# Analysis of Protein S C4b-Binding Protein Interactions by Homology Modeling and Inhibitory Antibodies†

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ABSTRACT: A monoclonal antibody (mAb 6F6) directed against the β-chain of C4b-binding protein (C4BP) was previously shown to inhibit the binding of protein S to C4BP. To localize the epitope of this antibody, 23 overlapping synthetic peptides (15-mers) covering the entire sequence (1–235) of the β-chain of C4BP were used. When the immobilized peptides were screened for their ability to bind mAb 6F6, only peptide β(51–65) showed high-affinity binding. The apparent affinity of mAb 6F6 for immobilized peptide β-(51–65) was somewhat similar to that for native C4BP with $K_d \sim 1$ nM for C4BP and ~9 nM for peptide β(51–65). Peptide β(51–65) inhibited the binding of the mAb 6F6 to immobilized C4BP with half-maximal inhibition at 30 μM peptide. Clotting assays of protein S anticoagulant cofactor activity using a factor Xa-1-stage assay with activated protein C allow measurement of free protein S in solution since only free protein S is active. Studies using such clotting assays showed that preincubation of C4BP with either mAb 6F6 or polyclonal anti-β(31–45) antibodies inhibited the formation of the complex between C4BP and protein S. Previous studies showed that, although peptide β(51–65) itself does not inhibit complex formation, peptide β(31–45) does bind directly to protein S and does inhibit protein S binding to C4BP. The three-dimensional structure of the first SCR (residues 2–60) of the C4BP β-chain was made on the basis of homology modeling. In the computer graphics model, residues 51–60 are spatially proximate to residues 31–45, suggesting that the inhibitory effect of mAb 6F6 on complex formation is most likely due to the antibody's steric hindrance of protein S access to β-chain residues 31–45. Thus, these anti-β-chain antibody results are very consistent with the hypothesis that the first SCR of the β-chain of C4BP provides a binding site for protein S.

The human plasma protein, C4b-binding protein (C4BP),[1] accelerates the decay of the classical pathway C3-convertase of complement activation and acts as a cofactor in the cleavage of C4b by factor I (Scharfstein et al., 1978). C4BP has lipid-binding properties and is also known as a proline-rich lipoprotein (Matsuguchi et al., 1989). Several forms of C4BP ($M_r$ 570 000) circulate in plasma. The major form is composed of seven apparently identical α-chains ($M_r$ 72 000) and one β-chain ($M_r$ 45 000) (Hillarp & Dahlbäck, 1988; Chung et al., 1985). This form of C4BP in plasma is in a 1:1 complex with protein S, a vitamin K-dependent protein that is an antithrombotic protein in the protein C pathway (Griffin et al., 1992; Dahlbäck, 1983). Protein S in complex with C4BP is not active as a cofactor for activated protein C, whereas free protein S is active (Dahlbäck, 1986; Bertina et al., 1985). The β-chain is necessary for the binding to protein S (Hessing et al., 1990; Hillarp & Dahlbäck, 1988). Residues 31–45 of the first short consensus repeat (SCR) of the β-chain (C4BP β1 SCR) contain a binding site for protein S (Fernández & Griffin, 1992, 1994). Previously, a panel of monoclonal anti-C4BP antibodies was studied in relation to their inhibitory activity in the binding of C4BP to protein S (Hessing et al., 1991). In this study, synthetic peptides representing the entire sequence of the β-chain of C4BP were used to identify the epitopes of antibody mAb 6F6 as β-chain residues 51–65, and a computer graphics molecular model was used to describe the location of residues 51–65 relative to residues 31–45.

## EXPERIMENTAL PROCEDURES

*Materials.* Streptavidin conjugated to alkaline phosphatase (SAAP), biotinylated anti-mouse IgG, and immunopure *p*-nitrophenyl phosphate (p-NPP Kit) (Pierce, Rockford, IL), biotin *N*-hydroxysuccinimide ester (NHS-d-biotin) (Clontech, Palo Alto, CA), and bovine serum albumin (BSA) (Calbiochem, La Jolla, CA) were obtained as indicated. Normal human citrate-anticoagulated pooled plasma (NHP) was purchased from George King Bio-Medical, Inc. (Overland Park, KS). All other reagents were of the highest quality available.

*Peptide Synthesis.* Twenty-three pentadecapeptides were synthesized as described (Fernández et al., 1993) covering the entire sequence of the β-chain of C4BP. Peptides overlapped by five residues.

*Protein Purification.* All proteins were of human origin. Protein S, protein S-depleted plasma, and activated protein C were purified as described (Heeb et al., 1993; Mesters et al., 1991). C4b-binding protein was purified as described (Fernández & Griffin, 1994). Protein concentration was determined by absorbance at 280 nm. An absorption coef-

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[1] Abbreviations: C4BP, C4b-binding protein; NHP, normal human plasma; mAb, monoclonal antibody; b-protein S, biotinylated protein S; b-C4BP, biotinylated C4b-binding protein; IFA, incomplete Freund's adjuvant; BSA, bovine serum albumin; TBS, Tris-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SAS, saturated ammonium sulfate; ELISA, enzyme-linked immunosorbent assay; SCR, short consensus repeat.

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ficient of 9.5 and 14.1 was used for protein S and C4BP, respectively (DiScipio & Davie, 1979; Perkins et al., 1986). Factor Xa was obtained from Enzyme Research Laboratories, South Bend, IN.

*Electrophoretic and Immunochemical Techniques.* Polyacrylamide gel analysis using sodium dodecyl sulfate (SDS–PAGE) was performed according to the method of Laëmmli (1970). The gels were stained with Coomassie brilliant blue G-250 or with the silver stain method. Western blotting was essentially performed as described (Schwarz et al., 1987). Gradient gels from 4% to 15% (Bio-Rad) were used to run samples in different lanes containing 200 ng of a partially reduced and carboxymethylated form of C4BP. The gels were transferred to nitrocellulose paper and blocked with 1% casein in Tris-buffered saline (TBS; 0.05 M Tris, 0.1 M NaCl, pH 7.4). Nitrocellulose blots were incubated with 1 μg of different mAb anti-C4BP/mL for 1 h and visualized with 1 μg of biotinylated anti-mouse IgG/mL followed by 1 μg/mL strepavidin conjugated to alkaline phosphatase and the p-NPP substrate kit (Pierce).

*Purification of mAb Anti-C4BP.* Balb/c mice were immunized by subcutaneous injection of purified C4BP and reduced and alkylated β-chain. After 2, 4, and 6 weeks they were boosted with the same amount of antigen in IFA. Three days after the final injection, spleen cells were fused with Ag8.653 myeloma cells. Fusion and hybridoma selection were performed according to standard procedures (Goding, 1986). Ascites from five different hybridomas (named 795, 1486, 1566, 1603, and 1337) were collected and precipitated with 45% SAS at 4 °C and centrifuged at 10 000 rpm for 15 min. The precipitate was resuspended in a buffer containing 0.02 M $Na_2CO_3$, pH 9.0, and purified on a Mono Q HH 10/10 column as described (Clezardin et al., 1985). The fractions were screened in an ELISA as described (Fernández et al., 1993), pooled, concentrated in a dialysis bag in 50% sucrose to give a concentration of approximately 1 mg/mL, and dialyzed.

*Affinity of Monoclonal Antibodies for Native C4BP.* The apparent dissociation constant ($K_d$) of the anti-C4BP mAb was determined by Scatchard analysis of data obtained using the following experimental procedure. Fifty microliters per well of immunoaffinity-purified antibody (10 μg/mL) was coated in 0.05 M $Na_2CO_3$ and 0.02% $NaN_3$, pH 9.0, for 1 h at 37 °C on microtiter plates, and subsequently, after the wells were washed, unoccupied sites were blocked with 200 μL of 10% BSA in TBS buffer for 1 h at room temperature. For negative controls, wells were blocked without any IgG coating. Biotinylated C4BP (50 μL, 0–20 μg/mL) was incubated in the wells for 2 h at room temperature. After the wells were washed three times with 0.2% BSA and 5 mM $CaCl_2$ in TBS, bound biotinylated C4BP was detected as described above. Scatchard analysis and apparent dissociation constants were calculated using Enzfitter Software, Cambridge. Linear Scatchard plots were observed.

*Inhibition of Binding of Biotinylated Protein S to C4BP by Anti-C4BP mAb.* Plates were coated with purified C4BP (10 μg/mL) in 0.02 M $Na_2CO_3$, pH 9.0, buffer and blocked with 200 μL per well of 2% BSA in TBS. Monoclonal anti-C4BP antibodies (50 μL, 0–400 μg/mL) were incubated in the wells for 2 h at room temperature. Ten microliters per well of biotinylated protein S was added to the plate, and the solution was incubated for another hour at room temperature. After three washes with 200 μL per well of washing buffer (0.2% BSA in TBS, 5 mM $CaCl_2$, and 0.02% Tween-20), 50 μL per well of SAAP (1 μg/mL) was incubated for 30 min at room temperature. After the plates were washed extensively, 100 μL per well of p-NPP (5 mg/mL) was added to the wells, and the $\Delta A_{405}$/min was measured in a BIOTEK Microplate reader.

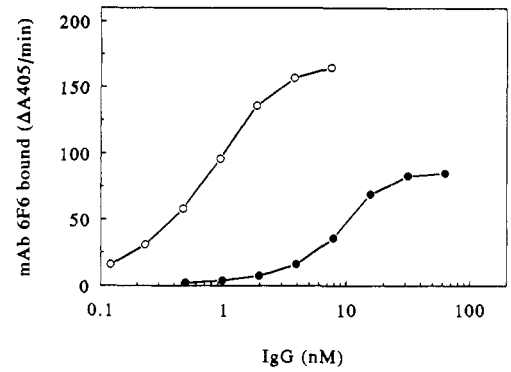



FIGURE 1: Binding of the anti-β-chain mAb 6F6 to immobilized peptide β(51–65) and immobilized native C4BP. Peptide (closed circles) or purified C4BP (open circles) was immobilized on a microtiter plate and incubated with the mAb 6F6. The amount of bound antibody was determined as described in Experimental Procedures.

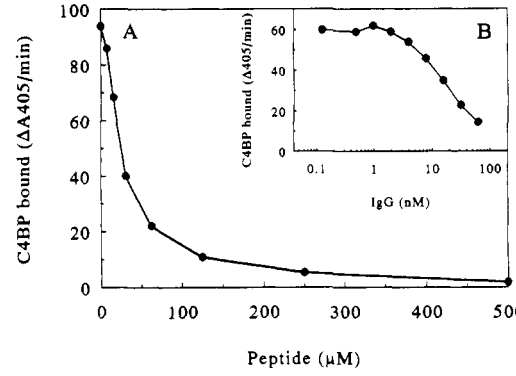



FIGURE 2: Inhibition of binding of C4BP to immobilized mAb 6F6 by peptide β(51–65) (A) or polyclonal anti-β(31–45) peptide antibody (inset B). mAb 6F6 was coated in wells on a plate and blocked as described in Experimental Procedures. Dilutions of β(51–65) peptide (A) were added to wells before incubation with C4BP. Polyclonal anti-β(31–45) antibody dilutions were preincubated with C4BP and then added to the corresponding wells. After incubation and washing, bound C4BP was detected with biotinylated anti-C4BP polyclonal antibody as described in Experimental Procedures.

*Solid-Phase Ligand Binding of Anti-C4BP mAb to Immobilized Synthetic Peptides or Native C4BP (Figure 1).* Microtiter plates were coated with β-chain synthetic peptides (20 μM, 50 μL per well) or native C4BP (10 μg/mL, 50 μL per well) in a buffer of 0.02 M $Na_2CO_3$, pH 9.0, overnight at 4 °C. The wells were then washed and blocked with 2% BSA in TBS (200 μL per well) for 2 h at room temperature. The plate was extensively washed and then incubated with 50 μL per well of a solution containing 0–10 μg/mL IgG in TBS containing 0.2% BSA, 5 mM $CaCl_2$, and 0.02% Tween-20 (dilution buffer) for 2 h at room temperature. After the plate was washed three times with the dilution buffer, the wells were incubated with biotinylated anti-mouse IgG (1 μg/mL, 50 μL per well) for 1 h at room temperature, washed three times, and incubated with streptavidin–alkaline phosphatase (SAAP) (1 μg/mL, 50 μL per well) for 30 min at room temperature. p-NPP at 5 mg/mL in 0.1 M ethanolamine, pH 9.0, was used to develop the plate (100 μL per well) after the plate was washed six times with dilution buffer (Figure 1).

*Inhibition of Binding of mAb 6F6 to C4BP by Peptide β(51–65) (Figure 2A).* A microtiter plate was coated with mAb 6F6 (RU-BP6F6) (10 μg/mL, 50 μL per well) in a pH 9.0, 0.02 M $Na_2CO_3$ buffer and blocked using the same conditions as above. Different concentrations of peptide β-(51–65) (50 μL per well) were incubated in the wells for 2 h at room temperature. After the plates were washed, C4BP (2 μg/mL, 50 μL per well) was incubated in each well for 1 h at room temperature. Biotinylated polyclonal anti-C4BP IgG (1 μg/mL) was used for antibody detection at 50 μL per well for 1 h at room temperature. The developing step with SAAP and substrate was the same as described above.

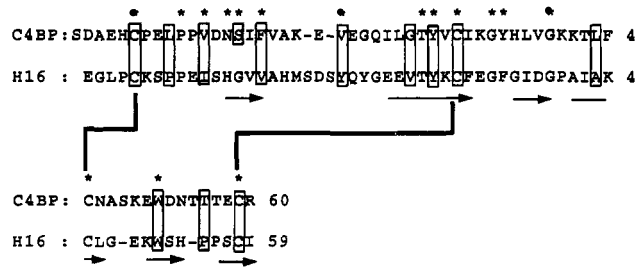
*Inhibition of Binding of mAb 6F6 to C4BP by Polyclonal Anti-Peptide β(31–45) Antibodies (Figure 2B).* A microtiter plate was coated with mAb 6F6 (10 μg/mL) and blocked using the same conditions as described above. A preincubation mixture containing different concentrations of polyclonal anti-peptide β(31–45) and C4BP (1 μg/mL final concentration) was incubated for 2 h at room temperature. Fifty microliters per well of this mixture was placed in wells of the microtiter plate containing immobilized mAb 6F6, and the mixture was incubated 1 h at room temperature. After the plate was washed, biotinylated anti-C4BP IgG (1 μg/mL) was incubated in the wells for 1 h at room temperature. The developing steps were the same as described above.

*Factor Xa-1-Stage Clotting Assays.* A factor Xa-1-stage clotting assay was used to quantitate free protein S in order to measure the ability of the anti-peptide antibodies to inhibit formation of the protein S–C4BP complex. All coagulation assays were performed in an Electra 700 automatic coagulation timer (Medical Laboratory Automation, Inc., Mount Vernon, NY) and were done in duplicate. For the factor Xa-1-stage assays, purified C4BP (0–70 nM final) was preincubated with or without antibodies (0–187 nM IgG final) in 40 μL of TBS–BSA for 1 h at 37 °C. Then 20 μL of purified protein S (80 nM final) in TBS–BSA containing 30 mM $CaCl_2$ was added for another 20-min incubation at 37 °C. Then the following were added: 100 μL of rabbit brain cephalin at 200 μg/mL, 100 μL of protein S-depleted plasma, 20 μL of 600 nM APC, and 15 μL of 4.13 nM factor Xa; and the time for clot formation at 37 °C was recorded. The standard curve for protein S anticoagulant cofactor activity yielded a straight line when prolongation of the clotting time was plotted versus protein S concentration (10, 20, 40, 60, 80 nM) on a log–log plot.

*Homology Modeling. (A) Sequence Alignment.* The sequence of C4BP β1, the first SCR of the β chain, and the 16 SCR repeat (H16) of factor H were manually aligned. The calculation of the solvent accessibility of the side chain was carried out on the structure of H16, and this information and the location of the secondary structure elements and disulfide bridges were taken into consideration in the sequence alignment (Table 1). This method avoids, when possible, introduction of insertions and deletions in the secondary structure elements (Greer, 1990). The sequence of C4BP β1 shares several identical or similar key residues with H16. These residues are suggested to dictate the three-dimensional structure of the SCR family of proteins.

*(B) Model Building and Computational Mutagenesis.* The initial model was built using the NMR structure of H16 kindly provided by Iain Campbell (Norman et al., 1991). The appropriate side-chain substitutions were introduced into the structure of H16, using the molecular graphics program InsightII and Homology (Biosym Technologies, Inc.) in order to match the sequence of C4BP β1. Values for side-chain torsion angle (χ) in H16 were used for each substitution as needed. However, if the C4BP β1 side chain was longer than the H16 side chains, an extended conformation was used for the remaining χ angles. The serine 1 of C4BP β1 has no counterpart in the template structure and was not modeled. The deletion in region 18–21 of C4BP β1 relative to H16 was refined by energy minization after residues Lys20–Glu21 and Glu21–Val22 were connected. For the two insertion regions, at residues 49 and 55 (Table 1), a search (Jones & Thirup, 1986) through the high-resolution protein structure available in the Brookhaven Protein Data Bank (Bernstein et al., 1977) was used, and the fragment which best accommodated the insertion in the structure was introduced in the model.

Table 1: Sequence Alignment of the H16 SCR and the C4BP β1 SCR[a]




[a] Arrows represent the β-sheet in the NMR structure of the H16 SCR. The star symbols represent the conserved residues in the SCR family or important residues involved in the folding. Disulfide bridges are shown as a thick black line. The black boxes indicate residues not accessible to the solvent in the NMR structure of the H16 SCR and in the energy-minimized model structure of the C4BP β1 SCR.

*(C) Energy Minimization.* No severe overlaps between atoms were detected during the construction of the model. The minor steric clashes and bond strain due to the introduction of the new side chains and loop building were regularized using the molecular simulation program Discover (Biosym Technologies, Inc.). Hydrogen atoms were added to the C4BP model structure according to standard geometries. The refinement of the structure was carried out in stages (Mackay et al., 1989). The first step was performed using a high constraining force constant on the protein heavy atoms ($K$ = 1000 kcal $mol^{-1}$ $Å^{-2}$) and energy minimized with the steepest descent algorithm until the maximum Cartesian derivative was less than 10 kcal $mol^{-1}$ $Å^{-1}$. This enabled relaxation of all built atoms without significant disruption of the overall structure. Three additional cycles of templates forcing ($K$ = 500, 100, and 50 kcal $mol^{-1}$ $Å^{-2}$) followed. Finally, unconstrained energy minization was carried out until the maximum derivative was less than 1 kcal $mol^{-1}$ $Å^{-1}$. All calculations were performed using a 20-Å nonbond cutoff and a dielectric constant of 1.

## RESULTS

*Mapping of the Epitope of the Anti-β-Chain mAb.* Since monoclonal antibodies (mAb) provide useful probes of protein S–C4BP interactions, the previously reported mAb 6F6 (Hessing et al., 1991) that inhibits complex formation was used for studies to define the β-chain epitope of mAb 6F6. An anti-α-chain mAb 8C11 that does not inhibit protein S–C4BP complex formation was used as a control. To localize the epitopes of mAb 6F6 in the β-chain of C4BP, 23 synthetic overlapping pentadecapeptides representing the entire sequence of residues 1–235 of the β-chain were used for initial screening (see Table 2). Peptides were immobilized on microtiter plates, and antibody binding was tested as described

Table 2: Binding of mAb 6F6 to Synthetic Peptides Representing Sequences of the β-Chain of C4BP[a]

| sequence no. | amino acid sequence | $\Delta A_{405}$ obsd |
|---|---|---|
| β(1–15) | SDAEHCPELPPVDNS | 0.67 |
| β(11–25) | PVDNSIFVAKEVEGQ | 0.63 |
| β(21–35) | EVEGQILGTYVCIKG | 0.84 |
| β(31–45) | VCIKGYHLVGKKTLF | 0.70 |
| β(41–55) | KKTLFCNASKEWDNT | 0.68 |
| β(51–65) | EWDNTTTECRLGHCP | 46.32 |
| β(61–75) | LGHCPDPVLVNGEFS | 0.82 |
| β(71–85) | NGEFSSSGPVNVSDK | 0.70 |
| β(81–95) | NVSDKITFMCNDHYI | 0.73 |
| β(91–105) | NDHYILKGSNRSQCL | 1.31 |
| β(101–115) | RSQCLEDHTWAPPFP | 0.83 |
| β(111–125) | APPFPICKSRDCDPP | 0.64 |
| β(121–135) | DCDPPGNPVHGYFEG | 0.33 |
| β(131–145) | GYFEGNNFTLGSTIS | 0.40 |
| β(141–155) | GSTISYYCEDRYYLV | 0.59 |
| β(148–160) | CEDRYYLVGVQEQ | 0.65 |
| β(151–165) | RYYLVGVQEQQCVDG | 0.75 |
| β(161–175) | QCVDGEWSSALPVCK | 1.91 |
| β(171–185) | LPVCKLIQEAPKPEC | 0.79 |
| β(181–195) | PKPECEKALLAFQES | 0.69 |
| β(191–205) | AFQESKNLCEAMENF | 0.87 |
| β(201–215) | AMENFMQQLKESGMT | 1.01 |
| β(211–225) | ESGMTMEELKYSLEL | 0.94 |
| β(221–235) | YSLELKKAELKAKLL | 0.56 |

[a] β-Chain peptides (20 μM) were coated on a microtiter plate, and following washing, wells were incubated with mAb 6F6. Bound IgG was detected after washing according to Experimental Procedures.

in Experimental Procedures. mAb 6F6 recognized the peptide EWDNTTTECRLGHCP (residues 51–65) (β51–65) of the β-chain with high affinity. None of the other available monoclonal antibodies including anti-α-chain mAb 8C11 mAbs 795, 1337, 1486, and 1556 recognized any of the synthetic β-chain peptides in these solid-phase experiments. Figure 1 shows the dose–response for the binding of mAb 6F6 to immobilized peptide β(51–65), giving a apparent $K_d$ of 9.3 nM and the dose–response for the binding of mAb 6F6 to native C4BP with an apparent $K_d$ of 1.05 nM. The apparent dissociation constant of immobilized anti-α-chain mAb 8C11 for C4BP was 3.3 nM (data not shown).

The ability of peptide β(51–65) to inhibit the binding of mAb 6F6 to C4BP was studied. Solid-phase assays of C4BP binding to immobilized mAb 6F6 showed that peptide β(51–65) was an effective inhibitor of this binding with half-maximal inhibition ($IC_{50}$) at 30 μM peptide (Figure 2A).

To study the spatial proximity of the epitope of mAb 6F6, i.e., residues 51–65 of the β-chain, to a binding site for protein S previously shown to involve β-chain residues 31–45 (Fernández & Griffin, 1992, 1994), immunoaffinity-purified polyclonal anti-peptide β(31–45) antibodies were prepared as previously described (Fernández & Griffin, 1994) and were tested for their ability to inhibit binding of C4BP to immobilized mAb 6F6. The polyclonal anti-β(31–45) antibodies effectively blocked mAb 6F6 binding with half-maximal inhibition at 13 nM IgG (Figure 2B).

Clotting experiments using factor Xa-1-stage assays and purified proteins were used to study the inhibition of protein S anticoagulant APC cofactor activity by C4BP and the neutralization of this inhibition by mAb 6F6. In control experiments, there was a stoichiometric linear inhibition of anticoagulant cofactor activity of 80 nM purified protein S by different concentrations (0–80 nM) of purified C4BP, and C4BP at 80 nM inhibited more than 90% of protein S activity (data not shown). The inhibitory effect of 80 nM C4BP on the activity of 80 nM purified protein S was abrogated by mAb 6F6 and polyclonal β(31–45) antibody (Figure 3). The

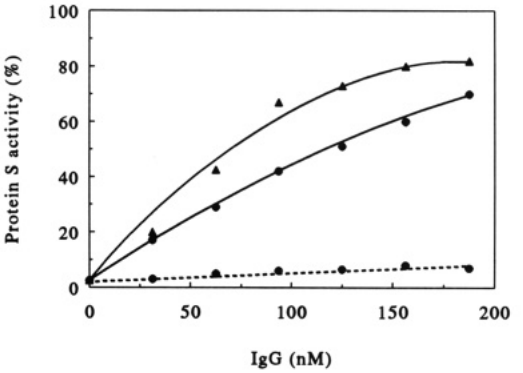



FIGURE 3: Neutralization of the inhibitory activity of C4BP by mAb 6F6 and by polyclonal anti-β(31–45) peptide antibodies. C4BP was preincubated with varying concentrations of antibodies and then added to purified protein S (80 nM protein S, 80 nM C4BP) as described in Experimental Procedures. Then the anticoagulant cofactor activity of protein S was determined. Curves: mAb 6F6 (circles, solid line), polyclonal anti-β(31–45) antibodies (triangles, solid line), and, as control, mAb 8C11 (circles, dashed line).

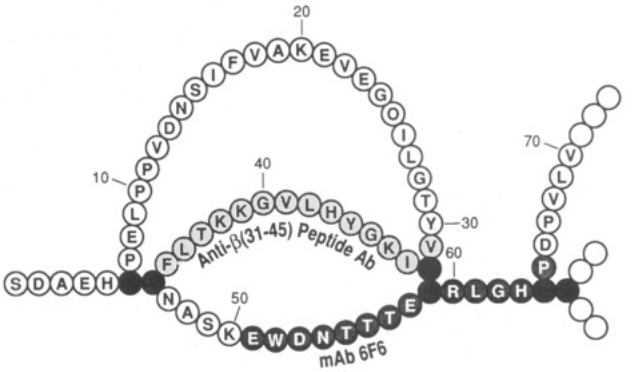



FIGURE 4: Primary sequence of the first SCR domain of the β-chain of C4BP (residues 1–60) with disulfide bonds suggested in homology to other SCRs of known structure (Norman et al., 1991).

anti-β(31–45) antibody protected protein S from C4BP neutralization and gave an 82% observed protein S activity at a 190 nM final concentration of IgG, and mAb 6F6 also protected protein S from C4BP neutralization and gave 85% observed protein S activity (Figure 3). The control anti-α-chain mAb 8C11 (190 nM) did not affect the inhibition of protein S activity by C4BP; i.e., there was less than 10% protein S anticoagulant cofactor activity at 90 nM IgG (Figure 3).

*Modeling of the First SCR Domain of the β-Chain of C4BP.* To define the three-dimensional relationship between the mAb 6F6 epitope, β(51–65), and the protein S binding site, β(31–45), a homology model of the first SCR domain of the β-chain was prepared. On the basis of the known NMR structure of H16, a three-dimensional model of the C4BP β1 SCR was constructed. Table 1 shows the sequence alignment of H16 SCR with the C4BP β1 SCR. It can be seen that all the residues which should be of importance for conferring the three-dimensional structure of H16 (Norman et al., 1991) were conserved in C4BP β1 SCR sequence (Table 1). These residues form a very compact hydrophobic core wrapped in a β-stranded sheet sandwich in the three-dimensional model structure (Figure 5). The conserved residues involved in the core structure are primarily Cys6–Cys46 and Cys32–Cys59 in C4BP β1 SCR. Other residues involved in the interior core are Leu9, Val12, Phe17, Val22, Tyr30, Leu44, and Trp52. Those residues are present in the structure of H16 SCR (Table 1, Figure 5). Residue Phe37 of H16 SCR is accessible to the solvent and is expected to interact with the adjacent SCR in

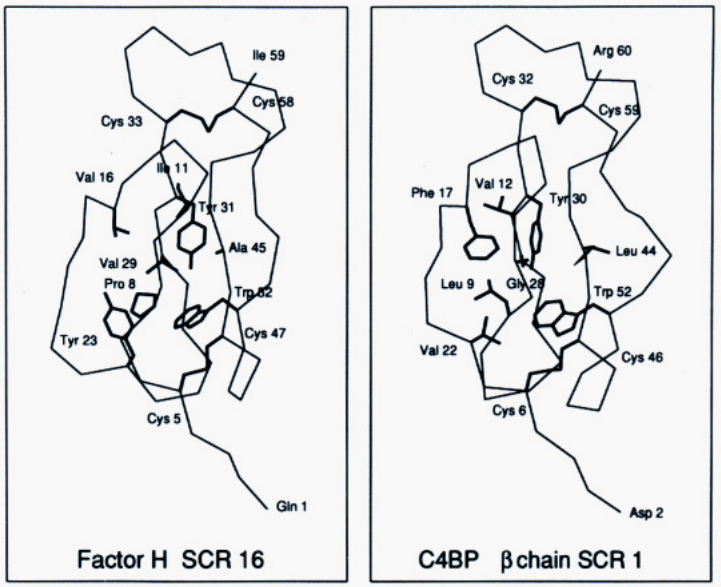



FIGURE 5: Cα backbone representing the NMR structure of the 16th SCR of factor H (H16) and of the β1 SCR of the C4BP homology model and highlighting the conserved side chains of the core residues. Residues involved in the formation of the compact hydrophobic core are Cys5–Cys47, Tyr23, Trp52, Pro8, Val29, Ala45, Tyr31, Ile11, Val16, and Cys33–Cys58 in H16 and are respectively Cys6–Cys46, Val22, Trp52, Leu9, Gly28, Leu44, Tyr30, Val12, Phe17, and Cys32–Cys59 in the β1 SCR of the C4BP.

factor H. Similarly, histidine 13 in H16 SCR is also suggested to be involved in the interaction with the second adjacent SCR. These two residues are often replaced by a Tyr and Asn pair as in the case in the C4BP β1 module. As shown in Table 1, the sequence alignment introduces a deletion of two residues in region 20–22 of C4BP β1 SCR relative to H16 SCR. This region represents a loose turn structure in H16 SCR; thus the structure based on this deletion was easily refined by energy minimization. Two insertions of one residue each were introduced in the C-terminal region of C4BP β1 SCR, involving residues Ser49 and Thr55. Ser49 lies in a turn structure, while Thr55 is located between two β-strand structures. These two insertions did not disturb the overall structure or the hydrophobic core of the model. The Ramachandran plot (data not shown) indicates that the $\phi$ and $\psi$ angles of the energy-minmized structure of the C4BP β1 SCR have acceptable conformation. The solvent accessibility calculations for the minimized model structure show that all the hydrophobic residues involved in the core are buried in the protein interior and that charged residues are all accessible to the surface (Table 1). A pair of spatially proximate residues involving Glu28 and Lys46 of H16 SCR are replaced by the adjacent pair of residues Leu27 and Phe45 on the surface of the C4Bp β1 SCR model structure, thus replacing a charge–charge interaction by an hydrophobic one. This area is close to peptide 31–45 involved in the interaction with protein S and might have a functional role by offering an hydrophobic surface in addition to its structural stabilizing effect.

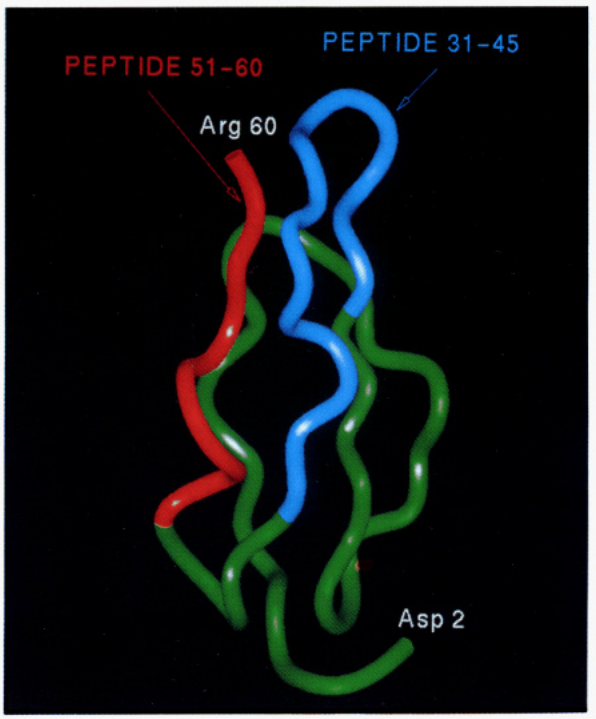



FIGURE 6: Ribbon representation of C4BP β1 SCR (residues 2–60) showing locations of peptides β(31–45) in blue and β(51–60) in red. Residues 31–45 represent a binding site for protein S, and residues 51–60 represent, at least in part, the epitope of mAb 6F6.

## DISCUSSION

Monoclonal antibodies are potentially useful in characterizing and identifying protein regions involved in protein–protein interactions. Monoclonal antibody mAb 6F6 directed against the β-chain of C4BP was previously shown to inhibit the binding of protein S to C4BP (Hessing et al., 1991). To localized the epitope of this antibody, 23 overlapping synthetic peptides (15-mers) covering the entire sequence (1–235) of the β-chain of C4BP were used (Fernández & Griffin, 1994).

When the immobilized peptides were screened for their ability to bind mAb 6F6, only peptide β(51–65) showed high-affinitybinding. The apparent dissociation constant of mAb 6F6 for peptide β(51–65) ($K_d$ = 9.3 nM) was comparable to that of native C4BP ($K_d$ = 1 nM) (Figure 1). In contrast to peptide β(31–45), which bound protein S and inhibited complex formation, peptide β(51–65) was not an effective inhibitor of the binding of protein S to C4BP, and protein S did not bind to immobilized peptide β(51–65) (Fernández & Griffin, 1994). To confirm that the epitope of mAb 6F6 involves β(51–65), it was shown that peptide β(51–65) inhibits

the binding of mAb 6F6 to immobilized C4BP with half-maximal inhibition at 30 $\mu$M peptide (Figure 2A).

Clotting assays of protein S anticoagulant activity using a factor Xa-1-stage assay with activated protein C allow measurement of free protein S since only free protein S is active as a cofactor for activated protein C. Studies showed that preincubation of mAb 6F6 with C4BP abrogated the formation of the complex between C4BP and protein S on the basis of clotting assays. The control mAb 8C11 directed against the $\alpha$-chain of C4BP did not block the complex formation of protein S and C4BP as measured using solid-phase binding assays and clotting assays (Figure 3).

To assess the spatial proximity of the epitope of mAb 6F6 comprising residues 51–60 of the $\beta$-chain and residues 31–45 involved in the binding site of protein S, a computer graphics homology model of the first SCR of the$\beta$-chain (residues 2–60) of C4BP was developed. SCR modules are normally recognized on the basis of a consensus sequence including a framework of four conserved cysteine residues that are disulfide linked in a 1–3 and 2–4 pattern (Figure 4). The three-dimensional structure of H16 is based on a $\beta$-sandwich arrangement, with one face comprised of three $\beta$-strands hydrogen bonded to form a triple-stranded $\beta$-sheet at its center and two separate $\beta$-strands forming the other face (Norman et al., 1991). On the basis of sequence alignment of C4BP $\beta$-chain residues 1–60 with those of the H16 SCR (Table 1), it was observed that all the residues which should be of major importance for dictating a typical SCR three-dimensional structure were conserved in the C4BP $\beta$1 SCR module. The observed high degree of homology of hydrophobic residues and of cysteines buried in the core interior and the fact that all the charged residues are accessible to the solvent suggest that the homology model very reasonably represents the structure of C4BP $\beta$1 SCR (Figure 5). On the basis of this model, the region comprising $\beta$-chain residues 31–45 which bind protein S is three-dimensionally proximate to residues 51–60 which contribute to the epitope of the anti-$\beta$-chain mAb 6F6 (Figure 6) as these sequences involve adjacent strands in the $\beta$-sheet sandwich. mAb 6F6 competed for the binding of rabbit polyclonal anti-(31–45) antibodies to C4BP with an $IC_{50}$ of 20 nM IgG (Figure 2B). These results and inspection of the model (Figure 6) suggest that the inhibitory effect of mAb 6F6 on the complex formation is most likely caused by antibody's steric hindrance of the access of protein S to residues 31–45 of the $\beta$1 SCR. Other less simple explanations, e.g., mAb 6F6-induced conformational changes in the $\beta$1 SCR, are possible though much less likely. Although it is certainly possible that the sequence of $\beta$(51–65) contributes to binding protein S, the sequence of $\beta$(31–45) is likely to be much more important thermodynamically since the latter but not the former peptide inhibits binding to C4BP. Overall, these findings and previous results (Fernández & Griffin, 1994; Härdig et al., 1993) emphasize the essential role of the first SCR of the C4BP $\beta$-chain in providing a high-affinity binding site for protein S.

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