Characterization of mini-protein S, a recombinant variant of protein S that lacks the sex hormone binding globulin-like domain

Merel VAN WIJNEN*†[1], Jeanette G. STAM*, Glenn T. G. CHANG*, Joost C. M. MEIJERS*†, Pieter H. REITSMA‡[2], Rogier M. BERTINA‡ and Bonno N. BOUMA*†

* Department of Haematology, G.03.647, University Hospital Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands, † Institute of Biomembranes, Utrecht University, The Netherlands, and ‡ Thrombosis and Haemostasis Research Center, Department of Hematology, Leiden University Hospital, P.O. Box 9600, 2300 RC Leiden, The Netherlands

Protein S is a vitamin K-dependent glycoprotein involved in the regulation of the anticoagulant activity of activated protein C (APC). Also, an anticoagulant role for protein S, independent of APC, has been described. Protein S has a unique C-terminal sex hormone binding globulin (SHBG)-like domain that represents about half of the molecule. To define the role of this domain in APC cofactor activity and in binding to C4b-binding protein (C4BP), we have constructed a recombinant protein S molecule of N-terminal residues 1–242 that lacks the SHBG domain (mini-protein S). A panel of monoclonal antibodies directed against the N-terminal region of protein S recognized plasma-derived protein S, wild-type recombinant protein S and mini-protein S with similar affinities, whereas a monoclonal antibody that recognizes an epitope in the SHBG domain did not detect mini-protein S. Mini-protein S did not bind to C4BP in a solid-phase binding assay, and the cofactor activity of mini-protein S was not inhibited by preincubation with C4BP. In a plasma coagulation assay, the cofactor activity of mini-protein S was lower than wild-type or plasma-derived preparations. In contrast, no difference in APC cofactor activities was observed when the preparations were tested in purified systems that monitor the APC-mediated degradation of factors Va or VIIIa. In conclusion, we constructed a protein S molecule that fails to bind C4BP and still displays cofactor activity for APC. This confirms the role of the C-terminal SHBG region in C4BP binding and demonstrates that N-terminal residues 1–242 are sufficient for the expression of APC cofactor activity in a system using purified components. In plasma, however, the C-terminal SHBG region plays a role in the expression of optimal APC cofactor activity.

INTRODUCTION

The physiological importance of protein S as an anticoagulant factor is demonstrated by the thrombotic tendency of protein S-deficient patients [1]. Protein S functions as a cofactor for activated protein C (APC) in the degradation of factors Va and VIIIa [2–6]. Recently, it has been reported that protein S stimulated the inactivation of membrane-bound factor Va by specific acceleration of the APC-mediated cleavage at Arg^{306} [7]. For the inactivation of factor VIIIa, it was postulated that factor V and protein S act as synergistic cofactors for APC in the degradation of factor VIIIa [8,9]. This synergistic effect is suggested to be required for expression of maximal protein S anticoagulant activity in plasma. Another possible explanation for the thrombotic tendency of protein S-deficient patients is the observation that protein S can also express anticoagulant activity, independent of APC. Protein S inhibits prothrombinase and tenase activity, independent of APC, both in plasma and in purified systems using phospholipid vesicles, human endothelial cells or human platelets [10–14].

Unlike other vitamin-K dependent coagulation factors, protein S contains a C-terminal sex hormone binding globulin (SHBG)-like domain that represents about half of the molecule. The SHBG domain has been shown to contain the binding site for the complement regulatory C4b-binding protein (C4BP) [15–18], and upon binding of C4BP to protein S the cofactor activity of protein S for APC is lost [19,20]. Also it has been suggested that the factor V binding site of protein S is located on or in the vicinity of the SHBG region [10] and that this interaction may be important for the APC-independent inhibition of prothrombinase activity by protein S [10,12].

To study the role of the SHBG domain in the binding of protein S to C4BP and in the anticoagulant properties of protein S, we have expressed a protein S molecule in eukaryotic cells that consists of the N-terminal residues 1–242 and lacks the C-terminal SHBG domain (designated mini-protein S).

MATERIALS AND METHODS

Materials

Purified recombinant factor VIII was a gift from Dr. D. Pittman (Genetics Institute, Cambridge, MA, U.S.A.). Purified human α-thrombin, protein C, protein C activator purified from *Agkistrodon contortrix contortrix* (ACC) and anti-ACC polyclonal antibody were provided by Dr. W. Kisiel (Department of Pathology, University of New Mexico, Albuquerque, NM, U.S.A.). Recombinant desulphato-hirudin was a gift from Dr. R. B. Wallis (Ciba-Geigy Pharmaceuticals, Hornsham, U.K.). Monoclonal antibodies CLB-PS25, CLB-PS13 and CLB-PS18 were gifts from Dr. J. A. van Mourik (Central Laboratory of the

Abbreviations used: APC, activated protein C; SHBG, sex hormone binding globulin; C4BP, C4b-binding protein; Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; TSR, thrombin-sensitive region; $B_{\frac{1}{2}max}$, half maximal binding; APTT, activated partial thromboplastin time; BHK, baby hamster kidney; ACC, *Agkistrodon contortrix contortrix*; PNGase, peptide *N*-glycosidase.

[1] To whom correspondence should be addressed, at the Department of Haematology.

[2] Present address: Laboratory for Experimental Internal Medicine, Academic Medical Center, Amsterdam, The Netherlands.

Netherlands Red Cross Blood Transfusion Services, Amsterdam, The Netherlands). CNBr-activated Sepharose, Fast Flow Q-Sepharose and PD-10 Sephadex G-25 columns were obtained from Pharmacia (Uppsala, Sweden). Pefabloc™ and cephalin-kaolin activated partial thromboplastin time [(APTT)-reagent] were obtained from Boehringer Mannheim (Mannheim, Germany). BSA, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine were purchased from Sigma (St. Louis, MO, U.S.A.). Factor VIII Coatest and chromogenic substrates S2366 and S2238 were obtained from Chromogenix (Mölndal, Sweden). Peptide *N*-glycosidase (PNGase) F was obtained from New England Biolabs (Beverly, MA, U.S.A.). Polyclonal peroxidase-conjugated antibodies against protein S were from Dako (Glostrup, Denmark).

Protein S constructs

Construction of wild-type protein S was performed by *Bam*HI–*Bgl*II digestion of the vector pSVPS [21] carrying the protein S cDNA sequence. The isolated fragment was cloned into the *Bam*HI site of the expression vector pZEM229R and designated pZEM229R-PS. The PZEM229R vector was provided by Dr. E. R. Mulvihill (Zymogenetics Inc, Seattle, WA, U.S.A.). Construction of mini-protein S was achieved by the substitution of Val^{243} of the mature protein with a stop codon by performing site-directed mutagenesis according to Kunkel [22] using the oligonucleotide primer: 5′-CAG AAG AGT TGT GAG TAA GTT TCA GTG TGC CTT-3′. Underlined nucleotides were altered. The sequence of the mutated protein S cDNA was confirmed by dideoxy sequencing. After *Cel*II–*Spe*I digestion the fragment with the stop codon was ligated into *Cel*II–*Spe*I-digested pZEM229R-PS.

Cell culture, transfection and purification of recombinant protein S

Transfection and expression in baby hamster kidney (BHK) cells was performed as described [23]. Expression of γ-carboxylated protein S was initiated by growing the cells for 3 days in conditioned medium (Optimem, Gibco BRL, Paisley, U.K.) supplemented with 10 μg/ml vitamin K1 (Konakion Roche, Mijdrecht, The Netherlands). Wild-type and mini-protein S were purified from the culture medium according to the method of Grinnell et al. [24]. Briefly, conditioned medium was collected and benzamidin/HCl (20 mM), Pefabloc™ (0.2 mm), and EDTA (5 mM) were added. After adsorption to an anion-exchange column (Fast Flow Q-Sepharose) protein S was eluted with Tris-buffered saline (TBS: 50 mM Tris/HCl pH 7.4/150 mM NaCl) containing 5 mM $CaCl_2$. To concentrate the protein S pool, EDTA (final concentration 10 mM) was added to the eluate, which was subsequently applied to a second Fast Flow Q-Sepharose column. Protein S was eluted with 50 mM Tris/HCl, pH 7.4/500 mM NaCl. Thrombin-cleavage of 500 μg wild-type or mini-protein S was performed with 1 unit/ml α-thrombin in 50 mM Tris/HCl, pH 7.4/500 mM NaCl/5 mM EDTA at 37 °C. After 2 h, 50 units of hirudin were added to inactivate thrombin and the mixture was applied to a Fast Flow Q-Sepharose column. Recombinant thrombin-cleaved protein S was purified as described above. Protein S concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions with BSA as a standard.

Determination of γ-carboxyglutamic acid (Gla) content

Gla analysis was performed by Dr. C. Vermeer (Department of Biochemistry, University of Limburg, Maastricht, The Netherlands) according to the method of Kuwada and Katayama [25].

Purification of proteins

Human protein S was purified from prothrombin-complex concentrates as described [26]. C4BP was immunopurified from human plasma according to the method of Hessing et al. [27]. Human protein C was activated with ACC (100:1) at 37 °C as described [28]. The generation of APC activity was monitored by the measurement of the rate of hydrolysis of the chromogenic substrate S2366. Activation was terminated by the addition of a polyclonal antibody (purified IgG) against ACC. Antibody–ACC complexes were removed by passing the mixture over protein-G-Sepharose and APC was analysed on SDS/PAGE. Human factor X was purified from plasma as described by Hackeng et al. [26] and activated with immobilized Russell's viper venom according to the method of Bock et al. [29]. Prothrombin was purified from human prothrombin-complex concentrates as previously described [4]. Factor V was purified from human plasma as described [30] and activated according to the method of Hackeng et al. [26]. All proteins appeared homogeneous on SDS/PAGE and protein concentrations were based on A_{280} readings using the following absorption coefficients ($A^{1\%}_{1\,cm}$): protein S, 9.5 [31]. C4BP, 14.1 [32]; protein C, 14.5 [33]; factor X, 11.6 [34]; prothrombin, 14.2 [35]; and factor V, 9.6 [36].

Recognition of protein S by monoclonal antibodies

Recombinant or plasma-derived protein S was coated on to the wells of microtitre plates in TBS overnight at 4 °C. Plates were washed with TBS/0.1 % (v/v) Tween-20, blocked with TBS/0.1 % Tween-20/3 % (w/v) BSA and incubated with protein-G-purified monoclonal antibodies (0–80 nM) for 2 h at room temperature. Plates were washed four times with TBS/0.1 % Tween-20 and bound IgG was detected with peroxidase-conjugated rabbit anti-mouse antibodies. Half maximal binding values were calculated by Scatchard analysis of the data with Enzfitter software (Leatherbarrow, R. J., 1987, Enzfitter, Elsevier, Biosoft, Amsterdam, The Netherlands).

C4BP-protein S binding assay

The binding of protein S to immobilized C4BP was performed as previously described [37]. Microtitre plates were coated with monoclonal antibody RU-8C11, which recognizes an epitope in the α-chain of C4BP (1 μg/ml in 50 mM sodium bicarbonate, pH 9.6). Plates were washed with TBS/3 mM $CaCl_2$/0.1 % Tween-20 and blocked with the same buffer containing 3 % BSA. All further dilutions were performed in this buffer, also with 3 % BSA. C4BP (1 μg/ml) was incubated for 1 h at 37 °C and, after washing, increasing concentrations of protein S were incubated for 2 h at 37 °C. Plates were washed and bound protein S was detected with a mixture of biotinylated monoclonal antibodies CLB-PS25 (1 μg/ml) and CLB-PS18 (1 μg/ml). The non-specific binding of protein S was determined in the absence of C4BP. Half maximal binding ($B_{\frac{1}{2}max}$) values were calculated as described above.

Preparation of phospholipid vesicles

Phospholipid vesicles containing 20 % phosphatidylserine, 40 % phosphatidylcholine and 40 % phosphatidylethanolamine were prepared as described by Brunner et al. [38], with some modifications. After evaporation of chloroform, phospholipids were dissolved in 10 mM Tris, pH 7.2/100 mM NaCl/0.2 mM EDTA/0.02 % (w/v) NaN_3/48 mM sodium deoxycholate. Ves-

Table 1 Recognition of mini, wild-type and plasma-derived protein S by monoclonal antibodies

Mini, wild-type and plasma-derived protein S were immobilized on microtitre plates and incubated with increasing concentrations of monoclonal antibody. Bound IgG was detected with peroxidase-conjugated rabbit anti-mouse IgG. $B_{\frac{1}{2}max}$ values were determined with Scatchard analysis. The means ± S.D. of three independent experiments performed in duplicate are shown.

Monoclonal antibody	Epitope	Calcium-dependent	Protein S preparation [$B_{\frac{1}{2}max}$ (nmol/l)]		
			Mini	Wild/type	Plasma
CLB-PS 25	Gla	−	0.34 ± 0.05	0.24 ± 0.04	0.28 ± 0.02
CLB-PS 13	Gla	+	0.29 ± 0.12	0.15 ± 0.03	0.19 ± 0.09
RU-PS 1G7	TSR	+	5.7 ± 2.5	3.9 ± 1.2	2.3 ± 0.8
CLB-PS 18	EGF	−	0.24 ± 0.11	0.57 ± 0.24	0.53 ± 0.07
RU-PS 3D9	EGF	+	0.47 ± 0.26	0.36 ± 0.13	0.37 ± 0.06
S20	SHBG	−	No binding	7.6*	5.6*

* $n = 1$.

icles were obtained by applying this mixture to a PD-10 Sephadex G-25 column. The phospholipid content of the fractions was determined by phosphate analysis [39].

Protein S cofactor activity in plasma

Protein S cofactor activity was determined with an APTT assay using a KC-10 coagulometer (Amelung, Lemgo, Germany) as described [21]. For this assay, plasma deficient in protein S and C4BP was prepared by immunoadsorption as described previously [10].

Effect of protein S on APC-catalysed inactivation of factor Va

Factor Va (3 nM) was incubated with 0.1 nM APC, 25 nM protein S and saturating amounts of phospholipid vesicles in TBS/3 mM $CaCl_2$/0.3 % (w/v) BSA at 37 °C. At different time intervals, 2 μl aliquots were taken and factor Va activity was determined from the rate of prothrombin activation in 120 μl mixtures containing 15 nM factor Xa, 0.5 μM prothrombin and 25 μM phospholipid vesicles. After 3 min the reaction was stopped by the addition of 3 μl of 0.5 M EDTA. Under these conditions the rate of prothrombin activation was constant in time and linearly dependent on the amount of factor Va. Thrombin activity was determined from the rate of hydrolysis of S2238, using purified thrombin as a standard.

Effect of protein S on APC-catalysed inactivation of factor VIIIa

Human factor VIII (19 nM) was activated by α-thrombin (0.24 units/ml) at room temperature in TBS/3 mM $CaCl_2$/0.3 % BSA. Maximal activation of factor VIII was achieved after 2 min, as measured by a one-stage clotting assay using factor VIII-deficient plasma. Hirudin (70 units) was added to stop the activation. After 5 min, 0.04 nM factor VIIIa was incubated with 2 nM APC, 70 nM protein S and saturating amounts of phospholipid vesicles in TBS/3 mM $CaCl_2$/0.3 % BSA at 37 °C. At different time intervals 20 μl aliquots were taken and factor VIIIa activity was immediately determined from the rate of factors VIIIa–IXa catalysed factor X activation in 100 μl mixtures using the factor VIII Coatest (Chromogenix). The rate of factor X activation was proportional to the amount of factor VIIIa. Owing to the instability of factor VIIIa, activation of factor VIII was performed before each experiment.

Binding of protein S to phospholipids

Phospholipid vesicles were coated overnight onto the wells of microtitre plates in 50 mM sodium bicarbonate, pH 9.6, at 4 °C. Plates were washed with TBS/3 mM $CaCl_2$/0.3 % BSA and blocked for 1 h with the same buffer containing 3 % BSA (binding buffer). Increasing concentrations of protein S were incubated in binding buffer (100 μl/well) for 3 h at room temperature. Plates were washed three times with binding buffer and bound protein S was detected with biotinylated CLB-PS18, a monoclonal antibody that recognizes an epitope in the epidermal growth factor (EGF)-like region of protein S (Table 1). Non-specific binding was determined in wells where phospholipids were absent and ranged between 0 and 25 % of the total binding.

RESULTS

Expression and purification of recombinant wild-type and mini-protein S

BHK cells were transfected with expression plasmids containing either wild-type or mini-protein S. Protein S antigen was detected in the culture medium of clones transfected with each construct and the antigen level was approximately 1–3 μg/ml after 72 h of culture. Recombinant protein S was purified according to the method of Grinnell et al. [24]. This purification includes a calcium-dependent 'pseudo affinity' elution from an anion-exchange column which separates incompletely γ-carboxylated protein S from fully γ-carboxylated forms. The elution profiles for both recombinant protein S preparations were similar; all recombinant protein S could be eluted with TBS containing 5 mM $CaCl_2$. No detectable amount of protein S was present in the subsequent high-salt wash. In agreement with this observation, the amount of γ-carboxylated glutamic acid residues in wild-type and mini-protein S was found to be similar (7 ± 1 and 6 ± 2 Gla/mol respectively).

The purified proteins were analysed by SDS/PAGE under reducing conditions and showed apparent molecular masses of 90 and 30 kDa for wild-type and mini-protein S respectively (Figure 1A, lanes 3 and 5). Both recombinant proteins were obtained with a purity of 90–100 % and contained a similar fraction of cleaved protein S (approximately 10 %) as analysed by gel scanning and densitometry. Recombinant wild-type pro-

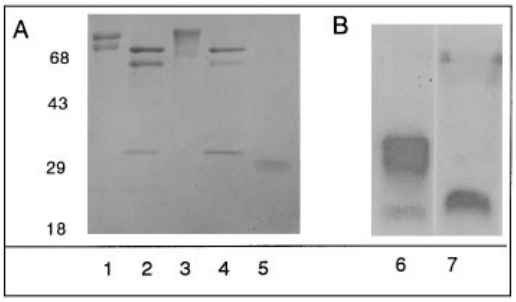

Figure 1 SDS/PAGE and immunoblot of purified protein S preparations

Recombinant mini-protein S, wild-type protein S and plasma-derived protein S (2 μg each) were analysed on a 12.5% SDS/PAGE gel under reducing conditions. Proteins were revealed by Coomassie Brilliant Blue staining (**A,** lanes 1–5) or immunoblotting (**B,** lanes 6 and 7) using monoclonal antibody CLB-PS18. Lane 1, plasma-derived protein S; lane 2, plasma-derived protein S treated with PNGase F; lane 3, wild-type protein S; lane 4, wild-type protein S treated with PNGase F; lane 5, mini-protein S; lane 6, mini-protein S; lane 7, thrombin-cleaved mini-protein S. Molecular mass markers (in kDa) are shown on the left. The low-molecular mass bands in lanes 2 and 4 represents PNGase F.

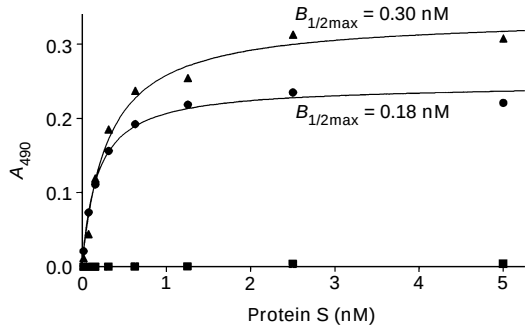

Figure 2 Binding of protein S to C4BP

Mini-protein S (■), wild-type protein S (▲) and plasma-derived protein S (●) were incubated with immobilized C4BP. Bound protein S was measured with biotinylated monoclonal antibodies CLB-PS18 and CLB-PS25. $B_{\frac{1}{2}max}$ values were calculated by Scatchard analysis. Each point represents the mean of triplicate determinations.

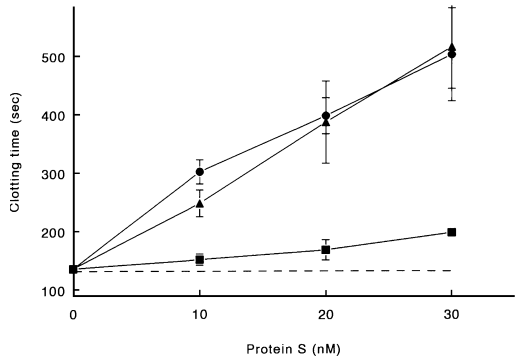

Figure 3 APC-cofactor activity of protein S

Prolongation of the clotting time was measured in an APTT coagulation assay using a KC-10 coagulometer (Amelung). Mini-protein S (■), wild-type protein S (▲) and plasma-derived protein S (●) were preincubated with protein S-C4BP-depleted plasma, APTT reagent and APC (final concentration 11 nM). Clotting was initiated by the addition of $CaCl_2$ and the clotting time was measured. Protein S concentrations denote final concentrations in the clotting assay. The means of at least four independent experiments ± S.D. are shown. The dotted line represents the clotting time obtained with APC alone or with APC and thrombin-cleaved protein S preparations.

tein S has a slightly higher molecular mass than plasma-derived protein S, but both proteins comigrate after treatment with PNGase F, indicating that the differences in migration patterns were due to a difference in *N*-linked glycosylation (Figure 1A, lanes 2 and 4). This has also been described for protein S expressed in other eukaryotic systems [21,24,40]. PNGase F treatment of mini-protein S did not change the migration pattern of the protein (results not shown). This observation confirmed the localization of *N*-linked glycosylation sites in the SHBG region of the protein S molecule [41]. Upon thrombin cleavage, the 30 kDa band of mini-protein S was converted into a 20 kDa fragment (Figure 1B, lane 7), which represents the EGF-like modules, and a 10 kDa fragment (not visible in Figure 1) containing the γ-carboxyglutamic acid-rich (Gla) domain, aromatic stack region and thrombin-sensitive region (TSR).

Recognition of plasma-derived, wild-type and mini-protein S by monoclonal antibodies

The reactivity of mini-protein S, wild-type protein S and plasma-derived protein S with monoclonal antibodies directed against different epitopes in protein S was studied in a solid-phase binding assay. Immobilized protein S was incubated with increasing concentrations of monoclonal antibody. The concentrations at which $B_{\frac{1}{2}max}$ occurred were determined by Scatchard analysis and were similar for mini-protein S, wild-type and plasma-derived protein S when antibodies were used that were directed against epitopes in the Gla-domain, TSR and the EGF-like modules (Table 1). This indicates that deletion of the SHBG region did not affect the conformation of epitopes of protein S that are recognized by these antibodies. As expected, a monoclonal antibody directed against an epitope in the SHBG region (S20) did not recognize mini-protein S (Table 1).

Binding of protein S to C4BP

To study the interaction of plasma-derived protein S, wild-type protein S and mini-protein S with C4BP, binding assays were performed using immobilized C4BP. The amount of bound protein S was detected with monoclonal antibodies CLBPS-18 and CLBPS-25, which both recognize the protein S forms (see Table 1). The protein S concentrations at which $B_{\frac{1}{2}max}$ occurred were calculated by Scatchard analysis. As shown in Figure 2, wild-type protein S and plasma-derived protein S have similar affinities for C4BP ($B_{\frac{1}{2}max}$ = 0.30 nM and 0.18 nM respectively), whereas binding of mini-protein S to C4BP was not detectable.

APC cofactor activity in a plasma system

To study the effect of the deletion of the SHBG-region on the APC cofactor activity of protein S, plasma-derived protein S, wild-type protein S and mini-protein S were compared in an APTT coagulation assay (Figure 3). In this assay, protein S prolongs the clotting time of protein S- and C4BP-depleted plasma in a dose-dependent manner only in the presence of APC. As shown in Figure 3, wild-type protein S displayed the same APC cofactor activity as plasma-derived protein S. This indicates that the difference in carbohydrate side-chains between wild-type and plasma-derived protein S did not affect the biological activity of recombinant protein S *in vitro*. Mini-protein S was active as a cofactor for APC, but the concentration of mini-protein S that was needed to obtain the same prolongation of the clotting time was found to be 8-fold higher. Similar results were obtained when additional phospholipid vesicles (20 % phosphatidylserine, 40 % phosphatidylcholine, 40 % phosphatidylethanolamine, 0–45 μM) were added together with cephalin to increase the amount of negatively charged phospholipids in the coagulation assay (results not shown). In a more defined assay, the factor Xa one-stage clotting test, the APC cofactor activity of mini-protein S was also decreased to the same extent when compared to wild-type protein S (results not shown). To show that the observed prolongation of the clotting time was specific for protein S, the protein S preparations were digested with thrombin. Cleavage of protein S resulted in a loss of cofactor activity, and no protein S-dependent prolongation of the clotting time was observed in the absence of APC in both clotting assays.

Effect of C4BP on protein S cofactor activity

The effect of C4BP on the cofactor activity of protein S was assessed with the APTT (Figure 4). Preincubation of wild-type protein S with C4BP for 1 h at room temperature resulted in a dose-dependent inhibition of protein S cofactor activity as measured in the APC-induced prolongation of the APTT. Protein

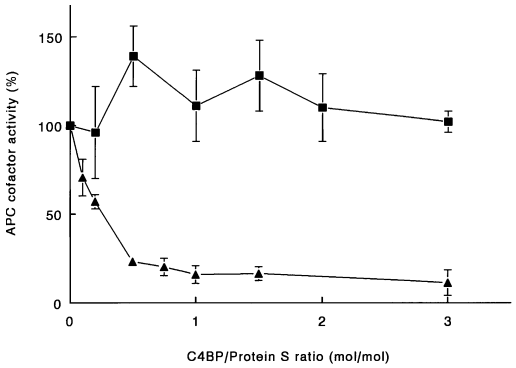

Figure 4 Inhibition of APC-cofactor activity of protein S by C4BP

Mini-protein S (■) and wild-type protein S (▲) were incubated with C4BP for 1 h at 37 °C and added to protein S-C4BP-depleted plasma to measure the protein S-dependent prolongation of the clotting time as described in Figure 3. The results are expressed as percentage of the clotting time obtained in the absence of C4BP and are the means ± S.D. of at least four measurements.

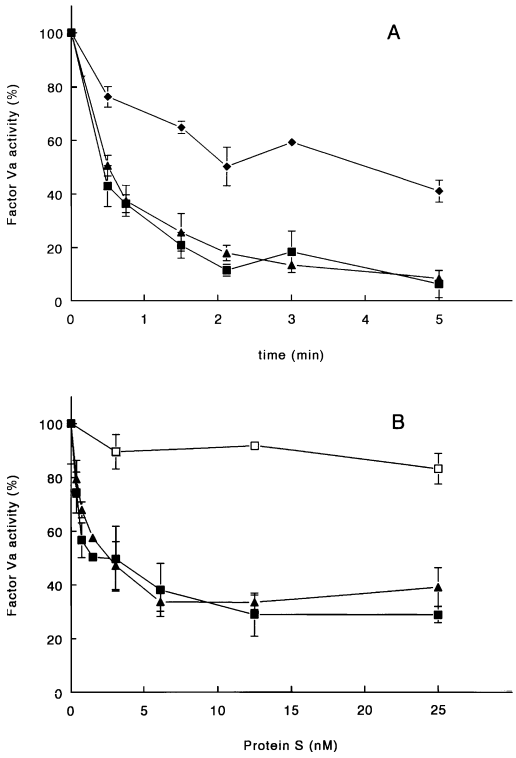

Figure 5 Effect of protein S on APC-catalysed inactivation of factor Va

Factor Va (3 nM) was incubated with 0.1 nM APC and phospholipid vesicles at 37 °C in the absence or presence of 25 nM protein S. At the indicated time-intervals (**A**) or after 2 min of incubation (**B**), factor Va activity was determined as described in the Materials and methods section. (**A**) No protein S (◆), wild-type protein S (▲), mini-protein S (■). Results are expressed as a percentage of factor Va activity without the addition of APC and protein S. (**B**) Wild-type protein S (▲), mini-protein S (■), thrombin-cleaved mini-protein S (□). Results are expressed as a percentage of activity of factor Va incubated with APC. The means ± S.D. of at least three independent experiments are shown.

S cofactor activity was almost completely blocked at equimolar concentrations of protein S and C4BP, suggesting the formation of a 1:1 complex between C4BP and protein S. The APC

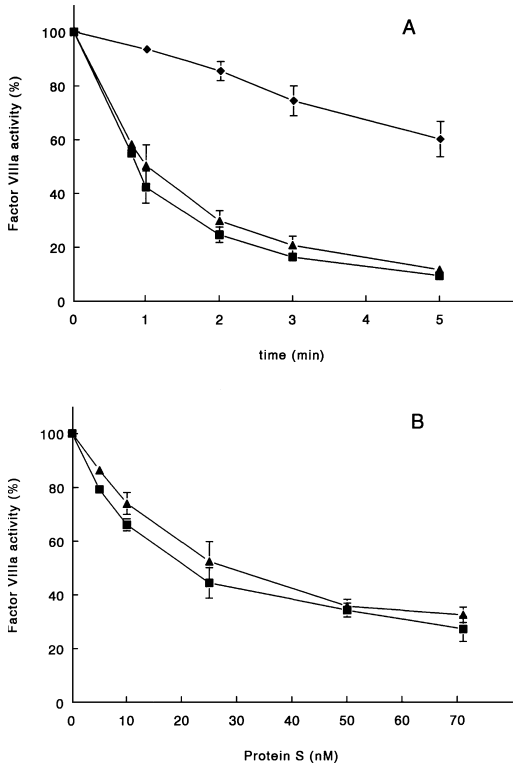

Figure 6 Effect of protein S on APC-catalysed inactivation of factor VIIIa

Factor VIIIa (0.04 nM) was incubated with 2 nM APC and phospholipid vesicles at 37 °C in the absence or presence of 70 nM protein S. At the indicated time-intervals (**A**) or after 2 min (**B**), factor VIIIa activity was determined as described in the Materials and methods section. (**A**) No protein S (◆), wild-type protein S (▲), mini-protein S (■). Results are expressed as a percentage of factor VIIIa activity without the addition of APC and protein S. Throughout the incubation period at 37 °C, no significant spontaneous loss of factor VIIIa activity was observed. (**B**) Wild-type protein S (▲), mini-protein S (■). Results are expressed as a percentage of factor VIIIa activity in the presence of APC. The means ± S.D. of at least three independent experiments are shown.

cofactor activity of mini-protein S was not affected, even by preincubation with a 3-fold molar excess of C4BP.

Effect of protein S on APC-catalysed inactivation of factor Va

Recently, it has been reported that protein S accelerates factor Va inactivation by APC by selectively promoting the cleavage of the factor Va heavy-chain at Arg^{306} [7].

Time courses of APC-catalysed inactivation of factor Va in the absence and the presence of protein S are shown in Figure 5(A). When 3 nM factor Va was inactivated with 0.1 nM APC and 25 nM wild-type or mini-protein S, factor Va inactivation was accelerated to the same extent by both recombinant protein S preparations. The enhancement of APC-catalysed factor Va inactivation was also studied as a function of the protein S concentration (Figure 5B). Mini-protein S and wild-type protein S showed similar dose–response curves when residual factor Va activity was measured after 2 min. The APC cofactor activity of mini-protein S was lost when mini-protein S was digested by thrombin.

Effect of protein S on APC-catalysed inactivation factor VIIIa

To study the effect of mini- and wild-type protein S on the APC-

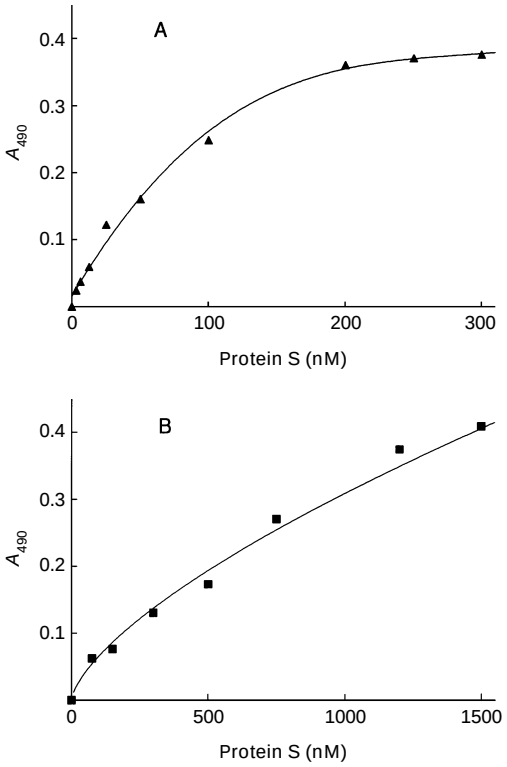

Figure 7 Binding of protein S to phospholipids

Wild-type protein S (**A**) and mini-protein S (**B**) were incubated on immobilized phospholipids for 2 h, after which phospholipid-bound protein S was detected with biotinylated monoclonal antibody CLB-PS18.

catalysed inactivation of factor VIIIa, factor VIIIa (0.04 nM) was incubated with 2 nM APC and 70 nM wild-type or mini-protein S. Time courses of factor VIIIa inactivation are shown in Figure 6(A). Both wild-type and mini-protein S accelerated factor VIIIa inactivation to the same extent. The APC-catalysed factor VIIIa inactivation as a function of protein S concentration resulted in identical dose–response curves for mini-protein S and wild-type protein S, when residual factor VIIIa activity was measured after 2 min (Figure 6B).

Taken together, these experiments show no effect of a deletion of the SHBG-region on protein S cofactor activity in systems using purified components.

Binding of protein S to phospholipids

Since the interaction of protein S with the phospholipid surface is necessary for the activity of protein S, we measured the binding of mini- and wild-type protein S to immobilized phospholipids. The binding of wild-type protein S was saturable and resulted in a $B_{\frac{1}{2}\max}$ of approximately 65 nM (Figure 7A). In contrast, the binding of mini-protein S to phospholipids was not saturable up to 1.5 μM (Figure 7B), indicating a lower affinity of the truncated protein S for phospholipids. Binding of both protein S preparations to phospholipids was completely inhibited by the addition of EDTA (results not shown).

Since the affinity of mini-protein S for phospholipids was decreased, the discrepancy between the APC cofactor activity of mini-protein S measured in plasma and in purified systems might be the result of either limiting amounts of phospholipids in the APTT-based assay or saturating amounts of phospholipids in the experiments with purified components. However, when the concentration of phospholipids in both assays was varied, no effect on the cofactor activity of mini-protein S was observed (results not shown).

DISCUSSION

Protein S is unique among vitamin-K dependent proteins because it contains a C-terminal region that is homologous to steroid-binding proteins [42]. This SHBG-like domain mediates binding to C4BP [15–18]. To study the contribution of the C-terminal SHBG domain to the binding of protein S to C4BP and to the anticoagulant function of protein S, we have expressed a recombinant protein S molecule that lacks this domain (mini-protein S). The protein was expressed in BHK cells and its purification included a calcium-dependent elution from an anion-exchange column [24], yielding a 90–100 % pure protein with an apparent molecular mass of 30 kDa (Figure 1).

Solid-phase binding studies showed that the binding of wild-type protein S and plasma-derived protein S to C4BP was not significantly different. In contrast, binding of mini-protein S to C4BP could not be detected, even at very high mini-protein S concentrations. Also APC-cofactor activity of mini-protein S in plasma was not inhibited by preincubation with C4BP (Figures 2 and 4). This confirms that the binding site for C4BP is located in the SHBG-like domain of protein S.

The APC cofactor activity of mini-protein S was studied in a system with purified components. Mini-protein S accelerated APC-catalysed inactivation of factor Va and factor VIIIa equally as efficiently as wild-type protein S (Figures 5 and 6). This indicates that N-terminal residues 1–242 are sufficient for APC cofactor activity. The APC cofactor activity of mini-protein S and wild-type protein S was also studied in plasma. In the plasma system, using an APTT-based assay, mini-protein S expressed significant APC cofactor activity that was completely lost after treatment with thrombin. However, the APC cofactor activity of mini-protein S was 8-fold lower than that of wild-type and plasma-derived plasma-derived protein S (Figure 3). Because all protein S preparations contained similar amounts of thrombin-cleaved material, the relatively low APC cofactor activity of mini-protein S is not related to the presence of more thrombin-cleaved mini-protein S. The possibility that the reduced cofactor activity of mini-protein S was due to degradation of the protein during the APTT measurement was excluded by blotting experiments (results not shown). In APTT-based assays, the effect of APC and protein S on the clotting time is complex and not well understood. Therefore we also used a factor Xa clotting time-based assay. In the presence of a fixed amount of APC and increasing concentrations of protein S, this clotting assay specifically measures the effect of protein S on the inactivation of factor Va. In this assay, the APC cofactor activity of mini-protein S was similarly reduced when compared with wild-type protein S (results not shown). Studies performed with protein S–factor IX chimeras, in which the SHBG-like domain was replaced by the factor IX protease module [43], also showed a decreased APC cofactor activity in a clotting assay. This implies a functional role for the SHBG-like domain in the expression of APC cofactor activity of protein S in plasma.

Because the interaction of protein S with a phospholipid surface is important for the expression of APC cofactor activity, we also studied the (calcium-dependent) binding of mini-protein S to immobilized phospholipids. Mini-protein S showed a reduced affinity for binding to phospholipids when compared with wild-type protein S (Figure 7). This suggests that the SHBG domain contributes to the optimal exposure of the phospholipid-

binding properties of the protein S molecule. Deletion of this domain may affect the stability of the conformation of the N-terminal epitopes involved in phospholipid binding, although the epitopes in the Gla-domain that are recognized by two anti-protein S monoclonal antibodies are conserved (Table 1). This suggests that the discrepancy between the APC cofactor activity of mini-protein S measured in plasma and in purified systems might be the result of the presence of other phospholipid-binding components in plasma that compete with mini-protein S for the phospholipid surface. The observation that higher concentrations of phospholipids do not affect the APC cofactor activity of mini-protein S in the APTT-based assay, and observations from a previous study [44] that the affinity for a membrane surface does not always correlate with the APC cofactor activity of protein S in this clotting test, indicates that the expression of a lower APC cofactor activity of mini-protein S in the coagulation assay is not necessarily the result of a lower affinity for phospholipids. In the system with purified components, the binding of protein S to phospholipids is not the rate-limiting step in the expression of APC cofactor activity, since the low affinity of mini-protein S does not have any effect on the cofactor activity. The presence of the other factors (APC, factor VIIIa, factor Va) probably augments protein S binding to the membrane surface. The cofactor activity of protein S in our system with purified components therefore is dependent on the interaction with APC or with the substrates factor Va and VIIIa. This is in agreement with the observations by Bakker et al. [3], who showed that protein S stimulates APC-catalysed factor Va inactivation, independent of the phospholipid concentration and composition. Also, the stimulation of the binding of APC to membranes by protein S is of minor importance in the human system [45]. The observation that mini-protein S expresses normal cofactor activity in the factor Va and factor VIIIa inactivation experiments demonstrates that the SHBG-like region does not play a role in the interaction between protein S and APC. This is in agreement with the data that the interaction between APC and protein S is mediated through the EGF modules of protein S [46–48].

These results suggest that additional components determine the expression of APC cofactor activity of protein S in plasma. The low cofactor activity of mini-protein S, as observed in the factor Xa one-stage assay and the APTT-based assay, demonstrates that the APC cofactor activity of mini-protein S in the inactivation of both factors Va and VIIIa in plasma is disturbed. In plasma, protein S abolishes the ability of factors Xa and IXa to protect factors Va and VIIIa from inactivation by APC [6,49,50]. Since the effect of protein S on the inactivation of factor Va and factor VIIIa by APC in the purified system is studied in the absence of these two factors, we cannot exclude that the lower cofactor activity of mini-protein S in plasma is the result of a disturbed elimination of the protective effect of factors Xa and IXa. Another explanation is the observation that protein S and factor V are synergistic cofactors for the inactivation of factor VIIIa by APC [8,9,51]. The absence of the binding site for factor V on mini-protein S may abolish this synergistic effect, resulting in a decreased APC cofactor activity for factor VIIIa. Preliminary data support this hypothesis, because we found the synergistic effect of factor V on mini-protein S in the APC-catalysed factor VIII inactivation to be significantly decreased (K. Váradi, M. van Wijnen, H. P. Schwarz and B. N. Bouma, unpublished work).

In conclusion, we have expressed a truncated protein S molecule that lacks the SHBG-like domain and still has cofactor activity for APC. The recombinant protein S variant fails to bind C4BP, which confirms that the binding site for C4BP is located in the SHBG-like domain of protein S. The expression of APC cofactor activity in systems using purified components demonstrates that N-terminal residues 1–242 are sufficient for APC cofactor activity. Interestingly, the C-terminal SHBG region of the protein S molecule seems to be required for the expression of optimal APC cofactor activity in plasma. This phenomenon is presently under investigation.

The authors thank Dr. J. A. van Mourik for monoclonal antibodies CLB-PS13, CLB-PS25, and CLB-PS18, Dr. C. Vermeer for the Gla/Glu-analysis, Dr. W. Kisiel for human α-thrombin, protein C, purified ACC snake venom and anti-ACC snake venom antibodies, Dr. D. Pittman for recombinant factor VIII, Dr. R. B. Wallis for recombinant hirudin, and the personnel of the Red Cross Blood Bank of Utrecht for providing us with fresh-frozen plasma. This work was supported in part by a grant from the Netherlands Heart Foundation (93.108) and a fellowship of the Royal Netherlands Academy of Arts and Sciences. J.C.M.M. is an Established Investigator of the Netherlands Heart Foundation.

REFERENCES

1 Borgel, D., Gandrille, S. and Aiach, M. (1997) Thromb. Haemost. **78**, 351–356
2 Kalafatis, M. and Mann, K. G. (1993) J. Biol. Chem. **268**, 27246–27257
3 Bakker, H. M., Tans, G., Janssen Claessen, T., Thomassen, M. C., Hemker, H. C., Griffin, J. H. and Rosing, J. (1992) Eur. J. Biochem. **208**, 171–178
4 Koedam, J. A., Meijers, J. C., Sixma, J. J. and Bouma, B. N. (1988) J. Clin. Invest. **82**, 1236–1243
5 Walker, F. J. (1981) J. Biol. Chem. **256**, 11128–11131
6 Solymoss, S., Tucker, M. M. and Tracy, P. B. (1988) J. Biol. Chem. **263**, 14884–14890
7 Rosing, J., Hoekema, L., Nicolaes, G. A. F., Christella, M., Thomassen, L. G. D., Hemker, H. C., Varadi, K., Schwarz, H. P. and Tans, G. (1995) J. Biol. Chem. **270**, 27852–27858
8 Shen, L. and Dahlback, B. (1994) J. Biol. Chem. **269**, 18735–18738
9 Lu, D., Kalafatis, M., Mann, K. G. and Long, G. L. (1996) Blood **87**, 4708–4717
10 Hackeng, T. M., van't Veer, C., Meijers, J. C. and Bouma, B. N. (1994) J. Biol. Chem. **269**, 21051–21058
11 Heeb, M. J., Rosing, J., Bakker, H. M., Fernandez, J. A., Tans, G. and Griffin, J. H. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 2728–2732
12 Heeb, M. J., Mesters, R. M., Tans, G., Rosing, J. and Griffin, J. H. (1993) J. Biol. Chem. **268**, 2872–2877
13 Koppelman, S. J., Hackeng, T. M., Sixma, J. J. and Bouma, B. N. (1995) Blood **86**, 1062–1071
14 van't Veer, C., Hackeng, T. M., Biesbroeck, D., Sixma, J. J. and Bouma, B. N. (1995) Blood **85**, 1815–1821
15 Walker, F. J. (1989) J. Biol. Chem. **264**, 17645–17648
16 Nelson, R. M. and Long, G. L. (1992) J. Biol. Chem. **267**, 8140–8145
17 Fernandez, J. A., Heeb, M. J. and Griffin, J. H. (1993) J. Biol. Chem. **268**, 16788–16794
18 Chang, G. T., Maas, B. H., Ploos van Amstel, H. K., Reitsma, P. H., Bertina, R. M. and Bouma, B. N. (1994) Thromb. Haemost. **71**, 461–467
19 Betina, R. M., van Wijngaarden, A., Reinalda Poot, J., Poort, S. R. and Bom, V. J. (1985) Thromb. Haemost. **53**, 268–272
20 Dahlback, B. (1986) J. Biol. Chem. **261**, 12022–12027
21 Chang, G. T., Ploos van Amstel, H. K., Hessing, M., Reitsma, P. H., Bertina, R. M. and Bouma, B. N. (1992) Thromb. Haemost. **67**, 526–532
22 Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 488–492
23 Meijers, J. C., Davie, E. W. and Chung, D. W. (1992) Blood **79**, 1435–1440
24 Grinnell, B. W., Walls, J. D., Marks, C., Glasebrook, A. L., Berg, D. T., Yan, S. B. and Bang, N. U. (1990) Blood **76**, 2546–2554
25 Kuwada, M. and Katayama, K. (1983) Anal. Biochem. **131**, 173–179
26 Hackeng, T. M., Hessing, M., van't Veer, C., Meijer Huizinga, F., Meijers, J. C., de Groot, P. G., van Mourik, J. A. and Bouma, B. N. (1993) J. Biol. Chem. **268**, 3993–4000
27 Hessing, M., Kanters, D., Hackeng, T. M. and Bouma, B. N. (1990) Thromb. Haemost. **64**, 245–250
28 Klein, J. D. and Walker, F. J. (1986) Biochemistry **25**, 4175–4179
29 Bock, P. E., Craig, P. A., Olson, S. T. and Singh, P. (1989) Arch. Biochem. Biophys. **273**, 375–388
30 Kane, W. H. and Majerus, P. W. (1981) J. Biol. Chem. **256**, 1002–1007
31 DiScipio, R. G. and Davie, E. W. (1979) Biochemistry **18**, 899–904
32 Perkins, S. J., Chung, L. P. and Reid, K. B. (1986) Biochem. J. **233**, 799–807
33 Kisiel, W. (1979) J. Clin. Invest. **64**, 761–769
34 Di Scipio, R. G., Hermodson, M. A., Yates, S. G. and Davie, E. W. (1977) Biochemistry **16**, 698–706
35 Bajaj, S. P., Rapaport, S. I. and Prodanos, C. (1981) Prep. Biochem. **11**, 397–412

36 Nesheim, M. E., Myrmel, K. H., Hibbard, L. and Mann, K. G. (1979) J. Biol. Chem. **254**, 508–517
37 Hessing, M., Vlooswijk, R. A. A., Hackeng, T. M., Kanters, D. and Bouma, B. N. (1990) J. Immunol. **144**, 204–208
38 Brunner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta **455**, 322–331
39 Rouser, G., Fkeischer, S. and Yamamoto, A. (1970) Lipids **5**, 494–496
40 Malm, J., Cohen, E., Dackowski, W., Dahlback, B. and Wydro, R. (1990) Eur. J. Biochem. **187**, 737–743
41 Bertina, R. M., Ploos van Amstel, H. K., van Wijngaarden, A., Coenen, J., Leemhuis, M. P., Deutz Terlouw, P. P., van der Linden, I. K. and Reitsma, P. H. (1990) Blood **76**, 538–548
42 Gershagen, S., Fernlund, P. and Lundwall, A. (1987) FEBS Lett. **220**, 129–135
43 He, X., Shen, L., Smith, K. J. and Dahlback, B. (1995) Blood **86**, 359a
44 van Wijnen, M., Stam, J. G., van't Veer, C., Meijers, J. C., Reitsma, P. H., Bertina, R. M. and Bouma, B. N. (1996) Thromb. Haemost. **76**, 397–403
45 Bangalore, N., Drohan, W. N. and Orthner, C. L. (1994) Thromb. Haemost. **72**, 465–474
46 Dahlback, B., Hildebrand, B. and Malm, J. (1990) J. Biol. Chem. **265**, 8127–8135
47 He, X., Shen, L. and Dahlback, B. (1995) Eur. J. Biochem. **227**, 433–440
48 Hackeng, T. M., Dawson, P. E., Kent, S. B. H. and Griffin, J. H. (1995) Thromb. Haemost. **73**, 1253
49 Nesheim, M. E., Canfield, W., Kisiel, W. and Mann, K. G. (1992) J. Biol. Chem. **257**, 1443–1448
50 Regan, L. M., Lamphear, B. J., Huggins, C. F., Walker, F. J. and Fay, P. J. (1994) J. Biol. Chem. **269**, 9445–9452
51 Varadi, K., Rosing, J., Tans, G., Pabinger, I., Keil, B. and Schwarz, H. P. (1996) Thromb. Haemost. **76**, 208–214

Received 14 August 1997/2 October 1997; accepted 17 October 1997