhibit the interaction between C4BP and PS¹²¹. Recombinant PS lacking these amino acids displays weaker binding to C4BP than wild-type protein, but the results suggested that other areas of PS are required for optimal binding to occur⁹³. A PS deletion mutant lacking residues 583–635 also demonstrated markedly reduced affinity to C4BP²⁴. In another study using synthetic peptides it was demonstrated that residues 420–434 of the SHBG-like domain provided an essential C4BP-binding site, which was subsequently confirmed by mutagenesis studies^{44, 45}. In yet another report based on phage display technology, residues 447–460 of PS were suggested to bind C4BP. This region was then further investigated with a combination of synthetic peptide inhibition and alanine-scanning mutagenesis. A recently developed 3D homology model of the SHBG-like domain of PS was used to analyze these and earlier data, and a groove present on the interface between the two LG-like domains was proposed as forming a possible binding site for C4BP³⁴. This is

in agreement with the observation that both the LG-type domains of PS are required for optimal binding to C4BP³⁹.

Chimeric molecules composed of various combinations of α - and β -chain CCP domains were used to localize the PS-binding site to β -chain CCP1 (Fig. 2)^{64, 67}. CCP2 has also been demonstrated to contribute to a small extent to the PS-binding site¹¹⁸. To elucidate the structural background for the involvement of CCP2 in PS-binding, a number of recombinant β -chain variants having mutations in CCP2 were tested. The mutations covered most of the possible binding surfaces of CCP2 and they all bound equally well as recombinant wild-type β -chain to PS¹²⁵. Taken together, the results suggest that the role of CCP2 in PS-binding is to direct and stabilize CCP1 rather than be a direct part of the binding site. Site-directed mutagenesis was used to identify the residues Ile¹⁶, Val¹⁸, Val³¹ and Ile³³ residing at the CCP1 surface as crucial for PS-binding, with secondary contributions from Leu³⁸ and Val³⁹¹²⁴. In addition, positively charged Lys⁴¹ and Lys⁴² contributed slightly to the interaction with PS. The fact that mostly hydrophobic amino acids are involved in the PS-C4BP interaction is in full agreement with our observation that the binding is not sensitive to ionic strength¹⁰ and has a very high affinity.

POTENTIAL ROLE OF THE PS-C4BP COMPLEX ON THE SURFACE APOPTOTIC CELLS

Due to the phospholipid-binding ability of PS, it has been hypothesized that PS is able to localize C4BP to areas where negatively charged phospholipids are exposed, thus providing local control of the complement. In support of this concept, a PS-dependent association of C4BP with negatively charged phospholipid vesicle membranes has been demonstrated¹⁰⁷. The C4BP bound to the phospholipid vesicles via PS could still interact with C4b, indicating that C4BP retained its ability to inhibit complement also when bound to the membrane. However, this does not seem to be the case for platelet-derived microparticles, which are shed from activated platelets and which expose negatively charged phospholipids. Although free PS bound to the microparticles, no binding could be demonstrated for the PS-C4BP complex, suggesting that other membrane surface components may interfere with binding of the complex³⁵. These studies prompted an investigation of the possible interaction of C4BP-PS with apoptotic cells, which early during the apoptotic process expose negatively charged phosphatidylserine on their surface (Fig. 5). Using Jurkat T cells and neutrophils, apoptosis-dependent binding of C4BP to the cells was demonstrated in the presence, but not in the absence of PS¹²³. The binding was calcium-dependent and was blocked by monoclonal antibodies directed against the Gla-domain of PS. The C4BP that was bound via PS to the apoptotic cells was able to interact with C4b, suggesting that it will still be able to inhibit complement. The PS-mediated binding of C4BP to apoptotic cells was not cell-type specific, supporting a physiological role of the C4BP-PS complex in the regulation of complement on the sur-

face of apoptotic cells¹²². The current hypothesis, which remains to be tested, is that C4BP inhibits activation of the classical pathway that would otherwise be the consequence of C1-binding to apoptotic cells.

Binding of C1 to apoptotic keratinocytes rendered apoptotic by irradiation or Sindbis virus infection has been clearly demonstrated⁷³. C1-binding is important for the removal of apoptotic cells via an as yet unknown receptor, as shown by the impaired ability of C1 knockout animals to remove apoptotic cells *in vivo*¹¹⁶. The fact that almost all patients lacking C1 are affected by SLE supports the hypothesis of C1 being important for the removal of apoptotic cells²¹. SLE is characterized by the presence of autoantibodies against cell components present on apoptotic cells that normally are not exposed for a prolonged time to the immune system²³. The potential role of C1q in the clearance of apoptotic cells was further demonstrated when C1q and MBL were shown to stimulate uptake of apoptotic cells by macrophages⁹⁶. The collagenous tails of C1q and MBL bind to calreticulin on the macrophage and give a signal via surface molecule CD91 for ingestion of the apoptotic cell by macropinocytosis⁹⁶.

Under physiological conditions, apoptosis is a well-controlled process, characterized by a lack of induction of inflammatory responses in the surrounding tissues. This suggests that the cell must be protected from the assembly of later complement components and that anaphylatoxin release must be prohibited. Therefore, there is a need for a strong complement inhibitor on the surface of apoptotic cells. C4BP is the perfect candidate for this function, particularly in view of findings that endogenous, membrane-bound inhibitors such as MCP and DAF are down-regulated during apoptosis⁶⁹. However, detailed functional studies of C4BP on apoptotic cells are needed.

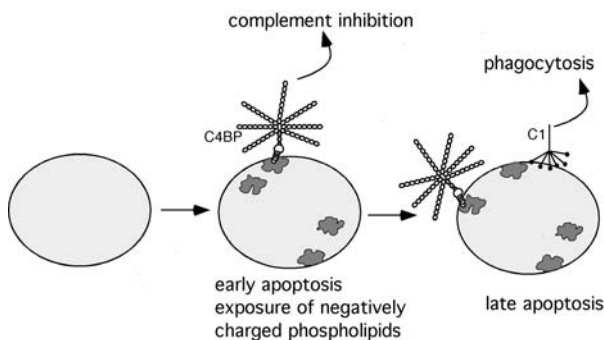


Figure 5. The role of C4BP in the removal of apoptotic cells. In early apoptosis, negatively charged phospholipids are exposed and C4BP is localized to the cell surface via PS. C4BP inhibits the classical pathway, which would otherwise be initiated after binding of C1. C1 interacts with yet unknown receptor on macrophages and facilitates phagocytosis of the cell.

ACTIVATION OF B CELLS

It was demonstrated recently that C4BP binds directly to CD40 expressed on the surface of human B cells. CD40 is a member of the tumor necrosis factor receptor superfamily and its engagement with CD40 ligand results in the upregulation of the surface expression of a number of markers, the proliferation of B cells, and the rescue of germinal center B cells from apoptosis⁴. In addition, CD40 ligation, in the presence of the cytokines interleukin (IL)-4 and -13, induces immunoglobulin isotype switching to IgE. Binding of C4BP to CD40, although at a site that differs from that used by CD40 ligand, induces proliferation, upregulation of CD54 (ICAM-1) and CD86, as well as IL-4-dependent IgE isotype switching in nor-

mal B cells²². These effects were not observed for B cells from patients with CD40 deficiency, showing that the functional effects are indeed due to binding of C4BP to CD40. Circulating B cells may not be important targets for activation by plasma C4BP, as only a small fraction of the total B cells in the body are present in the circulation. Furthermore, human serum poorly supports CD40 activation of B cells *in vitro*²². It is possible that other circulating plasma proteins may compete with C4BP for binding to circulating B cells. A potential candidate is serum amyloid P (SAP) component (see below). However, it was demonstrated that C4BP co-localizes with B cells in the secondary lymphoid follicles in human tonsils²². That C4BP was not detected in T cell-rich areas suggests that C4BP does not exude non-specifically into the extra-vascular space and may have specific reason to interact with B cells in germinal centers. These observations suggest that C4BP is indeed an activating ligand for CD40 and establish a novel interface between complement and B cell activation. The interaction is mediated by the α -chain of C4BP, as the recombinant protein lacking the β -chain had identical effects as C4BP purified plasma containing β -chain as well as PS. Detailed studies of the functional importance and structural requirements for the interaction for both C4BP and CD40 are required.

INTERACTION OF C4BP WITH HEPARIN

Each of the C4BP α -chains contains a binding site for heparin, the resulting affinity being quite high due to the polymeric structure of C4BP^{57, 104}. The interaction between C4BP and C4b can be inhibited by heparin, suggesting that the C4b and heparin-binding sites overlap¹²⁰. To study the binding between C4BP and heparin, affinity chromatography (Heparin-Sepharose) and surface plasmon resonance (Biacore) using biotinylated heparin immobilized on streptavidin chips were employed. The heparin-binding ability of C4BP is compromised by the removal of CCP2 and by insertion of two alanines between CCP1 and CCP2. In contrast to the dramatic effects on C4b-binding, deletion of CCP3 and CCP1 have only minor effects on heparin-binding, suggesting CCP2 to be the most important for the interaction (Fig. 2)¹³. Furthermore, the binding is nearly abolished in the C4BP mutants lacking positively charged amino acids at the CCP1/CCP2 interface¹⁷. At present, the physiological relevance of the interaction between C4BP and heparin is unclear. Possibly, C4BP interacts with structurally related molecules of heparan sulphate present on cell surfaces. Screening of several malignant cell lines resulted in the observation that the ovarian adenocarcinoma cell lines SK-OV-3, Caov-3,

and SW626 were capable of binding C4BP⁶². Functional tests showed that tumor cell-bound C4BP retained its cofactor activity for FI-mediated inactivation of C4b, thus increasing the control of classical pathway activation on the surfaces of these cells. The C4BP-binding moiety was not identified and it is possible that binding was mediated by heparan sulphate present on the surface.

C4BP was shown to interact with low-density lipoprotein receptor-related protein (LRP), which is an endocytic receptor involved in the catabolism of several plasma proteins with heparin-binding properties¹²⁶. The binding appears to be mediated by a heparin-binding site on the α -chain of C4BP, since the recombinant molecule composed of α -chains binds LRP and the monoclonal antibodies directed against the heparin-binding region block the interaction. In cellular degradation experiments, LRP-expressing cells bound and degraded C4BP, and the initial clearance of C4BP in mice was delayed upon injection of receptor-associated protein¹²⁶. Therefore, LRP may at least in part mediate catabolism of C4BP.

INTERACTION OF C4BP WITH SAP

SAP is a multimeric protein present in both plasma and tissues and composed of 5 identical 25 kDa subunits held together by non-covalent forces. SAP belongs to the pentraxin family together with a strong acute-phase reactant, C-reactive protein. However, the concentration of SAP does not increase in response to an acute-phase reaction. SAP is known to have several ligands (heparin, DNA, phospholipid, and lipopolysaccharide) and the interactions to be calcium-dependent³⁸. First, it was demonstrated that C4BP and fibronectin are the two proteins in serum that bind to a column with immobilized SAP³⁷, and the authors concluded that the C4BP-SAP interaction does not seem to occur in fluid phase in serum³. Later on it was shown that the interaction between C4BP and SAP requires the presence of calcium ions and the proteins form a 1:1 stoichiometric complex in fluid phase both in a system with purified components and in serum¹⁰⁸. The SAP-C4BP interaction was demonstrated using several methods, including light scattering, gel filtration, and ultracentrifugation¹⁰⁸. The self-association of SAP and its interactions with phospholipids or C4BP were mutually exclusive, with binding of C4BP being favored. Therefore, the interaction with C4BP in plasma could be important for inhibition of the self-association of SAP.

Recently, a combination of crossed immunoelectrophoresis, size exclusion chromatography, and

native polyacrylamide electrophoresis was used to show that SAP in serum, analyzed under native analysis conditions and free of immobilizing antibodies, does not have any major protein ligand. However, when the protein was aggregated by immobilized antibodies, C4BP and fibronectin clearly bound to SAP¹¹⁰. It appears therefore that the presence of 1:1 SAP-C4BP complexes in serum is a matter of controversy. Binding of SAP is localized to carbohydrate moieties in the central core of C4BP (Fig. 2) and is entirely independent of the binding surface required for interaction with PS⁵³. Although binding sites for SAP and C4b are separated in space, binding of SAP to C4BP inhibits the ability of the latter to serve as an FI cofactor in the cleavage of C4b⁵². This suggests that formation of SAP-C4BP in some conditions could influence complement regulation by C4BP.

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 simply hijack the host's complement regulators and subsequently down-regulate complement activation (for review see⁷⁵). C4BP can be captured by several bacterial pathogens and in some cases it is possible to directly correlate the binding with resistance of bacteria to complement-mediated killing. Inhibition of complement by C4BP leads to decreased opsonization of the bacteria with C3b, which in turn results in a decrease in phagocytosis, which is the major weapon against the pathogens (Fig. 6).

In recent years a number of interactions between pathogens and C4BP have been characterized on the structural level. It appears that most pathogens prefer-

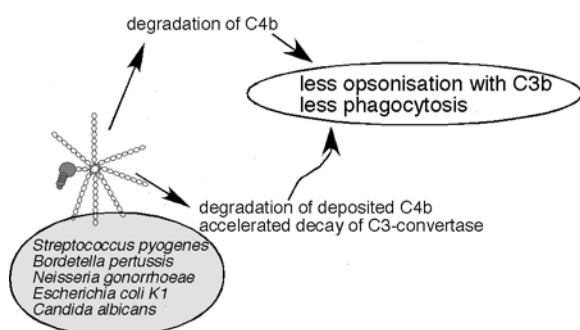


Figure 6. Pathogens capturing C4BP and mechanisms of protection from complement-mediated lysis and phagocytosis. C4BP bound to the surface of a pathogen inhibits classical C3-convertase, which leads to a decrease in opsonization and less efficient phagocytosis.

entially bind to the N-terminal, well-exposed part of the α -chain. M proteins of *Streptococcus pyogenes* interact with CCP1-CCP2 of the α -chain, and their binding sites overlap to some extent with the binding site for C4b (Fig. 2)^{1,9}. The interaction is based essentially on non-ionic/hydrophobic contacts, which yields binding of high affinity. Filamentous hemagglutinin from *Bordetella pertussis*, an etiologic factor of whooping cough, is another surface protein known to interact with C4BP⁸. There is, however, at least one more, as yet elusive component on the bacterial surface that contributes to this interaction. The binding is very similar to that between C4BP and C4b. It is based on ionic interactions and requires a cluster of charged amino acids at the CCP1/CCP2 interface of the α -chain⁸⁷.

Apart from binding FH to sialylated lipooligosaccharide and porin Por1A^{100, 101}, bacteria also employ porin molecules (Por1A and Por1B) to bind C4BP⁹⁹. The C4BP-Por1B interaction is ionic in nature (inhibited by high salt concentration 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, implying that CCP1 contains porin-binding sites (Fig. 2). Using allelic exchange to construct strains with hybrid porin molecules it was demonstrated that the N-terminal loop (loop 1) of Por1A together with loops 5 and 7 of Por1B are required for C4BP-binding. Furthermore, the pilC subunit of type IV pili from *Neisseria gonorrhoeae* binds CCP1-2 of human C4BP¹⁵. Inhibition of C4BP-binding to serum-resistant Por1A and Por1B strains in a serum bactericidal assay using Fab fragments against C4BP CCP1 results in complete killing of otherwise fully serum-resistant strains, underscoring the role of C4BP in mediating gonococcal serum resistance⁹⁹.

Finally, *Escherichia coli* K1, responsible for meningitis in neonates, binds C4BP. It was demonstrated that CCP3 of the C4BP α -chain interacts hydrophobically with the N-terminal part of the outer membrane protein A (OmpA) molecule (Fig. 2)⁹⁸. A compelling observation in this study was that the synthetic peptides corresponding to CCP3 sequences block the binding of C4BP to OmpA and also significantly enhance the serum bactericidal activity. The number of human pathogens shown to bind C4BP and/or FH is increasing and the mechanism of protection from complement seems to be widely distributed, implying that it has great survival value.

CONCLUDING REMARKS

C4BP is a truly remarkable protein with a number of important functions. As no deficient patients or ani-

mal knockouts are available it is possible that C4BP will reveal even more functions in the future. The protein is an unusual link between the complement and coagulation systems. C4BP is important for the inhibition of unwanted or excessive complement activation and the controlled removal of apoptotic cells

without evoking inflammatory reactions. A number of human pathogens have developed the ability to capture C4BP to their surfaces and protect themselves from the adverse effects of the complement system.

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