A Region of Vitamin K-dependent Protein S That Binds to C4b Binding Protein (C4BP) Identified Using Bacteriophage Peptide Display Libraries*

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Vitamin K-dependent protein S, a blood coagulation inhibitor, interacts with the C4b-binding protein (C4BP) in human plasma with high affinity ($K_D = 0.1 \text{ nM}$). Identification of a portion of protein S that binds to C4BP has been approached using random libraries of 6- and 15-mer peptides displayed on bacteriophage surfaces. Bacteriophage binding to the $\beta$-chain of C4BP were selected in several rounds of affinity purification with intervening amplification in E. coli. Homology searches of the affinity purified peptide sequences against protein S led to the identification of four regions in protein S that were similar to several of the selected peptides. These regions were synthesized as linear peptides and tested in inhibition experiments. Only one distinct peak (around position 450) was observed when the homology scores versus human protein S sequence were averaged over all affinity purified peptides. A synthetic peptide comprising residues 439–460 in human protein S was found to inhibit protein S binding to C4BP. The same result was found with two overlapping peptides (residues 447–468 and 435–468, respectively) in a second set of synthetic peptides. Direct binding of the peptides to C4BP was inferred from titrations monitored by recording the near UV circular dichroism spectra or the polarization of tryptophan fluorescence. The results suggest that residues 447–460 constitute a portion of protein S that is important for the interaction with C4BP. These findings may have implications for patients suffering from thrombosis, due to the lack of free protein S, by directing the design of drugs that disrupt protein S binding to C4BP.

Protein S (molecular mass = 75 kDa; see Fig. 1A) participates in the anticoagulant pathway as a cofactor to activated protein C. C4BP (see Fig. 1B) is a regulator of the classical complement pathway and is a highly glycosylated multimeric protein of high molecular mass (570 kDa). The primary structures of the two proteins reveal that they consist of multiple modules. Protein S contains one Gla-module, a thrombin-sensitive disulfide loop, four EGF-like modules and a C-terminal domain that is homologous to the sex hormone binding globulin (SHBG). The SHBG domain contains three glycosylation sites, two of which are conserved throughout different species (3–8). C4BP consists of 7–8 polypeptide chains that are linked together by disulfide bonds between cysteines located in the C-terminal part of each chain. The major isoform has seven $\alpha$-chains, each containing eight short consensus repeat (SCR) modules, and a single $\beta$-chain containing three SCR modules. One of the minor isoforms of C4BP has no $\beta$-chain and does not interact with protein S (9–11).

The structures of protein S and C4BP are known at the electron microscopy level (12, 13), showing that C4BP looks like a spider with the SCR modules arranged as beads on a string; whereas protein S is more compact. High resolution structures are available for several homologous modules in other proteins, for example EGF-like modules from factor IX and X (14–16), a pair of EGF-like modules from fibrinogen (17), a Gla-EGF module pair from factor X (18), and three single and one pair of SCR modules from factor H (19–21). Recent additions to the field are crystal structures of coagulation factor IX (22) and of factor VII in complex with tissue factor (23).

The interaction between C4BP and protein S is so strong ($K_D = 0.1–0.6 \text{ nM}$ in 150 mM NaCl, 2 mM Ca$^{2+}$, pH 7.5) (8, 24–27) that the entire pool of $\beta$-chain-containing C4BP in human plasma is complexed to protein S. In normal individuals, the total protein S concentration exceeds that of the $\beta$-chain-containing isoforms of C4BP by 30%, ensuring an uncomplexed protein S fraction with anticoagulant activity. The cause for thrombosis in some patients is a consequence of total protein S concentrations not exceeding the $\beta$-chain-containing C4BP isoform levels and protein S complexed to C4BP has no anticoagulant activity. C4BP is an acute-phase protein, and its concentration can increase as much as 4-fold during the acute-phase inflammatory response. It has recently been demonstrated, however, that it is primarily the concentration of the C4BP isoform lacking the $\beta$-chain that increases during the acute-phase inflammatory response, resulting in a maintained anticoagulation system in a normal individual and indicating a differential regulation of the $\alpha$- and $\beta$-chains (28). It has been
suggested that the biological role for the interaction between protein S and C4BP is to anchor the complex of C4BP and C4b to phospholipid membranes at the site of injury (where the coagulant and anticoagulant protein complexes are bound via their Gla-modules) to prevent inflammation. Thus, it is important to identify the domains that are mediating this protein-protein interaction.

Electron microscopy studies suggest that protein S binds to the β-chain of C4BP (13). Binding of protein S to the β-chain has also been shown using recombinant truncated β-chain proteins containing SCR modules 1–3 (29) or SCR 1–2 (30) as well as a construct with the first SCR of the β-chain fused to SCR 2–8 of the α-chain (30). A study using synthetic peptides has lead to the proposal that residues 31–45 in the first SCR-module of the C4BP β-chain are essential for the interaction (31). A structural prediction has been presented for the β-chain of C4BP (31, 32), revealing as a potential binding site for protein S a solvent-exposed hydrophobic patch in the first SCR-module lined with a positively charged area on an otherwise negatively charged surface.

The portion of protein S that binds to C4BP has not been conclusively specified. Many attempts have been made using either synthetic peptide fragments or site-directed mutagenesis of the protein S molecule or chimeric proteins. The smallest part that has been demonstrated to bind C4BP with full native affinity is the SHBG-like domain, as inferred from binding studies using protein hybrids of protein S and coagulation factor IX (27). Two synthetic peptides have been reported to compete with protein S for binding to C4BP when used in very large excess over protein S, one comprising residues 413–433 (33) and one comprising residues 605–614 (34, 35). Truncated protein S variants lacking residues 607–635 or 577–635 have drastically reduced affinities for C4BP (36). A 10^3-fold reduction in affinity has been reported for a similar variant lacking residues 583–635 (37), meaning either that some residues in the 583–635 sequence are directly involved in the binding to C4BP or that the C-terminal 52 residues are necessary for the correct folding or presentation of another part that contains the binding site. Other mutants that have been found to have reduced affinity for C4BP have an extra alanine inserted at position 611 or Cys-598 deleted (36). It is also known that bovine and human protein S (82% identical; see Refs. 3 and 4) bind to human C4BP with about the same affinity, whereas the homologous proteins SHBG (26% identical to the SHBG-like domain of human protein S; see Ref. 38) and growth arrest-specific protein 6 (Gas 6) (44% identical to human protein S; see Ref. 39) do not show any detectable binding to C4BP.

We have chosen to address the problem of identifying the C4BP binding site in protein S using the phage display method. Sequences with high affinity for the C4BP binding site in protein S using the phage display method was amplified from the 2 Y. Härdig, unpublished result.

**MATERIALS AND METHODS**

**Proteins**—C4BP was purified from human plasma as described (1). Two recombinant proteins called SCR-β1 and Rec. α, respectively, were used in the screening procedure. SCR-β1 was expressed in a prokaryotic expression system and contained the two N-terminal SCR modules from the C4BP β-chain, whereas Rec. α, which was expressed in an eukaryotic expression system, was composed of C4BP molecules lacking the β-chain. Both recombinant proteins were expressed and purified as described (11, 30). Avidin, streptavidin, and BSA were from Pierce (Rockford, Illinois). Biotinylation of Proteins—Prior to biotinylation, buffer amines were removed from the C4BP and SCR-β1 by concentration and multiple washes with phosphate-buffered saline buffer in Centricon concentrators (Grace, Beverly, Massachusetts) with molecular mass cut-offs of 30 kDa, for C4BP, and 3 kDa, for SCR-β1. NHS-LC-biotin was first dissolved in H2O and then added to the proteins. The C4BP concentration was 0.2 mg/ml, and a 10-fold molar excess of NHS-LC-biotin was added. The SCR-β1 concentration was 0.3 mg/ml, and a 5-fold molar excess of NHS-LC-biotin was added. The mixture was allowed to react for 30 min at room temperature, and the unreacted NHS-LC-biotin was removed by separation on a desalting column or by multiple washes in the concentrators. The amount of biotinylation was 0.5 biotin/protein for SCR-β1, and 2–4 biotin/protein for C4BP, as judged by using 2,4'-hydroxyazobenzene/benzonic acid reagent included in the NHS-LC-biotinylation kit from Pierce.

**Phage Display Peptide Libraries**—The 6-mer phage display library was amplified from the 2 Y. Härdig, unpublished result. The 15-mer library was amplified from the 2 Y. Härdig, unpublished result. The randomized peptide sequences were tailed to the N terminus (between residues 5 and 6) of the phage coat protein pIII.

**Peptide Synthesis and Purification**—The linear peptides (BD4, BD6, and SL1-SL7) were synthesized on a Milligen 9050 Plus synthesizer (continuous flow peptide synthesis) using Fmoc chemistry with active esters (pentafluorophenyl esters). The first amino acid in the synthesis (the C-terminal amino acid) was coupled to the resin PEG-PS SupportTM from Millipore (polyethylene glycol-polystyrene). After the synthesis, the resin was rinsed and dried. The peptide was released from the resin by cleavage for 2 h under N2 gas in the darkness using 92–95% TFA containing different scavengers depending on the amino acid composition of the peptide. The resin was removed by filtering and washed with concentrated TFA. After concentration, the peptide was precipitated and washed 4 times in cold diethyl ether. The ether was evaporated, and the peptide was dissolved in 0.1% TFA/H2O (or in 50–75% acetic acid for the SL1, SL2, SL4, SL6, and SL7 peptides that were difficult to dissolve in 0.1% TFA). The peptide was purified on an HPLC (Waters 600E System Controller, Waters 486 Tunable Absorbance Detector) C8 column (Kromasil 5, 100A C8, 250 × 21.2 mm) using a linear gradient of 0.1% TFA/H2O and 0.1% TFA, 80% acetonitrile/H2O. The peptide was concentrated by speedvac and lyophilization.

**Peptide Purification**—The peptides BD4 and BD6 were reduced (in 0.1 M Tris, pH 8.3, with 0.1 M diithiothreitol and 6 μM guanidine-HCl for 2 h at room temperature at a peptide concentration of 10 mg/ml) prior to HPLC purification (as described above). After purification, they were folded to form a disulfide bond between the two cysteines in each peptide (in 0.1 M Tris, pH 8.3, with 1 mM EDTA, 3 mM cysteine-HCl, and 0.01 M Na2SO4 for 16 h at room temperature at a peptide concentration of 0.1 mg/ml). The peptides were subjected to a second HPLC purification (as above) after folding.

**Chemicals**—All chemicals were of the highest grade commercially available. Buffers and all other solutions were autoclaved or sterile-filtered prior to use. Sterilized labware was used throughout. The following abbreviations for autoclaved buffers are used in the text: TBS, 50 mM Tris, 0.15 M NaCl, 2 mM CaCl2, pH set to 7.5 with HCl; TBS/Tween, TBS with the addition of 0.5% Tween; TBS/NaNO2, TBS with the addition of 0.02% NaNO2; HC, 10 mM Hepes, 0.15 mM NaCl, 3 mM EDTA, 0.005% Tween 20, pH 7.4; and phosphate-buffered saline, 0.137 mM phosphate buffer, pH 7.0 with 0.15 M NaCl. The sensorscops CMS and amine coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-

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The text continues with more detailed descriptions and experimental methods related to the study of C4BP and its interactions with protein S, including biopanning experiments and the use of phage display libraries to identify binding sites.
After discarding the avidin/streptavidin solution, the remaining protein adsorption sites on the plastic were blocked by BSA; 400 μl of a solution containing 1 mg/ml BSA and 10 μg/ml avidin or streptavidin was added and allowed to react for 1 h. Dishes were then washed 6 times with TBS/Tween. In the first three rounds (“P+L+” method), the biotinylated ligate was allowed to react for 2 h with the immobilized avidin/streptavidin (in 400 μl TBS/Tween) prior to 6-fold washing (TBS/Tween), addition of 0.1 μg biotin (in 400 μl TBS/Tween), and addition of the phages (1011 physical particles in 100 μl of TBS/NaN3), whereas in rounds four and five (“PL+” method), the biotinylated ligate was allowed to equilibrate for 24 h with the phages (1011 physical particles in 100 μl of TBS/NaN3) prior to dilution with 400 μl of TBS/Tween and binding to streptavidin/avidin on the dish. After 10 washes with TBS/Tween, the binding phages were eluted with 400 μl of 0.2 M glycine buffer at pH 2.2. The eluate was neutralized by mixing with 75 μl of 1 M Tris/HCl, pH 9.1. The phage eluate from each round of biopanning was amplified in E. coli and purified from the culture supernatant using two PEG precipitation steps to provide the input phages for the next round of biopanning. The number of transducing units were counted for input and output phages of each round to provide an estimate of the yield in each round of biopanning.

Selective Removal of Peptides Binding to the C4BP α-chain—C4BP interacts with many different proteins. In addition to the high affinity protein S-binding site on the β-chain, there are lower affinity binding sites on the α-chain for C4b, serum amyloid P component, heparin, and factor VIII. A fraction of the captured peptides might thus bind to sites on the α-subunit. Therefore, in an extra (sixth) round of biopanning, a large excess of recombinant C4BP containing only α-chains (Rec. α) was added to the C4BP phage mixture to capture α-chain binding phages. The input phages were in 10-fold excess over intact C4BP, and the Rec. α was in 100-fold molar excess over the α-chains in intact C4BP. The same amount of Rec. α was added in one of the washes. In contrast to intact C4BP, the recombinant protein was not biotinylated whereby, in principle, predominantly β-chain binding phages should remain attached to the dish after extensive washing.

Affinity Purification Using Magnetic Beads—Affinity purification of the phage-bound 6- and 15-mer libraries against C4BP was also performed using magnetic beads. Biotinylated C4BP was coupled to streptavidin-coated magnetic beads (Dynabeads M-280, 112.06, Dynal AS, Oslo, Norway). The beads were separated from buffers in the different steps using a magnetic particle concentrator (Dynal MPC-M, Dynal AS, Oslo, Norway). The beads were first washed three times with TBS/Tween. 90 μg of biotinylated C4BP was mixed with 2 mg beads in 400 μl of TBS/NaN3, 1 mg/ml BSA for 30 min at room temperature. The beads were then washed once, 1 μl of 10 μM biotin was added, and beads were then washed four times with TBS/Tween. 10 μl of an original phage display library or 100 μl of an amplified library was mixed with C4BP-coupled beads in 400 μl of TBS/Tween and allowed to equilibrate overnight on a rocker. Four rounds were carried out, the first two rounds with 500 μl beads and the last two with 30 μl beads. Rec. α was added in rounds three and four. The beads were washed 10-fold with TBS/Tween prior to 400 μl of 0.2 M glycine buffer at pH 12. Neutralization with 75 μl of 1 M Tris/HCl, pH 9.1, amplification in E. coli, and purification using two PEG precipitation steps provided input phages for the next round.

DNA Sequencing—The amino acids sequences of affinity purified phage-bound peptides were derived by sequencing the DNA corresponding to the N-terminal part of gene III coat protein, which is 29 residues C-terminal to the insert. Sequencing was performed with the PRISM Sequence Terminator double-stranded DNA sequencing kit (Applied Biosystems) using an Applied Biosystems Model 373 DNA sequencer.

Peptide Inhibition of the Protein S-C4BP Interaction in Microtiter Plates—Microtiter plates were coated with C4BP, 50 μl/well, 10 μg/ml in 0.075 M sodium carbonate buffer, pH 9.6. The plates were incubated overnight at 4 °C and then washed with TBS, pH 7.5, containing 0.1% Tween 20. After quenching (TBS, pH 8.0, containing 0.05% Tween 20, 3% fish gelatin, and 0.02% NaN3, 100 μl/well, for 30 min) and washing, 10 μl of dilutions of the peptides (0.1–3000 μM) or plasma-purified human protein S in TBS containing 10 mM EDTA were added together with a trace amount of 125I-labeled protein S in a final volume of 50 μl and left at 4 °C overnight. The wells were then washed, and the amount of bound protein S detected using a γ-counter.

Peptide Inhibition of the Protein S-C4BP Interaction, Surface Plasma Resonance Studies—The surface plasma resonance studies were performed using a BIACore™ apparatus from Pharmacia Biosensor AB. Immobilization of C4BP to the dextran-coated gold surface of a sensorchip was performed at a flow rate of 5 μl/min, using HC as flow buffer. Equal volumes of 0.1 M NHS and 0.1 M N-ethyl-N’-(3-diethylaminopropyl)carboxyldiimide were first mixed, after which 30 μl of the mixture was flown over the sensorchip surface to activate the carboxymethylated dextran. C4BP was then injected over the sensorchip (40 μl of a 60 μg/ml solution in 10 mM NaHAc, at pH 4.75), after which unreacted NHS-ester groups were blocked by 15 μl of 1 M ethanolamine, pH 8.5. The system was regenerated by addition of 15 μl of 0.1 M HCl, which removes all non-covalently bound molecules. The immobilized amount of C4BP was 8000 response units (RU). Protein S association was monitored with 50 nm human protein S in HC buffer in a continuous flow of 1 μl/min during 45 min. The ability of each peptide to inhibit the protein S binding was studied by following the association to C4BP for mixtures of protein S and peptide. In BIACore™, the total amount of bound material is measured, and since the peptides are 25-fold smaller than protein S, inhibition of protein S binding by peptide binding will drastically lower the observed response during the association phase. The percent protein S bound, X, was calculated as

\[ X = \frac{S_{\text{max}} - 0.04 S_{\text{max}}}{0.96 S_{\text{max}}} \]  

where \( S_{\text{max}} \) is the measured response and \( S_{\text{max}} \) is the response obtained in the absence of peptide.

Peptide Binding to C4BP: Fluorescence Polarization Studies—Fluorescence spectra were recorded on a SPEX Fluorolog spectrometer using a 1 × 1-cm cuvette. The excitation bandwidth was 2 nm, and the emission bandwidth was 3 nm. Emission spectra between 300 and 425 nm (step 1 nm) were recorded with excitation at 270, 275, 280, 285, and 290 nm. For polarization measurements, two polarizers were placed in the excitation path and emission light path. The components with the polarizers set parallel and perpendicular to one another were recorded separately, with excitation at 270 nm and emission at 325–345 nm (step 2.5 nm). Each component was taken as the average of 10 scans.

Peptide Binding to C4BP: CD Spectroscopy Studies—Near UV circular dichroism (CD) spectra were recorded on a JASCO-720 spectropolarimeter using a thermostated cuvette with a 1-cm path length. Spectra were recorded between 300 and 250 nm, using a wavelength step of 1 nm, response time of 4 s, and scan rate of 10 nm/min. Four scans were recorded and averaged for each spectrum.

Homology Search—Homology search was performed with the homemade program HOMOFILE. This program tests all possible alignments of a peptide with a protein sequence. The output is a file that lists, for each residue in the protein (as starting position of the alignment), the identity, near identity, and high similarity scores. The identity is taken as strict identity, and the criteria for near identity and high similarity are listed in Table I. The output files were either imported one by one to KaleidagraphTM for plotting or averaged by another homemade program, AVEHOM, the output of which is a file that lists, for the starting position of each alignment, the average identity, near identity, and high similarity scores.

| Table I Criteria for homology scoring |
|--------------------------------------|
| Residue | Near identity | High similarity |
| A        | G             | P             |
| B        | G             | P             |
| C        | G             | P             |
| D        | F             | I             |
| E        | L             | H             |
| F        | L             | F             |
| G        | P             | L             |
| H        | A             | F             |
| I        | E             | V             |
| J        | H             | P             |
| K        | F             | W             |
| L        | F             | F             |
| M        | D             | E             |
| N        | D             | R             |
| O        | Q             | S             |
| P        | K             | E             |
| Q        | Q             | S             |
| R        | S             | N             |

\[ X = (S - 0.04 S_{\text{max}})/0.96 S_{\text{max}} \] (Eq. 1)
**RESULTS**

Human C4BP was used to affinity purify phage-bound peptides from two different phage display libraries, one with displayed hexapeptides and one with pentadecapeptides. Both libraries contain inserts at the N terminus of the coat protein pIII of M13. These inserts are randomized at the genetic level, and each phage contains up to five copies of one particular peptide in 6-mer or 15-mer library. In parallel, phage-bound peptides, SL2, SL6, and SL7, prevent the binding of protein S to C4BP, whereas none of the other peptides had any effect on the protein S-C4BP interaction. Half-maximum inhibition was seen at 100–200 μM for the three inhibiting peptides.

**Peptide Inhibition of the Protein S-C4BP Interaction, Surface Plasmon Resonance**—The ability of the synthetic peptides to inhibit the binding of human protein S to C4BP was also studied using surface plasmon resonance on a BIAcore™ system. For six of the peptides (SL1, SL3, SL4, SL5, BD4, and BD6), we observed the same response as with protein S alone, even when the peptides were in 6000-fold molar excess over protein S (300 μM peptide, 50 nM protein S). However, three peptides, SL2, SL6, and SL7, prevent the binding of protein S to C4BP with half-maximum inhibition at 30–120 μM peptide concentration (Fig. 3B).

Residues 447–460 are in common in all three peptides with inhibitory action.

**Peptide Binding to C4BP, Fluorescence Polarization Studies**—The interaction of the SL6 and SL7 peptides with the C4BP fragment SCR-β2, was studied by fluorescence spectroscopy. SCR-β2 contains two tryptophan residues, and one tryptophan is present in each of the SL6 and SL7 sequences. The emission spectra of SCR-β2 alone, SL6 alone and a 1:1 mixture are shown in Fig. 4A. Two titrations of SCR-β2 with SL6 and SL7, respectively, were monitored using polarizers and recording the parallel ($I_\parallel$) and perpendicular ($I_\perp$) emission components separately. The polarization, $p$, was calculated as

$$p = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$$

(Eq. 2)

The polarization of a 15 μM SCR-β2 solution as a function of SL7-concentration is shown in Fig. 4B. There is an initial increase in the polarization up to ~1 molar equivalent of added peptide, and then $p$ gradually decreases due to the appearance of free peptide. The data was fitted by assuming a 1:1 stoichiometry and that the observed polarization is a weighted average of the polarizations of free peptide, free SCR-β1,2, and the SCR-β1,2peptide complex, where the weighting takes into account the different numbers of chromophores in the three spe-

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**Additional Information**

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**Supplementary material may be obtained from the authors.**
cies. The variable parameters in the fit were $P_S, P_L, P_C, C_S$, and $K_D$, where $P_S, P_L,$ and $P_C$ are the polarizations of free SCR-$\beta_{1,2}$, free peptide, and the SCR-$\beta_{1,2}$-peptide complex, respectively; $C_S$ is the total SCR-$\beta_{1,2}$ concentration; and $K_D$ is the dissociation constant of the SCR-$\beta_{1,2}$-peptide complex. $C_L$ is the total peptide concentration. The fitted equation was derived as,

$$p = \left\{ \frac{P_L C_L + \frac{C_S[2P_L K_D + (3P_S - P_L) Y]}{K_D + Y}}{C_L + 2 C_S} \right\} (C_L + 2 C_S)$$  \hspace{0.5cm} (Eq. 3)

where

$$Y = -0.5(K_D + C_S - C_L) + \sqrt{0.25(K_D + C_S - C_L)^2 + C_S K_D}$$  \hspace{0.5cm} (Eq. 4)

and yielded good fits to the experimental points as shown in Fig. 4B. Computer analysis was performed separately for each of the two titrations (SCR-$\beta_{1,2}$ with SL6 and SL7, respectively). The best fits in both cases were obtained with values of $K_D$ between 0.01 and 0.1 $\mu$M. The error square sum of the fits were, however, only 2–3-fold higher for a $K_D$ of 1 $\mu$M. The dissociation constant of the SCR-$\beta_{1,2}$-peptide complex obtained from the polarization titrations can therefore not be specified more precisely than $K_D \approx 1$ $\mu$M. The titration with SL6 was performed using an independent batch of SCR-$\beta_{1,2}$ and initially had a lower value of the polarization. However, the increase on adding peptide was of similar magnitude, and the overall shape of the curve was very similar to that shown in Fig. 4B. We believe the small difference in starting polarization is due to variations in the amount of correctly folded SCR-$\beta_{1,2}$ in different batches.

**FIG. 3.** Peptide inhibition of the protein S-C4BP interaction showing the amount of protein S bound (relative to the amount bound in the absence of peptide) versus concentration of the nine peptides listed in Table II, SL1 (∗), SL2 (∗), SL3 (∗), SL4 (+), SL5 (○), SL6 (●), SL7 (▲), BD4 (△), and BD6 (◇). A, equilibrium binding assay using immobilized C4BP in microtiter wells and radiolabeled human protein S. B, surface plasmon resonance assay on a BIAcoreTM sensorchip. The amount of protein S bound, $X$, was calculated from the observed signal intensity, $S$, compared with the maximum signal intensity in the absence of peptide, $S_{\text{max}}$, as $X = (S - 0.04)/0.96 S_{\text{max}}$.

**Peptide Binding to C4BP-CD Spectroscopy Studies—**The interaction between SCR-$\beta_{1,2}$ and different peptides was studied using CD spectroscopy. As shown in Fig. 5A, the near UV spectrum of an equimolar mixture of SCR-$\beta_{1,2}$ and SL6 is distinctly different from the sum of spectra recorded for SCR-$\beta_{1,2}$ and SL6 individually. The difference spectrum (mixture minus spectral sum) has a positive peak around 270–280 nm. Similar results were obtained also with the peptides SL2 and SL7 but not with the peptides BD4 and BD6. The peak intensity, $\Theta_j$, of the difference spectrum as a function of added peptide is shown in Fig. 5B for SL6, SL7, BD4, and BD6. Curves calculated using the following equation,

$$\Theta_j = \Theta_{\text{max}}(V_j/V)(Y/Y + K_D)$$  \hspace{0.5cm} (Eq. 5)

where

$$Y = -0.5(K_D + C_S - C_L) + \sqrt{0.25(K_D + C_S - C_L)^2 + C_S K_D}$$  \hspace{0.5cm} (Eq. 6)

agreed well with data for SL6, SL7, and SL2 (data not shown) when $K_D$, the dissociation constant of the SCR-$\beta_{1,2}$-peptide complex, was around 1 $\mu$M (Fig. 5B). In Equation 5, $\Theta_{\text{max}}$ is the ellipticity difference at saturation, $C_L$ is the total peptide concentration, and $C_S$ is the total SCR-$\beta_{1,2}$ concentration. $V$ is the total volume. $V_j$ is the total volume before the start of the titration.
DISCUSSION

The phage display technique is fruitful for mapping the epitopes recognized by monoclonal antibodies (40), and in the study of protein-protein, protein-DNA (42, 43), and intra-protein (44) interactions. The technique has also proven useful in assessing protease specificity (45), criteria for protein folding (46), as well as designing D-peptide drugs (47, 48). The amino acid sequence identified in a phage display experiment toward a specific target is not always unambiguously assigned to a given stretch of the protein that binds to the target in vivo. One reason for this is that the native binding sequence often does not have the highest possible affinity but rather the most suitable affinity for its biological context. Another reason is that the library, although very diverse, is not 100% complete, and the natural binding sequence might not be present. A third reason is that the library is not completely random on the amino acid level due to codon usage considerations and constraints. A fourth reason is that the native binding epitope may not be a contiguous primary sequence, rather, the binding epitope may be influenced by the three-dimensional structure. With these obstacles in mind, we have made an attempt to identify the regions in protein S that have the highest homologies with the phage displayed sequences that were selected by affinity purification in the present work.

Visual inspection of individual selected display peptides both to each other or to protein S failed to provide a convincing alignment, in contrast to what is commonly observed in other phage display experiments. A more objective alignment of each individual peptide sequence to protein S, using homology searches (HOMOFILE) followed by averaging the scores obtained for all peptides (AVEHOM), revealed a peak around position 450 (Fig. 2), which is significantly higher than the noise.

Surprisingly, few of the phage display peptides selected by affinity purification against C4BP or SCR-β1,2 showed any striking similarity to the first disulfide loop in the SHBG-like domain of protein S or to the second disulfide loop, both of which have been previously suggested as likely locations of the C4BP binding site (33–35). Therefore, to specifically address this discrepancy, two peptides corresponding to these disulfide loops were synthesized (BD4, residues 405–437, and BD6, residues 595–628) and tested for inhibition of the protein S-C4BP interaction. Consistent with the phage display results, we found that these peptides have no inhibitory effect even when employed in 6000-fold excess over protein S. This is further corroborated by the failure of BD4 and BD6 to show any binding to SCR-β1,2 in binding experiments monitored by CD spectroscopy.

Certain experimental details may account for the conflicting BD4 and BD6 peptide binding results obtained by Walker (34), Fernández and Griffin (33), and by those reported herein. The level of confirmation of peptide purity was lower in the previous
Indeed, one of the three glycosylation sites in protein S (Asn-458) lies within the C4BP-binding region identified here, and another one (Asn-468) is close by (Fig. 6). The glycosylation sites at residues 458 and 468 are found in several species (human, rhesus monkey, bovine, mouse, porcine, rabbit, and rat), whereas the third site at Asn-489 is present only in human and monkey protein S. Gas 6 and the SHBG are two proteins that are homologous to protein S but do not bind to C4BP.2 An alignment of the amino acid sequences of these proteins with protein S show distinct differences in the 447–460 region (Fig. 6). Gas 6 contains a 4-amino acid insertion and SHBG has a one residue deletion and a different pattern of hydrophobic and hydrophilic residues. In addition, both Gas 6 and SHBG lack the consensual sequence (N-X-S/T) for carbohydrate attachment at the positions corresponding to residues 458, 468, and 489 in protein S. Our future studies will investigate the role that glycosylation plays in the C4BP-protein S interaction. The N-linked carbohydrate at position 458 is not essential for binding to C4BP. A variant of human protein S, the so-called protein S-Heerlen, which lacks this carbohydrate moiety due to a Ser→Pro mutation at position 460 (49), still interacts with C4BP (50).

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