Interaction between Protein S and Complement C4b-binding Protein (C4BP)

AFFINITY STUDIES USING CHIMERAS CONTAINING C4BP β-CHAIN SHORT CONSENSUS REPEATS

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Human C4b-binding protein (C4BP) is a regulator of the complement system and plays an important role in the regulation of the anticoagulant protein C pathway. C4BP can bind anticoagulant protein S, resulting in a decreased cofactor function of protein S for activated protein C. C4BP is a multimeric protein containing several identical α-chains and a single β-chain (C4BPβ), each chain being composed of short consensus repeats (SCRs). Previous studies have localized the protein S binding site to the NH2-terminal SCR (SCR-1) of C4BPβ. To further localize the protein S binding site, we constructed chimeras containing C4BPβ SCR-1, SCR-2, SCR-3, SCR-1+2, SCR-1+3, and SCR-2+3 fused to tissue-type plasminogen activator. Binding assays of protein S with these chimeras indicated that SCR-2 contributes to the interaction of protein S with SCR-1, since the affinity of protein S for SCR-1+2 was up to 5-fold higher compared with SCR-1 and SCR-1+3. Using an assay that measures protein S cofactor activity, we showed that cofactor activity was decreased due to binding to constructs that contain SCR-1. SCR-1+2 inhibited more potently than SCR-1 and SCR-1+3. SCR-3 had no additional effect on SCR-1, and therefore the effect of SCR-2 was specific. In conclusion, β-chain SCR-2 contributes to the interaction of C4BP with protein S.

C4b-binding protein (C4BP)1 is an important regulator of the complement system (1–5). It accelerates C2a decay from the classical pathway C3-convertase (C4b2a) complex (1, 6) and promotes factor I-mediated degradation of C4b (1–3, 7). C4BP also has a high affinity for anticoagulant vitamin K-dependent protein S, and together they form a noncovalent 1:1 stoichiometric complex (8–11). Binding of protein S to C4BP results in a decreased cofactor function of protein S for anticoagulant activated protein C (APC) in the degradation of coagulation factors Va and VIIIa (12–14). Complex formation between protein S and C4BP has no effect on the inhibition of complement activation. C4BP is a multimeric glycoprotein (M9, 530,000–570,000), composed of six or seven identical α-chains, and approximately 80–85% of C4BP contains an additional single β-chain that binds protein S (8, 15, 16). In their COOH-terminal regions, the α- and β-chains contain cysteine residues that form the interchain disulfide bridges in the so-called core region (17). In electron microscopy studies, C4BP has an octopus-like appearance (18–20). Under normal conditions, approximately 60% of total protein S is bound to C4BP, and 40% is free (21). During an acute phase response, C4BP levels can increase up to 4-fold. Due to a mechanism of differential regulation of α- and β-chain expression, an increase of α-chains predominates during such an acute phase response, and hence free protein S is held at stable levels (21). The α-chains (M9, 70,000) are composed of eight homologous domains called short consensus repeats (SCRs) (17, 22). SCRs are commonly found structures in complement regulatory proteins such as factor H and decay activation factor, in which the SCR units have complement acceleration factor, in which the SCR units have complement C3b/C4b binding properties (23). However, noncomplement regulatory proteins have also been found containing SCR units such as β2 glycoprotein I and the β-subunit of coagulation factor XIII, in which the function of the SCR units are unknown (for reviews, see Refs. 23 and 24). The β-chain (M9, 45,000) is composed of three SCR units, and previous studies have shown the protein S binding site to be localized within the NH2-terminal SCR unit (SCR-1) of the β-chain (25–28). In this study, recombinant chimeras were constructed composed of each individual β-chain SCR unit and combinations of SCR units (SCR-1+2, SCR-1+3, and SCR-2+3) fused to the NH2 terminus of a modified tissue plasminogen activator (tPA) in which the serine residue was replaced by an alanine residue (29). This inactive tPA module is well characterized and has been proven in previous studies to be a useful tool to investigate the function of protein domains (29–31). The aim of this study was to investigate the role of each individual β-chain SCR unit in the interaction between protein S and C4BP. Studies using chimeric SCR-tPA constructs show that SCR-2 of C4BP β-chain is involved in the interaction of protein S with SCR-1.

**EXPERIMENTAL PROCEDURES**

Proteins—C4BP was immunopurified from human plasma as described by Hessing et al. (32). Protein S was purified and activated as described previously (33). Protein S was purified from prothrombin concentrate as described by Hackeng et al. (34).

Chimeric SCR-tPA Constructs—Each individual β-chain SCR unit and adjacent SCR units (SCR-1+2 and SCR-2+3) were amplified using wild type recombinant C4BP β-chain. This construct was made by PCR amplification from a human liver cDNA library using oligonucleotides C4BPβF (5′-TTTGAATTCTATTACATCTGCTCAGCT-3′) and C4BPβR (5′-TTTGAATTCATTTACATCTGCTCAGCTGTA-3′). After amplification, the PCR product was cleaved with

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1 The abbreviations used are: C4BP, complement C4b-binding protein; C4BPβ, C4BP β-chain; APC, activated protein C; SCR, short consensus repeat; tPA, tissue plasminogen activator; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.
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### TABLE I

Oligonucleotide sequences used for PCR amplification

The template used for each PCR amplification is shown at the bottom of the table. Wild type recombinant C4BPβ is depicted as pcDNA3-C4BPβ. Recombinant C4BPβ lacking the second NH2-terminal SCR unit is depicted as pcDNA3-C4BPβΔSCR2. In the left column, forward primers are denoted by the letter F, and backward primers are denoted by the letter R. In the second column, nucleotide sequences of the primers are shown. Boldface, BglII restriction site; Underlined, XhoI restriction site. On top of the six outmost right columns, the different β-chain SCR unit chimeras are depicted by numbers equal to the corresponding β-chain SCR units contained within the chimeras. Combinations of template DNA and primers used for amplification of the different chimeras are indicated by dots.

| Primer   | Sequence                     | SCR-tPA construct |
|----------|------------------------------|-------------------|
|          |                              | 1 | 2 | 3 | 1&2 | 1&3 | 2&3 |
| C4BPβ1F  | 5'TTT AGA TCT GAG CAC TGT CCA GAG CTT CCT CT3' | • | • | • | • | • | • |
| C4BPβ1R  | 5'T TCC TCG AGT GCG CCA CTC AGT AGT GGT GT3'  | • | • | • | • | • | • |
| C4BPβ2F  | 5'TTT AGA TCT TGG GGC CAC TGT CCT GAT CT3'  | • | • | • | • | • | • |
| C4BPβ2R  | 5'T TCC TCG AGT ACT TTT GCA GAT GGG AAA GG3' | • | • | • | • | • | • |
| C4BPβ3F  | 5'TTT AGA TCT AGG GAC TGT GAC CCT CCT GGG G3' | • | • | • | • | • | • |
| C4BPβ3R  | 5'T TCC TCG AGT CAA CTT GCA GAC TGG AAG TG3' | • | • | • | • | • | • |
| Template  | pcDNA3-C4BPβ | | | | | | |
|          | pcDNA3-C4BPβΔSCR2 | | | | | | |

EcoRI (underlined) and cloned in EcoRI-cleaved expression vector pcDNA3 (Invitrogen, Leek, The Netherlands). The sequence and orientation of the amplified region of this construct was confirmed by dyeoxy sequencing. This construct was designated pcDNA3-C4BPβ. PCR strategies for amplification of SCR units from pcDNA3-C4BPβ were based on the intron/exon organization of the C4BP gene as described (35) with the primers depicted in Table I. For the amplification of SCR-1+3, a modified C4BPβ was used that lacked SCR-2 (pcDNA3-C4BPβΔSCR-2 in Table I) that will be described elsewhere. After amplification, PCR products were cleaved with BglII/HindIII and cloned in BglII/HindIII-cleaved expression vector ZpL7 containing a modified TPA (30). These chimeric SCR-tPA constructs were designated SCR-1, SCR-2, SCR-3, SCR-1+2, SCR-1+3 and SCR-2+3, respectively. The sequence of the amplified regions of all constructs was confirmed by dyeoxy sequencing.

Cell Culture, Transfection, and Purification of Recombinant Constructs—Transfection of baby hamster kidney cells was performed as described previously (36). Expression of all constructs was performed in conditioned medium (CHO-II-SFM; Life Technologies, Inc., Paisley, U.K.), and harvested medium was stored at -20 °C until needed for further use. Purification of chimeric SCR-tPA constructs was performed as described previously (29) using a monoclonal antibody against tPA. Concentrations of chimeric SCR-tPA constructs were determined using an ELISA system that determines tPA concentration (ImulysiteTM TPA; Biopool, Umeå, Sweden). Purified constructs were applied to 10% SDS-PAGE under reducing and nonreducing conditions and stained by Coomassie Brilliant Blue or transferred to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) for standard Western blotting procedures using sheep anti-tPA antibodies (1 μg/ml, Enzyme Research Laboratories Inc.) followed by a polyclonal peroxidase-conjugated antibody against sheep antibodies (Dako, Glostrup, Denmark).

Binding of Protein S to Immobilized Chimeric SCR-tPA Constructs—The binding of protein S to immobilized chimeric SCR-tPA constructs was performed as follows. Microtiter plates (96-well vinyl assay plates; catalog no. 2595, Costar, Cambridge, MA) were coated overnight at 4 °C with a polyclonal antibody against tissue plasminogen activator (5 μg/ml; ImulysiteTM-TPA; Biopool AB) in coat buffer (15 mM Na2CO3, 10 mM H2O, 35 mM NaHCO3, pH 9.6), 50 μl/well. Plates were washed three times with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.1% (v/v) Tween 20. Plates were blocked for 2 h at 37 °C with TBS containing 3% (w/v) bovine serum albumin (blocking buffer), 100 μl/well. CHO-II-SFM supernatant derived from baby hamster kidney cells transfected with the constructs described above was then added to the wells and incubated for 2 h at 37 °C in blocking buffer containing 0.1% Tween 20, 50 μl/well. After washing three times with TBS containing 0.1% Tween 20, increasing concentrations of protein S were added to the wells and incubated for 2 h at 37 °C in blocking buffer containing 0.1% Tween 20 and 5 mM CaCl2, 50 μl/well. Plates were washed three times with TBS containing 0.1% Tween 20, and bound protein S was detected using a polyclonal peroxidase-conjugated antibody against protein S (1.3 g/liter IgG; Dako), 1:200 diluted in blocking buffer containing 0.1% Tween 20 and 5 mM CaCl2, 50 μl/well. After washing three times with TBS containing 0.1% Tween 20, staining solution consisting of 0.4 mg/ml o-phenylenediamine and 0.002% H2O2 in 100 mM phosphate, 50 mM citric acid buffer (pH 5.0) was added to the wells (100 μl/well). The reaction was stopped by adding 50 μl/well 1 M H2SO4, and absorbance was measured at 490 nm in a Vmax microtiter plate reader (Molecular Devices, Menlo Park, CA). Values were corrected for background absorbance.

Binding of Chimeric SCR-tPA Constructs to Immobilized Protein S—Microtiter plates were coated overnight at 4 °C with a polyclonal antibody against protein S (3 g/liter IgG; Dako), 1:1000 diluted in coat buffer, 50 μl/well. Plates were washed three times with TBS containing 0.1% Tween 20. Plates were blocked for 2 h at 37 °C with blocking buffer, 100 μl/well. Purified human protein S (1 μg/ml) was added to the wells and incubated in blocking buffer containing 0.1% Tween 20 and 5 mM CaCl2, 50 μl/well. Plates were washed three times with TBS containing 0.1% Tween 20. Plates were blocked for 2 h at 37 °C with blocking buffer, 100 μl/well. Purified human protein S (1 μg/ml) was added to the wells and incubated in blocking buffer containing 0.1% Tween 20 and 5 mM CaCl2, 50 μl/well. Plates were washed three times with TBS containing 0.1% Tween 20, and increasing concentrations of purified chimeric SCR-tPA constructs were added and incubated for 2 h at 37 °C in blocking buffer containing 0.1% Tween 20 and 5 mM CaCl2, 50 μl/well. After washing the plates three times with TBS containing 0.1% Tween 20, sheep anti-tPA antibodies (1 μg/ml; Enzyme Research Laboratories Inc.) were added in blocking buffer containing 0.1% Tween 20 and 5 mM CaCl2, 50 μl/well. After 1 h of incubation at 37 °C, plates were washed three times with TBS containing 0.1% Tween 20. Bound sheep antibodies against tPA were detected by adding a polyclonal peroxidase-conjugated rabbit antibody against sheep antibodies (Dako), 1:1000 diluted in blocking buffer containing 0.1% Tween 20 and 5 mM CaCl2, 50 μl/well. After washing three times with TBS containing 0.1% Tween 20, the wells were developed and measured as described above. Values were corrected for background absorbance.

Stoichiometry of the Interaction between Protein S and SCR-tPA Constructs—A fluid phase binding assay was used to investigate the stoichiometry of the interaction between protein S and the SCR-tPA constructs that bound to protein S. For this assay, 5 μg of purified

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rabbit antibodies directed against human protein S (Dako) was coupled to 2.5 ml of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions. Fluid phase binding was performed by allowing increasing concentrations of SCR-tPA constructs to bind to 10 nM human protein S in a volume of 100 µl of TBS containing 5% bovine serum albumin and 10 mM CaCl₂. Binding was performed overnight at 4°C with constant rotation. Then 100 µl of rabbit anti-human protein S-Sepharose beads in TBS was added (final CaCl₂ concentration 5 mM). After 90 min of incubation with constant rotation at room temperature, total protein S was removed from the incubation mixture by spinning down the Sepharose beads in an Eppendorf centrifuge for 5 min at 14,000 rpm. The supernatant was analyzed with a polyclonal tPA ELISA (Imulysé™, tPA, Biopool AB) for nonbound (free) SCR-tPA constructs. As a control, the supernatant was also analyzed with a polyclonal protein S ELISA to confirm that all of the protein S was precipitated by the Sepharose beads. In this polyclonal ELISA, rabbit anti-protein S antibodies were used as catching antibodies, and peroxidase-conjugated rabbit anti-protein S antibodies were used as detecting antibodies (antibodies from Dako).

**Competition Experiments Using Chimeric SCR-tPA Constructs for Binding of Protein S to Immobilized C4BP**—Microtiter plates were coated overnight at 4°C with a monoclonal antibody against C4BPa (5C11) in coat buffer, 2 µg/ml, 50 µl/well. Plates were washed three times with TBS containing 0.1% Tween 20. Plates were blocked for 2 h at 37°C with blocking buffer, 100 µl/well. Purified human C4BP (2 µg/ml) was incubated in blocking buffer for 1 h at 37°C. Purified human protein S (0.5 nM) was preincubated with chimeric SCR-tPA constructs (0–400 nM) for 1 h at 37°C in blocking buffer containing 5 mM CaCl₂. After washing the plates three times with TBS containing 0.1% Tween 20, aliquots of 50 µl from the preincubation mixtures were applied to the plates and incubated for 2 h at 37°C. After washing three times with TBS containing 0.1% Tween 20, bound protein S was detected using a polyclonal peroxidase-conjugated antibody against protein S (1.3 g/liter IgG; Dako), 1:2000 diluted in TBS was added (final CaCl₂ concentration 5 mM). After 90 min of incubation with constant rotation at room temperature, total protein S was removed from the incubation mixture by spinning down the Sepharose beads in an Eppendorf centrifuge for 5 min at 14,000 rpm. The supernatant was analyzed with a polyclonal tPA ELISA (Imulysé™, tPA, Biopool AB) for nonbound (free) SCR-tPA constructs as a control. The supernatant was also analyzed with a polyclonal protein S ELISA to confirm that all of the protein S was precipitated by the Sepharose beads. In this polyclonal ELISA, rabbit anti-protein S antibodies were used as catching antibodies, and peroxidase-conjugated rabbit anti-protein S antibodies were used as detecting antibodies (antibodies from Dako).

**Protein S Cofactor Activity in Plasma**—Protein S cofactor activity was determined with an activated partial thromboplastin time-based assay using a KC-10A microcoagulometer (Amelung, Lemgo, Germany). For this assay, plasma deficient in protein S and C4BP was prepared by immunoadsorption as described previously (37). Purified human protein S (160 nM) was preincubated for 30 min at 37°C with serial dilutions of chimeric SCR-tPA constructs in TBS containing 0.3% (w/v) bovine serum albumin plus 3 mM CaCl₂. Aliquots of 12.5 µl from the preincubation mixtures were added to a mixture of 25 µl of plasma deficient in protein S and C4BP and 12.5 µl of 240 nM activated protein C solution (final APC concentration 30 nM). After adding 25 µl of PTT reagent (Roche Molecular Biochemicals), coagulation was initiated by adding 25 µl of 25 mM CaCl₂ (final volume 190 µl). In the range of protein S used in this assay (0–20 ng protein S), there was a linear relationship between clotting time and protein S concentration. Values were corrected for background absorbance.

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**RESULTS**

**Expression and Purification of Chimeric SCR-tPA Constructs**—In order to study the role of each individual C4BPα SCR unit in the interaction of C4BP with protein S, chimeras were constructed of β-chain SCR units fused to the NH₂ terminus of a modified tPA. Baby hamster kidney cells were transfected with the expression vector containing the chimeric SCR-tPA. After purification of the chimeric constructs with an immunoadsorption procedure using a polyclonal antibody against tPA (Fig. 2, A and B), the chimeric SCR-tPA constructs appeared as diffuse bands, which is probably caused by heterogeneous glycosylation of the proteins, because the β-chain of C4BP and the tPA module are both highly glycosylated (17, 29). Constructs containing single β-chain SCR units had molecular weights of approximately 73,000 with the exception of SCR-3, which had an estimated molecular weight of 65,000. Constructs containing two β-chain SCR units had molecular weights of approximately 80,000. The chimeric SCR-tPA constructs were also detected by Western blotting using a polyclonal antibody against tPA (Fig. 2, A and B).
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Binding of Protein S to Immobilized Chimeric SCR-tPA Constructs—The interaction of chimeric SCR-tPA constructs with protein S was investigated using a direct binding assay in which protein S was allowed to bind to immobilized chimeric SCR-tPA constructs (Fig. 3). The results are expressed as a percentage of maximum binding (B_{max}) for each construct. Apparent dissociation constants of the binding of protein S to chimeric SCR-tPA constructs were 10.3 nM for SCR-1, 1.9 nM for SCR-1+2, and 13.7 nM for SCR-1+3, respectively. Protein S did not bind to SCR-2, SCR-3, SCR-2+3, or tPA. The apparent dissociation constant for the binding of protein S to plasma C4BP was 1.7 nM, which is identical to published values (10). This indicates that protein S has an affinity for SCR-1+2 that is comparable with the affinity for plasma C4BP, whereas the affinity of protein S for SCR-1 and SCR-1+3 was approximately 5 times lower.

Binding of Chimeric SCR-tPA Constructs to Immobilized Protein S—A binding assay was performed in which chimeric SCR-tPA constructs were allowed to bind to immobilized protein S. The results of the binding experiments of chimeric SCR-tPA constructs to immobilized protein S are presented in Fig. 4. Binding of each chimeric SCR-tPA construct is expressed as a percentage of B_{max} to protein S. The apparent dissociation constants for the binding of chimeric SCR-tPA constructs to protein S are 207.4 nM for SCR-1, 24.9 nM for SCR-1+2, and 124.0 nM for SCR-1+3, respectively. SCR-2, SCR-3, SCR-2+3, and tPA did not bind to protein S. The apparent dissociation constant for the binding of plasma-purified C4BP to protein S was 2.9 nM, which is in the range of previously published values between 2 and 5 nM (28). This implies that in this system, SCR-1+2 has an affinity for protein S approximately 5–10 times lower compared with plasma-purified C4BP.

Stoichiometry of the Interaction between Protein S and SCR-tPA Constructs—The stoichiometry of the interaction between protein S and the SCR-tPA constructs that bound to protein S was analyzed using a fluid phase binding assay. Increasing concentrations of SCR-tPA constructs were allowed to bind to protein S, after which total protein S was immunoprecipitated using rabbit anti-protein S antibodies coupled to Sepharose beads. After immunoprecipitation, no protein S could be detected in the supernatant using a polyclonal protein S ELISA. Free SCR-tPA in the supernatant was determined using a polyclonal tPA ELISA. Bound SCR-tPA was calculated by subtracting values for free SCR-tPA from the concentrations of SCR-tPA added. Values for bound SCR-tPA were plotted against free SCR-tPA (data not shown), and the B_{max} of each SCR-tPA construct was calculated. The stoichiometry of the interaction of protein S with each protein S-binding SCR-tPA construct is presented in Table II as the ratio of B_{max} and the protein S concentration used. The values for B_{max}/protein S displayed in Table II are presented as means of two separate experiments. One protein S molecule was found to bind to 1.0 molecule of SCR-1, 1.0 molecule of SCR-1+2, and 1.1 molecules of SCR-1+3, respectively. These findings show that each SCR-tPA construct that is able to bind to protein S (SCR-1, SCR-1+2, and SCR-1+3) contains one binding site for protein S, confirming the existence of a single binding site for protein S on the β-chain of C4BP.

Competition Experiments Using Chimeric SCR-tPA Constructs for Binding of Protein S to Immobilized C4BP—Chimeric SCR-tPA constructs were allowed to compete with immobilized plasma purified C4BP to bind protein S. In these experiments, purified human protein S (0.5 nM) was preincubated with increasing concentrations of chimeric SCR-tPA constructs and then allowed to bind to immobilized purified human C4BP. Bound protein S was expressed as a percentage of maximum binding in the absence of chimeric SCR-tPA constructs in Fig. 5. Preincubation of protein S with SCR-1+2 resulted in inhibition of protein S binding, and a 50% inhibition of protein S binding was observed at 22 nM SCR-1+2. SCR-1 and SCR-1+3 had only a minor effect on the binding of protein S to C4BP, and a 50% inhibition of protein S binding was observed at 212 and 179 nM, respectively. In agreement with the results of the direct binding assays (Fig. 3 and 4), SCR-2, SCR-3, SCR-2+3, and tPA did not compete for protein S binding.

Protein S Cofactor Activity in Plasma—The effect of the SCR constructs on the cofactor activity of protein S was tested by preincubation of 20 nM protein S (final concentration) with increasing concentrations of chimeric SCR-tPA constructs. Subsequently, C4BP- and protein S-depleted plasma was added, and the clotting time was determined in the presence of 30 nM APC. The residual cofactor activity of protein S after preincubation with chimeric SCR-tPA constructs is shown in Fig. 6. SCR-1+2 yielded a 50% inhibition of protein S cofactor activity at a concentration of approximately 70 nM (SCR-tPA: protein S ratio of 3.5). A 50% inhibition of protein S cofactor activity by SCR-1 and SCR-1+3 was obtained at 320 nM SCR-1 (SCR-tPA:protein S ratio of 16) and 210 nM SCR-1+3 (SCR-tPA:protein S ratio of 10.5), respectively. Preincubation of protein S with SCR-2, SCR-3, SCR-2+3, or tPA had no effect on
the cofactor activity of protein S. The inhibition of protein S cofactor activity by SCR-1+2 is in concert with the competition experiments shown in Fig. 5. The maximum inhibitory effect in both experiments is accomplished at approximately 300 nM SCR-1+2. At these concentrations, maximum binding of SCR-1+2 occurred in the direct binding assays of chimeric SCR-tPA constructs to immobilized protein S shown in Fig. 4. This suggests that the decrease in protein S cofactor activity can be attributed to the complex formation of protein S with SCR-1, SCR-1+2, and SCR-1+3, respectively.

### DISCUSSION

The binding site of protein S on C4BP has been localized before on the β-chain of C4BP (16, 32, 38), and residues within the first NH₂-terminal SCR unit were shown to be important for the interaction between protein S and C4BP (25, 26, 28, 39). In this report, the possible role of the second and third β-chain SCR unit in this interaction was investigated using chimeric constructs containing β-chain SCR units fused to a modified tPA module. This tPA module is well characterized and has been proven in previous studies to be a useful tool to investigate the function of protein modules (29–31). The SCR units in these SCR-tPA chimeras were recognized by several monoclonal antibodies directed against the β-chain of C4BP. The binding of protein S to the SCR-tPA constructs and the e3ffection on the cofactor function of protein S for activated protein C due to the interaction with these chimeric SCR constructs were investigated.

Binding assays of chimeric SCR-tPA constructs and protein S showed that constructs containing SCR-1 bind to protein S with a stoichiometry of 1:1 (Table II), whereas constructs lacking SCR-1 did not bind to protein S (Figs. 3 and 4). These studies confirm previous results that have localized the protein S-binding site to the NH₂-terminal SCR (SCR-1) of C4BPβ (25, 26, 28, 39). The binding of protein S to immobilized SCR-1+2 occurred with the same affinity as for binding to plasma C4BP, whereas the binding of SCR-1+2 to immobilized protein S occurred with an affinity that was approximately 5–10 times lower compared with plasma C4BP. The reason for this discrepancy is not known.

Interestingly, constructs containing SCR-1 but lacking SCR-2 (SCR-1 and SCR-1+3) had an affinity that was approximately 5 times lower for protein S than SCR-1+2. This was found in the binding of protein S to immobilized SCR-tPA constructs (Fig. 3) as well in the binding of SCR-tPA constructs to immobilized protein S (Fig. 4). These results were confirmed by competition experiments in which soluble chimeric SCR-tPA constructs were allowed to compete with immobilized C4BP for binding to protein S (Fig. 5). A possible explanation for the difference found between SCR-1 and SCR-1+2 is that SCR-2 in the latter construct could function as a spacer, localizing SCR-1 distant from the tPA module and thereby yielding better binding properties of this SCR module. However, such an additional SCR unit is also present in the construct SCR-1+3, while this construct had a binding affinity for protein S as low as SCR-1 alone. This implies that the contribution of SCR-2 in the interaction between protein S and SCR-1+2 was specific and cannot be explained by a spacer function alone. Instead, SCR-2 could cause a conformational change in SCR-1 that yields a higher 

![FIG. 5. Competition of chimeric SCR-tPA constructs with immobilized human C4BP for protein S binding.](image)

![FIG. 6. Inhibition of protein S cofactor activity by chimeric SCR-tPA constructs.](image)
affinity binding of SCR-1 to protein S. Alternatively, when SCR-2 is adjacent to SCR-1 and SCR-1 is bound to protein S, SCR-2 may bind to protein S as well, thereby yielding a higher affinity binding of SCR-1+2 to protein S.

In previous studies using synthetic peptides, a peptide comprising β-chain SCR-1 residues 34–42 was able to completely inhibit the binding of a monoclonal antibody directed against residues 420–434 of protein S (26), and also C4BP was found to bind to a peptide comprising protein S residues 408–434 (40), implying that β-chain residues 34–42 bind near residues 420–434 of protein S. In another study, a synthetic peptide comprising protein S residues 605–614 was shown to inhibit the binding of protein S to C4BP, and in addition C4BP was shown to bind also to this immobilized peptide (41). Interestingly, deletion variants of protein S lacking residues 607–635 (42) and residues 583–635 (43) were shown to have markedly reduced affinities for binding to C4BP. Peptides comprising a third region in protein S that inhibit binding of protein S to C4BP have been found (44). It is tempting to speculate that C4BPβ SCR-1 and SCR-2 each have their own binding region within protein S.

It is known that the β-chain is highly glycosylated (17) and that the multiple carbohydrate side chains present in the β-chain of C4BP are not involved in the protein S binding (27). As shown in Figs. 1 and 2, the SCR-tPA chimeras are also highly glycosylated. Since the carbohydrate side chains are not involved in the interaction between protein S and C4BP, a possible different glycosylation of the chimeric SCR-tPA chimeras is excluded as the cause of the different binding affinities reported here for the interactions between the chimeric SCR-tPA constructs and protein S.

The finding that β-chain SCR-2 is involved in the interaction of protein S with SCR-1 is not in agreement with the results of Härđig and Dahlbäck, who found that SCR-1 of C4BPβ bound to protein S with an affinity comparable with plasma-purified C4BP (28). In their study, Härđig and Dahlbäck composed chimeras of C4BP α-chains with one, two, or three of the NH₂-terminal SCR modules replaced by the β-chain counterpart. Hence, next to the β-chain SCR-1 unit in all of their constructs, an additional second NH₂-terminal SCR unit from the α-chain or β-chain was present, and this may explain the different results compared with our study. It is possible that the second NH₂-terminal SCR unit derived from the α-chain in their constructs was able to exert a function comparable with SCR-2 of C4BPβ, yielding the same affinity of protein S for all of the α-chain chimeras. A possible explanation for this may be found in the homology between the SCR units from the α-chain (22) and the β-chain (17). A specific sequence present in the second NH₂-terminal SCR unit of both the α-chain and the β-chain may result in an optimal folding of β-chain SCR-1 or, alternatively, may contain sites that contribute to the binding of protein S to β-chain SCR-1.

A role of the α-chains in the interaction with protein S was previously proposed in studies by Suzuki and Nishioka (45). In their study, a COOH-terminal core fragment derived from C4BP (Mₑ 160,000) was found to contain a protein S binding site, and after reduction and carboxymethylation of the COOH-terminal fragment, a peptide of Mₑ 2500 corresponding to Ser⁴⁴⁷–Tyr⁴⁶⁷ was identified as the protein S binding region. Possibly, this region (within the eighth SCR unit of the α-chain) may also contain a sequence homologous to the sequence discussed above that in combination with β-chain SCR-1 could contribute to the binding affinity of β-chain SCR-1 with protein S. Hessing et al. (20) have identified monoclonal antibodies directed against the α-chain that inhibited the binding of protein S to C4BP. The inhibitory effect of these antibodies on the interaction between protein S and C4BP is most likely to be due to steric hindrance but does not exclude the possibility that the α-chains can also play a role in the interaction between C4BP and protein S.

In our activated partial thromboplastin time-based coagulation assay, SCR-1+2 had the strongest effect in the inhibition of cofactor activity of protein S (Fig. 6). Maximum inhibition of protein S cofactor activity was accomplished at concentrations at which also maximum binding was accomplished in the direct binding assays (Fig. 4). Hence, inhibition of protein S cofactor activity can be attributed to the complex formation between chimeric SCR-tPA constructs and protein S. Nishioka and Suzuki (46) have shown that protein S in complex with C4BP is still able to bind to APC, excluding the possibility that C4BP prevents binding of protein S to APC by steric hindrance. Studies using fluorescence resonance energy transfer have shown that protein S alters the active site location of APC closer to the membrane surface, and as a mechanism, a change in topography and/or conformation of the active site of APC has been postulated (47). A possible mechanism for the decrease in protein S cofactor activity due to the complex formation of protein S with C4BP could be that C4BP prevents protein S to induce this topographical and/or conformational change in the active site of APC. The mechanism by which C4BP prevents this change in topography and/or conformation could be by altering the conformation of protein S or vice versa, preventing protein S from changing into a conformation that is necessary to express cofactor activity. In the mechanism of inhibition of protein S cofactor activity, it is most likely that the SCR-tPA chimeras SCR-1, SCR-1+2, and SCR-1+3 act in a way comparable with C4BP. The exact contribution of the SCR-tPA constructs in the decrease in cofactor activity of protein S for APC in the inhibition of factors Va and VIIIa remains to be elucidated.

We conclude that the second NH₂-terminal SCR unit of C4BPβ contributes to the interaction between protein S and the first NH₂-terminal SCR unit of the C4BPβ. This is the first time that an SCR unit other than β-chain SCR-1 has been shown to be involved in the interaction of C4BP with protein S.

REFERENCES
1. Gigli, I., Fujita, T., and Nussenzweig, V. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6596–6600
2. Fujita, T., and Nussenzweig, V. (1979) J. Exp. Med. 150, 267–276
3. Fujita, T., Gigli, I., and Nussenzweig, V. (1978) J. Exp. Med. 148, 1044–1051
4. Nussenzweig, V. J., and Melton, R. (1981) Methods Enzymol. 80, 124–133
5. Scharfstein, J., Ferreira, A., Gigli, I., and Nussenzweig, V. (1978) J. Exp. Med. 148, 267–222
6. Daha, M. R., and van Es, L. A. (1980) Immunol. Lett. 21, 2051–2054
7. Fujita, T., and Tamura, N. (1983) J. Exp. Med. 157, 1239–1251
8. Dahlback, B. (1991) Thromb. Haemostasis 66, 49–61
9. Dahlback, B., Frohnn, B., and Nelsestuen, G. (1990) J. Biol. Chem. 265, 16082–16087
10. Dahlback, B. (1983) Biochem. J. 209, 847–856
11. Dahlback, B., and Stenflo, J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2512–2516
12. Dahlback, B. (1986) J. Biol. Chem. 261, 12022–12027
13. Bertina, R. M., van Wijngaarden, A., Reinalda Poot, J., Poort, S. R., and Bom, V. J. (1985) Thromb. Haemostasis 53, 268–272
14. Comp, P. C., and Esmon, C. T. (1984) New Engl. J. Med. 311, 1525–1528
15. Griffin, J. H., Gruber, A., and Fernández, J. A. (1992) Biochem. J. 280, 851–856
16. Hillarp, A., Hessing, M., and Dahlback, B. (1989) J. Biol. Chem. 264, 4531–4536
17. Hillarp, A., and Dahlback, B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1183–1187
18. Dahlback, B., Smith, C. A., and Muller-Eberhard, H. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3461–3465
19. Dahlback, B., and Muller-Eberhard, H. J. (1984) J. Biol. Chem. 259, 11631–11634
20. Hessing, M., Katers, D., Heijnen, H. F., Hackeng, T. M., Sixma, J. J., and Bouma, B. N. (1991) Eur. J. Immunol. 21, 2077–2085
21. Garcia de Frutos, P., Alim, R. I., Härđig, Y., Zöller, B., and Dahlback, B. (1994) Blood 84, 815–822
22. Chung, L. P., Bentley, D. R., and Reid, K. B. M. (1985) Biochem. J. 230, 133–141
23. Reid, K. B. M., Bentley, D. R., Campbell, R. D., Chung, L. P., Sim, R. B., Kristensen, T., and Tack, B. F. (1986) Immunol. Today 7, 230–234

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24. Reid, K. B. M., and Day, A. J. (1989) *Immunol. Today* 10, 177–180
25. Fernández, J. A., Villoutreix, B. O., Hackeng, T. M., Griffin, J. H., and Bouma, B. N. (1994) *Biochemistry* 33, 11073–11078
26. Fernández, J. A., and Griffin, J. H. (1994) *J. Biol. Chem.* 269, 2535–2540
27. Hardig, Y., Rezaie, A., and Dahlback, B. (1993) *J. Biol. Chem.* 268, 3033–3036
28. Hardig, Y., and Dahlback, B. (1996) *J. Biol. Chem.* 271, 20861–20867
29. Mejiers, J. C. M., Mulvihill, E. R., Davie, E. W., and Chung, D. W. (1992) *Biochemistry* 31, 4680–4684
30. Hillarp, A., Pardo-Manuel, F., Ruiz, R. R., Rodríguez de Córdoba, S., and Dahlback, B. (1993) *J. Biol. Chem.* 268, 15017–15023
31. Herwald, H., Renne, T., Mejiers, J. C. M., Chung, D. W., Page, J. D., Colman, R. W., and Müller-Esterl, W. (1996) *J. Biol. Chem.* 271, 13061–13067
32. Koedam, J. A., Meijers, J. C. M., Sixma, J. J., and Bouma, B. N. (1988) *Clin. Invest.* 82, 1226–1243
33. Hackeng, T. M., Hessing, M., van’t Veer, C., Meijer-Huizinga, F., Mejiers, J. C. M., de Groot, P. G., van Mourik, J. A., and Bouma, B. N. (1993) *J. Biol. Chem.* 268, 3993–4000
34. Hillarp, A., Pardo-Manuel, F., Ruiz, R. R., Rodríguez de Córdoba, S., and Dahlback, B. (1993) *J. Biol. Chem.* 268, 15017–15023
36. Mejiers, J. C. M., Davie, E. W., and Chung, D. W. (1992) *Blood* 79, 1435–1440
37. Hackeng, T. M., van’t Veer, C., Mejiers, J. C. M., and Bouma, B. N. (1994) *J. Biol. Chem.* 269, 21051–21058
38. Hillarp, A., and Dahlback, B. (1988) *J. Biol. Chem.* 263, 12759–12764
39. Villoutreix, B. O., Fernández, J. A., Teleman, O., and Griffin, J. H. (1995) *Protein Eng.* 8, 1253–1258
40. Fernández, J. A., Heeb, M. J., and Griffin, J. H. (1993) *J. Biol. Chem.* 268, 16788–16794
41. Walker, F. J. (1989) *J. Biol. Chem.* 264, 17645–17648
42. Nelson, R. M., and Long, G. L. (1992) *J. Biol. Chem.* 267, 8140–8145
43. Chang, G. T., Maan, B. H., Ploos van Amstel, H. K., Reitsma, P. H., Bertina, R. M., and Bouma, B. N. (1994) *Thromb. Haemostasis* 71, 641–647
44. Linse, S., Hardig, Y., Schultz, D. R., and Dahlback, B. (1997) *J. Biol. Chem.* 272, 14658–14665
45. Suzuki, K., and Nishioka, J. (1988) *J. Biol. Chem.* 263, 17034–17039
46. Nishioka, J., and Suzuki, K. (1990) *J. Biol. Chem.* 265, 9072–9076
47. Yegneswaran, S., Wood, G. M., Esmon, C. T., and Johnson, A. E. (1997) *J. Biol. Chem.* 272, 25013–25021