Binding Site for C4b-Binding Protein in Vitamin K-Dependent Protein S Fully Contained in Carboxy-Terminal Laminin-G-type Repeats. A Study Using Recombinant Factor IX-Protein S Chimeras and Surface Plasmon Resonance†

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ABSTRACT: The interaction between vitamin K-dependent protein S and the C4b-binding protein (C4BP) was studied using surface plasmon resonance and genetic engineering. The affinity, as well as association and dissociation rates of the complex, was measured for human and bovine protein S at five different calcium concentrations. The binding to C4BP of six protein hybrids containing different parts of coagulation factor IX and protein S was studied in the absence and presence of calcium. The results show that dissociation of the human protein S−C4BP complex is extremely slow in the presence of ≥10 μM calcium ($k_{off} = 7 \times 10^{-6}$ s^{-1}) and the association rate constant is $k_{on} = 7 \times {}^{4}$ M^{-1} s^{-1}. Human and bovine protein S were found to bind to human C4BP with the same affinity, $K_D = 0.1$ nM, but the rates of association and dissociation were higher for the bovine protein S ($k_{on} = 2 \times {}^{5}$ M^{-1} s^{-1}, $k_{off} = 2 \times 10^{-5}$ s^{-1}). In the absence of calcium, the affinity for C4BP was reduced by a factor of 65 for human protein S and by a factor of 40 for bovine protein S. The decreased affinity could be mainly attributed to an increased off-rate (12−17-fold), while the on-rate decreased 3−4-fold. The studies using chimeric proteins show that the portion of protein S that is responsible for binding to C4BP is fully contained in the two laminin-G-type repeats, which are homologous to the sex hormone binding globulin (SHBG). All hybrids that contain the laminin-G-type repeats bind to C4BP with the same affinity as recombinant protein S, whereas hybrids lacking these repeats show no detectable binding to C4BP. The present data also suggest that the effect of calcium on the C4BP-binding properties is mediated by calcium binding site(s) in the laminin-G-type repeats.

Protein S is a vitamin K-dependent protein which is present in human plasma at a concentration of 20−25 mg/L (0.35 μM) [DiScipio *et al.*, 1977; Stenflo & Jönsson, 1979; Dahlbäck, 1983a; reviewed in Dahlbäck (1995)]. It has anticoagulant properties, functioning as a non-enzymatic cofactor to activated protein C (APC)[1] in the degradation of the activated forms of coagulation factor V and factor III (Walker, 1980). In addition, it expresses APC-independent anticoagulant effects (Heeb *et al.*, 1993; Hackeng *et al.*, 1994). The physiological importance of protein S as an anticoagulant is demonstrated by the relationship between inherited protein S deficiency and thromboembolic disease [reviewed in Dahlbäck (1995)].

In human plasma, approximately 60% of protein S is non-covalently complexed to C4b-binding protein (C4BP) (Dahlbäck & Stenflo, 1981; Dahlbäck, 1983b). Upon complex formation, protein S loses its anticoagulant function (Dahlbäck, 1986). C4BP is a high molecular weight glycoprotein (M_r approximately 570 000) functioning as a regulator of the classical complement pathway. It is a cofactor to factor I in the degradation of C4b, and in addition it serves as a decay accelerating factor for the C4b−C2a complex [Fujita *et al.*, 1978; reviewed in Dahlbäck (1991)].

The primary structures of human, monkey, porcine, bovine, rabbit, murine, and rat protein S have been elucidated (Lundwall *et al.*, 1986; Greengard *et al.*, 1995; Dahlbäck *et al.*, 1986; He & Dahlbäck, 1993; Yasuda *et al.*, 1995; Chu *et al.*, 1994). Before secretion, protein S undergoes several post-translational modifications including vitamin K-dependent γ-carboxylation of glutamic residues, β-hydroxylation of aspartic acid and asparagine residues, and attachment of N-linked carbohydrate chains. Mature protein S (Figure 1A) is a single-chain (75 kDa) mosaic protein composed of

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[1] Abbreviations: APC, activated protein C; BIA, biospecific interaction analysis; C4BP, complement C4b-binding protein; CCP, complement control protein; DMEM, Dulbecco Eagle's modified medium, EDC, *N*-ethyl-*N*′-(3-(diethylamino)propyl)carboxydiimide; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; Gas 6, growth arrest-specific protein 6; Gla, γ-carboxyglutamic acid; NHS, *N*-hydroxysuccinimide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RU, response unit; SCR, short consensus repeat; SDS, sodium dodecyl sulfate; SHBG, sex hormone binding globulin; SPR, surface plasmon resonance; TSR, thrombin sensitive region.

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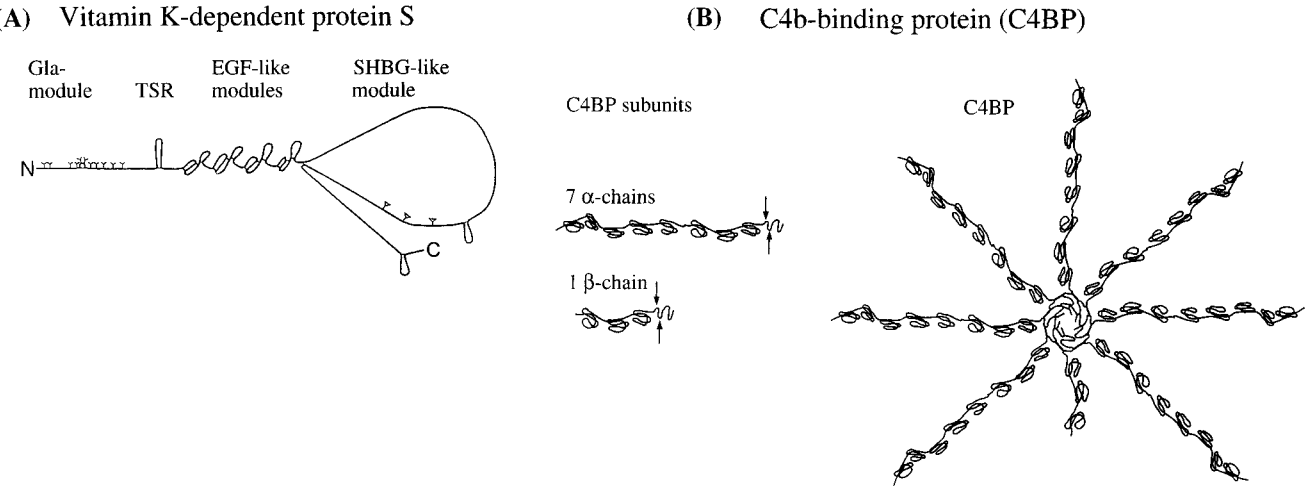

FIGURE 1: Schematic picture showing the modular arrangement of protein S and C4BP. (A) Protein S containing the N-terminal Gla-module which is rich in γ-carboxyglutamic acid residues, the thrombin sensitive disulfide loop, four EGF-like modules with high-affinity calcium binding sites, and the SHBG-like domain which contains two laminin-G-type repeats. (B) This drawing of C4BP is based on electron microscopy of C4BP and NMR-derived three-dimensional structures of SCR modules in other proteins. The angle between two consecutive SCR modules is arbitrarily drawn. The seven α-subunits are identical and built from eight complement control protein (CCP) modules, also called short consensus repeat (SCR) modules, plus the C-terminal linking domain. The β-chain consists of three SCR modules and a linking domain.

multiple modules. Starting from the NH_2-terminus, it contains a γ-carboxyglutamic acid (Gla)-rich module, a thrombin-sensitive region (TSR), four epidermal growth factor (EGF)-like modules, and two laminin-G-type modules. The two laminin-G-type modules are often called the SHBG-like domain due to homology to sex hormone binding globulin (SHBG). In human protein S, the laminin-G-type modules contain three potential sites for N-linked carbohydrate side chains (Lundwall *et al.*, 1986). The Gla-module binds several calcium ions with low affinity and interacts with negatively charged phospholipid in a calcium dependent manner (Nelsestuen *et al.*, 1978; Schwalbe *et al.*, 1989; Sunnerhagen *et al.*, 1995). In addition, the protein contains four high-affinity calcium binding sites, some of which are located in the EGF-like modules (Dahlbäck *et al.*, 1990a,b). The integrity of the TSR has been shown to be essential for the APC−cofactor activity (Suzuki *et al.*, 1983). Moreover, both TSR and the first EGF-like module have been shown to be important for the APC−cofactor function of protein S (Dahlbäck *et al.*, 1990a; He *et al.*, 1995).

C4BP (Figure 1B) is composed almost entirely of modules of the short consensus repeat (SCR) type [also called complement control protein (CCP) modules or Suchi modules; reviewed in Dahlbäck (1995)]. In human plasma, the majority of C4BP consists of seven α-chains and a single β-chain [Scharfstein *et al.*, 1978; Hillarp & Dahlbäck, 1988; reviewed in Dahlbäck (1991)]. Each α-chain contains a binding site for the complement protein C4b, whereas the protein S binding site is located on the β-chain (Härdig *et al.*, 1993; Fernández & Griffin, 1994; Härdig & Dahlbäck, 1996). Each α-chain contains eight SCR modules and carries one binding site for the activated complement protein C4b. The β-chain contains three SCR modules, and the protein S binding site is demonstrated to be fully contained in the most N-terminal SCR-module (Härdig & Dahlbäck, 1996). C4BP has a spider-like structure with seven α-chains and one β-chain linked together by disulfide bonds near their carboxy-termini, and the SCR modules within each subunit are arranged as beads on a string (Dahlbäck *et al.*, 1983; Dahlbäck & Müller-Eberhardt, 1984). Recent ^{1}H NMR structures of single SCR modules (Norman *et al.*, 1991; Barlow *et al.*, 1992) have shown that the 60 amino acids in an SCR module are folded into an elongated domain of five β-strands. The N- and C-termini are located on opposite ends of the module, in agreement with the beads-on-a-string-like appearance. A ^{1}H NMR solution structure of a pair of SCR modules (Barlow *et al.*, 1993) indicates flexibility in the inter-module region with the relative orientation of the two modules specified to within a few degrees.

Reported values for the equilibrium binding constant for the interaction between protein S and C4BP range from 2×10^9 to 1×10^{10} M^{-1} in the presence of 2 mM calcium (Nelson & Long, 1992; Schwalbe *et al.*, 1990; Dahlbäck *et al.*, 1990b; Greengard *et al.*, 1995). Dissociation of the protein S-C4BP complex is a slow process, as shown in the absence of added metal ions using agarose gel electrophoresis to separate the free and C4BP-bound protein S at certain intervals after adding a tracer of radioactive protein S (Dahlbäck, 1983b). The kinetics of the association and dissociation of the complex has not been previously reported for the interaction in the presence of calcium, except for bovine protein S that was bound to phospholipid vesicle surfaces (Schwalbe *et al.* 1990). Several studies have aimed at localizing the region in protein S which interacts with C4BP, but no consensus has emerged (Fernández *et al.*, 1993; Walker, 1989; Weinstein & Walker, 1990; Nelson & Long, 1992; Chang *et al.*, 1992). These studies suggest different non-overlapping parts of the SHBG-like domain to be involved, but it has not been clearly shown if the binding site is fully contained in this part of protein S. Indeed, the calcium-dependence of the protein S-C4BP interaction has lead to the speculation that calcium-binding EGF-like modules in protein S may play a role in the interaction (Dahlbäck *et al.*, 1990b,c).

In the present work we have made measured the association and dissociation rate constants for the protein S−C4BP interaction at five different calcium concentrations, using surface plasmon resonance. To elucidate which modules of protein S contribute to the C4BP-binding site, we have expressed several different recombinant chimeras between

coagulation factor IX and protein S. Using surface plasmon resonance we demonstrate that the C4BP-binding site is fully contained within the laminin-G-type repeats comprising the SHBG-like domain of protein S.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of highest grade commercially available. The sensor chips CM5 and amine coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-(diethylamino)propyl)carboxydiimide (EDC), and ethanolamine hydrochloride were from Pharmacia Biosensor AB (Uppsala, Sweden). The surfactant Tween 20 was from Riedel de Haen (Seelze, Holland). DNA sequencing and the *T7 gene in vitro* mutagenesis kits were from United States Biochemicals Corporation. Molecular weight markers for SDS gels were from Pharmacia (Stockholm, Sweden), and hygromycin B was from Calbiochem. Lipofectin, Optimem medium, fetal calf serum, and Dulbecco Eagle's modified medium (DMEM) were from Gibco Corp. Chemicals used for amino acid analysis were obtained from Beckman Instruments, and those used for protein sequence determination were from Applied Biosystems. [^{125}I]-labeled sodium iodide was obtained from Du Pont.

Plasma Proteins. Protein S from human and bovine plasma and C4BP from human plasma were purified as described (Dahlbäck, 1983a,b).

cDNA Clones for Recombinant Proteins. To express wild-type bovine protein S, site-directed mutagenesis was used to introduce a missing codon (He *et al.*, 1995) in a cDNA clone for full-length bovine protein S (Dahlbäck *et al.*, 1986), which was missing one nucleotide, an A in the EGF2 module. *Bam*HI sites were introduced at both the 5′-end and the 3′-end of the full-length human cDNA clone as described (He *et al.*, 1995). Full-length human factor IX cDNA clones were the kind gifts of Dr. Darrel W. Stafford (Department of Biology, University of North Carolina) (Lin *et al.*, 1990) and Dr. Kathryn Berkner (ZymoGenetics, Inc).

Construction of Factor IX−Protein S Chimeras. The modular arrangements of the six hybrid proteins based on factor IX and protein S hybrids are outlined in Figure 2. Two different strategies were used for the construction of chimeras. Chimeras I−III were made using a polymerase chain reaction (PCR)-based strategy using factor IX/protein S hybrid oligonucleotides, whereas chimeras IV−VI were constructed utilizing unique restriction enzyme cleavage sites created by site-directed mutagenesis. Sequences of the hybrid oligonucleotides used for construction of chimeras I−VI are shown in Table 1. PCR amplification of the target DNA was performed essentially as described by Saiki *et al.* (1988). Each reaction was carried out in 100 μL volume and contained 0.5 μM of each primer, 200 μM each of the deoxyribonucleotide triphosphates (dNTPs: dATP/dCTP/dTTP/dGTP), and 2.5 units of AmpliTaq DNA Polymerase (Perkin Elmer). The buffer used was 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 8.4. The PCR reaction was performed on a Thermal Cycler (Perkin Elmer) using a "Step-Cycle" program set to denature at 94 °C for 1 min, anneal at 60 °C for 1 min, and extend at 72 °C for 1 min for a total of 30 cycles. The PCR-based strategy for construction of chimeras I−III is outlined in Figure 3. After amplification, DNA was prepared by preparative agarose gel electrophoresis (Sambrook *et al.*, 1989). The final chimeric cDNAs were

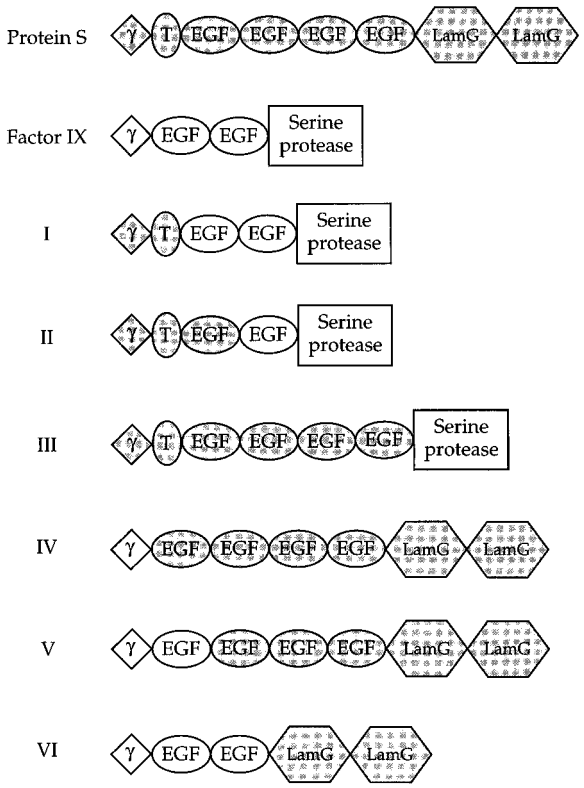

FIGURE 2: Schematic drawing showing the composition of modules from protein S and factor IX in chimeric proteins I−VI.

cleaved with *Bam*HI and cloned into the *Bam*HI site of the pUC18 vector. The plasmids were propagated in *Escherichia coli* DH 5α and sequenced (Sanger *et al.*, 1977).

Chimeras IV−VI were constructed using unique restriction enzyme cleavage sites which were introduced into the human factor IX and bovine protein S cDNA by site-directed mutagenesis, using the T7 gene in vitro mutagenesis kit from United States Biochemicals Corporation (Figure 3). The amino acid sequences of protein S and factor IX were back-translated into ambiguous DNA sequences according to methods described previously (Lin *et al.*, 1990; Little & Mount, 1984). In protein S, *Kpn*I sites were created between TSR and EGF1 and between EGF4 and SHBG in bovine protein S as previously described (He *et al.*, 1995). In a similar manner, *Kpn*I sites were generated between Gla and EGF1 and between EGF2 and the serine protease modules in human factor IX. *Sac*I sites were introduced between EGF1 and EGF2 in bovine protein S and between EGF1 and EGF2 in human factor IX. The oligonucleotide primers 7−9 for factor IX and primers 10−12 for protein S were designed for this purpose (Table 1). Primer 7 (corresponding to amino acid sequence Try 42-Pro 55 in factor IX) created a *Kpn*I site in factor IX between Gla and EGF1. This led to a change in factor IX at position 49 (Asp49Tyr). Primer 8 (corresponding to amino acid sequence Gly 79-Gly 93) created a *Sac*I site in factor IX between EGF1 and EGF2. This led to changes in factor IX (Asp85Glu and Val 86Leu). Primer 9 (corresponding to amino acid sequence Gln 121-Arg 134) created a *Kpn*I site in factor IX between EGF2 and a serine protease module without change of amino acid sequence. Primer 10 (corresponding to amino acid sequence Ser 71-Asp 83 in bovine protein S) created a *Kpn*I site in bovine protein S between TSR and EGF1. This led to a change in bovine protein S (Asp78Tyr). Primer 11 (corre-

Table 1: Sequences of Oligonucleotides Used in Construction of Protein S/Factor IX Chimeras[a]

PCR strategy		
Primer A		5'-CTC GGA TCC CAA GCC TCC GCC CGT TTC GC-3' (*Bam* HI)
Primer 1		5'-(GGA CTC ACA CTG ATC TCC)(AAT GGC ATT GAC ACA GCT)-3' (Factor IX / Protein S)
Primer 2		5'-(AGC TGT GTC AAT GCC ATT)(GGA GAT CAG TGT GAG TCC)-3' (Protein S / Factor IX)
Primer 3		5'-(GTT ACA TGT TAC ATC TAA)(TTC ACA CTT TTC TCC TTG)-3' (Factor IX / Protein S)
Primer 4		5'-(CAA GGA GAA AAG TGT GAA)(TTA GAT GTA ACA TGT AAC)-3' (Protein S / Factor IX)
Primer 5		5'-(TGG AAA TGG CAC TGC)(CTC ACA ACT CTT CTG ATC)-3' (Factor IX / Protein S)
Primer 6		5'-(GAT CAG AAG AGT TGT GAG)(CCA GCA GTG CCA TTT CCA)-3' (Protein S / Factor IX)
Primer B		5'-CTC GGA TCC TTG GAA ATC CAT CTT TC-3' (*Bam* HI)
Site-directed mutagenesis		
Primer 7	Factor IX	5'-GAT TGG ACT CAC ACT GGT ACC CAT CAA CAT ACT GCT TCC-3' (*Kpn* I)
Primer 8	Factor IX	5'-ATT CTT AAT GTT ACA TGT GAG CTC TAA TTC ACA GTT CTT TC-3' (*Sac* I)
Primer 9	Factor IX	5'-GAA GTC CTG TGA ACC AGC GGT ACC ATT TCC ATG TGG AAG AGT-3' (*Kpn* I)
Primer 10	Protein S	5'-CAG AGG ATT ACA CTG GTA CCA AAAT GGC ATT GAC ACA GC-3' (*Kpn* I)
Primer 11	Protein S	5'-GGA TCT TTG CAT TCA TTG AGC TCA GAT TCA CAC TTT CCT C-3' (*Sac* I)
Primer 12	Protein S	5'-AAG GAA GGC ACA CTG GTA CCG CTG GCT CAC AAC TCT TCT GAT GTT-3' (*Kpn* I)

[a] Restriction site sequences are underlined. The sequences complimentary to the protein S and factor IX cDNAs are given in parentheses.

sponding to amino acid sequence Lys 113-Ile 126) created a *Sac*I site in bovine protein S between EGF1 and EGF2. This led to the replacements Asp117Glu and Ile118Leu. Primer 12 (corresponding to amino acid sequence Gln 238-Leu 252) created a *Kpn*I site in bovine protein S between EGF4 and SHBG without alteration of the amino acid sequence.

A 1386 base pair *Bam*HI fragment of factor IX and a 2021 base pair *BamH*I-*Sac*I fragment of bovine protein S were subcloned into M13mp18, and single-strand templates were prepared using standard methods (Sambrook *et al.*, 1989). Site-directed mutagenesis was accomplished by the "gapped duplex" method (Vandeyar *et al.*, 1988; Raleigh & Wilson, 1986). The M13mp18 clones carrying the factor IX or protein S cDNAs inserts were annealed with each specific oligonucleotide and subjected to second-strand synthesis with T4 DNA polymerase and ligation with T4 DNA ligase. The DNA constructs were transfected into competent *E. coli* SDM (mcrA$^-$ mcrB$^-$) cells. Single-stranded DNA from individual resultant plaques was isolated and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using M13 universal primer and factor IX or protein S specific primers. The protein S cDNA inserts were isolated from double-stranded phage DNA, and appropriate restriction fragments were used to construct full-length human and bovine protein S cDNA (in pUC18) containing the desired *Kpn*I sites and *Sac*I sites using standard techniques. Chimeras IV−VI were created by exchange of *Bam*HI-*Kpn*I fragments or *Bam*HI-*Sac*I fragments between factor IX and protein S.

All chimeric cDNA was isolated after *Bam*HI digestion and subcloned into the *Bcl*I site of the expression plasmid pGT-h, which was a kind gift from Dr. B. W. Grinnell (Lilly Research Laboratories, Eli Lilly Company, Indianapolis, IN) (Berg *et al.*, 1992). The six chimeras as well as wild-type protein S or factor IX cDNA expression plasmids were prepared by CsCl gradient ultracentrifugation (Sambrook *et al.*, 1989).

Cell Culture and Expression of Recombinant Proteins. The adenovirus-transfected human kidney cell line 293 was grown in DMEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL of penicillin and streptomycin, 10 μg/mL vitamin K1. Transfection was performed using the lipofectin method (Felgner *et al.*, 1987). Briefly, DNA (2−4 μg) was diluted to 0.1 mL with sterile water. Lipofectin was added (1 μg/μL), and samples were left at room temperature for 10−15 min. Cell monolayers (40−50% confluence in a 5 cm Petri dish) were washed twice in serum-free Optimem medium (Gibco). The DNA/lipid mixture was diluted to 1 mL in Optimem medium, added to the cells, and incubated overnight (16−20 h) to obtain stable transfectants. The cells were fed with 2 mL of complete medium containing 10% calf serum and left to recover for another 48−72 h. They were then trypsinized and seeded into 10 cm dishes with selection medium (DMEM containing 10% serum and 200 μg/mL hygromycin B) at 1:5 (Grinell *et al.*, 1990). Hygromycin-resistant colonies were obtained after 3−5 weeks of selection, picked with cloning cylinders (20 colonies on average in each DNA transfection), and

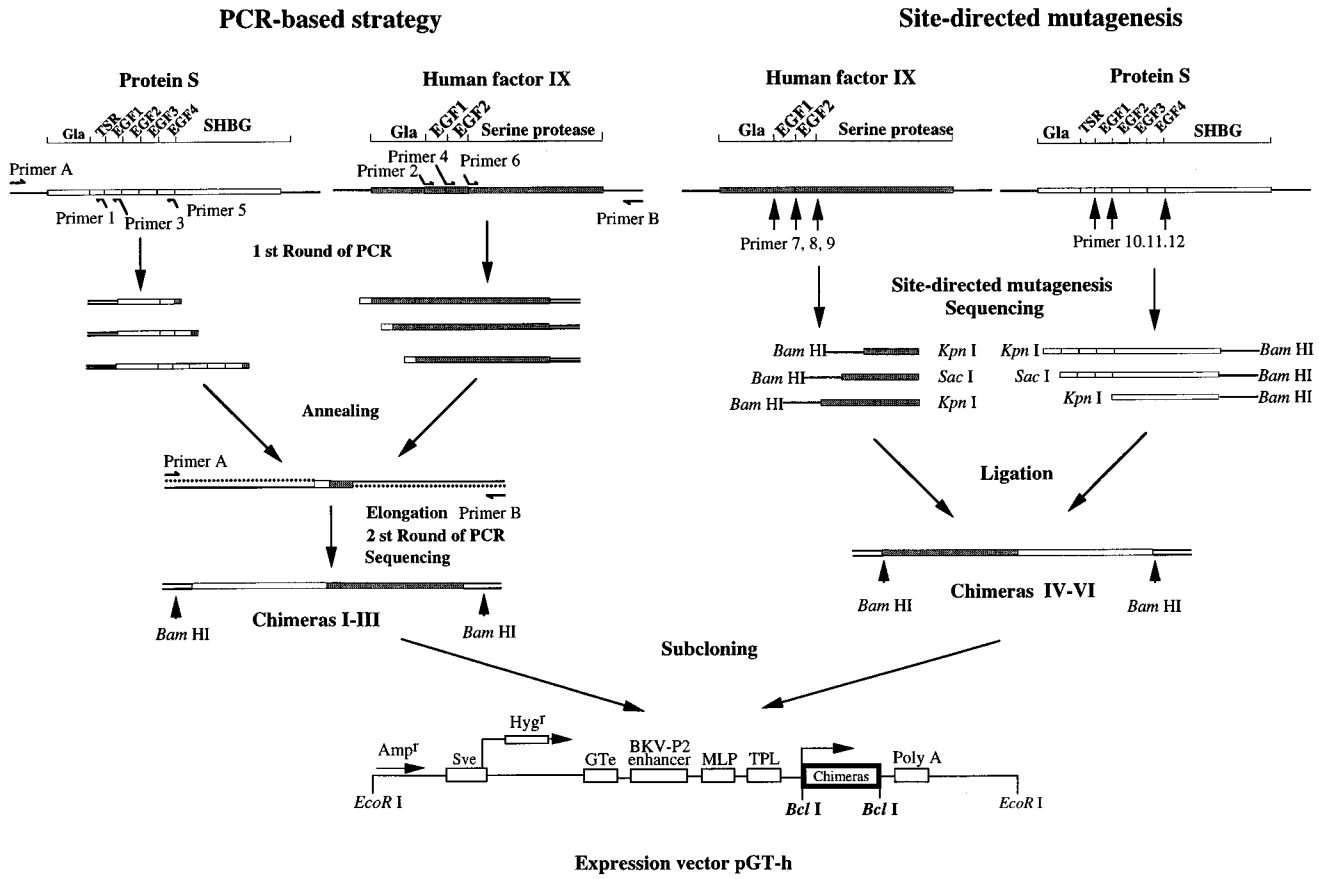

FIGURE 3: PCR-based strategy for construction of chimeric proteins I−III and the construction of hybrids V−VI using unique restriction enzyme cleavage sites created by site-directed mutagenesis.

grown to confluence and the media screened for chimera expression with enzyme-linked immunosorbent assay (ELISA) using either HPS-21 or MAb-7. HPS-21 is a monoclonal antibody with an epitope in the Gla-module of protein S (Dahlbäck et al., 1990a), and Mab-7 is directed against factor IX (Smith & Ono, 1984). High-producing clones were isolated and grown to confluence in the presence of 10 μg/mL vitamin K1.

Purification of Recombinant Wild-Type Protein S and Chimeras I−VI. Chimeras I−III were each purified from approximately 20 L of tissue culture medium using a method previously developed for the purification of human/bovine protein S chimeras (He *et al.*, 1995). Recombinant factor IX and chimeras IV−VI were purified using the same method with the exception that the Ca^{2+}-dependent factor IX antibody Mab-7 (Smith & Ono, 1984) was used instead of HPS-21 (Mab-7 was coupled to Affigel 10 at 4 mg/mL). The concentrations of the purified proteins were determined with amino acid composition analysis after acid hydrolysis.

Enzyme-Linked Immunosorbent Assay (ELISA). Quantification of recombinant wild-type and chimeric proteins was performed by ELISA using a mixture of anti-human/bovine protein S antiserum or a factor IX antiserum as catching antibodies and biotinylated monoclonal antibody HPS-21 (for chimeras I−III) or MAbs-7 (chimeras IV−VI) as detecting antibodies essentially as described previously (He *et al.*, 1995).

Polyacrylamide Gel Electrophoresis and Amino Acid Sequencing. Polyacrylamide (10−15%) slab gel electrophoresis (PAGE) was run in the presence of 0.1% sodium dodecyl sulfate (SDS) under reducing and non-reducing conditions, and proteins were visualized by silver-staining (Morrissey, 1981). After SDS−PAGE, the proteins were electroblotted onto polyvinylidene difluoride membranes and their N-terminal amino acid sequences were determined using an Applied Biosystems gas-phase sequenator as described (Matsudaira, 1987).

Surface Plasmon Resonance Studies. The interaction between protein S or chimeric proteins and C4BP was studied by surface plasmon resonance technology, using a BIAcore biosensor system (Pharmacia Biosensor AB), and the data were evaluated as suggested by O'Shannesy (1993, 1994). Immobilization of C4BP to the sensor chip was performed at a constant flow rate of 5 μL/min, using Hepes/KOH, pH 7.4, with 0.15 M NaCl and 0.05% Tween 20 as flow buffer. The small amount of Tween 20 in the buffer was used to prevent clogging of the tubes in the BIAcore apparatus. Equal volumes of 0.1 M NHS and 0.1 M EDC were first mixed, and 30 μL of the mixture was allowed to flow over the sensor chip surface to activate the carboxymethylated dextran (6 min); 40 μL of 60 μg/mL C4BP in 10 mM NaHAc at pH 4.75 was then injected over the sensor chip (8 min), after which unreacted NHS-ester groups were deactivated by 15 μL of 1 M ethanolamine (pH 8.5, 3 min). The system was regenerated by 15 μL of 0.1 M HCl (3 min) to remove all non-covalently bound molecules.

Protein S (or hybrid proteins) association to and dissociation from the immobilized C4BP was studied at several different solution conditions. The flow buffer was 10 mM Hepes/KOH, pH 7.4, with 0.15 M NaCl, 0.05% Tween 20, and in different sets of experiments it contained either 3.4 mM EDTA, 10 μM $CaCl_2$, 100 μM $CaCl_2$, 1.0 mM $CaCl_2$,

or 10 mM $CaCl_2$. Aliquots of protein S stock solution (6.25 μM protein S in 50 mM Tris, 0.15 M NaCl) were diluted in the flow buffer, and 45 μL was injected during the association phase at a constant flow rate of 3 μL/min. The association phase was followed for 15 min and repeated using several different protein concentrations at each $CaCl_2$ or EDTA concentration. The dissociation phase was monitored during 30 h with flow buffer at a constant flow of 3 μL/min. Flow rates of 1, 3, and 6 μL/min were tested and gave identical results.

Evaluation of Surface Plasmon Resonance Data. The association reaction studied by the use of BIAcore occurs between component B (here C4BP) immobilized to the gold dextran-coated sensor chip surface and component A in solution (here protein S or chimeras I−VI). Component A is in constant flow (constant concentration and constant flow rate) during the association phase, and AB complex formation leads to a change in refractive index of the sensor chip surface, which is reported continuously in terms of response units (RU). The response is proportional to the total mass of molecules bound at the surface. The dissociation process is initiated by change to a constant flow of protein-free buffer, and a decrease in the response corresponds to component A dissociating from the immobilized component B into the buffer. In addition to the response changes due to association and dissociation of component A, the signal changes abruptly when protein injection starts or ends, due to changes in refractive index, so the first few points of each phase are omitted in the fitting procedure.

Dissociation Phase. The dissociation phase can in the simplest case be described by first-order kinetics. The decrease of AB complex with time (t) is

$$\mathrm{d[AB]}/\mathrm{d}t = -k_{\mathrm{off}}\,[\mathrm{AB}] \tag{1}$$

and the data analysis involves fitting the function

$$R(t) = C\exp(-k_{\mathrm{off}}\,t) \tag{2}$$

to the relative response, $R(t)$, measured as the response in RU minus the base line RU (before injection of protein). The variable parameters in the fit are the constant C [$C = R(t{=}0)$] and the dissociation constant, k_{off}.

Association Phase. During the association phase the AB complex increases as a function of time according to

$$\mathrm{d[AB]}/\mathrm{d}t = k_{\mathrm{on}}[\mathrm{A}][\mathrm{B}] - k_{\mathrm{off}}\,[\mathrm{AB}] \tag{3}$$

Since component A is in constant flow, we can replace [A] with the concentration, c, of this component:

$$\mathrm{d[AB]}/\mathrm{d}t = k_{\mathrm{on}}c[\mathrm{B}] - k_{\mathrm{off}}\,[\mathrm{AB}] \tag{4}$$

For a 1:1 stoichiometry (as for the protein S−C4BP interaction; Dahlbäck, 1983b), one A molecule bound to each B molecule, the concentration of the B component at time t will be

$$[\mathrm{B}] = B_0 - [\mathrm{AB}] \tag{5}$$

where B_0 is the initial concentration of B. Substitute of this expression for [B] in eq 4 yields

$$\mathrm{d[AB]}/\mathrm{d}t = k_{\mathrm{on}}c\{B_0 - [\mathrm{AB}]\} - k_{\mathrm{off}}\,[\mathrm{AB}] \tag{6}$$

The change in the measured quantity, R, is proportional to the change in [AB] ($R = \alpha$[AB], where α is a proportionality constant). The value of [AB] at complete saturation is equal to B_0 for a 1:1 stoichiometry, and the measured value at complete saturation would be $R_{\mathrm{max}} = \alpha B_0$.

$$\mathrm{d}R/\mathrm{d}t = k_{\mathrm{on}}c\{R_{\mathrm{max}} - R\} - k_{\mathrm{off}}\,R \tag{7}$$

which can be rearranged to

$$\mathrm{d}R/\mathrm{d}t + R(k_{\mathrm{on}}c + k_{\mathrm{off}}) = k_{\mathrm{on}}cR_{\mathrm{max}} \tag{8}$$

Solving this first-order differential equation yields

$$R(t) = R_0 + (R_{\mathrm{max}}ck_{\mathrm{on}}/(ck_{\mathrm{on}} + k_{\mathrm{off}}))(1 - \exp(-(ck_{\mathrm{on}} + k_{\mathrm{off}})t)) \tag{9}$$

where R_0 is the R value at time 0. This is generally not the same as the base line value because any change in solvent composition changes the background R value. The association rate constant is evaluated by fitting eq 9 to the association phase data, using predetermined values of c and k_{off} (c by acid hydrolysis of the protein stock solution, and k_{off} by analysis of dissociation data, cf. above).

RESULTS

Expression and Characterization of Recombinant Factor IX−Protein S Chimeras. Recombinant factor IX, bovine protein S, and the seven factor IX-protein S chimeras were expressed in human 293 cells. On average, 20 clones were processed from each cDNA clone. Cells were grown to confluence in medium supplemented with 10 μg/mL of vitamin K1, and the level of protein secreted to the culture medium was measured by ELISA and found to vary between 0.8 and 8.6 μg/mL/24 h. The different recombinant proteins were purified and analyzed by SDS−PAGE. Under both reducing and non-reducing conditions, the recombinant proteins migrated as single bands. The apparent molecular weights of the wild-type and chimeric proteins were in agreement with those expected based on their module composition. Amino-terminal protein sequence analysis yielded the expected sequences of recombinant wild-type factor IX, protein S, and their chimeras (data not shown).

Binding of Protein S to C4b-Binding Protein. Association and dissociation rates of the protein S−C4BP interaction were measured with surface plasmon resonance technology, using BIAcore. Human C4BP was immobilized to an activated dextran layer on a BIAcore sensor chip gold surface using amine coupling. The immobilized amount of C4BP was usually between 3000 and 5000 RU, with 1 RU corresponding to 1 ng/mm^2. 50−100 individual protein S binding experiments were performed on each immobilization, with intervening washes using 0.1 M HCl to release bound protein S. The reproducibility in these experiments was found to be very high, and we observed no effect on the measured rate constants even after 100 binding experiments.

The association and dissociation rate constants for immobilized C4BP interacting with plasma-derived human or bovine protein S were determined at five different Ca^{2+} concentrations. A first series of experiments was performed with relatively high protein S concentration (200 nM) during the association phase, and the dissociation phase was followed for 30 h. The values of k_{off} were estimated by

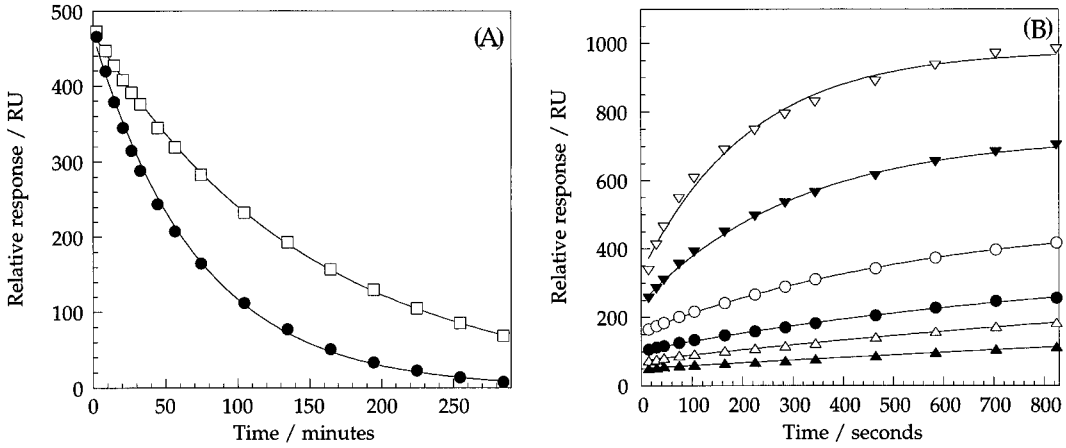

FIGURE 4: Computer analysis of SPR data. (A) Dissociation phase data. The quality of fit is illustrated by showing a few representative points from the dissociation phases of sensorgrams for bovine (●) and human (□) protein S in the presence of 3.4 mM EDTA. The curves are obtained by computer fitting to the data points using eq 2. (B) Association phase data acquired in the presence of 1 mM Ca^{2+}. The quality of fit is illustrated by showing a few representative points from the association phases of sensorgrams for 1.5 (▲), 3 (△), 6.25 (●), 12.5 (○), 50 (▼) and 200 nM (▽). The curves are obtained by computer fitting to the data points using eq 9.

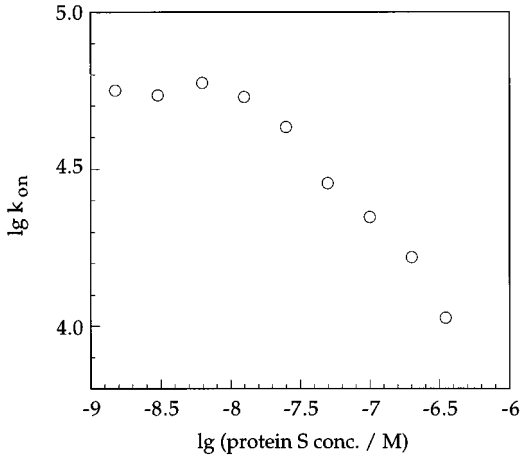

FIGURE 5: (A) Apparent association (k_{on}) rate constant as a function of human protein S concentration.

computer fitting to the data acquired during the dissociation phase using eq 2 as exemplified in Figure 4A. The base line stability over 30 h was tested and found reliable. A second set of experiments was performed to find a range of protein S concentrations yielding consistent values for the association rate constant, k_{on}. In these experiments, the association phase was followed for 15 min. The data obtained at 1−12 nM protein S gave consistent results (Figure 5), whereas a further increase in protein S concentration led to an reduction in the apparent association rate constant. This may be a consequence of the tendency of protein S to aggregate at higher concentration, or alternatively the apparent association constant is diffusion limited at higher protein S concentration. A third series of experiments was performed with triplicate experiments at 1.5, 3, 6, and 12 nM protein S at each of the five calcium concentrations. Computer fitting to the association data using eq 9 produced good fits as illustrated in Figure 4B. The k_{on} values obtained at 1.5, 3, 6, and 12 nM protein S were averaged, and the association and dissociation rate constants are summarized in Table 2. The obtained values of the association and dissociation rate constants at 10 μM, 100 μM, 1.0 mM, and 10 mM Ca^{2+} agreed within experimental error (Figure 6), and the values in Table 1 are averaged over these four Ca^{2+} concentrations. However, the values obtained at 3.4 mM EDTA deviate significantly from the values in Ca^{2+} containing buffer and are reported separately. This is indicative of a significant Ca^{2+}-dependence of the interaction between protein S and C4BP.

We found that the dissociation rate constant for the complex between human protein S and human C4BP is very low, $k_{off} = 7 \times 10^{-6}$ s^{-1}, in the presence of ≥10 μM Ca^{2+}. Together with an association rate constant of, $k_{on} = 7.3 \times 10^4$ M^{-1} s^{-1}, this yielded a very high affinity, $K_D = k_{off}/k_{on} = 0.10$ nM. Both the association and dissociation rate constants were higher in the case of bovine protein S; $k_{on} = 2 \times 10^5$ M^{-1} s^{-1}, and $k_{off} = 2 \times 10^{-5}$ s^{-1}, resulting in the same affinity for human C4BP as observed for human protein S ($K_D = 0.10$ nM). When Ca^{2+} was removed (measurements in buffer with 3.4 mM EDTA), the affinity for C4BP was reduced by a factor of 65 for human protein S and by a factor of 40 for bovine protein S. The decreased affinity could be mainly attributed to an increased off-rate (12−17-fold), while the on-rate decreased 3−4-fold.

As a control experiment, protein S solutions (1.5−12 nM) were injected over a sensor chip surface with no C4BP immobilized. There was an initial steep rise in response, followed by a flat phase, and then after injection of protein S there was a steep decrease and the signal returned to the original response value. The magnitude of the initial rise was similar to that observed for the same protein S solution injected over a sensor chip with C4BP. The results of the control experiment suggest that there is no significant binding of protein S to the dextran matrix on the sensor chip.

Binding of Chimeras to C4b-Binding Protein. Six hybrid proteins have been constructed for assessment of the parts of protein S that are responsible for C4BP binding. The chimeras are composed of different parts of bovine protein S and human factor IX, as outlined in Figure 2. The binding to immobilized C4BP of these chimeras as well as recombinant factor IX and protein S was studied using surface plasmon resonance technique as described above for human and bovine protein S. Recombinant bovine protein S was found to interact with human C4BP with the same affinity as bovine protein S purified from plasma in the presence EDTA. However, in the presence of Ca^{2+}, the recombinant form bound C4BP with 6-fold lower affinity than the plasma form. Recombinant bovine factor IX, and the chimeras

Table 2: Dissociation, k_{off}, and Association, k_{on}, Rate Constants for Protein S Binding to Immobilized C4BP

protein	solution condition[a]	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	log K	K_D (nM)
human protein S	3.4 mM EDTA	$(1.20 \pm 0.05) \times 10^{-4}$	$(1.85 \pm 0.07) \times 10^{4}$	8.19	6.5
	0.01−10 mM Ca^{2+}	$(7.1 \pm 1.1) \times 10^{-6}$	$(7.3 \pm 0.2) \times 10^{4}$	10.01	0.10
bovine protein S	3.4 mM EDTA	$(2.56 \pm 0.10) \times 10^{-4}$	$(6.5 \pm 0.2) \times 10^{4}$	8.40	4.0
	0.01−10 mM Ca^{2+}	$(2.2 \pm 0.2) \times 10^{-5}$	$(2.12 \pm 0.07) \times 10^{5}$	9.98	0.10

[a] Substance added to 10 mM Hepes/KOH, pH 7.4, with 0.15 M NaCl and 0.05% Tween 20.

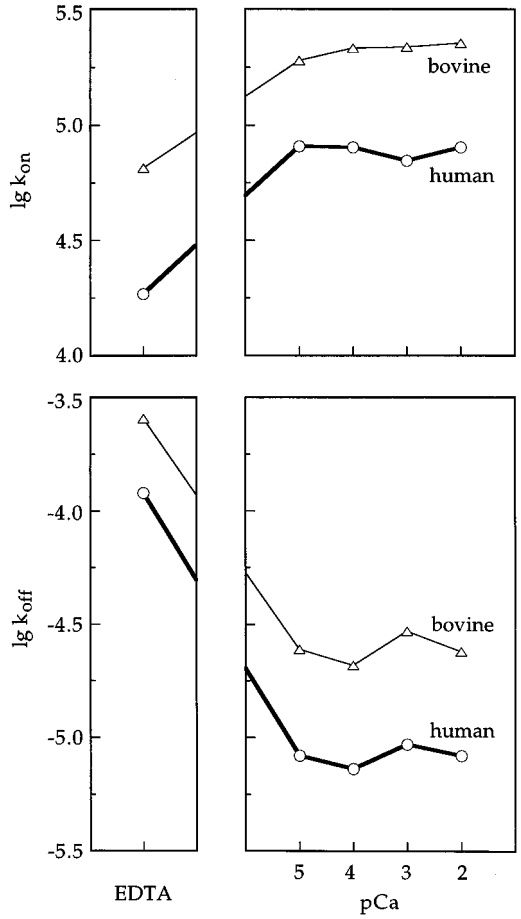

FIGURE 6: Apparent association (k_{on}) and dissociation (k_{off}) constants as a function of calcium concentration, for binding of human (○) and bovine (△) protein S to immobilized human C4BP.

I−III, which have N-terminal portions of protein S combined with the C-terminal parts of factor IX, showed no observable binding to C4BP in the SPR experiment, neither in the presence of Ca^{2+} nor EDTA. However, strong interaction with the immobilized C4BP was observed for recombinant bovine protein S as well as the chimeras (IV−VI) which all contain the SHBG domain of protein S and zero, one, or four of its EGF-like modules. This suggests a vital importance of the laminin-G-type repeats for the interaction between protein S and C4BP. The on- and off-rates in 2 mM Ca^{2+} and 3.4 mM EDTA, respectively, as obtained from computer fits to the association and dissociation phase data for hybrids IV−VI and recombinant bovine protein S are summarized in Table 3.

DISCUSSION

In the present work we have used surface plasmon resonance (SPR) to study the effects on the protein S−C4BP interaction due to three types of perturbations: (1) changes in solvent conditions, (2) species variants, and (3) substitution of protein domains. The SPR technique is feasible for protein−protein interactions with association rates between 10^2 and 10^6 M^{-1} s^{-1} and dissociation rates in the range 10^{-3} to 10^{-6} s^{-1}, under the conditions that immobilization of one component does not perturb the interaction and that there is no binding of the fluid phase protein to the dextran matrix on the sensor chip. The precision in both on- and off-rates can be quite high if they fall within the above ranges. The method can in such cases be especially useful for assessing *changes* in binding parameters due to different types of perturbations. The interaction between protein S and C4BP has a suitable on-rate to be studied with this method, and the off-rate is at the lower limit. It is reasonable to believe that immobilization of C4BP perturbs the protein S-binding function to a very low extent because of the spider-like structure of C4BP, with the protein S-binding site located on the shortest of the eight "arms". If the site of immobilization is randomly chosen, there is a 7−19 times higher probability of immobilization via an α-chain than via the β-chain, with the higher number taking into account the larger number of SCR modules in the α-chain as compared to the β-chain. The protein S-binding site on the vast majority of immobilized C4BP molecules is thus likely to have essentially the same properties as in solution.

The affinity for protein S-binding to C4BP is considerably lower in the presence of EDTA than in the presence of Ca^{2+} (≥ 10 μM). When Ca^{2+} is removed from human protein S, we find that the affinity for C4BP is reduced by a factor of 65, and that this is a reflection of a significantly increased off-rate (a factor of 17) and slightly reduced on-rate (a factor of 4). Thus, the major role of Ca^{2+}-ions in enhancing the interaction is to increase the life-time of the C4BP−protein S complex, once formed, while a more minor role is to increase the rate of complex formation. Protein S contains four high-affinity calcium binding sites that are saturated at free calcium concentrations exceeding 10 μM, and at least some of these sites are present within the four EGF-like modules (Dahlbäck *et al.*, 1990c). The requirement of as little as 10 μM Ca^{2+} to induce the full calcium effect on the rate constants, and thus stabilize the interaction with C4BP, indicates that the interaction with C4BP is coupled to Ca^{2+}-binding to one or more of these high-affinity calcium sites in protein S but not to Ca^{2+} binding to the lower affinity ($K_D \approx 1-3$ mM) sites in the Gla module of protein S. The Ca^{2+}-binding properties of C4BP have, to our knowledge, not been investigated.

The binding analyses using hybrid proteins containing different domains of factor IX and protein S were based on the assumption that factor IX does not interact with C4BP, which was confirmed in the present study. All three chimeras that contain the SHBG-like domain from protein S bound C4BP with the same affinity as the recombinant bovine protein S, whereas none of the chimeras lacking the SHBG-like domain showed any observable binding to C4BP. This is the first direct evidence that the laminin-G-type

Table 3: Dissociation, k_{off}, and Association, k_{on}, Rate Constants for Binding of Factor IX−Protein S Hybrids to Immobilized C4BP

protein	solution condition[a]	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	log K	K_D (nM)
r-bovine protein S	3.4 mM EDTA	$(3.2 \pm 0.2) \times 10^{-4}$	$(5.9 \pm 0.2) \times 10^{4}$	8.23	5.4
	2 mM Ca^{2+}	$(7.4 \pm 1.1) \times 10^{-5}$	$(1.3 \pm 0.2) \times 10^{5}$	9.24	0.57
chimera IV	3.4 mM EDTA	$(3.3 \pm 0.1) \times 10^{-4}$	$(6.1 \pm 0.2) \times 10^{4}$	8.27	5.4
	2 mM Ca^{2+}	$(7.1 \pm 0.2) \times 10^{-5}$	$(1.2 \pm 0.2) \times 10^{5}$	9.23	0.59
chimera V	3.4 mM EDTA	$(3.6 \pm 0.2) \times 10^{-4}$	$(5.7 \pm 0.2) \times 10^{4}$	8.20	6.3
	2 mM Ca^{2+}	$(6.2 \pm 0.1) \times 10^{-5}$	$(1.0 \pm 0.2) \times 10^{5}$	9.21	0.62
chimera VI	3.4 mM EDTA	$(3.3 \pm 0.2) \times 10^{-4}$	$(6.0 \pm 0.2) \times 10^{4}$	8.26	5.5
	2 mM Ca^{2+}	$(6.3 \pm 0.1) \times 10^{-5}$	$(1.1 \pm 0.2) \times 10^{5}$	9.24	0.57

[a] Substance added to 10 mM Hepes/KOH, pH 7.4, 0.15 M NaCl and 0.05% Tween 20.

repeats of protein S contains the binding site(s) for C4BP. Previous studies using inhibitory peptides and protein S mutants have been controversial and pointed to different non-overlapping parts as essential binding sites (Walker, 1989; Weinstein & Walker, 1990; Nelson & Long, 1992; Chang *et al.*, 1992; Fernández *et al.*, 1993). The present hybrid protein data show that the binding site for C4BP is *fully* contained within the two laminin-G-type repeats of protein S. The same type of modules are found in several proteins involved in protein−protein interactions, for example, laminin (Nissinen *et al.*, 1991), the sex hormone binding globulin SHBG (Gershagen *et al.*, 1987), agrin (Tsim *et al.*, 1992), slit (Rothberg & Artavanis-Tsakonas, 1992), neurexin (Ushkarev *et al.*, 1992), and growth arrest specific protein 6 (Gas 6; Manfioletti *et al.*, 1993). It has been found for some of these proteins that the laminin-G-type repeats contain binding sites for other proteins or receptors, for example, in slit (Rothberg & Artavanis-Tsakonas, 1992). Gas 6 binds to and stimulates the receptor tyrosine kinases axl (Varnum *et al.*, 1995) and sky/Tyro3 (Ohashi *et al.*, 1995). It has recently been shown that bovine protein S interacts with the human Tyro 3 and that the laminin-G-type repeats contain the portion of protein S interacting with the receptor (Nyberg *et al.*, 1996). The three-dimensional fold has to our knowledge not been elucidated for any laminin-G-type module, but the five modules in laminin appear as five globular domains on electron micrograms (Beck *et al.*, 1990).

For all four proteins that contain the laminin-G-type repeats of protein S, hybrids IV, V, and VI and recombinant bovine protein S, the same change in binding parameters is observed when going from 3.4 mM EDTA to 2 mM Ca^{2+}. This is especially striking for hybrid VI which lacks all four EGF-like modules from protein S, some of which contain high-affinity Ca^{2+}-binding sites. The Ca^{2+}-induced increase in affinity for C4BP is therefore not likely to be dependent on Ca^{2+}-binding sites in the EGF-like modules in protein S. More likely, the coupling between Ca^{2+} binding and C4BP binding relies on Ca^{2+} binding sites present within the SHBG-like domain of protein S. Our future studies aim at localizing and characterizing these calcium sites, which may be unique to protein S or present also within laminin-type repeats in other proteins. Interestingly, for the protein SHBG there are reported effects of calcium ions on dimerization and steroid binding properties (Bocchinfuso & Hammond, 1994; Damassa *et al.*, 1996).

In the presence of EDTA, the recombinant bovine protein S binds C4BP with similar affinity as protein S purified from bovine plasma. However, if we compare the values obtained in the presence of Ca^{2+} the recombinant protein binds C4BP with 6-fold lower affinity than the plasma protein. This was a consistent observation which we have no explanation for. However, the important point is that there are no differences between wild-type protein S and the chimeras IV, V, and VI, i.e., the binding site is fully contained within the laminin-G-type modules.

CONCLUSIONS

The C4BP binding portion of protein S is fully contained in the laminin-G-like repeats. There is a thermodynamic coupling between the C4BP binding function and high-affinity Ca^{2+} binding to protein S. The present study has also provided accurate data for the affinity as well as association and dissociation kinetics of the reaction between C4BP and human or bovine protein S.

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