The Amino-terminal Module of the C4b-binding Protein \( \beta \)-Chain Contains the Protein S-binding Site* \\
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Human C4b-binding protein (C4BP) is composed of multiple \( \alpha \)-chains associated with a single \( \beta \)-chain. Each chain is composed of homologous, tandemly arranged repeats of so-called short consensus repeats (SCRs). We have previously shown that the three SCR modules of the \( \beta \)-chain contain a high affinity binding site for anticoagulant vitamin K-dependent protein S. On the basis of experiments using synthetic peptides, residues 31–45 of the amino-terminal SCR (SCR-1) in the \( \beta \)-chain were suggested to be involved in protein S binding, but it is not known whether SCR-1 contains the entire protein S-binding site. To address this question, two different truncated forms of the \( \beta \)-chain (\( \beta \)1,2 and \( \beta \)2,3) were expressed in a prokaryotic expression system. The \( \beta \)1,2 construct (SCR-1 + SCR-2) contained the high affinity binding site for protein S in contrast to \( \beta \)2,3 (SCR-2 + SCR-3), which did not bind protein S. Unfortunately, it was not possible to express SCR-1 alone in this system. To further elucidate whether the protein S-binding site is fully contained in SCR-1 or whether SCR-2 is also required, recombinant \( \alpha/\beta \)-chain chimeras were constructed. These chimeras were composed of \( \alpha \)-chains with one, two, or three of the amino-terminal SCR modules replaced by the \( \beta \)-chain counterpart and were expressed in a eukaryotic expression system. All recombinant variants were retained within the cells and could be extracted in biologically active forms. The three \( \alpha/\beta \)-chain chimeras bound protein S equally well, with a \( K_D \) of \( \sim 2.3 \times 10^{-9} \) M\(^{-1} \) as compared with 2.1 \( \times 10^{-9} \) M\(^{-1} \) for plasma-purified C4BP. These results show that the entire protein S-binding site on C4BP is contained within \( \beta \)-chain SCR-1.

C4b-binding protein (C4BP)\(^1\) is a high molecular weight glycoprotein that regulates both the complement system, by binding to complement component C4b, and the protein C anticoagulant system, through its interaction with protein S (1–5). Protein S is an anticoagulant vitamin K-dependent protein that functions as a cofactor to activated protein C in its degradation of coagulation factor Va and factor VIIIa (6). Approximately 70% of protein S in human plasma circulates in complex with C4BP. Only free protein S acts as an activated protein C cofactor, and deficiency of free protein S is associated with increased risk of thromboembolic disease (7). C4BP is composed of two kinds of subunits, the \( \alpha \)- and \( \beta \)-chains (Fig. 1A). Most of the C4BPs in human plasma contain seven \( \alpha \)-chains and one \( \beta \)-chain, but other isoforms also exist (8). Each chain is composed of internally homologous repeats called short consensus repeats (SCRs), complement control proteins, or Sushi domains. The \( \alpha \)-chain contains eight SCRs, and the \( \beta \)-chain contains three (9, 10). Each SCR is composed of \( \sim 60 \) amino acids and contains several conserved residues including four cysteines, which form intradomain disulfide bridges (Cys1–Cys3 and Cys2–Cys4). In addition, each chain contains a carboxyl-terminal non-repeat region with two cysteines, which link the chains together through disulfide bridging. SCR modules are found in many complement and non-complement proteins. Some SCRs are involved in ligand binding, while others seem to function merely as spacers. Using a recombinant truncated \( \beta \)-chain that was expressed in a prokaryotic system, we previously showed that the protein S-binding site was located within the three SCR modules of the \( \beta \)-chain (11). A peptide comprising amino acids 31–45 of the C4BP \( \beta \)-chain (located in SCR-1) has been reported to inhibit the binding of protein S to C4BP (12). This peptide also bound directly to protein S with a \( K_D \) that was \( \sim 60 \) times weaker than that of plasma C4BP-protein S binding (13). Furthermore, antibodies against this peptide inhibited the binding of protein S to C4BP. These results suggest that amino acids 31–45 contain an important part of the binding site for protein S, but it is not known whether this region of SCR-1 constitutes the entire binding site or whether other parts of the \( \beta \)-chain are involved in forming the binding site.

To further characterize the C4BP-protein S interaction, two different truncated forms of the \( \beta \)-chain were expressed in a prokaryotic expression system and tested for their protein S binding capacity. A recombinant protein composed of SCR-1 + SCR-2 bound protein S equally well as plasma-purified C4BP, whereas a construct containing SCR-2 + SCR-3 did not bind protein S. However, it was not possible to further localize the protein S-binding site in this system. We were unable to express SCR-1 by itself, and due to folding problems, the system was unsuitable for site-directed mutagenesis. Attempts to express truncated or intact \( \beta \)-chain in various eukaryotic expression systems were also unsuccessful. Instead, recombinant C4BP cDNA chimeras composed of C4BP \( \alpha \)-chains with SCR-1, SCR-1 + SCR-2, or SCR-1 + SCR-2 + SCR-3 replaced by corresponding SCRs from the \( \beta \)-chain were constructed and expressed in eukaryotic cells. Protein S binding studies using these recombinant proteins showed that \( \beta \)-chain SCR-1 contains the entire protein S-binding site and that neither \( \beta \)-chain

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1 The abbreviations used are: C4BP, C4-binding protein; SCR, short consensus repeat; mAb, monoclonal antibody; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
SCR-2 nor SCR-3 contributes to the high affinity binding between C4BP and protein S.

MATERIALS AND METHODS

Proteins

C4BP (14) and protein S (15, 16) were purified from human plasma. Concentrations of purified proteins were estimated by measuring absorbance at 280 nm. The extinction coefficients (ε) were used for intact C4BP (17) and 9.5 for protein S (18), whereas we chose to use 10 as the extinction coefficient for the recombinant proteins that were expressed in the prokaryotic expression system. The monoclonal antibody HPC4 was a kind gift from Dr. C. T. Esmon (Oklahoma Medical Research Foundation). The polyclonal antibodies against intact C4BP were prepared and characterized as described previously (19). Monoclonal antibody 36 (mAb 36) was raised against the third SCR of the C4BP β-chain, and its epitope has been shown to be located in the third SCR of the β-chain, whereas mAb 64 and mAb 96 recognized the C4BP α-chain. Protein S and C4BP were radiolabeled with 125I (Amersham Corp.) using IODO-BEAD™ and purified as described previously (19).

Cloning Procedure

Prokaryotic Expression—The β-chain cDNA constructs were made using polymerase chain reaction (PCR) technology (20). Full-length human C4BP β-chain cDNA was used in the PCR amplification (10). The following oligonucleotides were synthesized using an Applied Biosystems Model 381A DNA synthesizer: 1, TACTGCAGCAGACCTGCACAGGCTCTCC; 2, TACTGCAGATCTAATCCCTGAGTGGGA; 3, CTCGCAAGCACTGTCTGACATGGTGCT; and 4, TACTGCAGTTAGATCAAGCTGAGTGGGAA. Oligonucleotides 1 and 2 were used to amplify the DNA encoding SCR-1 + SCR-2 of the β-chain (β1,2), and oligonucleotides 3 and 4 to amplify SCR-1 + SCR-3 (β2,3). Oligonucleotide 1 contained a SalI/HindIII site (underlined), which introduced an amino-terminal Asp in the recombinant protein, while oligonucleotide 3 contained a NdeI site (underlined). Oligonucleotides 2 and 4 contained one PstI site (underlined) and one stop codon (boldface) each. The DNA fragments were subcloned into the pBluescript vector (Stratagene), sequenced using an Applied Biosystems Model 373 DNA sequencer, and fragments were subcloned into the pBluescript vector cut with the same enzymes, and sequenced. Similar procedures were repeated for the β2,3 and β2,3 constructs, but with SnaB1 being replaced by BsmI. Finally, the constructs were cloned into HindIII- and NotI-cleaved pCDNA3 vector (Invitrogen).

Expression and Characterization of Recombinant Proteins

Prokaryotic Expression—The truncated β-chains were expressed in the bacterial strain J221 as described previously (11). Briefly, 1 ml of an overnight culture of the transformed bacteria in LB medium containing 50 µg/ml ampicillin was inoculated to 25 ml of the same medium and grown to an A600 of 0.8. This culture was then grown in 1 liter of LB medium to the same cell density. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration, 1 mM), and incubation was continued for 12 h at room temperature. After centrifugation of the bacteria (10,000 × g, 30 min), the periplasmic extract was prepared by hypotonic shock of the bacterial pellet in 100 ml of H2O for 30 min at 4 °C and centrifugation as described above. The periplasmic extract and the supernatant obtained after the first centrifugation were mixed and supplemented with 20 mM Tris-HCl (pH 7.5), 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.02% NaN3, 2 mM CaCl2, and 50 mg/liter soybean trypsin inhibitor. Glutathione (reduced form; 1 mM) was added to facilitate the formation of correctly folded recombinant proteins. The recombinant proteins were purified using monoclonal antibody HPC4 coupled to Affi-Gel as described previously (11).

Eukaryotic Expression—AV 12-664 cells (a Syrian hamster cell line) were grown in Dulbecco's modified Eagle's medium (ICN) supplemented with fetal bovine serum (10%, v/v; Integro B.V.), penicillin (50 IU/ml; ICN), streptomycin (50 µg/ml; ICN), and glutamine (4 mM; ICN) at 37°C in 5% CO2. The cells were transfected using the calcium phosphate method as described earlier (23). The cells were grown to confluency and lysed with a single detergent buffer (50 mM Tris-HCl (pH 8.0), 0.15 mM NaCl, 1% Nonidet P-40, 0.02% NaN3, 100 µg/ml phenylmethylsulfonyl fluoride, and 2 µg/ml leupeptin).

Pulse-Chase Analysis—A pulse-chase experiment was performed to check for expression and secretion of recombinant β1,2. A pulse containing 300 µCi of [35S]Cys and [35S]Met (Amersham Corp.) in Dulbecco's modified Eagle's medium was added to each 100-mm plate containing 90% confluent cells and left for 30 min. The cells were lysed either directly after the pulse or after a 240-min chase with unlabeled complete medium. The medium and the cell lysates were preincubated for 2 h at 4°C with polyclonal rabbit IgG (3 µg) and protein A-Sepharose (50 µl, 40 mg/ml) (Pharmacia Biotech Inc.) to reduce nonspecific binding. The protein A-Sepharose was then pelleted by centrifugation for 2 min; a polyclonal antibody to C4BP was added; and the mixture was

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**Fig. 1. Schematic representation of intact C4BP and the constructs expressed in the prokaryotic system.**

- **A**: Intact C4BP containing seven α-chains and one β-chain. Each oval represents an SCR module. The shaded modules are those that were expressed in the prokaryotic system.
- **B**: C4BP β-chain SCRs. B1, B2, B3, and B4 represent an SCR module. The α-chain SCRs.

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The protein A-Sepharose was then pelleted by centrifugation for 2 min; a polyclonal antibody to C4BP was added; and the mixture was...
incubated overnight at 4 °C. The next day, protein A-Sepharose was added at the same concentrations as above, and after another 2-h incubation, the immunocomplexes were pelleted by centrifugation for 2 min. The pellet was washed three times in ice-cold lysis buffer and dissolved in sample buffer. After SDS-PAGE of the samples, the gel was dried, and the labeled proteins were detected using a PhosphorImagerTM SI (Molecular Dynamics, Inc.) (24, 25).

Characterization of Recombinant Proteins by Monoclonal Antibodies—Microtiter wells were coated with three different monoclonal antibodies (mAb36, mAb67, and mAb96) (50 μl, 10 μg/ml) in 75 mM sodium carbonate buffer (pH 9.6) (coating buffer) at 4°C overnight. The next day, the wells were washed three times in Tris-buffered saline (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl) containing 0.1% Tween 20 (washing buffer). The plates were then quenched with 1% bovine serum albumin in Tris-buffered saline for 30 min at room temperature; after washing, plasma-purified C4BP and the recombinant proteins in their cell lysate were added at a concentration of 1 μg/ml, and the plates were incubated for 2 h at room temperature. After incubation, the plates were washed; incubated with biotinylated mAb67; washed again; and then developed using streptavidin, biotinylated horseradish peroxidase, and 1,2-phenylenediamine (Dakopatts AB) according to the manufacturer's instructions.

Determination of Concentrations of Recombinant α/β-Chain Chimeras

Microtiter wells were coated with mAb 67 (50 μl, 10 μg/ml) in coating buffer, washed, and quenched as described above. After washing, a trace amount of 125I-labeled C4BP was added together with increasing amounts of unlabelled C4BP (0.017–35 nM) or recombinant proteins (cell lysate) in Tris-buffered saline (final volume, 50 μl). After overnight incubation, the wells were washed, and the bound radioactivity was measured in a γ-counter. The concentration of recombinant C4BP was calculated against the standard of plasma-purified C4BP.
Protein S Binding to SCR-1 of the C4BP β-Chain

Blotting Techniques
Purified β1,2 and β2,3 and cell lysates from cells expressing β1α, β2α, or β3α were subjected to SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corp.) using a semidy Electric Blotter (J C Biotechnological Instruments ApS). After blotting, the membranes were quenched with Tris-buffered saline containing 3% fish gelatin and 0.02% NaN₃, for 1 h. Western Blotting—The membranes were incubated with monoclonal antibody HPC4 (which was directed against the epitope fused to β1,2 and β2,3) or with a polyclonal antibody to C4BP (β1α, β2α, and β3α), washed in washing buffer, and then incubated either with an alkaline phosphatase-conjugated rabbit anti-mouse antiserum (Dakopatts AB) (β1,2 and β2,3) or with an alkaline phosphatase-conjugated swine anti-rabbit antiserum (Dakopatts AB) (β1α, β2α, and β3α). After a final washing procedure, the blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 0.1 M Tris-HCl, 5 mM MgCl₂, (pH 9.5).

Ligand Blotting—After quenching with fish gelatin, the membranes were incubated with 125I-labeled human protein S in 2 mM CaCl₂ for 120 min. After washing in washing buffer containing 2 mM CaCl₂ for 3 × 10 min, the signals were detected and quantified using a PhosphorImager.

Binding Assays
Direct Binding—Recombinant proteins expressed in the prokaryotic system were tested for direct binding to protein S essentially as described previously (11). Plasma-purified C4BP was immobilized in microtiter wells, and the wells were quenched as described above. 125I-Labeled protein S was then allowed to compete with increasing concentrations of unlabeled protein S (1.9–120 nM) for binding to the immobilized proteins, and the amount of bound protein S was measured in a γ-counter. Direct binding of the recombinant αβ-β-β-β-chain chimeras to protein S was measured in the following way. mab67 (50 μl, 10 μg/ml) was immobilized in microtiter wells; the wells were quenched; plasma-purified C4BP or cell lysates containing the various recombinant proteins (1 μg/ml; final volume, 50 μl) were added; and the wells were incubated for 2 h. The rest of the assay was identical to that described for measuring direct protein S binding to β1,2 and β2,3.

Competition Assay—The ability of the recombinant proteins to displace protein S binding to immobilized C4BP was tested essentially as described previously (11). Plasma-purified C4BP was immobilized in microtiter wells. After quenching with 1% bovine serum albumin, increasing amounts of plasma-purified C4BP (0.03–400 nM) or of the various recombinant proteins (0.0008–1000 nM) were added together with a trace amount of 125I-labeled protein S. After an overnight incubation, the amount of bound protein S was detected as described above.

RESULTS
Prokaryotic Expression of β1,2 and β2,3—Two different truncated forms of the C4BP β-chain were expressed in a prokaryotic expression system using the pN-III-pelB vector. β1,2 was composed of SCR-1 + SCR-2 (Glu⁶⁹–Arg⁹⁰) of the β-chain, and β2,3 was composed of SCR-2 + SCR-3 (Gly⁹²–Ile⁷⁷) (Fig. 1B). One liter of culture medium yielded ~1 mg of recombinant protein. The recombinant proteins were analyzed by SDS-PAGE and shown to have a Mᵣ of ~14,000 (Fig. 3A). After reduction, the respective Mᵣ values were 18,500 (β1,2), and 16,000 (β2,3).

Protein S Binding to β1,2 and β2,3—In a ligand blot assay using 125I-protein S, unreduced β1,2 gave a positive binding signal, whereas β2,3 did not bind protein S (Fig. 3B). Reduction of recombinant β1,2 was followed by a total loss of protein S binding capacity. To compare protein S binding to the recombinant truncated β-chain with that to plasma-purified C4BP, two different competition assays were performed. In the first assay, immobilized β1,2 bound to protein S with high affinity and with a half-maximum binding of 9 nM (as compared with 5 nM for

Fig. 3. Analysis of β1,2 and β2,3 by Western and ligand blotting. After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane. A, Western blot. The proteins were detected with monoclonal antibody HPC4. Lanes 1 and 3, β2,3; lanes 2 and 4, β1,2. B, ligand blot. The membrane was incubated with 125I-protein S, and the signal was detected using a PhosphorImager. Lanes 1 and 3, β1,2; lanes 2 and 4, β2,3.

Fig. 4. Protein S binding to β1,2 and β2,3. A, direct binding assay. Increasing concentrations of protein S were allowed to compete with a trace amount of 125I-labeled protein S for binding to immobilized plasma-purified C4BP or to the recombinant proteins. Each point represents the mean value from three different experiments. Binding is expressed as percent of the maximum binding observed in each experiment. B, competition assay. Immobilized plasma-purified C4BP was allowed to compete with increasing concentrations of C4BP, β1,2, or β2,3 in fluid phase for binding to 125I-labeled protein S. Each point represents the mean value from three different experiments. 100% binding was estimated in the absence of fluid-phase competitor. ⬤, wells coated with plasma-purified C4BP; □, wells coated with β1,2; ■, wells coated with β2,3.
Protein S Binding to SCR-1 of the C4BP β-Chain

plasma-purified C4BP), whereas β2,3 did not bind protein S (Fig. 4A). In the second assay, β1,2 completely inhibited the binding of protein S to C4BP, whereas β2,3 did not interfere with the C4BP-protein S interaction (Fig. 4B).

Eukaryotic Expression of β1α, β2α, and β3α—cDNA constructs composed of the C4BP α-chain with one, two, or three of its amino-terminal SCRs replaced by corresponding SCRs from the β-chain were expressed in a eukaryotic expression system. β1α was composed of the leader peptide and the amino-terminal asparagine from the α-chain (Met48–Asn1), SCR-1 and the amino acids between SCR-1 and SCR-2 from the β-chain (Cys56–His63), and SCR-2–SCR-8 and the carboxyl-terminal region from the α-chain (Cys65–Leu549) (Fig. 2A). Similarly, β2α contained SCR-1, SCR-2 and the amino acids between SCR-2 and SCR-3 from the β-chain (Cys56–Asp121), and SCR-3 and the carboxyl-terminal region from the α-chain (Cys127–Leu549), whereas β3α contained the three amino-terminal SCRs from the β-chain (Cys56–Cys179), whereas the region between β-chain SCR-3 and α-chain SCR-4, together with the remaining part of the construct, was derived from the α-chain (Glu187–Leu549).

After transfection of the cells, none of the recombinant proteins were detected in the medium. A pulse-chase experiment was performed in which cells transfected with β1α, intact α-chain, or intact expression vector (mock-transfected cells)

were labeled with [35S]Met and [35S]Cys and then chased with unlabeled medium as described under "Materials and Methods" (Fig. 5). After immunoprecipitation, the recombinant proteins were analyzed using a PhosphorImager. The reduced recombinant proteins were captured inside the cells and not exported to the medium. Directly after the pulse, both β1α and recombinant intact α-chain were found only in the cell lysate. After a 4-h chase experiment with unlabeled medium, a small amount of intact α-chain was detected in the cell lysate (most of it was found in the medium), whereas recombinant β1α was present only in the cell lysate. Like intact α-chain, β1α appeared as two distinct bands on unreduced SDS-PAGE. In both cases, these bands correspond to molecules containing seven and eight disulfide-bridged chains.

Neither cell lysate nor medium from mock-transfected cells contained any C4BP.

The cell lysates were also subjected to SDS-PAGE and Western blotting and were developed with a polyclonal antibody against the α-chain to confirm that the bands detected in the pulse-chase experiment corresponded to recombinant C4BP (Fig. 6). The reduced recombinants were of approximately the same size as the plasma-purified C4BP α-chain (70 kDa).

To investigate whether the recombinant proteins were correctly folded, they were tested for binding to three different monoclonal antibodies against C4BP in an enzyme-linked immunosorbent assay system. mAb 67, which recognized all three recombinant proteins, and mAb 96, which recognized only β1α, were both raised against intact C4BP. A third monoclonal antibody, mAb 36, which has earlier been shown to bind to SCR-3 of the β-chain,2 was reacted only with the recombinant protein containing this module, i.e. β3α.

To determine the concentrations of β1α, β2α, and β3α in the cell lysates, [125I]-labeled plasma-purified C4BP was allowed to compete with the recombinant proteins for binding to mAb 67, and the concentrations were calculated using the standard of plasma-purified C4BP. From each 100-mm dish, 1–20 μg of recombinant protein was obtained.

Protein S Binding to α/β-Chain Chimeras β1α, β2α, and β3α—The protein S binding capacity of the recombinant α/β-chain chimeras was analyzed using a ligand blot assay (Fig. 7). Cell lysates containing β1α, β2α, or β3α were subjected to SDS-PAGE. The proteins were transferred to a membrane and incubated with [125I]-protein S. Plasma-purified C4BP, purified recombinant C4BP composed of intact α-chains, and cell lysate from mock-transfected cells were used as controls. Bound [125I]-protein S was quantified using a PhosphorImager, and it was found that the signals from the three recombinant α/β-chains

FIG. 6. Analysis of recombinant proteins by Western blotting. Plasma-purified C4BP and cell lysates from the transfected cells were reduced and applied to 10% SDS-polyacrylamide gel. The gel was then subjected to Western blotting using a polyclonal antiserum against C4BP as described under "Materials and Methods." Lane 1, plasma-purified C4BP; lane 2, cell lysate from mock-transfected cells; lanes 3–5, cell lysates from cells transfected with β1α, β2α, and β3α, respectively.
were equally strong and approximately four times stronger than the signal derived from plasma-purified C4BP, whereas neither recombinant intact α-chain nor cell lysate from mock-transfected cells bound protein S. Reduced proteins did not bind protein S (data not shown).

To confirm that β-chain SCR-1 contains the entire protein S-binding site, both a direct binding assay and a competition assay were performed. Protein S binding to immobilized β1α, β2α, and β3α was saturable and of high affinity. Scatchard analysis of the data showed that the affinity constants for protein S binding to the recombinant chimeras and to plasma-purified C4BP were almost identical (protein S binding to the recombinant chimeras and to plasma-purified C4BP) in displacing binding of protein S to immobilized plasma-purified C4BP, whereas cell lysate from mock-transfected cells did not interfere with the C4BP-protein S interaction (Fig. 8B).

**DISCUSSION**

Human C4b-binding protein forms a high affinity, noncovalent complex with anticoagulant vitamin K-dependent protein S (3–5). We previously demonstrated that the protein S-binding site was contained within the three SCR modules of the β-chain (11). In peptide binding experiments, amino acids 31–45, located in SCR-1 of the β-chain, were found to constitute an important part of the binding site for protein S (12). Homology modeling (based on the NMR structures of SCR-15 and SCR-16 of factor H) suggested that the β-chain was mainly covered by a negative contour, but that an area in the carboxyl-terminal region of SCR-1 (including amino acids 31–45) had a positive electrostatic potential (13). A hydrophobic patch that was accessible to the solvent was also found in this region. Several hydrophobic residues are also found in SCR-3, however; and the homology modeling study did not rule out the possibility that some of these residues are in close contact with SCR-1 and thus are able to interact with protein S. In the study presented here, two truncated forms of the β-chain (lacking SCR-1 and SCR-3, respectively) were expressed in a prokaryotic expression system, and it was shown that deletion of SCR-3 had no effect on protein S binding, whereas deletion of SCR-1 resulted in a total loss of protein S binding. The binding assays showed that protein S bound to C4BP with somewhat higher affinity than to β1.2. However, the protein S binding of β1.2 was equally strong compared with that of a recombinant protein composed of all three β-chain SCRs described earlier, and the difference compared with plasma-purified C4BP could probably be explained by the fact that not all of the recombinant proteins produced in the prokaryotic expression system were correctly folded (11). Attempts to express SCR-1 by itself in this system or intact or truncated variants of the β-chain in various eukaryotic expression systems have been unsuccessful. To investigate whether SCR-1 contained the entire protein S-binding site, overlapping PCR was used to construct recombinant α-chains with one, two, or three of the amino-terminal SCRs replaced by the β-chain counterpart. Cells transfected with these cDNA constructs were unable to export the recombinant proteins from the cells. A possible explanation for these difficulties in expressing and exporting the β-chain is that the β-chain contains information important for the regulation of the amount of β-chain-containing C4BP that is produced by the cell or that the presence of α-chainSCR-1 is a prerequisite for export of the protein. The polymeric form of the recombinant proteins had a molecular weight comparable to that of plasma-purified C4BP, which shows that the polymerization process is independent of the three amino-terminal SCRs of the α-chain. The constructs were recognized by three different monoclonal antibodies that bound only to unreduced C4BP, thus strongly suggesting that the recombinant proteins were correctly folded. The recombinant α/β-chain chimeras contained one β-chain SCR-1 on each α-chain and therefore provided seven or eight protein S-binding sites on each molecule, as compared with the single protein S-binding site on plasma-purified C4BP. This

![Fig. 7. Ligand blotting.](Image)

Plasma-purified C4BP, recombinant intact α-chain, and cell lysate from the transfected cells were applied to 5% SDS-polyacrylamide gel, transferred to a membrane, and subjected to ligand blotting using 125I-protein S. Lane 1, plasma-purified C4BP; lane 2, recombinant intact α-chain; lane 3, cell lysate from mock-transfected cells; lanes 4–6, cell lysates from cells transfected with β1α, β2α, and β3α, respectively.

![Fig. 8. Protein S binding to β1α, β2α, and β3α.](Image)

A, results of the direct binding assay performed as described under “Materials and Methods.” Each value represents the average of three determinations. The data were plotted according to Scatchard (26). ○, plasma-purified C4BP; ■, β1α; □, concentration of bound protein S divided by the concentration of unbound protein S; ●, concentration of bound protein S. B, competition between the recombinant proteins and plasma-purified C4BP for protein S binding. Increasing concentrations of fluid-phase C4BP or fluid-phase recombinant proteins (cell lysates) competed with immobilized C4BP for binding a trace amount of 125I-labeled protein S. 100% binding was estimated in the absence of fluid-phase competitor. Results are shown of three different binding experiments using plasma-purified C4BP (○), β1α (■), β2α (●), and β3α (▲).
was reflected in the binding studies, in which the recombinant proteins bound approximately four times more protein S than did plasma-purified C4BP. The observation that all protein S-bindingsitesonthechimericmoleculeswerenotavailableis consistent with results of studies of the C4b-C4BP interaction that showed that, even though each C4BP molecule contains six or seven binding sites for C4b, only four of them are occupied at physiological ionic strength, whereas binding of additional C4b is probably sterically hindered (27). The binding studies performed in this work clearly showed that the amino-terminal SCR of the β-chain (SCR-1) contains the entire protein S-binding site. As we have earlier been unable to express the β-chain satisfactorily, further characterization of the protein S-binding site using site-directed mutagenesis has not been possible. The β1α chimeric protein that we have described here is a new way of expressing the SCR-1 module from the β-chain, which will be useful in determining which amino acids in SCR-1 are involved in the C4BP-protein S interaction.

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