Expression and characterization of a recombinant C4b-binding protein lacking the β -chain

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C4b-binding protein (C4BP) is a high-molecular-mass glycoprotein which contains binding sites for complement component C4b, anti-coagulant vitamin K-dependent protein S and serum amyloid P component (SAP). The major form of C4BP in plasma is composed of seven identical α -chains and a single β -chain. We have expressed full-length cDNA for the α -chain in a eukaryotic expression system and characterized functional properties of non- β -chain-containing C4BP. During synthesis, recombinant α -chains polymerized into two different high-molecular-mass C4BP forms which were composed of seven or eight α -chains. Recombinant C4BP bound C4(H₂O) (used instead of C4b) equally as well as native C4BP, functioned equally as well as factor I cofactor in the degradation of C4(H₂O) and bound to SAP. In

contrast, the recombinant C4BP did not bind protein S and therefore did not inhibit the ability of protein S to function as a cofactor to activated protein C. Tunicamycin treatment of the transfected cells prevented N-linked glycosylation, but did not affect polymerization of the α -chains into a high-molecular-mass C4BP. The non-glycosylated C4BP had comparable properties to glycosylated C4BP in several functional assays. These results demonstrate polymerization of C4BP α -chains to be independent both of the β -chain and of the N-linked carbohydrates. Moreover, N-linked carbohydrates and the β -chain were neither required for the ability of C4BP to bind C4b and to function as factor I cofactor nor for the interaction with SAP.

INTRODUCTION

Human C4b-binding protein (C4BP) (M_r 570 000) is composed of seven α -chains (M_r 70 000) and one β -chain (M_r 45 000) [1–3]. Both chains contain internally homologous modules, denoted short consensus repeats (SCRs), Suchi modules or complement control protein modules (CCP). Each SCR is approximately 60 amino acids long and has a framework of conserved amino acid residues, including four cysteines which form internal disulphide bridges. The α -chain is composed of eight SCRs [4], and the β -chain three [5]. Each α -chain has four potential N-linked glycosylation sites, located at Asn-173, Asn-240, Asn-458 and Asn-480, but amino acid sequencing has shown position 240 not to be glycosylated [4]. The β -chain contains five sites for N-linked glycosylation [5]. The spider-like shape of C4BP is the result of polymerization and disulphide bridging (involving cysteines in non-repeated C-terminal parts) of multiple chains during synthesis [2]. In human plasma, the most common form of C4BP is composed of seven α -chains and one β -chain, but variants with seven α -chains and no β -chain or with six α -chains and one β -chain also exist [6].

C4BP contains multiple binding sites for the complement component C4b, one on each α -chain [1,2]. It regulates the classical pathway C3-convertase (C4b,C2a) by accelerating the decay of C2a from the complex, and by functioning as a cofactor to factor I in the degradation of C4b [1,7–10]. In human plasma, essentially all β -chain-containing C4BP molecules are bound to vitamin K-dependent protein S, the interaction being bimolecular, non-covalent and of high affinity [11–15]. Free protein S (approximately 30 % of plasma protein S) functions as a cofactor to activated protein C (APC) in its degradation of coagulation

factors Va and VIIIa, whereas binding of protein S to C4BP is associated with loss of its anti-coagulant properties [16,17]. The protein S-binding site is located within the three SCR modules of the β -chain [3,18,19] and protein S binding to C4BP does not interfere with the capacity of C4BP to bind C4b [20]. C4BP also binds serum amyloid P component (SAP), which is a member of the pentraxin family of proteins [13,21,22]. SAP is homologous to the acute-phase protein C-reactive protein (CRP) [23,24]. Its biological role is incompletely understood, but it has been suggested to be involved in regulation of the classical complement pathway [25,26]. The complex between C4BP and SAP has a 1:1 stoichiometry and C4BP bound to SAP binds protein S equally as well as free C4BP [13]. Binding of SAP to C4BP inhibits the factor I cofactor function of C4BP [27].

To elucidate whether the α -chain sequence contained all the information required for synthesis and polymerization of subunits, recombinant C4BP α -chains were expressed in eukaryotic cells. Recombinant C4BP lacking N-linked carbohydrates was synthesized during treatment with tunicamycin, an inhibitor of the dolichol phosphate-mediated glycosylation [28]. The complement regulatory properties of recombinant C4BP, as well as its ability to bind SAP and protein S, were characterized.

MATERIAL AND METHODS

Proteins

The following proteins were purified from human plasma according to respective references: C4BP [12,29], protein S [30,31], factor I [32], C4 [33] and SAP [24]. Bovine protein S [34] and protein C [35] were purified from bovine plasma as described.

Abbreviations used: C4BP, C4b-binding protein; CRP, C-reactive protein; SCR, short consensus repeat; SAP, serum amyloid P component; TBS, Tris-buffered saline; TE, Tris-EDTA; APC, activated protein C.

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Figure 1 Characterization of recombinant C4BP

Plasma-derived C4BP (lane 1), recombinant C4BP containing no β -chain (lane 2) and recombinant C4BP containing neither β -chain nor N-linked carbohydrates (lane 3) were analysed on SDS/PAGE. (a) and (b), unreduced and reduced proteins respectively, on silver-stained gels; (c) reduced proteins subjected to Western blotting developed with a polyclonal antiserum against C4BP.

Protein C was activated by thrombin and purified by SP-Sephacrose chromatography [36]. C4(H₂O), which was used instead of C4b in the factor I-mediated degradation assay and in the C4BP ligand-binding studies, was prepared by repeated freezing and thawing of purified C4 [37,38].

SAP was coupled to CNBr-Sephacrose (0.5 mg/ml gel matrix) and stored in Tris-buffered saline (TBS; 50 mM Tris/HCl, 150 mM NaCl, pH 7.5) containing 1 mM CaCl₂ and 0.02% NaN₃. The monoclonal antibody 104 (MK 104) against C4BP, was coupled to Affi-Gel 10 (6 mg/ml) according to the manufacturer's instructions. MK 104 reacts with an epitope which is located close to, or in, the third SCR of the α -chain (A. Hillarp and B. Dahlbäck, unpublished work). The polyclonal antiserum which was used in the ELISA was directed against the N-terminal part of the α -chain [39].

Concentrations of purified proteins were estimated by measuring absorbance at 280 nm. The following absorption coefficients ($\epsilon_{1\%}^{1\text{cm}}$) were used: intact C4BP, 14.1 [40]; human protein S, 9.5 [41]; bovine protein S, 10.0 [42]; protein C, 14.5 [43]; factor I, 14.3 [32]; C4, 8.3 [44] and SAP, 18.2 [45]. For the recombinant C4BP, the same absorption coefficient as for intact C4BP was used.

Protein S, intact and recombinant C4BP, and C4(H₂O) were radiolabelled with ¹²⁵I using IODO-BEADS (Bio-Rad) and purified as described previously [39].

Cloning procedure

A full-length α -chain cDNA clone (isolated from a λ gt-11 library from Clontech) was cleaved with *Eco*RI and *Xba*I. Since the α -chain cDNA contains an internal *Eco*RI site, this resulted in two α -chain fragments, one being 1.6 kb and the other 0.5 kb. Both fragments were isolated and the largest was ligated to *Eco*RI/*Xba*I-cleaved pBluescript^{RII} vector (Stratagene). The resulting construct was cut with *Eco*RI and ligated to the 0.5 kb α -chain fragment. The insert was recovered after cleavage with *Hind*III and *Not*I and then ligated to pcDNA I Neo vector (Invitrogen), a eukaryotic expression vector which renders the transfected cells neomycin resistant.

Cell line and culture conditions

AV 12-664 cells (a Syrian hamster cell line) were grown in Dulbecco's modified Eagle's medium (GIBCO), supplemented with fetal-calf serum (10%, v/v), penicillin (50 IU/ml), streptomycin (50 μ g/ml) and glutamine (4 mM), at 37 °C in 5% CO₂.

DNA transfection

AV 12-664 cells were grown to 50% confluency in 100-mm-diam. Petri dishes under conditions described above. For every Petri dish to be transfected, 10 μ g of DNA was mixed with 215 μ l of TE (10 mM Tris, 1 mM EDTA, pH 8.0) and 25 μ l of 2.5 M CaCl₂ and incubated for 10 min at room temperature. Aliquots (250 μ l) of 2 $\times$ Hepes-buffered saline (0.274 M NaCl, 0.01 M KCl, 3 mM Na₂HPO₄, 12 mM dextrose, 42 mM Hepes) were then added and the DNA solution incubated for another 20 min. After changing to fresh cell medium, the DNA solution was added and the cells were incubated overnight at 37 °C. The DNA-containing medium was replaced with fresh medium and the cells were again incubated at 37 °C. After 48 h, the cells were split 1:8 and allowed to grow in selection medium (400 μ g/ml G-418 sulphate; Geneticin, GIBCO). When the transfected cells reached confluency, they were checked for C4BP expression, using an ELISA.

Inhibition of N-linked glycosylation

Transfected cells were grown to confluency in G-418-containing medium. The selection medium was then replaced with medium containing both G-418 and 10 μ g/ml tunicamycin (Sigma). After 24 h, this medium was thrown away and replaced with new medium containing G-418 and tunicamycin. Medium was then collected every third day until the cells died.

Determination of C4BP concentration by ELISA

An ELISA, with a polyclonal antibody against C4BP as primary antibody and a biotinylated monoclonal antibody (MK 104) against the α -chain as secondary antibody, was performed essentially as described previously [15]. The concentration of recombinant C4BP in the medium was calculated against a standard of C4BP purified from plasma.

Purification of the recombinant protein

Medium from the transfected cells was collected every third day and stored at -20 °C until required for purification. It was centrifuged for 30 min at 10000 *g*, and mixed with MK 104 coupled to Affi-Gel 10 (50 ml), overnight. The gel was packed in a column (1.5 cm $\times$ 40 cm), equilibrated with TBS, pH 7.5, and washed with 50 mM Tris/HCl, 1.0 M NaCl, pH 7.5. The recombinant protein was eluted with 3 M guanidine hydrochloride and dialysed against TBS.

Electrophoretic and blotting techniques

Plasma-derived and recombinant C4BP were run on 5% (unreduced proteins) or 5–15% (reduced proteins) PAGE in the presence of SDS, as previously described [46,47]. The protein was then visualized with silver staining [48] or transferred to nitro-

cellulose membrane and detected using the polyclonal antisera described above [39].

Ligand-binding assays

Protein S-binding assays [18] and C4(H₂O)-binding assays [27] were performed as described previously.

C4(H₂O)-degradation assay

The C4(H₂O)-degradation assay was performed essentially as described previously [20,27].

SAP–Sephacrose binding

Samples (100 μ g) of normal or recombinant C4BP were incubated with 2 ml of SAP–Sephacrose (0.5 mg/ml) in TBS containing 2 mM CaCl₂ and 0.1 % BSA overnight at 4 °C. The Sepharose was then packed in a column, washed with TBS containing 2 mM CaCl₂ and the bound C4BP was eluted with 50 mM Tris/HCl, 1 M NaCl and 2 mM EDTA. The same ELISA as described above was used to quantify the amount of C4BP in the different fractions.

Clotting assay

Human plasma (100 μ l) was mixed with 100 μ l of activated partial thromboplastin time (APTT) reagent (Organon Teknika Corp.) for 5 min. Samples (100 μ l) of a mixture containing bovine protein S (1 μ g/ml), and bovine APC (1 μ g/ml) in 20 mM Tris/HCl containing 50 mM NaCl, 0.1 % BSA and 30 mM CaCl₂ were added and the clotting time measured. Protein S was incubated with recombinant C4BP (0–100 μ g/ml) or plasma purified C4BP (0–100 μ g/ml) in 2 mM CaCl₂ for 30 min at 37 °C before addition to the protein C solution.

RESULTS

Assembly of recombinant C4BP α -chains into a high-molecular-mass protein

From 1 l of medium, approximately 5 mg of recombinant C4BP was obtained after purification on the MK 104 column. On SDS/PAGE, both plasma-derived C4BP and its recombinant counterpart demonstrated two high-molecular-mass bands (Figure 1a). In plasma C4BP, these bands correspond to molecules containing seven α -chains and one β -chain (upper band) and six α -chains and one β -chain or seven α -chains and no β -chain (lower band) [6]. The positions on the gels of the major band of recombinant C4BP indicated that the majority of the recombinant molecules contain seven α -chains. A minor recombinant C4BP species with slightly higher molecular mass presumably represented molecules containing eight α -chains. After reduction, the recombinant α -chain had a slightly lower relative molecular mass than the α -chain of plasma C4BP (approximately 70000), suggesting the amount of carbohydrates in the two C4BP forms were similar but not identical (Figure 1b). The α -chain of the C4BP which was obtained from the tunicamycin-treated cells had a lower relative molecular mass (approximately 62000) and cleavage with endoglycosidase F did not further reduce the molecular mass (results not shown). Figure 1(b) also shows the position of the β -chain from plasma C4BP at M_r 45000. The different C4BP forms were also analysed by Western blotting, which confirmed that the silver-stained protein bands represented C4BP (Figure 1c).

Figure 2 C4b binding

Binding of C4b to intact C4BP and to the recombinant C4BP forms was measured as described in the Materials and methods section. (a) Direct C4b binding; (b) competition for C4b binding. Each point represents the mean value ($\pm$ S.D.) from three different experiments. $\bullet$, intact C4BP; $\blacksquare$, recombinant C4BP; $\square$, recombinant C4BP from tunicamycin-treated cells.

Figure 3 C4(H₂O)-degradation assay

The capacity of intact and recombinant C4BP to function as a cofactor to factor I were compared. 1, intact C4BP; 2, recombinant C4BP; 3, recombinant C4BP from tunicamycin-treated cells; 4, control without C4BP or recombinants. A and B represent experiments with 100 and 150 nM of the C4BP forms respectively.

Functional properties of recombinant C4BP

The recombinant C4BP forms were analysed for their C4(H₂O)-binding capacity, both in a direct binding assay (Figure 2a) and in a competitive assay (Figure 2b). In the first assay, C4(H₂O) was immobilized in microtitre wells and binding of the different radiolabelled C4BP forms tested. In the second assay, the different unlabelled forms of C4BP were allowed to compete with ¹²⁵I-labelled plasma-derived C4BP for binding to immobilized C4(H₂O). The two recombinant C4BP forms bound C4(H₂O) almost as well as plasma-derived C4BP did, suggesting that the N-linked carbohydrates are not involved in the C4BP–C4b interaction. C4(H₂O) bound to C4BP is a substrate for the enzyme factor I, and the two peptide-bond cleavages of the α -chain result in three end products, C4d and two smaller fragments [10,37,38]. Both the carbohydrate-containing and the non-glycosylated recombinant C4BP forms were found to express factor I-cofactor activity in a C4(H₂O)-degradation assay (Figure 3).

Figure 4 C4BP binding to SAP-Sepharose

Samples (100 μ g) of native C4BP or the two recombinants were passed through a 2 ml SAP-Sepharose column. The column was washed with TBS containing 2 mM CaCl_2 and the bound C4BP eluted with 0.5 ml portions of 50 mM Tris/HCl/1 M NaCl/2 mM EDTA. Each fraction contained 0.25 ml. An ELISA was performed to measure the C4BP concentrations.

This suggested that neither the β -chain nor the N-linked carbohydrates of the α -chain are required for expression of factor I-cofactor activity.

Binding of the two recombinant C4BP forms to SAP-Sepharose was compared with that of plasma-derived C4BP. All three forms of C4BP were found to bind to the SAP-Sepharose in the presence of 2 mM CaCl_2 (Figure 4). This demonstrates that the β -chain is not involved in SAP binding and that the N-linked carbohydrate is not a prerequisite for SAP binding to C4BP.

Figure 5 Protein S binding

Binding of protein S to intact C4BP and to the recombinants was measured as described in the Materials and methods section. (a) Direct protein S binding; (b) competition for protein S binding. Each point represents the mean value ($\pm$ S.D.) from three different experiments. ●, intact C4BP; ■, recombinant C4BP; □, recombinant C4BP from tunicamycin-treated cells.

Figure 6 Clotting assay

A clotting assay was performed as described in the Material and methods section. 100% on the y-axis corresponds to approximately 90 s. Control experiments performed without bovine APC gave a clotting time of approximately 50 s. Each point represents the mean value from three different experiments. ●, intact C4BP; ■, recombinant C4BP; □, recombinant C4BP from tunicamycin-treated cells.

Two different protein S-binding assays were performed to investigate whether the α -chains contain one or more low-affinity binding sites for protein S. However, neither a direct binding assay nor a competition assay demonstrated any interaction between protein S and recombinant C4BP lacking the β -chain (Figure 5). The observation that recombinant C4BP did not bind protein S was consistent with results of a functional coagulation-based assay. Plasma-derived C4BP inhibited the APC-cofactor activity of protein S, whereas the two recombinant C4BP forms had no effect on the anti-coagulant function of protein S (Figure 6).

DISCUSSION

Many complement and non-complement proteins contain one or more SCRs [49–54]. Several proteins are mainly composed of multiple SCRs (such as C4BP, factor H, complement receptors 1 and 2, membrane cofactor protein and decay accelerating factor), while others contain other types of modules in addition to the SCRs, e.g. C1r, C1s and factor B. C4BP is unique among the SCR-containing proteins in containing two different SCR-containing subunits, the α - and the β -chain.

C4BP in plasma is heterogeneous in subunit composition. The major form (70 % of plasma C4BP) 'C4BP-high', contains seven α -chains and one β -chain, whereas 'C4BP-low' is composed of either six α -chains and one β -chain or seven α -chains and no β -chain [6]. The molecular mechanisms which are involved in the polymerization process, i.e. the assembly of the multiple α -chains into the high-molecular-mass C4BP molecule, are completely unknown. It is in this respect noteworthy that the majority of recombinant C4BP contained seven α -chains, suggesting this to be a preferred number of chains. As one of the naturally occurring forms of C4BP in plasma lacks the β -chain, it has been proposed that the β -chain is not required for polymerization of the C4BP α -chains. However, the possibility that the β -chain has been cleaved off after synthesis has not been ruled out. The present results clearly demonstrate that the α -chains contain information required for the assembly of six to eight α -chains into a high-molecular-mass, functionally active C4BP. Moreover, the polymerization process is obviously not dependent on the presence of N-linked carbohydrate side-chains.

Recently, we demonstrated that a truncated recombinant β -chain, composed of the three SCR units, bound protein S with only slightly lower affinity than intact C4BP [18]. This suggested that the protein S-binding site was located in the β -chain but did not exclude the possibility that the α -chains were also involved in the protein S binding. Other investigators have suggested that each of the α -chains contains a protein S-binding site and that the multiple closely spaced α -chains create a high-affinity protein S-binding site [55]. The present data rules out this possibility as there was no detectable interaction between protein S and recombinant C4BP composed of only α -chains.

Electron microscopy suggested that the C4b-binding site was located in the N-terminal part of the α -chain [2], but a binding site in SCRs 6 and 7 has also been proposed [56,57]. Even though the β -chain is not directly involved in the C4b-binding and factor I-cofactor function, the β -chain may have an indirect complement-regulating function *in vivo* because it binds protein S. The C4BP–C4b–protein S–SAP complex binds to negatively charged phospholipid membranes through the vitamin K-dependent part of protein S and this may be instrumental in controlling the complement system on phospholipid surfaces [13].

The recombinant C4BP from the tunicamycin-treated cells bound C4(H₂O) with affinity comparable to recombinant C4BP from non-treated cells and to C4BP from plasma. All three C4BP forms also functioned as factor I cofactors in the degradation of C4(H₂O). This demonstrated that the N-linked carbohydrates were not important for these functions. As discussed, C4BP also interacts with SAP, which is a glycoprotein with lectin-like properties. Relatively little is known about the function and purpose of this interaction but it is known that binding to C4BP prevents the aggregation of SAP and it has been suggested that C4BP has a SAP-transporter function. Recently our laboratory demonstrated that SAP down-regulates the factor I-cofactor function of C4BP [27]. The SAP-binding site on C4BP is not yet known and we have now shown that neither the β -chain nor the N-linked carbohydrates are required for the C4BP–SAP in-

teraction. This was surprising, since SAP is known to bind to specific monosaccharides [58]. The fact that absence of the N-linked carbohydrate moiety did not preclude C4BP binding to SAP–Sepharose calls for a more detailed characterization of the binding of SAP to C4BP.

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