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Protein S binding in relation to the subunit composition of human C4b-binding protein

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The human regulatory complement component C4b-binding protein (C4BP) circulates in plasma either as a free protein or in a bimolecular complex with the vitamin K-dependent protein S. The major form of C4BP is composed of 7 identical, disulfide-linked 70 kDa subunits (α-chains), the arrangement of which gives the C4BP molecule a spider-like appearance. Recently, we identified a unique 45 kDa subunit (β-chain) in C4BP. We have now isolated a subpopulation of C4BP, which does not bind protein S. This C4BP species, which had a molecular weight slightly lower than that of the predominant form, was found to lack the β-chain. Another lower molecular weight form of C4BP was also purified. It contained the β-chain and was efficient in binding protein S. Its subunit composition was judged to comprise six α-chains and one β-chain. These results indicate C4BP in plasma to be heterogeneous at a molecular level vis-a-vis subunit composition and/or protein S binding ability and provide support for the concept that the β-chain of C4BP contains the single protein S binding site.

Complement regulation; C4b-binding protein; Blood coagulation; Protein S; Subunit composition; Protein-protein interaction

1. INTRODUCTION

Control of complement activation is mediated by a number of proteins, either circulating in plasma or associated with the cell membranes; see review by Law and Reid [1]. C4b-binding protein (C4BP), a plasma glycoprotein, accelerates the decay of the classical pathway C3-convertase and acts as cofactor to factor I, a serine protease, in the cleavage of C4b [2–6]. Approximately 50% of C4BP circulates in plasma in complex with the anticoagulant vitamin K-dependent protein S [7–9]. Protein S has a high affinity for negatively charged phospholipid membranes, and has been proposed to act as a mobile receptor for C4BP, attaching it to the surface of activated or injured cells [8–11]. In the regulation of the coagulation system, protein S is a cofactor to activated protein C in the degradation of the thrombin-activated factors V and VIII [12,13]. As only free protein S has cofactor activity, C4BP would seem to have a regulatory function in the coagulation system [14,15].

The major form of C4BP (M_r 570 000) in plasma is composed of 7 identical 70 kDa subunits (referred to here as α-chains), each of which contains one C4b-binding site [8,16–19]. Electron microscopy studies showed the subunit arrangement to give the C4BP molecule a spider-like structure [18,19]. The α-chain contains 8 homologous repeats, each approximately 60 amino acid residues long [17,20]. Similar, so-called short consensus repeats (SCR), have been found in other proteins, several of which have C3b or C4b binding properties [21,22]. Recently, we reported the presence of a novel disulfide-linked 45 kDa subunit (referred to here as the β-chain) in C4BP, and experimental data suggested it to contain the protein S binding site [23]. Amino acid and nucleotide sequencing of the β-chain show it to be homologous to the α-chain, containing three SCRs and a 60 amino acid residues long non-homologous carboxy-terminal region (Hillarp and Dahlbäck, submitted).

Two forms of C4BP differing slightly from each other in molecular weight, have been identified [2,4,7,8,16,17,23–25]. They have been designated C4BP-high and C4BP-low, and the difference at the molecular level between the two forms has not been elucidated. We have observed that approximately 80% of C4BP in plasma binds to barium citrate, probably due to interaction with protein S [8]. C4BP-low which was purified from the barium citrate supernatant was unable to bind protein S [8], but is was not investigated whether it contained the β-chain. Recently, we identified another low molecular weight form of C4BP which binds to barium citrate [23]. This form binds

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Abbreviation: SDS-PAGE, sodium dodecyl sylfate-polyacrylamide gel electrophoresis

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protein S and contains the β-subunit. Suzuki and Nishioka [26] have confirmed that C4BP-low, which can be purified from the supernatant after barium citrate adsorption, is unable to bind protein S. They suggested this C4BP form to be composed of truncated α-chains, and proposed the protein S binding site of C4BP to be located in the eighth SCR of the α-chain.

To correlate the protein S binding of C4BP to its subunit composition, we have now examined the C4BP-low form which does not bind protein S, for its β-chain content. We find this form of C4BP to lack the β-chain. This supports the concept that the single protein S binding site on the C4BP molecule is located on the β-chain.

2. EXPERIMENTAL

2.1. *Proteins*

C4BP was purified from fresh citrated human plasma in a 3-step procedure including barium citrate adsorption, polyethylene glycol precipitation and monoclonal antibody affinity chromatography. C4BP obtained from the barium citrate precipitate will be referred to here as C4BP-Ba, and that recovered from the barium citrate adsorbed plasma as C4BP-sup. To fresh citrated human plasma (obtained from the local blood bank) the following proteinase inhibitors were added: phenylmethanesulfonyl fluoride (1 mM), soybean trypsin inhibitor (50 mg/l), benzamidine (10 mM) and 6-amino-*n*-hexanoic acid (10 mM). The purification was performed either on an ice-bath or at +4°C. Barium chloride (1 M, 80 ml/l plasma) was added slowly to the plasma, which was then gently stirred for one hour. The barium citrate precipitate was collected by centrifugation at 10 000×*g* for 30 min. The pellet was dissolved in 0.2 M EDTA, pH 7.4, and insoluble material removed by centrifugation. C4BP, both that recovered in the barium citrate eluate and that remaining in the barium adsorbed plasma, was precipitated by the addition of solid polyethylene glycol 6 000 (5%, w/v). The precipitates were collected by centrifugation at 10 000×*g* for 30 min, dissolved in 50 mM Tris-HCl, 0.15 M NaCl, pH 8.0, containing 0.1% Tween 20, and dialyzed against the same buffer. Finally, C4BP was purified using a monoclonal antibody against C4BP (M104) coupled to Affi-gel 10 (BioRad). After washing of the column, first with dialysis buffer and then with 1 M NaCl containing 0.1% Tween 20, the bound C4BP was eluted with 0.1 M glycine, 0.5 M NaCl, pH 2.2. The eluate was immediately neutralized with Tris-base and dialyzed against 50 mM Tris-HCl, 0.15 M NaCl, pH 8.0. The solution was concentrated by ultrafiltration using a XM-50 membrane (Amicon) and stored at −70°C until used. The C4BP concentration was estimated from the absorbance at 280 nm using an extinction coefficient (1%, 1 cm) of 14.1 [27]. Human protein S was purified as previously described [28] and ^{125}I-labelled using Iodobeads (Pierce Chemical Co.) according to the manufacturer's instructions.

2.2. *Antisera*

Polyclonal antisera against the individual C4BP subunits were raised in rabbits. C4BP was reduced and alkylated and the α- and β-chains separated on a large 5–15% SDS-PAGE (100 μg protein per well). After electrophoresis and staining of the gel (1% Coomassie in H_2O for 30 min followed by destaining in H_2O), the two subunits were cut out, and the gel sections homogenized with a glass hand-homogenizer in 1 ml 0.15 M NaCl. The homogenates were mixed with 1 ml complete Freunds adjuvant and used to immunize rabbits. The antiserum against the α-chain only recognized the reduced and alkylated α-chain. Presumably due to a small amount of fragmented α-chains in the β-chain preparation, the antiserum against the β-chain reacted weakly with reduced and alkylated α-chain, though this did not adversely affect the interpretation of results. Both antisera reacted poorly with native C4BP.

The monoclonal antibody (M 104), used for affinity chromatography purification of C4BP and immunoblotting, recognized an epitope in the N-terminal part of the α-chain (Hillarp and Dahlbäck, to be published elsewhere).

2.3. *Electrophoretic and blotting techniques*

SDS-PAGE was performed as described previously [29], using Maizel's buffer system [30]. Two different linear gradient gels were used, 3–5% for intact C4BP and 5–15% for separation of the two subunits under reducing conditions. In the 'sequential' (unreduced/reduced) SDS-PAGE analysis, unreduced C4BP was applied to the first gel (3–5%), and the stained bands were cut out and applied to the second gel (5–15%) after reduction. The technique has been described in more detail elsewhere [23]. The gels were stained with Coomassie brilliant blue R-250, and the relative amounts of the observed isotypes of C4BP were estimated by laser densitometry using an Ultroscan 2202 apparatus (LKB). Immuno- and ligand blotting was performed with Immobilon (Millipore) as the membrane, using procedures previously described [31].

3. RESULTS AND DISCUSSION

The C4BP that was purified from the supernatant plasma after barium adsorption, C4BP-sup, and that recovered from the barium citrate precipitate, C4BP-Ba, were both analyzed by means of low-percentage SDS-PAGE (fig.1). The proteins were either stained with Coomassie or transferred to Immobilon membranes for immunoblotting or ^{125}I-protein S ligand blotting. Both preparations, C4BP-Ba (lane A) and C4BP-sup (lane B), appeared as doublets and their high

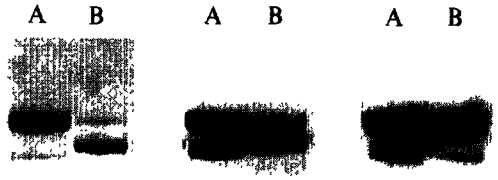

Fig.1. Different C4BP forms demonstrated with SDS-PAGE, immunoblotting and ^{125}I-protein S ligand blotting. The two different C4BP preparations C4BP-Ba (A) and C4BP-sup (B) were run on a 3–5% gradient SDS-PAGE. The gels were either stained with Coomassie brilliant blue, or the protein was transferred to Immobilon membranes and analyzed with a monoclonal antibody (M104) directed against the α-chain of C4BP (immunoblot). The ability of C4BP to bind protein S was tested using radiolabelled protein S (ligand blot). Approximately 10 μg of protein was applied to each lane.

molecular weight forms migrated to identical positions, and both were able to bind protein S. The two low molecular weight forms appeared to differ slightly from each other in molecular weight. The 'low' form in the C4BP-sup preparation had a slightly higher molecular weight than the corresponding form in C4BP-Ba. ^{125}I-labelled protein S ligand blotting clearly showed the 'low' form in the C4BP-Ba preparation to be able to bind protein S (lane A), whereas no protein S binding was observed to the predominant 'low' form in the C4BP-sup preparation (lane B). The weak signal obtained on ligand blotting, in the lower molecular weight region in lane B represents a molecular weight corresponding to the 'low' form seen in lane A. This suggests the presence of trace amounts of C4BP-low with intact protein S binding remaining in the supernatant after barium adsorption. This trace component was not visible on Coomassie stained gels or on the immunoblotting. The proportions of the Coomassie stained bands in the two preparations were estimated by densitometric scanning of the stained gels. The 'high' and 'low' forms constituted 85% and 15%, respectively, in the C4BP-Ba preparation, whereas in the C4BP-sup preparation corresponding values were 20% and 80%. Approximately 80% of C4BP in plasma binds to the barium citrate precipitate, leaving the remaining 20% in the supernatant [8]. From these data it can be estimated that 70–75% of plasma C4BP is of the 'high' form, and that the two 'low' forms each comprise 10–15%.

To ascertain whether protein S binding was correlated with subunit composition, sequential SDS-PAGE was performed (fig.2). In this analysis, the protein bands seen in the low percentage gel were cut out, reduced, and applied to high percentage SDS-PAGE, and subsequently immunoblotted using a polyclonal antiserum that reacted both with α- and β-chains. The β-chain was clearly demonstrated to be present in the three protein S-binding C4BP forms. However, the 'low' form in the C4BP-sup preparation, which did not bind protein S, contained no detectable β-chain. Also of note is the finding that the α-chains in the 4 lanes migrated to identical positions, indicating that they were identical in molecular weight. Another polyclonal antiserum, specifically recognizing the reduced and alkylated α-chain, did not react with the 45 kDa band in any of the lanes, showing that this band indeed represented a distinct molecular species, and not a degradation product of the α-chains (results not shown). These results indicate the presence in plasma of a subpopulation of C4BP lacking β-chain and protein S binding ability, and constitute support for the concept that the single protein S binding site on C4BP is located on the β-chain.

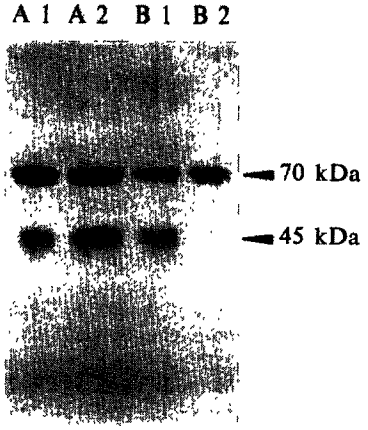

Fig.2. Presence of β-chain in different C4BP forms demonstrated by immunoblotting after 'sequential' SDS-PAGE. C4BP-Ba (A) and C4BP-sup (B) were applied to a 3–5% gradient SDS-PAGE. The 'high' (1) and 'low' (2) bands were cut out, reduced and applied to a 5–15% gradient SDS-PAGE. After electrophoresis, the separate C4BP chains were transferred to Immobilon and analyzed with an antiserum that recognized both chains. Approximately 50 μg of protein was applied to the first gel.

Suzuki and Nishioka [26] described a monoclonal antibody (MFbp 16) that failed to recognize C4BP-low purified from the barium citrate supernatant. However, it reacted with C4BP-low purified from the barium citrate precipitate. This suggests the C4BP-low remaining in the barium citrate supernatant to lack an antigenic structure which is present in the other C4BP forms. Suzuki and Nishioka concluded that the protein S binding site as well as the epitope for the monoclonal antibody was located in the carboxy-terminal of the α-chain, and that the difference between the 'high' and 'low' molecular weight forms of C4BP resides in the α-chain. However, our present results suggest an alternative explanation, i.e. that the MFbp 16 binds to the β-chain of C4BP. Suzuki and Nishioka suggested the α-chain of C4BP-low, purified from the barium supernatant, to be truncated. However, using a high resolution SDS-PAGE system, we found no difference in molecular weight between the α-chains of the different C4BP forms.

We propose that the molecular weight differences between the various C4BP forms may be explained by differences in subunit composition. The results of several approaches indicate the major C4BP form in plasma to contain 7 α-chains. High resolution electron microscopy findings suggest C4BP to contain 7 extended C4b-binding tentacles (each corresponding to one α-chain) and one smaller subunit, which bind protein S (corresponding to the β-chain) [19]. When chymotrypsin digestion of C4BP was monitored on SDS-PAGE, seven 48 kDa α-chain fragments were found to be released [18]. Perkins et al. [27], using X-ray scattering, reported the number of subunits to be 7.4 ± 1. These results are compatible with our conclusion that the major species of C4BP contains 7 α-chains and one β-

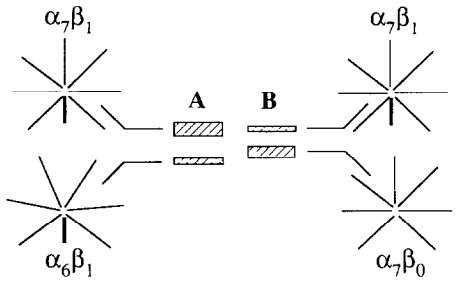

Fig.3. Schematic model of subunit composition of different C4BP forms. The SDS-PAGE pattern of C4BP-Ba (A) and C4BP-sup (B) from fig.1 and the proposed subunit composition of each form are shown.

chain [23]. In the chymotrypsin digestion study [18], 7 α-chain fragments were estimated to be released during digestion when the upper band in the C4BP doublet was used as starting point; starting from the lower band, the number of α-chains was estimated to be 6. These data, taken together with those of the present study, suggest the following conclusions regarding subunit composition: The 'high' forms of C4BP, which are identical in the two preparations used here, contain 7 α-chains and one β-chain; the C4BP-low form that is obtained from the barium citrate supernatant and which lacks the protein S binding site, contains 7 α-chains but no β-chain; finally, the protein S binding C4BP-low that is obtained from the barium precipitate, contains 6 α-chains and one β-chain. This interpretation is schematically illustrated in fig.3. It is probable that additional species of C4BP exist in plasma, which differs in subunit composition (e.g. 8 α-chains with or without one β-chain).

The β-chain and the protein S binding site share many characteristics [23,31]. Both are located close to the central core of C4BP, both are susceptible to chymotryptic digestion but can be protected by bound protein S, and both are present in one copy per C4BP molecule. The present findings provide further support for the conclusion that the single protein S binding site of C4BP is located on the β-chain.

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