REVIEW ARTICLE

The interaction between complement component C4b-binding protein and the vitamin K-dependent protein S forms a link between blood coagulation and the complement system

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INTRODUCTION

Blood coagulation is a complex of reactions, which involves several enzymes, cofactor proteins, phospholipid surfaces and Ca^{2+} ions. This waterfall or cascade system is initiated upon exposure of blood to tissue factor or negatively charged surfaces. Then the intrinsic or the extrinsic pathway of coagulation are initiated, leading to the formation of thrombin and fibrin. This clotting cascade prevents excessive bleeding upon injury (Furie & Furie, 1988; Mann *et al.*, 1988). Another example of a cascade system is the complement system, which is composed of two pathways: the classical pathway and the alternative pathway (Lachmann & Hughes-Jones, 1985). The classical pathway is activated by immune complexes and the alternative pathway by activating surfaces such as the surface of a micro-organism. One of the main functions of the complement system is the lysis of target cells (Muller-Eberhard & Miescher, 1985; Law & Reid, 1988). In recent years most of the components of both systems have been purified and the molecular interactions that are required for the catalytic events that occur at each step of the cascades have been characterized. Although both cascade systems are composed of many different proteins, there are only few interactions known between them. In fact, the interaction between complement component C4, C4b-binding protein (C4BP) and protein S constitutes a unique link between the complement and coagulation systems (Fig. 1). This review deals with the structural and functional relationships of C4, C4BP and protein S, and their regulation. In addition, the short consensus repeat (SCR) domains of C4BP will be discussed in context of other SCR-containing proteins. A SCR unit is approx. 60 amino acids long and demonstrates a typical consensus framework of conserved residues. The SCR unit can be defined as a single domain with a double-loop structure, formed by overlapping disulphide bonds.

THE PROTEIN C ANTICOAGULANT PATHWAY

In recent times it has been recognized that apart from extrinsic and intrinsic coagulation and fibrinolysis pathways (Furie & Furie, 1988; Mann *et al.*, 1988) there is another pathway involved in the regulation of the coagulation process, the so-called protein C pathway. The first step in the pathway is the conversion of protein C into its active form by thrombin. For this process a cofactor on the endothelial cell is necessary: thrombomodulin (Owen & Esmon, 1981; Esmon *et al.*, 1982). Thrombomodulin-bound thrombin is not able to act as a coagulant enzyme, but acts as an activator of protein C. Activated protein C (APC) is

a powerful inhibitor of the coagulation pathway, by inactivating coagulation factors Va (Kisiel *et al.*, 1977; Comp & Esmon, 1979; Walker *et al.*, 1979; Dahlbäck & Stenflo, 1980; Marlar *et al.*, 1982; Nesheim *et al.*, 1982; Suzuki *et al.*, 1983a; van de Waart *et al.*, 1984) and VIIIa (Vehar & Davie, 1980; Marlar *et al.*, 1982; Fulcher *et al.*, 1984; Eaton *et al.*, 1986; Walker *et al.*, 1987; Koedam *et al.*, 1988), whereas at the same time it stimulates fibrinolysis by inactivating the inhibitor of tissue plasminogen activator (Comp & Esmon, 1981; Sakata *et al.*, 1985; van Hinsbergh *et al.*, 1985). The expression of the anticoagulant activity of APC requires the presence of calcium ions and membranes that contain negatively charged phospholipids. In the absence of lipids, proteolysis of either factor V or VIII by APC is very slow. Plasma contains a component that accelerates the inactivation of factor V and VIII by APC (Walker, 1980, 1981a; Walker *et al.*, 1987; Koedam *et al.*, 1988). This cofactor activity was found to be associated with a vitamin K-dependent protein, which was arbitrarily named protein S (DiScipio & Davie, 1979; Stenflo & Johnsson, 1979) in reference to its first isolation in Seattle. Its effect was found to be dependent on the presence of membranes that contained negatively charged phospholipids (Walker, 1981a). Functional as well as binding studies indicated that protein S and APC were capable of forming a 1:1 molar complex on the surface of phospholipid vesicles (Walker, 1981a). However, so far no direct complex formation between protein S and APC, in solution or on phospholipid surfaces, has been demonstrated. APC can be inactivated in plasma by at least two protease inhibitors, the protein C inhibitor (Suzuki *et al.*, 1983b; Meijers *et al.*, 1988a) and α_1 -antitrypsin (Van de Meer *et al.*, 1987; Heeb & Griffin, 1988; Meijers *et al.*, 1988b). The anticoagulant activity of protein C has physiological significance because persons with severe protein C (or protein S) deficiency tend to have thrombosis (Griffin *et al.*, 1981; Bertina *et al.*, 1982; Esmon, 1984; Comp & Esmon, 1984; Schwarz *et al.*, 1984; Bertina, 1985; Kamiya *et al.*, 1986).

THE VITAMIN K-DEPENDENT PROTEIN S

Human protein S is a vitamin K-dependent plasma protein consisting of 635 amino acids in a single polypeptide chain and has an apparent molecular mass of 69 kDa. It has three potential N-glycosylation sites (Asn-458, -468 and -489), but it is not known which of the three sites are glycosylated (DiScipio *et al.*,

Abbreviations used: APC, activated protein C; CR1, complement receptor type 1; CR2, complement receptor type 2; C4BP, C4b-binding protein; DAF, decay-accelerating factor; EGF, epidermal growth factor; Gla, γ -carboxyglutamic acid; LEC-CAM, lectin-EGF-complement-cellular adhesion molecules; MCP, membrane cofactor protein; RCA, regulation of complement activation; SCR, short consensus repeat; SHBG, sexual-hormone-binding globulin.

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Fig. 1. Schematic illustration of a unique link between coagulation and the complement system

Thrombin (T) binds to thrombomodulin (TM) expressed on endothelial cells and activates protein C (PC). Activated protein C (APC) requires a cofactor protein S (PS) for optimal interaction with endothelial cells or platelets and subsequent degradation of the activated procoagulant cofactors, FVa and FVIIIa, to the inactive forms, FVi and FVIIIi. PS can form a 1:1 noncovalent complex with C4b-binding protein (C4BP), which is a cofactor for the inactivation of the C3-convertase (C4b2a) by factor I of the classical complement pathway. An interaction between C1q and immune complexes (Ag-Ab) is involved in the activation of the classical pathway, which triggers the activation of the other complement components C2, C4 and C3, leading to the polymerization of C5b-9 to form the membrane attack complex (MAC).

1977; Lundwall *et al.*, 1986). Table 1 lists the physical properties as well as the plasma concentration of protein S. Protein S serves as a cofactor in the inactivation of coagulation factor Va and VIIIa by activated protein C (APC) as described above. The protein S cofactor activity for APC is dependent on the presence

of negatively charged phospholipids (Nelsetuen *et al.*, 1978; Walker, 1981a) and is present on cell membranes, e.g. on the surface of platelets and endothelial cells (Walker, 1984a; Suzuki *et al.*, 1984; Harris & Esmon, 1985; Stern *et al.*, 1986a,b; Hessing *et al.*, 1989; Hackeng *et al.*, 1990). For lipids to be effective, they

Table 1. Properties of human protein S, C4 and C4BP

Property	Protein S	C4	C4BP
Carbohydrate content (%)	7.8	10	7–11
$A_{280}^{1\%}$	9.5	8.7	14.1
Function	Cofactor for APC	Constituent of C3 convertase	Cofactor for C4b cleavage
Gla residues	11	—	—
M (kDa)	69	197	530–570
pI	5.5	8.1	6.7
Polypeptide composition	1	3	6–8
Plasma concentration ($\mu\text{g/ml}$)	25	350	143

must contain phosphatidylserine. The structure of the fatty acid side chains of the phospholipids and the presence of cholesterol in lipid vesicles influences the cofactor activity of protein S (Walker, 1988). A new relationship between the anticoagulant protein C pathway and the autonomic nervous system has recently been suggested by the observation that noradrenaline-mediated endothelial cell stimulation resulted in loss of intracellular protein S and suppression of cell surface binding sites, thus modulating the APC activity on the vessel wall (Brett *et al.*, 1988).

Approximately half of the protein S in human plasma is present as a noncovalent complex (K_D 10^{-7} M) with complement component C4b-binding protein (C4BP), and the remaining half is present as a free protein (Dahlbäck & Stenflo, 1981). The protein S–C4BP complex exhibited slow kinetics of association and dissociation (Dahlbäck, 1983b). Only free protein S has cofactor activity for APC (Comp & Esmon, 1984, 1986; Bertina *et al.*, 1985; Dahlbäck, 1986). The mechanism of the inhibition of the cofactor activity of protein S by C4BP is not clearly understood. However, ternary complex formation between protein S, C4BP and APC has now been demonstrated, suggesting that the protein S–C4BP complex competes with free protein S for binding to APC (Nishioka & Suzuki, 1990). Protein S forms complexes with C4BP both in the presence of calcium (K_D 5×10^{-10} M) and in the presence of EDTA (K_D 10^{-7} M) (Dahlbäck, 1983b; Dahlbäck *et al.*, 1990b). The low K_D in the presence of calcium suggests that *in vivo* all protein S is bound to C4BP. Since this is not the case, Dahlbäck *et al.* (1990b) have suggested that a third unrecognized component might be involved in the interaction between protein S and C4BP.

Thrombin cleavage of protein S converts the single-chain protein to a disulphide-linked two-chain structure, which leads to decreased calcium and phospholipid binding and a parallel loss of the cofactor activity for APC (Dahlbäck, 1983a; Suzuki *et al.*, 1983c; Walker, 1984b; Morita *et al.*, 1986; Dahlbäck *et al.*, 1986b). The thrombin-mediated cleavage of protein S is inhibited by physiological calcium concentrations (Morita *et al.*, 1986; Mitchell *et al.*, 1986). At high calcium concentrations (5–10 mM) both native protein S and protein S cleaved by thrombin bind equally well to phospholipids (Schwalbe *et al.*, 1990). The thrombin cleavage sites in bovine protein S are localized at Arg⁵² and Arg⁷⁰ (Dahlbäck *et al.*, 1986b), whereas the cleavage sites in its human counterpart are not yet completely resolved. Malm *et al.* (1987) demonstrated that a monoclonal antibody against a calcium-dependent epitope in or close to the γ -carboxyglutamic acid (Gla) region of protein S inhibited both phospholipid binding and APC cofactor activity.

The recently determined amino acid sequences as predicted by the nucleotide sequences of cDNAs coding for human and

Fig. 2. Schematic presentation of structural domains in the human vitamin K-dependent coagulation factors (a) and the tentative secondary structure of protein S (b)

(ACT, activation region; AS, aromatic amino acid stack; DHS, catalytic triad with amino acids Asp, His and Ser; EGF, four domains homologous to the EGF precursors; Gla, vitamin K-dependent γ -carboxyglutamic acid-rich region; K, kringle domain; SHBG, the C-terminal loop similar to the sex hormone binding globulin; SS, signal sequence; T, thrombin-sensitive region; OH, hydroxy group of β -hydroxyasparagine or β -hydroxyaspartic acid. Modified from Lundwall *et al.* (1986).

bovine protein S demonstrate that, unlike the other vitamin K-dependent coagulation factors, protein S is not a serine protease (Dahlbäck *et al.*, 1986a; Lundwall *et al.*, 1986; Hoskins *et al.*, 1987; Ploos van Amstel *et al.*, 1987). Protein S is composed of discrete domains and contains several functionally important post-translationally modified amino acids (Dahlbäck *et al.*, 1986a; Stenflo *et al.*, 1987). Like the other vitamin K-dependent proteins (Fig. 2a), protein S contains a Gla-rich N-terminal region, which is terminated by a short hydrophobic region containing four aromatic residues, the so-called aromatic stack or H region (Park & Tulinsky, 1986). This is followed by a thrombin-sensitive region and four epidermal growth factor (EGF) homologous regions (Doolittle *et al.*, 1984), containing one β -hydroxyaspartic acid residue and three β -hydroxyasparagine residues each. β -Hydroxyaspartic acid was first recognized in protein C (Drakenberg *et al.*, 1983) and factor X (McMullen *et al.*, 1983a,b), whereas β -hydroxyasparagines were first found in protein S. The functions of these amino acid residues are not yet elucidated, but it has been suggested that they are important for Gla-independent high-affinity calcium binding (Sugo *et al.*, 1986; Stenflo *et al.*, 1987; Rees *et al.*, 1988; Dahlbäck *et al.*, 1990a). At the C-terminus a 387-amino-acid domain of unrecognized structure and unknown function is present, which is partly similar to the sexual hormone binding globulin (SHBG), an androgen-binding protein in plasma (Baker *et al.*, 1987; Gershagen *et al.*, 1987). Fig. 2(b) shows the tentative secondary structure and different domains of human protein S. The functional importance of the various regions in protein S is

only partly established. The Gla region is required for calcium and phospholipid binding (Sugo *et al.*, 1986; Furie & Furie, 1988; Dahlbäck *et al.*, 1990a). The EGF-like domains are suspected to contain the high-affinity Gla-independent binding sites, and a model in which APC and protein S interact via the first EGF-like domain and the thrombin-sensitive region has been proposed (Dahlbäck *et al.*, 1990a). No specific function has been assigned to the SHBG-like domain, although Walker (1989) has claimed that amino acids 605–614 of the protein S sequence are important for the interaction with C4BP.

Two genes homologous with the human protein S cDNA are located near the centromere on chromosome 3 (Ploos van Amstel *et al.*, 1987, 1988, 1990; Watkins *et al.*, 1988; Long *et al.*, 1988; Edenbrandt *et al.*, 1990). The protein S genes, PS α and PS β , span approx. 1200 nucleotides and show a high degree (96.5%) of similarity (Ploos van Amstel *et al.*, 1988, 1990) between their exonic sequences. The protein S gene, PS α , contains 15 exons that code for proteins S mRNA as derived from the reported protein S cDNAs, whereas the PS β gene was identified as a pseudogene (Long *et al.*, 1988; Edenbrandt *et al.*, 1990; Ploos van Amstel *et al.*, 1990; Schmidel *et al.*, 1990). The intron-exon organization of the PS α gene is similar to that of the other coagulation factors, i.e. each exon codes for a separate domain in the protein. Recently, Malm *et al.* (1990) reported on the expression of completely γ -carboxylated and β -hydroxylated recombinant human protein S that had full biological activity as cofactor for APC and was able to bind to C4BP.

C4b-BINDING PROTEIN

C4b-binding protein (C4BP) (previously named C3b-C4bINA cofactor) is a high- M_r plasma protein, which regulates the activation of the classical pathway of the complement system (Scharfstein *et al.*, 1978; Fujita *et al.*, 1978; Fujita & Nussenzweig, 1979; Gigli *et al.*, 1979; Nagasawa & Stroud, 1980; Nussenzweig & Melton, 1981). Table 1 lists the physical properties as well as the plasma concentration of C4BP. C4BP functions as a cofactor for factor I in the proteolytic degradation of C4b (Fujita *et al.*, 1978; Fujita & Nussenzweig, 1979; Gigli *et al.*, 1979; Nagasawa & Stroud, 1980; Fujita & Tamura, 1983) and in addition accelerates the decay rate of the C4b2a (EC 3.4.21.43) complex (Gigli *et al.*, 1979; Daha & van Es, 1980). The mechanism by which the binding of C4b to C4BP turns C4b into a substrate for factor I is unknown, but presumably involves a conformational change in C4b (Fujita & Tamura, 1983).

C4BP is present in human plasma in two major forms, antigenically identical, but of slightly different molecular mass (530 and 570 kDa) and net charge. Both forms (referred to as C4BP-high and C4BP-low) act as cofactors in the cleavage of C4b by factor I (Fujita & Nussenzweig, 1979). C4BP-high is the dominant form and accounts for up to 80% of C4BP in plasma (Dahlbäck, 1983b; Villiers *et al.*, 1981). The major form of C4BP (570 kDa) is composed of multiple identical α -chains of 70 kDa and one distinct β -chain of 45 kDa that are linked via disulphide bridges (Scharfstein *et al.*, 1978; Reid & Gagnon, 1982; Hillarp & Dahlbäck, 1988, 1990; Hillarp *et al.*, 1989; Hessing *et al.*, 1990c). Each of the α -chains forms a tentacle with the N -terminus located at the periphery of the tentacle, whereas the C -termini are located in the central core of C4BP (Dahlbäck & Muller-Eberhard, 1984; Nagasawa *et al.*, 1983; Perkins *et al.*, 1986). Electron microscopic studies of C4BP have suggested that C4BP has a spider-like structure with seven elongated tentacles extending from a central, ring-like core (Dahlbäck *et al.*, 1983; Dahlbäck & Muller-Eberhard, 1984). Solution X-ray scattering

studies suggested that C4BP in solution resembles a more rigid bundle of seven arms in contrast to the splayed-out images seen in electron micrographs (Perkins *et al.*, 1986). The binding sites for C4b are located at the peripheral half of each α -chain (Dahlbäck *et al.*, 1983; Fujita *et al.*, 1985) and it has been reported that up to 4.8 molecules of C4b can bind to one C4BP molecule (Scharfstein *et al.*, 1978). It has recently been suggested that there are four strong (K_D 0.8×10^{-7} M) and two weak sites for C4b on C4BP (Ziccardi *et al.*, 1984) and the addition of more C4b molecules is sterically hindered. However, the absorption coefficient used for C4BP of 9.3 (Villiers *et al.*, 1981) was lower than the one of 14.1 determined by Perkins *et al.* (1986), and a new interpretation of the less-than-full binding of C4b to C4BP would lead to an increase in the number of C4b molecules that can bind to one single C4BP molecule, which would be in agreement with the results of a hydrodynamic analysis of the C4BP structure (Perkins *et al.*, 1986). The physiological concentration of C4BP exceeds the observed K_D of the C4BP-C4b interaction, suggesting that any C4b molecule present in serum is bound to C4BP.

Limited proteolysis of C4BP by chymotrypsin results in the cleavage of two peptide bonds, at Tyr³⁹⁵ and Trp⁴²⁵, yielding two major fragments (Dahlbäck & Muller-Eberhard, 1984; Nagasawa *et al.*, 1983): a 48 kDa fragment constituting the major portion of the extended α -chain and a 160 kDa fragment representing the central core of C4BP. The central core contains carbohydrate chains and is composed of disulphide-linked 25 kDa peptides. The function of the carbohydrate chains attached to the central core of C4BP is unknown. The C4b-binding function and the factor I cofactor activity are located in the 48 kDa fragments (Nagasawa *et al.*, 1982). Reid & Gagnon (1982) showed that trypsin selectively cleaves a peptide bond in the middle of each α -chain of C4BP, generating fragments of 36–39 kDa. These two fragments are disulphide-linked, in contrast with the chymotrypsin digestion. Limited digestions of human C4BP with chymotrypsin, trypsin and pepsin (Chung *et al.*, 1985a) have indicated that the cofactor activity for factor I is located in the region of residues 177–322 and that the region 332–395 may be important for the binding to C4b (Chung & Reid, 1985). The localization of the C4b-binding site to these regions in the middle of the α -chains is in disagreement with previous results obtained from electron microscopic studies of the C4BP-C4b complex (Dahlbäck *et al.*, 1983), that demonstrated that C4b was bound to the N -terminal tips of the α -chains.

The complete amino acid sequence of one of the α -chains of C4BP was determined by a combination of cDNA and peptide sequencing (Chung *et al.*, 1985a,b; Lintin & Reid, 1986). Each α -chain was composed of 549 amino acid residues. The N -terminal 491 residues can be divided into eight homologous short consensus repeats (SCRs) (also known as Sushi-domains) of approx. 60 amino acids. SCRs have been found in other complement and non-complement proteins (Reid *et al.*, 1986; Kristensen *et al.*, 1987a) and because all proteins involved in the binding of C3b or C4b contain SCRs, the group of proteins that contain SCRs have been classified as the superfamily of C3b/C4b-binding proteins. The 58 C -terminal residues do not fall into the same pattern of internal homology as seen for the N -terminal 491 amino acids, and from structure-prediction studies it appears that they form a stable α -helix (Chung *et al.*, 1985b). The C -terminal region contains two cysteine residues thought to be involved in the interchain disulphide linkage between the α - and β -chains. This hypothesis is supported by the recently reported sequence of mouse C4BP (Kristensen *et al.*, 1987b), where the different subunits were found to be linked by noncovalent bonds in the C -terminal region which was without cysteines. Recently,

Fig. 3. Schematic model of C4BP

Upper part, the seven α -chains (70 kDa; red) and one β -chain (45 kDa) (hatched) are disulphide-linked to each other near their C-terminal ends. The interchain disulphides are tentatively placed between the two non-homologous cysteines in the subunits. Lower part, one α -chain demonstrating the eight SCRs (denoted 1–8; pink). The C-terminal, non-homologous, 58 amino acids are indicated by the hatched area. Chymotryptic (CT) cleavage sites are indicated by the arrows. The putative binding sites for C4b^{1,2} and protein S³ according to Chung & Reid (1985) and Suzuki & Nishioka (1988) respectively, are indicated. More recently, it became obvious that the β -chain is important for the binding of protein S (Hillarp & Dahlbäck, 1988; Hillarp *et al.*, 1989; Hillarp & Dahlbäck, 1990; Helsing *et al.*, 1990c).

a 5' cDNA clone for human C4BP was isolated and sequenced to complete the cDNA sequence coding for residues 1–32, thus confirming the protein sequence data. From this sequence a leader peptide sequence was derived, which was 32 residues in length and showed a high degree of hydrophobicity typical of documented leader sequences (Lintin *et al.*, 1988). Recently, Matsuguchi *et al.* (1989) isolated and analysed the cDNA for proline-rich protein and revealed that it is identical with that of the α -chain of C4BP. Proline-rich protein (Sata *et al.*, 1976) is a glycoprotein present in chylomicrons with lipid binding properties, but with no clear function.

Recently, Hillarp & Dahlbäck (1990) determined the primary structure of the β -chain of C4BP. The β -chain consisted of 235 amino acids and the calculated molecular mass was found to be 26.4 kDa, which was in contrast with the estimated mass of 45 kDa derived from gel electrophoresis. The difference was caused by glycosylation of the mature molecule. The β -chain can be divided into three SCRs, each of 57–60 residues in length with the typical pattern of conserved amino acids shared by all SCR-containing proteins. As a consequence, the β -chain of C4BP is similar to the α -chain of C4BP and in addition the C-terminal end of the β -chain is rather similar to the corresponding region of the α -chain. The two cysteines in the C-terminal regions of both α - and β -chains are probably required to form the C4BP polymer by interchain disulphide bridges. The overall similarity

between the two subunits of C4BP probably reflects a close evolutionary relationship between them, suggesting that the different domains are utilized in a similar manner. However, it remains to be investigated what the ligand specificity determines.

The gene for human C4BP spans about 40 kb and was assigned to the human chromosome 1, band 1q32 long with the genes encoding complement receptor 1 and 2 (CR1 and CR2), decay-accelerating factor (DAF), factor H and membrane cofactor protein (MCP) (Rodrigues de Cordoba *et al.*, 1985; Weis *et al.*, 1987; Lublin *et al.*, 1987; Rey Campos *et al.*, 1987; Hing *et al.*, 1988; Carrol *et al.*, 1988; Bora *et al.*, 1989; Pardo-Manuel *et al.*, 1990), which also regulate complement activation, as part of the RCA (regulation of complement activation) gene cluster (Rodrigues de Cordoba & Rubinstein, 1987; Hourcade *et al.*, 1989). Recently, Pardo-Manuel *et al.* (1990) demonstrated that the genes for the α - and β -chains of C4BP are closely linked within the RCA gene cluster in a head-to-tail arrangement, supporting the concept that the C4BP α and C4BP β genes are the result of a gene duplication event.

In 1981, Dahlbäck & Stenflo demonstrated the existence of the C4BP–protein S complex. Protein S and C4BP formed a 1:1 non-covalent complex with an apparent K_D of $(1.6–4.5) \times 10^{-7}$ M (Dahlbäck, 1984). C4BP was found to contain one single binding site for protein S, which was distinct from the multiple binding sites for C4b and the binding of protein S to C4BP had no direct

effect on the C4b-binding function of C4BP (Dahlbäck & Hildebrand, 1983). The protein S binding site on C4BP has been localized near the central core (Dahlbäck *et al.*, 1983; Hillarp & Dahlbäck, 1987; Nukatsuka & Nagasawa, 1987; Suzuki & Nishioka, 1988) on the distinct disulphide-linked β -chain (Hillarp & Dahlbäck, 1988, 1990; Hillarp *et al.*, 1989; Hessing *et al.*, 1990c). The β -chain was sensitive for proteolysis by chymotrypsin and when cleaved the protein S binding ability is lost. When occupied with protein S the β -chain was protected from cleavage by chymotrypsin (Hillarp & Dahlbäck, 1988). In contrast, Suzuki & Nishioka (1988) localized the protein S binding site on C4BP at a 2.5 kDa peptide corresponding with Ser⁴⁴⁷-Tyr⁴⁶⁷ of each α -chain. This region is located in the most C-terminal unit of the eight SCRs of each α -chain of C4BP (Chung *et al.*, 1985a,b). In 1983, only C4BP-high was found to be able to bind protein S (Dahlbäck, 1983a), but the interpretation of the complex formation between protein S and C4BP was somewhat complicated by the fact that there seemed to exist two different populations of C4BP, differing in protein S binding ability. At present, it has become clear that approx. 80 % of C4BP in plasma can bind to barium citrate via its interaction with protein S and that both the adsorbed C4BP-high and -low form are able to bind protein S and contain the β -chain. C4BP-high and -low forms isolated from the supernatant of barium citrate adsorbed plasma were incapable of binding protein S and were lacking the β -chain (Hillarp *et al.*, 1989; Hessing *et al.*, 1990c). The identification of C4BP molecules without β -chain and protein S binding ability supports the concept that a single protein S binding site is localized on the β -chain. However, as no direct complex formation between an isolated β -chain and a protein S molecule was found (Hillarp & Dahlbäck, 1988; Hillarp *et al.*, 1989; Hessing *et al.*, 1990c), it cannot be excluded that the protein S binding site is much more extended than the size of one epitope on the β -chain. The central core domain at the C-terminal ends of the α -chains might support or facilitate the noncovalent interaction with protein S. Fig. 3 summarizes the present available data regarding the C4BP structure and its functional domains. It has been proposed that the molecular mass differences between the various C4BP molecules are due to a variable polymerization of a number of α - and β -chains during the biosynthesis (Hillarp *et al.*, 1989; Hessing *et al.*, 1990c). However, this hypothesis remains to be investigated.

So far, no clear physiological function for the C4BP-protein S complex has been found. However, protein S bound to C4BP was found to be resistant to chymotrypsin, leukocyte elastase and cathepsin G (Nukatsuka & Nagasawa, 1987), which could suggest that binding of protein S to C4BP may be significant for the protection of protein S from proteolytic inactivation during inflammatory responses. More recently, Schwalbe *et al.* (1990) obtained evidence that protein S in complex with C4BP retained its affinity for negatively charged phospholipids, which supports a previous hypothesis of Dahlbäck (1984) that protein S-mediated localization of C4BP to injured or activated cell surfaces forms an alternative mechanism for the control of local classical complement activation. Several other complement regulator proteins, like CR1, DAF and MCP have indeed been found on cell surfaces.

SHORT CONSENSUS REPEATS IN C4BP AND RELATED PROTEINS

In recent years the increase in determination of primary structures has revealed that about half of all complement proteins, many of them interacting with C3 or C4, contain two or more structural elements, referred to as SCRs. The number of proteins containing SCRs is continuously growing and at present com-

prises several complement and non-complement proteins (Table 2). SCRs are always arranged in tandem and in some cases the complete primary sequence consists of SCR units. Each SCR is approx. 60 amino acids long and the typical consensus framework of conserved residues includes four cysteines, two prolines, one tryptophan and several other partially conserved glycines and hydrophobic residues (Reid *et al.*, 1986). The SCR unit can be defined as a single domain with a double-loop structure, formed by overlapping disulphide bonds in a 1-3 and 2-4 fashion (Fig. 4) and is encoded by a single exon (Janatova *et al.*, 1989; Hillarp & Dahlbäck, 1990). Prediction of the secondary structure suggests that the SCR will take the form of a predominantly β -structure and a model based on antiparallel β -strands has been proposed (Perkins *et al.*, 1988). The presently available data suggest that proteins containing SCRs arranged in tandem have an elongated structure with compact SCR units resembling a long string of pearls (Reid *et al.*, 1986). Such an elongated structure might be advantageous for receptor proteins like C4BP, factor H, CR1 and 2 to increase their action radius for their respective ligands. Janatova *et al.* (1989) have localized the disulphide bonds in one of the SCRs of C4BP, which indicated that the intra-SCR disulphides are linked with the first and third and the second and fourth half-cystine in one SCR unit. The same pattern has been found in β_2 -glycoprotein I, a non-complement protein containing five SCRs (Lozier *et al.*, 1984). So far, no inter-SCR disulphide bonds have been found in any of the SCR-containing proteins. This is consistent with the observations that most of the SCRs are encoded by separate exons (Reid *et al.*, 1986; Kristensen *et al.*, 1987a). However, some variation in the principle of one exon per SCR has been observed (Barnum *et al.*, 1989).

The function of the SCRs is not yet elucidated, but it is tempting to believe that they provide the structural basis for the C3b and C4b binding capacity. On the other hand, there are many proteins belonging to this superfamily of SCR-containing proteins that are not involved in complement (Table 2). Many of these proteins are able to bind other proteins, but the involvement of SCR in these interactions has not been clarified. It has been suggested that the SCRs may be utilized in a variety of binding reactions in a manner similar to the immunoglobulins, or that a SCR unit may be responsible for a certain overall structure of the SCR-containing proteins (Reid *et al.*, 1986; Kristensen *et al.*, 1987a).

The identification of the adhesion molecules ELAM-1, MEL-14 and GMP-140 suggested the existence of a new gene family of proteins that are involved in carbohydrate-dependent interaction between circulating blood cells and the endothelium. These adhesion proteins consist of domains homologous to the lectins, SCR and EGF and therefore this family has been named lectin-EGF-complement-cellular adhesion molecules (LEC-CAM) (Stoolman, 1989). Also in this case is the function of the SCRs unknown, but they contain most or all of the invariant residues that constitute the consensus sequence of an SCR unit. In addition, the first half of each repeat contains two extra conserved cysteines, indicating a strong relationship between these three adhesion proteins. The same structural motifs as occur in the LEC-CAM family have been found in the proteoglycan versican (Zimmermann & Ruoslahti, 1989). Several other proteoglycans from various species contain lectins and SCRs, but these SCRs have no additional cysteines as in the LEC-CAM family.

In 1988, Kotwal & Moss described a 35 kDa secretory peptide from the vaccinia virus that was composed of four SCRs and showed a great similarity (38 % identity) with the α -chain of C4BP. It was suggested that this secretory peptide of the vaccinia virus inhibits complement activation to defend itself from host immune defence. Norris *et al.* (1989) have purified a 160 kDa

Table 2. SCR-containing proteins

Protein	Number of SCRs	References
β_2 -Glycoprotein Ib	5	Lozier <i>et al.</i> (1984)
Cartilage proteoglycan core (rat)	1	Doerge <i>et al.</i> (1986)
CR1	30	Klickstein <i>et al.</i> (1988)
CR2	16	Weis <i>et al.</i> (1988)
CRY (mouse)	5	Paul <i>et al.</i> (1989)
C1r	2	Journet & Tosi (1986)
C1s	2	Tosi <i>et al.</i> (1987)
C2	3	Bentley (1986)
C4BP- α	8	Chung <i>et al.</i> (1985b)
C4BP- β	3	Hillarp & Dahlbäck (1990)
C6	2	DiScipio & Hugli (1989)
C7	2	DiScipio <i>et al.</i> (1988)
DAF	4	Medof <i>et al.</i> (1987)
ELAM-1	6	Bevilacqua <i>et al.</i> (1989)
Factor B	3	Morley & Campbell (1985)
Factor C (horseshoe crab)	1	Tokunaga <i>et al.</i> (1987)
Factor H	20	Ripoche <i>et al.</i> (1988)
Factor XIII	10	Ichinose <i>et al.</i> (1986)
GMP-140	9	Johnston <i>et al.</i> (1989)
Haptoglobin	1	Kurosky <i>et al.</i> (1980)
Interleukin 2 receptor	2	Leonard <i>et al.</i> (1985)
MCP	4	Lublin <i>et al.</i> (1988)
MEK-14 (mouse)	2	Lasky <i>et al.</i> (1989)
Membrane protein (<i>Trypanosoma cruzi</i>)	?	Norris <i>et al.</i> (1989)
Secretory protein (vaccinia virus)	4	Kotwal & Moss (1988)
Thyroid peroxidase	1	Kimura <i>et al.</i> (1989)
Versican	1	Zimmermann & Ruoslahti (1989)

Fig. 4. A general consensus sequence of the SCR and its putative disulphide bonding

A typical SCR unit is about 60 amino acids long and is composed of several conserved residues. In this Figure the most commonly occurring residues and their relative positions are shown. These residues are conserved in at least 70% of the SCRs analysed. The four cysteine residues and the tryptophan residue are virtually always strictly conserved and found in 95–100% of the SCRs analysed. Several disulphide bridges have been determined in C4BP as well as in the non-complement protein, β_2 -glycoprotein Ib, containing five SCRs. Each circle represents an amino acid; black circles indicate the cysteine residues.

protein from the protozoan parasite, *Trypanosoma cruzi*, which is resistant to complement lysis. Although this 160 kDa protein was not completely sequenced, a cDNA clone expressing this protein hybridized with a human DAF cDNA, suggesting that this 160 kDa protein represents a member of the SCR-containing superfamily. These findings suggest that micro-organisms and parasites may be capable of evading complement attack by the synthesis of complement regulatory molecules in the invaded host.

COMPLEMENT COMPONENT C4

Complement C4 is a glycoprotein of 197 kDa consisting of three disulphide-linked α (88 kDa), β (72 kDa) and γ (32 kDa) chains (Porter & Reid, 1978, 1979; Muller-Eberhard & Lepow, 1965; Kerr, 1980; Bhakdi & Trannum Janssen, 1983; Lundwall *et al.*, 1981). Table 1 lists the physical properties as well as the plasma concentration of C4. Synthesized in the liver as a single chain polypeptide of 1706 amino acids (Belt *et al.*, 1984) it undergoes several modifications involving proteolytic excision of two intersubunit-linking peptides (Goldberger & Colten, 1980), sulphation (Karp, 1983a), glycosylation (Matthews *et al.*, 1982), and generation of a thiolester site in the α -chain (Karp, 1983b). The proteolytic cleavage by C1s in the *N*-terminal part of the α -chain of C4 leads to the generation of the activation peptide C4a (8 kDa) and the activated component C4b (181 kDa). C4b contains at least two binding sites. One is unstable and responsible for the binding of C4b to antibody-antigen aggregates and to acceptor sites on cell surfaces (Porter & Reid, 1978, 1979; Muller-Eberhard & Lepow, 1965). The other binding site of C4b is stable and specific for C2 (Kerr, 1980). C2 bound to C4b will be cleaved by C1s, resulting in the C4b2a complex which acts as the C3 convertase (Kerr, 1980). Cleavage of C4b by factor I and C4BP in the α' -chain of C4b leads to the generation of the intermediate product, C4b', after the first cleavage of the α' -chain, resulting in a 70 kDa and a 16 kDa α_4 -fragment of the α' -chain both still disulphide-linked to the other chains (see Fig. 5). The second cleavage results in the excision of C4d [45 kDa (α_2)], and the generation of a third fragment [27 kDa (α_3)], of the α' -chain still disulphide-linked to C4c (Nagasawa *et al.*, 1980). The C4BP binding site has been localized on the α' -chain of C4b and it has been demonstrated that this binding could be inhibited by monoclonal antibodies specific for the α' -chain (Hessing *et al.*, 1990b). The epitopes of these monoclonal antibodies were identified between the residues Ala⁷³⁸–Arg⁸²⁶ and it was concluded that the C4BP binding site is localized at the *N*-terminus of the α_3 -fragment of C4b (Hessing *et al.*, 1990d). As C4BP binds both to C4b and to the negatively charged heparin via its *N*-terminal 48 kDa chymotryptic tentacles (Hessing *et al.*, 1990a), which have been shown to contain basic residues between SCR 1 and 2 of the α -chains of C4BP (Chung *et al.*, 1985a,b; Janatova *et al.*, 1989), a cluster of seven acidic amino acids between residues 744–752 in C4b might be important for binding.

The activation of C4 is accompanied by a conformational change. C4 treated with nitrogen nucleophiles, chaotropic reagents or frozen-thawed (i.e. C4b-like C4 or iC4) undergoes the same conformational alterations as a result of the cleavage of the internal thiolester bond in the α -chain in C4 after activation. Both iC4 and soluble C4b are able to assemble the fluid-phase C3 convertase, are haemolytically inactive, form a complex with C4BP and are attacked by factor I (von Zabern *et al.*, 1982; von Zabern & Gigli, 1982). Human C4 is very polymorphic (Mauff *et al.*, 1983) and is the product of two coexpressed loci, C4A and C4B. It has long been recognized that the two isotypes of C4 differ dramatically in their efficiency of covalent binding to protein or carbohydrate, despite nearly identical structures (Belt

Fig. 5. Schematic representation of C4 and its cleavage fragments

The C1s and factor I (I) cleavage sites and the putative C4BP binding site are indicated by the arrows. Factor I cleaves two peptide bonds in the α' -chain of C4b, giving rise to the C4d (or α_2) fragment (45 kDa), which is released from the remaining part of the molecule (C4c). C4c consists of two α' -chain fragments: an N-terminal α_3 -fragment (27 kDa), and a C-terminal α_4 -fragment (16 kDa), both linked to the β - and γ -chain via disulphide bridges.

et al., 1984). C4A preferentially forms amide bonds with protein, while C4B binds more efficiently to hydroxyl groups of carbohydrate and to some extent protein (Isenman & Young, 1984; Law *et al.*, 1984). Little is known about the affinity of the interaction of the two isotypes with C4BP and if they are equally susceptible to cleavage by factor I.

BIOSYNTHESIS OF THE COMPLEMENT COMPONENTS C4BP, C4 AND THE VITAMIN K-DEPENDENT PROTEIN S

Protein S is synthesized and secreted by human hepatoma cell lines (Fair & Marlar, 1986), human and bovine endothelial cells (Fair *et al.*, 1986; Stern *et al.*, 1986a), megakaryocytes (Ogura

et al., 1987) and a human erythroleukemia cell line (Schwarz *et al.*, 1985a). In human platelets protein S is contained in the α -granules and is released by thrombin stimulation (Schwarz *et al.*, 1985b, 1986).

Human complement C4 and C4BP are synthesized in the liver, and Saeki *et al.* (1989) reported that the synthesis and secretion of C4BP by human hepatoma cells was enhanced by interleukin-6 and tumour necrosis factor, suggesting that C4BP is an acute phase protein. Funakoshi *et al.* (1988) established that also proline-rich protein (which may be identical to C4BP; see above) is an acute phase reactant and that the plasma concentration of proline-rich protein correlated with known acute phase reactants. Lappin & Whaley (1990) reported on the synthesis of C4BP by cultured human monocytes, which was increased after interferon

stimulation. C4BP has not been detected in human endothelial cells or platelets.

CLINICAL PERSPECTIVES

The importance of protein S as an anticoagulant has been demonstrated by the identification of several patients with heterozygous protein S deficiency associated with recurrent venous thrombosis (Comp & Esmon, 1984; Schwarz *et al.*, 1984; Broekmans *et al.*, 1985; Bertina, 1985; Kamiya *et al.*, 1986) or with abnormalities of bone mineral density (Pan *et al.*, 1990). The incidence of heterozygous protein S deficiency in patients with thrombotic disease is between 5 and 8% (Broekmans, 1985; Bertina, 1985; Gladson *et al.*, 1985). The disorder is probably inherited as an autosomal dominant trait (Schwarz *et al.*, 1984; Broekmans *et al.*, 1985; Bertina, 1985; Mannucci *et al.*, 1986). Recently, molecular alterations in the protein S genes have been reported in families exhibiting protein S deficiency (Ploos van Amstel *et al.*, 1989a,b; Schwarz *et al.*, 1989). Also an acquired protein S deficiency may be a risk factor for the development of thromboembolic complications and may occur in the nephrotic syndrome (Vigano-D'Angelo *et al.*, 1987), in systemic lupus erythematosus (Friedman *et al.*, 1987), during oral anticoagulation (Weiss, 1987; D'Angelo *et al.*, 1988), during pregnancy (Comp *et al.*, 1986; Rose *et al.*, 1986; Malm *et al.*, 1988a), during oral contraception (Boerger *et al.*, 1987; Huisveld *et al.*, 1987; Melissari & Kakkar, 1988), in sickle cell anemia (Francis, 1988), during chemotherapy for breast cancer (Rogers *et al.*, 1988), in liver disease and in disseminated intravascular coagulation (D'Angelo *et al.*, 1988).

There is little documentation of changes in C4BP levels in disease or trauma and so far no deficiency of C4BP has been reported. However, C4BP is hormonally regulated, with levels increasing slightly during pregnancy and in women using oral contraceptives (Comp *et al.*, 1986; Boerger *et al.*, 1987; Malm *et al.*, 1988a; Melissari *et al.*, 1988; Melissari & Kakkar, 1988). Saeki *et al.* (1989) described a moderate increase of C4BP levels in patients with acute pneumonia during acute phase and Barnum & Dahlbäck (1990) found that C4BP is elevated in systemic lupus erythematosus. In addition, Kusunoki *et al.* (1989) reported glomerular deposition of C4BP in patients with idiopathic membranous glomerulonephritis. Low or undetectable levels of protein S-C4BP complexes have been found in term and preterm infants (Malm *et al.*, 1988b; Schwarz *et al.*, 1988; Maolic *et al.*, 1988; Melissari *et al.*, 1988).

A complete deficiency of C4, albeit rare, is invariably correlated with severe immune complex disease and is often fatal (Hauptman *et al.*, 1986). Also the rather common partial deficiency in one of the isotypes of C4 is associated with an increased susceptibility to immune complex disease (Fielder *et al.*, 1983; Rittner *et al.*, 1984; Revielle *et al.*, 1985; Howard *et al.*, 1986). Whether the C4 deficiency is causative or simply serves as a marker for a linked disease susceptibility gene is not known.

CONCLUDING REMARKS

There are remarkably few interactions known between the coagulation and complement cascades. Although experiments *in vitro* have indicated that complement components can be cleaved by coagulation enzymes and vice versa, and while these enzymic cleavages resemble activation *in vivo*, the physiological importance of these observations remains uncertain (Bennet *et al.*, 1987). In this review I have tried to provide some insights into recent developments in a unique link between coagulation and complement pathways, namely the interaction between C4, C4BP

and protein S. Given the great importance of protein links in living systems, there will be no doubt that new important interactions between different cascade systems will be found.

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