Localization of a Hydrophobic Binding Site for Anticoagulant Protein S on the β-Chain of Complement Regulator C4b-binding Protein*

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Joanna H. Webb‡, Bruno O. Villoutreix§, Björn Dahlbäck¶, and Anna M. Blom‡

From the 2Division of Clinical Chemistry, Department of Laboratory Medicine, Lund University, University Hospital Malmö, Malmö S-205 02, Sweden and §INSERM U428, University of Paris V, 4 Avenue de L’Observatoire, 75006 Paris, France

C4b-binding protein (C4BP) is a plasma glycoprotein involved in regulation of the complement system. C4BP consists of seven α-chains and one unique β-chain, all constructed of repeating complement control protein (CCP) modules. The β-chain, made up of three CCPs, binds tightly to vitamin K-dependent protein S, a cofactor to anticoagulant activated protein C. When bound to C4BP, protein S loses its activated protein C cofactor function. In this study, we have mutated potentially important amino acids located at the surface of CCP1 of the β-chain to probe the protein S-CCP interaction. The substitutions were designed after analysis of a homology-based three-dimensional structure of the β-chain and were L27T/F45Q, I16S/V18S, V31T/I33N, I16S/V18S/V31T/I33N, L38S/V39S and K41E/K42E. The mutants were expressed in a prokaryotic system, purified using an N-terminal His-tag, refolded using an osido-shuffling system, and tested in several assays for their ability to bind protein S. Our data define Ile16, Val18, Val31, and Ile33 as crucial for protein S binding, with secondary effects from Leu30 and Val39. In addition, Lys31 and Lys32 contribute slightly to the interaction. Our results further confirm that surface hydrophobicity analysis may be used to identify ligand recognition sites.

C4b-binding protein (C4BP)1 is a regulator of the classical pathway of complement that also plays a role in the anticoagulant protein C pathway (1, 2). In the complement cascade, C4BP acts as a cofactor to factor I in the degradation of C4b (3). In addition, C4BP inhibits the formation and accelerates the decay of the classical C3 convertase pathway, i.e. the C4b2a complex (4). C4BP is a large plasma glycoprotein of 570 kDa consisting, for the main isoform, of seven α-chains and one β-chain, which are held together by a central core. Both the α- and β-chains are composed of multiple complement control protein (CCP) domains. A CCP domain is ~60 residues long and contains two disulfide bridges and a central antiparallel β-sheet (5). CCP domains are present in numerous proteins both within and outside the complement system (6). Some CCP-containing molecules have been investigated by NMR or x-ray crystallography, e.g. vaccinia virus complement control protein (7), β2-glycoprotein I (8), and CD46 (9). The knowledge of the three-dimensional structure of these domains enabled us to construct a homology-based model of C4BP.

The α-chains of C4BP, consisting of eight CCPs, bind complement protein C4b (1). A key recognition site for C4b on C4BP has been recently ascribed to a cluster of positively charged amino acids on the interface of CCP1-CCP2 of the α-chain (10). The unique C4BP β-chain, essentially made up of three CCPs, binds protein S, an anticoagulant molecule that acts mainly as cofactor to activated protein C in the degradation of coagulation factors Va and VIIIa. C4BP and protein S form a high affinity, noncovalent complex with a 1:1 molecular ratio, which is greatly enhanced by calcium (11). The C-terminal sex hormone globulin binding-like region of protein S is involved in the interaction with C4BP (12, 13). This domain in protein S is expected to have calcium-binding site(s), whereas it has never been shown or proposed that C4BP interacts with any metal ion (12, 13).

In plasma, ~70% of protein S is in complex with C4BP. Only free protein S functions as an activated protein C cofactor (11). In contrast, C4BP in complex with protein S can still exert its regulatory functions on the complement system (14). C4BP regulates the plasma availability of free protein S since the concentration of free protein S represents the molar excess of protein S over C4BP (15). The biological importance of the protein S-C4BP interaction is emphasized by the fact that only the concentration of free protein S can clearly be linked to thrombotic risk in patients suffering from protein S deficiencies (16). The physiological purpose of the interaction between C4BP and protein S is not yet fully understood. However, protein S, being a vitamin K-dependent protein, has a very high affinity for negatively charged phospholipids; and therefore, it could localize C4BP to surfaces where such phospholipids are exposed (17, 18).

Our group has shown that the β-chain of C4BP (19) contains the protein S-binding site (20); more precisely, CCP1 is required for binding to occur (21). It was recently suggested by van de Poel et al. (22, 23) that CCP2 also contributes to the binding to a small extent. van de Poel et al. used a different approach to study the binding. In their investigation, chimeras were constructed composed of individual CCP modules (or the different CCPs in combination) fused to the N-terminus of a modified tissue plasminogen activator. They found that CCP2 increased the affinity for protein S ~5-fold.
Fernández and Griffin (24) used synthetic peptides to probe the protein S-C4BP interaction and suggested residues 31–45 on C4BP to be important for protein S binding. This hypothesis was supported in a subsequent report (25), where it was found that preincubation of C4BP with monoclonal antibody 6F6 (directed against a region located nearby residues 31–45) inhibited the C4BP and protein S interaction. It was then concluded that the antibody interfered with the interaction most likely because of steric hindrance (25).

We have previously shown that binding of protein S to C4BP varied only to a small extent with the concentration of salt, in a manner implying a significant contribution from hydrophobic interactions, with minor roles played by electrostatic forces (26). These experiments were the first to confirm the hypothesis that a hydrophobic cluster at the surface of CCP1 may be the main binding site for protein S (27).

In this study, we have mutated potentially important amino acids located in this hydrophobic cluster on C4BP CCP1. The mutations were chosen based on the homology-based, computer-generated three-dimensional structure of the C4BP β-chain and previous experimental data. In addition, two lysesins that could be responsible for the slight electrostatic component seen in the interaction were also mutated and studied. Wild-type CCP1 and CCP2 of the β-chain and the mutants were expressed in a prokaryotic system, purified on a nickel-Sepharose column, and refolded. All recombinant proteins were then tested for their ability to bind protein S, as determined by their accessibility to the antibody (directed against a region located nearby residues 31–45) in an indirect enzyme-linked immunosorbent assay. A decrease in antibody binding indicated that the antibody interfered with the interaction most likely because of steric hindrance (25).

Structural Analysis of Recombinant Proteins

**Binding of Monoclonal Antibodies**—All recombinant proteins were tested for their ability to bind to seven monoclonal antibodies raised against the recombinant wild-type β-chain using a standard procedure. Microtiter plates were coated with 50 μg of purified antibody at 10 μg/ml in carbonate buffer (pH 9.6) at 4 °C overnight. The plate was then washed three times with wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (w/v) Tween 20, and 2 mM CaCl$_2$), quenched in wash buffer with 3% fish gelatin for 2 h, and washed as described above. Increasing amounts of recombinant β-chain (wild-type and mutant) plus trace amounts of $^{129}$I-labeled recombinant wild-type β-chain were added for 1 h at room temperature. The plate was then washed five times, and bound radioactivity was measured in a γ-counter.

**Gel Filtration**—All recombinant proteins were analyzed by gel filtration (Superox 12 HR 10/30, Amersham Pharmacia Biotech). Fifty μg of each protein was applied to the column, previously equilibrated with Tris-buffered saline (pH 8.0). The flow rate used was 0.5 ml/min. During each run, the absorbance of the eluate at 280 and 214 nm was continuously monitored.

**Mass Spectrometry**—Mass spectrometry, carried out at the Protein Analysis Center of the Karolinska Institute (Stockholm, Sweden), was performed on all recombinant proteins using quadrupole time-of-flight (Q-TOF) mass spectrometry (Micromass) (28). The proteins were dialyzed against 2% HAc. Samples were subsequently analyzed by nanoelectrospray mass spectrometry in 1% acetic acid and 60% acetonitrile.

**Circular Dichroism**—Recombinant proteins were dialyzed against 10 mM sodium phosphate (pH 7.4) before analysis. Approximately 50 μg of each protein was analyzed in the far-UV region (185–250 nm). The resolution was 1 nm; the speed was 10 nm/min; and the response was measured every 8 s. Sensitivity was 20 millidegrees.

**Proteins**—Human C4BP and protein S were purified as described before (29). The concentrations were determined by measuring the OD600 nm was ~0.7. Expression of protein was induced by the addition of the cell culture of the transformed bacteria grown in Luria broth containing 30 μg/ml kanamycin was used to inoculate 500 ml of cold phosphate-buffered saline. After incubation for 15 min at room temperature, with lysozyme added to a final concentration of 100 μg/ml, the bacteria were sonicated at 10 micron peak to peak and centrifuged in the same way. The pellet obtained was suspended in 6 mg guanidine HCl, 20 mM Tris-HCl (pH 8.0), and 10 mM reduced glutathione and sonicated and centrifuged as described above. The supernatant was then applied to a nickel-nitriolactric acid Superflow column (2.6 × 12 cm, QIA-GEN) equilibrated with the same buffer. The column was washed with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 20 mM imidazole, and the protein was eluted with 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 100 mM EDTA. Fractions containing protein were then collected and the absorbance at 280 nm was monitored. Tris-HCl (pH 8.3) and dithiothreitol were then added, both to a final concentration of 100 mM. After incubation for 2 h at 4 °C, the sample was diluted in 50 mM Tris-HCl (pH 8.3), 3 mM cysteine, and 0.3 mM cysteine so that the absorbance at 280 nm was equal to 0.1, and folding of the protein was accomplished by overnight dialysis at 4 °C against the same buffer. Iodoacetamide was then added to the dialyzed sample to a final concentration of 5 mM, and dialysis was continued overnight at 4 °C against 50 mM Tris-HCl (pH 8.0) and 10% glycerol. The dialyzed sample was then applied to a MonoQ column (2 ml, Amersham Pharmacia Biotech) equilibrated with the same buffer; protein was in the flow-through. The protein was concentrated using an Amicon concentrator. Finally, the protein was dialyzed against 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10% glycerol and stored at −70 °C until further use. Exact concentrations of recombinant protein were determined by analysis of amino acid composition after hydrolysis in 6 N HCl for 24 h.

**Materials and Methods**

**Cloning Procedure**

The prokaryotic expression vector used for expression of the recombinant C4BP β-chain was pET-26b (+) (Novagen). It carries an N-terminal pelB signal sequence and a C-terminal 6-His tag. CCP1 and CCP2 of the C4BP β-chain were cloned by polymerase chain reaction from full-length cDNA of the β-chain and a previous experimental data. In addition, two lysesins that could be responsible for the slight electrostatic component seen in the interaction were also mutated and studied. Wild-type CCP1 and CCP2 of the β-chain and the mutants were expressed in a prokaryotic system, purified on a nickel-Sepharose column, and refolded. All recombinant proteins were then tested for their ability to bind protein S. We found that substitution of four hydrophobic amino acids by polar residues in the first CCP of the β-chain decreased the apparent affinity for protein S 100-fold. Our results not only provide insights into the nature of the protein S-C4BP interaction, but also have implications for the prediction of binding sites at the surface of other CCP modules and may be valuable for the understanding of protein-protein recognition.
were run on 15% SDS-polyacrylamide gel under reducing (1 mm; 2 mm wash buffer, and bound radioactivity was determined in a triton-100 g). For radioligand blotting, the proteins were transferred from the gel to a polyvinylidene difluoride membrane. The membrane was then incubated for 1 h at room temperature in a quenching solution composed of buffer A (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 0.5% (v/v) Tween 20) supplemented with 3% fish gelatin. The buffer was changed to buffer A with 2 mM CaCl₂ and trace amounts of ¹²⁵I-labeled protein S, and the membrane was incubated overnight at 4 °C. The membrane was then washed with buffer A, dried, and exposed in a cassette. Finally, the membrane was scanned using a PhosphorImager. (Molecular Dynamics). In addition, the intensities of the bands detected were estimated by densitometry using ImageQuant software (Molecular Dynamics).

Binding Assays

Direct Binding—Microtiter plates were coated with 50 µl of protein (plasma-purified C4BP or recombinant wild-type or mutant β-chain) at 10 µg/ml in 75 mM sodium carbonate (pH 9.6) at 4 °C overnight. The plate was then washed three times with wash buffer and quenched in wash buffer with 3% fish gelatin for 2 h. The plate was washed as described above, and protein S was added at increasing concentrations (0–240 nM final concentration) with trace amounts of ¹²⁵I-labeled protein S at 4 °C overnight. Finally, the plate was washed five times with wash buffer, and bound radioactivity was determined in a γ-counter.

Competition Assay—Microtiter plates were coated with 50 µl of plasma-purified C4BP at 10 µg/ml in 75 mM sodium carbonate (pH 9.6) at 4 °C overnight. The plate was then washed three times with wash buffer, quenched in wash buffer with 3% fish gelatin for 2 h, and washed as described above. Increasing amounts of plasma-purified C4BP or recombinant β-chain (wild-type and mutant) plus trace amounts of ¹²⁵I-labeled protein S were added overnight at 4 °C. The next day, the plates were washed five times with the same buffer, and bound radioactivity was measured in a γ-counter.

RESULTS

Expression and Purification of Recombinant Proteins—To study a possible binding site for protein S on the C4BP β-chain, the following mutations were introduced in the first CCP of the β-chain: L27T/F45Q, I16S/V18S, V31T/I33N, I16S/V18S/V31T/I33N, K41E/K42E, and L38S/V39S. The mutations were chosen after analysis of a homology-based three-dimensional model of the β-chain (Fig. 1) and previous experimental data on the protein S-C4BP interaction (21, 24–27). The residues mutated in this study are shown in red. Two potential N-linked glycans (yellow) in the first CCP of C4BP are approximated as an oligosaccharide (five sugar units) core structure. The side chains of Asn⁴⁷ and Asn⁸⁴ are displayed in red and colored orange.

Characterization of Recombinant Proteins—Introduction of mutations did not affect the expression levels or electrophoretic mobilities of corresponding proteins compared with the wild-type protein. Furthermore, all constructs bound with similar apparent affinity to seven monoclonal antibodies raised against the recombinant wild-type protein. Results for two different antibodies are shown as an example of the binding curves obtained (Fig. 3).

The exact masses of all recombinant proteins (except L38S/V39S) were analyzed by mass spectrometry. All masses were the precise expected match given the change in mass due to the changes of amino acids (Table II).

Upon gel filtration, all proteins eluted with a major single peak at a volume of ~15 ml, corresponding to a protein size of 13 kDa, as judged by a standard curve obtained from proteins with a known molecular mass, indicating that aggregates were not present (data not shown). The amount of protein eluted in the major peak, compared with the total amount of protein eluted, varied between 63 and 99%, the lowest being for the L27T/F45Q mutant. The major eluted peak for the mutant with four hydrophobic amino acids mutated, I16S/V18S/V31T/I33N, contained 84% of the total eluted protein.

Circular dichroism analysis of all recombinant proteins gave very similar spectra, once again confirming that introductions of mutations did not cause folding changes. The signal was not possible to judge below 205 nm due to background noise. Results are presented as strength of the signal relative to the
Radioligand Blotting—To assess the binding of protein S to the various mutants, we used radioligand blotting, in which unreduced wild-type and mutant proteins immobilized on a polyvinylidene difluoride membrane were allowed to bind 125I-labeled protein S (Fig. 5, one representative experiment is shown). Mutant L27T/F45Q bound with similar or increased strength compared with the recombinant wild-type b-chain. I16S/V18S, V31T/I33N, I16S/V18S/V31T/I33N, and L38S/V39S all lost the binding ability for protein S as judged by the absence of bands on the blot after analysis by the PhosphorImager. The K41E/K42E mutant displayed weaker binding than the recombinant wild-type b-chain. For better quantification of differences between mutants, the intensities of all bands were estimated by measurement of density using ImageQuant software. The results are shown in Table III; each value represents the mean ± S.D. of three different experiments. No binding was detectable when proteins were reduced (data not shown), implying that the two characteristic disulfide bonds present in the CCP modules were appropriately formed and, as expected, are crucial for the domain folding and thus for the interaction with protein S.

Direct Binding Assay—To further confirm the results obtained by radioligand blotting, a ligand binding assay was performed (Fig. 6A). Increasing amounts of unlabeled and 125I-labeled protein S were added to immobilized plasma-purified C4BP or the recombinant b-chain (wild-type or mutant). After washing, bound radioactive protein S was measured using a γ-counter. The recombinant wild-type b-chain construct bound protein S with similar affinity as plasma-purified C4BP. This result also supports the structural integrity of the recombinant molecule and further confirms the fact that this expression system combined with the proper refolding technique leads to the production of a protein sharing the same characteristics as plasma-purified C4BP, but with the absence of glycosylation. It is known, however, that the carbohydrate side chains are not

![Figure 2](image1.png)  
**FIG. 2.** Analysis of the purified recombinant C4BP b-chain by SDS-PAGE. Recombinant proteins (wild-type and mutant) were separated by SDS-15% PAGE under reducing (~1 μg/well) (A) and nonreducing (~0.5 μg/well) (B) conditions. Proteins were then visualized using the silver staining method.

![Figure 3](image2.png)  
**FIG. 3.** Binding between recombinant constructs (wild-type and mutant) and two different monoclonal antibodies directed against C4BP b-chain CCP1 and CCP2. Recombinant constructs competed with 125I-labeled recombinant wild-type b-chain for binding to immobilized monoclonal antibody. 125I-Labeled recombinant wild-type b-chain binding was estimated at 100% in the absence of fluid-phase competitor. , recombinant wild-type b-chain; △, I16S/V18S/V31T/I33N; ○, I16S/V18S; ▲, V31T/I33N; ●, L27T/F45Q; ■, L38S/V39S; ▽, K41E/K42E. A, immobilized monoclonal antibody 42; B, immobilized monoclonal antibody 15.

**Table II**  
Determination of exact masses of recombinant proteins using quadrupole time-of-flight (Q-TOF) mass spectrometry

| Recombinant b-chain construct | Measured mass (Da) |
|------------------------------|--------------------|
| Wild-type                    | 14,404             |
| L27T/F45Q                    | 14,373             |
| I16S/V18S/V31T/I33N          | 14,368             |
| I16S/V18S                    | 14,366             |
| V31T/I33N                    | 14,407             |
| L38S/V39S                    | ND*                |
| K41E/K42E                    | 14,405             |

*Not determined.

C4BP or the recombinant b-chain (wild-type or mutant). After washing, bound radioactive protein S was measured using a γ-counter. The recombinant wild-type b-chain construct bound protein S with similar affinity as plasma-purified C4BP. This result also supports the structural integrity of the recombinant molecule and further confirms the fact that this expression system combined with the proper refolding technique leads to the production of a protein sharing the same characteristics as plasma-purified C4BP, but with the absence of glycosylation. It is known, however, that the carbohydrate side chains are not...
important for protein S binding, as a truncated recombinant wild-type β-chain composed of three CCP modules was able to bind to protein S in a similar fashion as plasma-purified C4BP (20), which is also confirmed in the present study. As in the radioligand blotting, the L27T/F45Q mutation seemed to increase the binding of C4BP to protein S. For the remaining mutants, I16S/V18S, V31T/I33N, I16S/V18S/V31T/I33N, and L38S/V39S, the binding was essentially lost. K41E/K42E again displayed weaker binding, never reaching more than ~70% of binding of 125I-labeled protein S, compared with the wild-type β-chain.

**Competition Assay**—In the competition assay, increasing amounts of plasma-purified C4BP or recombinant β-chain (wild-type or mutant) were allowed to compete with immobilized C4BP for binding of fluid-phase 125I-labeled protein S. After washing, bound radioactive protein S was measured using a γ-counter. The wild-type β-chain bound protein S equally well compared with plasma-purified C4BP (Fig. 6). In this assay, mutant K41E/K42E bound in a similar fashion compared with the wild-type β-chain (Fig. 6C). L38S/V39S displayed ~10-fold less apparent affinity (Fig. 6C), whereas I16S/V18S, V31T/I33N, and I16S/V18S/V31T/I33N had an ~100-fold lower apparent affinity (Fig. 6D).

**DISCUSSION**

In this study, we show that a key binding surface for protein S is centered on Ile16, Val18, Val31, and Ile33 on CCP1 of the β-chain of C4BP. This cluster of solvent-exposed hydrophobic residues on the first CCP of the β-chain, together with two lysines, became apparent during analysis of a predicted three-dimensional model structure for C4BP (26, 27) based upon the NMR structure reported by Norman et al. (5). Since relatively large patches of solvent-exposed hydrophobic residues tend to destabilize the native state of a protein, possibly by shifting the folding equilibrium toward denaturation (32), it could have been argued that this region of C4BP was not appropriately modeled and was a computational artifact. However, we have also predicted the structure of this first CCP using other experimental templates (e.g. β2-glycoprotein I, CD46, and vaccinia virus complement control protein) and found that this exposed hydrophobic cluster is essentially present in all models (data not shown). Because of this observation and the data showing that the protein S-C4BP interaction is not significantly altered by the presence of increasing concentrations of NaCl, it was most likely that the solvent-exposed cluster indeed plays a significant role in protein S binding. The mutations introduced were chosen based on the analysis of the homology-based three-dimensional structure of the β-chain of C4BP (Fig. 1). Substitution of solvent-exposed residues by more polar ones should be favorable to the protein stability and/or folding, and we do not expect that structural problems could be induced because of the mutations. For example, the Ile16, Val18, Val31, Ile33, and Leu38 cluster has a surface area of ~300 Å². With a hydrophobic solvation free energy of ~20 cal/mol/Å², the energetic cost of exposing a patch of 300 Å² is high and ~6 kcal/mol.

The substitutions used were I16S/V18S, V31T/I33N, I16S/V18S/V31T/I33N, L38S/V39S, L27T/F45Q, and K41E/K42E. Our results strongly suggest that the protein S-binding site is indeed composed of a hydrophobic patch containing Ile16, Val18, Val31, Ile33, Leu38, and Val39, but that Leu27 and Phe45 are not involved in the binding. The weak contribution of electrostatic forces, observed earlier (26), could be due in part to the pres-

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**TABLE III**

| Recombinant β-chain construct | Intensity % |
|-------------------------------|------------|
| Wild-type                     | 100        |
| L27T/F45Q                     | 153 ± 20   |
| I16S/V18S/V31T/I33N           | 2 ± 7      |
| I16S/V18S                     | <1         |
| V31T/I33N                     | 1 ± 3      |
| L38S/V39S                     | 6 ± 3      |
| K41E/K42E                     | 69 ± 23    |
ence of Lys41 and Lys42. Although these amino acids affect the binding, they do not, by themselves, seem to be crucial for the interaction. It is clear that the change of Ile16/Val18 or Val31/Ile33 is sufficient to abrogate the protein S-C4BP interaction.

The residues involved in protein S binding are shown in Fig. 7. These amino acids form a cluster located in the direct vicinity of the second CCP (Figs. 1 and 6). van de Poel et al. (22, 23) showed that CCP2 seems to have a weak contribution to the binding between protein S and C4BP. It is possible that the large sex hormone globulin binding-like domain of protein S not only binds to CCP1, but also interacts with CCP2 of the β-chain. Another explanation for the influence of CCP2 on the binding of protein S could be that the second CCP sterically facilitates the binding of protein S to CCP1. However, our data are in agreement with our previous results stating that CCP1 contains the key binding site for protein S (21), as the substitution of four residues dramatically alters the interaction, fully consistent with the structural observations.

The β-chain is glycosylated, and two consensus sequences for N-linked glycosylation are present on the first CCP module (33). The difference between the expected molecular mass of the β-chain based on its amino acid composition (26.4 kDa) and the apparent molecular mass as judged by SDS-PAGE (45 kDa) implies that most or all of the glycosylation sites are occupied (33). This is further emphasized by the fact that, after digestion with endoglycosidase F, an enzyme that removes N-linked car-
bohydrates, the apparent molecular mass on SDS-PAGE of the β-chain is 29 kDa (33). It is known that oligosaccharide moieties can contribute to protein-protein interactions (34). For instance, the N-linked glycan attached to the second CCP of CD46 is essential for virus binding (35). However, it has been shown that sugars are not important for the binding of protein S to C4BP (20). Therefore, assuming one or both Asn residues (at positions 47 and 54) to be glycosylated, the glycan should be located outside the key binding surface for protein S. To study the spatial arrangement of these sugars relative to the defined protein S-binding site, glycan core structures were modeled as shown in Figs. 1 and 7. The glycan molecule was taken from the x-ray structure of CD46 (9) and grafted onto C4BP Asn47 and Asn54. Because we show that the key binding residues for protein S are Ile14, Val18, Val11, and Ile50 and since the glycans are away from this region, the predicted structure and experimental data are again in full agreement.

Fernández and Griffin (24) used synthetic peptides to probe the protein S-C4BP interaction and suggested that residues 31–45 are important for protein S binding. Our results in part confirm their observations since we show that Leu38 and Val39 have great influence on the protein S-C4BP interaction. However, we show that also Ile60 and Val68 are crucial for the binding of protein S to C4BP. Furthermore, changing the hydrophobic residues Leu27 and Phe45 to polar residues did not lessen the binding to protein S. Rather, the substitution seemed to slightly enhance the binding, suggesting that these residues directly or indirectly repulse protein S.

The role of exposed hydrophobic residues in protein-protein interaction is not unique for the CCPs of C4BP. Our data are consistent with the analysis of other macromolecular interactions, as in many systems, a solvent-exposed hydrophobic cluster has been observed (36–38). For instance, it has been proposed upon analysis of the CD46 x-ray structure that the CD46-measles virus hemagglutinin interaction is dependent on a critical set of hydrophobic residues at the protein interfaces and that this reaction resembles the CD4-HIV gp120 interaction (9). Recent investigations have highlighted the importance of solvent-exposed hydrophobic patches (ranging from 200 to 1200 Å²) (39, 40). Many different types of binding surfaces are expected to be found in nature to allow proper folding of the molecules and to render high, medium, or low affinity as well as specificity. Thus, it is obvious that some protein interfaces are very rich in charged residues, whereas others display solvent-exposed hydrophobic clusters. The importance of hydrophobic contacts for protein interactions is appealing, as such interactions appear to glue two molecules together. However, these interactions may not be very specific and so should be complemented by hydrophilic interactions. This situation is expected in the protein S-C4BP interaction, as it seems that the key hydrophobic binding surface is supplemented by hydrophilic interactions. Long-range electrostatic interaction may not be the only force that can affect association. Important contributions to the binding free energy involve also desolvation (i.e. the removal of solvent from nonpolar and polar atoms). Indeed, when a large apolar surface is exposed to solvent, other long-range attractive forces are expected to contribute significantly to protein assemblies (41). There seem to be many reasons to maintain large or small hydrophobic clusters within a binding site area of transient or very stable molecular complexes, despite the overall energetic cost of such a structural feature.

Investigation of the protein S-C4BP interaction is of importance for numerous reasons. First, protein S deficiency is a risk factor for thrombosis, and a better understanding of the protein S-C4BP interaction could be valuable for better diagnosis and treatment of coagulation disorders. Second, CCP modules are present in numerous proteins involved in various important biological processes. Thus, analysis of a specific CCP domain could provide information that may be a general consensus for CCP modules. Third, a better understanding of protein-protein interaction guides the design of approaches aimed at the prediction of hot spots at the surface of a molecule. This is of importance because many research projects involve characterization of binding sites, which then helps the understanding of molecular mechanisms and thus the generation of new therapeutic compounds or diagnostic tools.

In conclusion, we have shown a large hydrophobic patch on the β-chain of C4BP to be crucial for binding of protein S to C4BP. In addition, Lys41 and Lys42 could contribute to the modest electrostatic component playing a role in this interaction. These data are the first reported that clearly pinpoint the specific amino acids on CCP1 responsible for the interaction between protein S and C4BP. Our data are also in agreement with what has been observed for other CCP-containing molecules as well as in several macromolecular assemblies. Furthermore, our investigation emphasizes the rational of using computer-based molecular modeling to predict the three-dimensional structure of a protein and its potential in the design of experiments aimed at a better understanding of the relationships between structure and function.

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FIG. 7. Outline of the protein S-binding site on C4BP. The C4BP CCP1 model is shown with the same orientation as for Fig. 1. The solvent-accessible area is rendered as a solid surface, and side chains of the residues important for protein S binding are colored green and blue (see “Discussion”). The glycan side chains are in yellow. N-term, N terminus.
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