Published in final edited form as:

Thromb Haemost. 2013 August ; 110(2): 275–282. doi:10.1160/TH12-12-0953.

Plasma protein S residues 37–50 mediate its binding to factor Va and inhibition of blood coagulation

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Summary

Protein S (PS) is an anticoagulant plasma protein whose deficiency is associated with increased risk of venous thrombosis. PS directly inhibits thrombin generation by the blood coagulation pathways by several mechanisms, including by binding coagulation factors (F) Va and Xa. To identify PS sequences that mediate inhibition of FVa activity, antibodies and synthetic peptides based on PS sequence were prepared and employed in plasma coagulation assays, purified component prothrombinase assays, binding assays, and immunoblots. In the absence of activated protein C, monoclonal antibody (Mab) S4 shortened FXa-induced clotting in normal plasma but not in PS-depleted plasma. Mab S4 also blocked PS inhibition of FVa-dependent prothrombinase activity in purified component assays in the absence or presence of phospholipids and inhibited binding of PS to immobilized FVa. Epitope mapping identified N-terminal region residues 37–67 of PS as this antibody's epitope. A peptide representing PS residues 37–50 inhibited FVa-dependent prothrombinase activity in a noncompetitive manner, with 50% inhibition observed at 11 μ M peptide, whereas a peptide with a D-amino acid sequence of 37–50 was ineffective. FVa, but not FXa, bound specifically to the immobilized peptide representing residues 37–50, and the peptide inhibited binding of FVa to immobilized PS. These data implicate PS residues 37–50 as a binding site for FVa that mediates, at least in part, the direct inhibition of FVa-dependent procoagulant activity by PS.

Keywords

anticoagulant; factor Va; monoclonal antibody; peptide; protein S; structure-function relationship

Introduction

Protein S (PS) is an essential anticoagulant plasma component, deletion of which leads to embryonic lethal coagulopathy in mice (1;2). In humans, homozygous PS deficiency leads

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Conflicts of interest

None declared.

to life-threatening thrombosis in neonates (3), requiring aggressive treatment, and heterozygous deficiency is associated with increased risk of venous thrombosis, and possibly increased risk of arterial thrombosis (4–6). Plasma PS is a 75 kDa glycoprotein that exists 40% (130 nM) in the free form, and 60% (200 nM) in a complex with C4b-binding protein (~500 kDa). PS serves as a cofactor for the anticoagulant protease, activated protein C (APC) during proteolytic inactivation of FVa and FVIIIa (7;8), but it also has direct anticoagulant activity, independent of APC, in plasma assays, prothrombinase assays, extrinsic FXase assays, APTT assays, on endothelial cells, and on platelets (9–12). Only the free form of PS has significant APC cofactor activity, but both the free and the complexed forms have direct anticoagulant activity and can inhibit the prothrombinase activity of FXa. About 2.5 % of the PS in blood resides in the alpha granules of platelets and is released when platelets are activated (13). Platelet PS can directly downregulate thrombin and FXa generation on platelets and microparticles, the major sites for blood coagulation reactions (14). We recently reported that PS infused without APC in a baboon thrombosis model inhibited platelet and fibrin deposition, suggesting that PS may have therapeutic potential (15).

PS inhibition of prothrombinase is due to its interaction with FXa (Kd app~18 nM)(10) and with FVa (Kd app~33 nM)(9). We discovered that most plasma PS contains Zn^{2+} that is necessary for efficient interaction with FXa and tissue factor (11). Zn^{2+} is lost during certain purification procedures, but not others, leading to variable activity reports from different labs. Zn^{2+} -deficient PS can enhance inhibition of extrinsic FXase by tissue factor pathway inhibitor (TFPI) (16), while Zn^{2+} -containing PS can inhibit extrinsic FXase independently of TFPI, largely by binding and inhibiting tissue factor (11).

Binding of PS to FXa was reported to be dependent on the thrombin-sensitive region (TSR) of PS or on the PS EGF-4 module (17;18). Binding of PS to FVa was reported to be dependent on a site within the 15 C-terminal amino acid residues of PS, consistent with the observation that PS in complex with the large C4b-binding protein that binds the C-terminal region of PS, does not bind well to FVa (19). Considering the large size of the FVa molecule, other FVa binding sites on PS may exist.

Here, using experiments that were performed mainly in the absence of PL to separate protein-protein interactions from protein-lipid interactions involving PS, we show that mAb S4 inhibits the direct anticoagulant activity of PS because it blocks binding of PS to FVa. Furthermore, epitope mapping showed that mAb S4 recognizes a specific sequence in the PS N-terminal region of residues 37–67 and that a synthetic peptide comprising PS residues 37–50 inhibits the procoagulant activity of FVa, suggesting that these PS residues are involved in binding and inhibiting FVa.

Materials and methods

Proteins and reagents

PS in citrated plasma was barium adsorbed, eluted with 32% saturated ammonium sulfate and dialyzed against Tris-buffered saline (TBS: 0.05 M Tris, 0.1 M NaCl, pH 7.4) (20). PS-C4b binding protein was separated from free PS by treatment with polyethylene glycol to a

final concentration of 4.4%. Free PS in the supernatant was immunoaffinity-purified on a column of mAb S7 coupled to Sepharose. After washing the column with TBS-1 mM sodium citrate, bound PS was eluted with 0.1 M glycine, 0.05 M NaCl, 1 mM citrate, pH 2.7. Eluted PS was adjusted to neutral pH, pooled, concentrated in an Amicon concentrator, and dialyzed twice against TBS. PS prepared by this method retains Zn^{2+} and direct anticoagulant activity. Two preparations were used for many of the experiments, denoted as PS and PS2.

Human prothrombin, thrombin (EC 3.4.21.5), and FXa (EC 3.4.21.6) were from Enzyme Research Laboratories. Human FV was purified and activated as described (21). Thrombin chromogenic substrate CBS 35.4 was from American Diagnostica, and FXa chromogenic substrate was from Centerchem. Phospholipid (PL) vesicles were 80% phosphatidyl choline, 20% phosphatidylserine, and were prepared by sonication (9). Normal and PS-depleted plasma (PSdP) was from George King Biomedicals or from Affinity Biologicals. Monoclonal antibodies against PS and goat anti-PS antibodies were prepared as described (9;19). Rabbit anti-FV antibodies were from Dako. Maxisorp, low-binding, and V-well microtiter plates were from Nunc. Biotinylated secondary antibodies, streptavidin-alkaline phosphatase, and phosphatase substrates were from Pierce. Chymotrypsin was from Worthington. Peptides were synthesized in the lab of Dr. Richard Houghten of the Torrey Pines Institute for Molecular Studies.

Prothrombinase assays

Assays were performed in v-well polypropylene plates at 23°C using several variations, the dilution buffer consisting of 0.05 M Hepes, 0.1 M NaCl, 0.5% BSA (HBS-BSA), 5 mM CaCl_2 , 0.02% NaN_3 , pH 7.4. In some experiments, PS was preincubated in this solution with and without neutralizing anti-PS mAb S4 for 30 min. For full prothrombinase experiments, 20 pM FVa and 25 μM PL were preincubated with or without PS, neutralized PS, or test peptide for 10 min prior to addition of 1 nM FXa, followed by 0.6 μM prothrombin (final concentrations). Aliquots were removed at 0.5 min intervals and quenched in TBS, 0.5% BSA, 10 mM EDTA, pH 8.2 in low binding flat-well plates. Then chromogenic substrate for thrombin was added to 0.2 mM final concentration, and the rate of increase in absorbance of the thrombin product was measured at 405 nm in a microtiter plate reader. Standard curves for converting mAU/min to nM thrombin were employed. In experiments where noted, FVa or PL were omitted, and aliquots were removed and quenched at longer intervals of 5–10 min.

Clotting assays

FXa-based clotting assays were performed without exogenous PL. Normal or PS-depleted plasma (PSdP) (30 μl) was mixed with 55 μl of HBS-BSA with or without increasing concentrations of PS and/or neutralizing anti-PS mAb S4 and 15 μl of FXa at specified final concentrations. Mixtures were incubated for 3 min at 37°C in an ST4 coagulometer (Diagnostica Stago), then recalcified with 25 μl of 33 mM CaCl_2 and the time to clotting was measured. Standard curves of variable FXa (up to 19 pM as 100% activity) without PS addition was used to convert clotting times to % FXa activity when PS was added.

FVa-based assays were performed as described for APC cofactor activity of mouse PS (1;22). Briefly, plasma supplemented with fibrinogen and PL was incubated 2 min with FVa, with or without APC and/or peptide PSP37, prior to addition of CaCl_2 .

Immunoblots and microtiter plate binding assays

Immunoblots, ELISAs for PS antigen (23), and plate binding assays on Maxisorp microtiter plates were developed with various antibodies as indicated in the figure legends, followed by biotinylated secondary antibodies, streptavidin alkaline phosphatase, and phosphatase substrate. Plates were blocked with I-Block (Tropix) except for FXa or FVa binding, where 3% hydrolyzed fish skin gelatin was used. The washing solution contained 0.1% porcine skin gelatin and 2 mM CaCl_2 in HBS. For immunoblots, PS (8.9 μM) was untreated or treated at 37°C for 20 min with 2.7 $\mu\text{g/ml}$ chymotrypsin and the reaction was terminated in SDS-PAGE sample buffer at 90°C. Separately, PS was untreated or treated with thrombin coupled to Sepharose (20% beads) as described for 30 min (24).

Results

Effect of mAb S4 on the anticoagulant activity of PS in plasma assays

Preliminary experiments indicated that anti-PS mAb S4 inhibited both the direct anticoagulant activity of PS and the APC cofactor activity of PS. To investigate primarily the direct anticoagulant activity of PS and its intermolecular interactions, we sought to separate it from the APC cofactor activity of PS by testing the ability of PS to inhibit prothrombinase activity in the absence both APC and PL, since PL are required for effective cofactor activity of PS in concert with APC.

Two different preparations of PS directly inhibited coagulation by prolonging FXa-1-stage clotting times in the absence of exogenous PL, with PS doses in the physiologic range (total plasma PS, 330 nM; free plasma PS, 130 nM) (Fig. 1A). The prolongation of clotting times by the two PS preparations (up to 100 sec at 379 nM) was abolished by mAb S4 (Fig. 1B). By use of a standard curve of decreasing concentrations of FXa in the absence of PS, clotting times were converted to apparent % FXa activity. The clotting assays were performed in the presence of blocking antibodies against APC in order to exclude any possible contribution of APC cofactor activity of PS. In previous studies, mAb S4 was tested in normal plasma, PSdP, and patient plasmas deficient in PS (23). MAb S4 shortened clotting times in normal plasma, but had negligible effect in PSdP, and intermediate effects in heterozygous PS-deficient patient plasmas. MAb S4 also blocked the ability of PS to prolong clotting times in PSdP in activated partial thromboplastin time (APTT) assays in which clotting was initiated with dilute APTT reagent rather than with FXa (23).

Effect of mAb S4 on the direct anticoagulant activity of PS in prothrombinase assays

It is recognized that plasma contains endogenous lipids that may or may not support PS anticoagulant activity in FXa-1-stage assays. Additional testing was therefore performed in purified component prothrombinase assays to investigate parameters important for the direct anticoagulant activity of PS in the absence of PL. Prothrombinase assays also enabled

evaluation of the effect of PS in the presence and absence of FVa. FVa is the cofactor for FXa that enhances its activity by two to three orders of magnitude (25).

The two preparations of PS inhibited prothrombinase activity (inhibited thrombin generation) in purified component assays in a dose-dependent manner in the absence of PL (Fig. 1C), with IC₅₀'s within the physiologic range of plasma PS. In the absence of PL, inhibition of prothrombinase by the two PS preparations was abolished by mAb S4 when FVa was present in the assays (Fig. 1D), but mAb S4 had no effect when FVa was absent (data not shown). In the presence of optimal PL (25 μ M, enough PL surface to support FXa, FVa, and PS binding), mAb S4 also negated inhibition of prothrombinase activity by PS if FVa was present, while an unrelated Ca²⁺-dependent anti-PS mAb (S1) had little effect on PS inhibition (Fig. 1D). In the presence of saturating PL, if FVa was absent, mAb S4 had a weak effect on PS inhibition (data not shown). This effect may have been partly due to mAb S4's interference with PS binding to PL. Control extrinsic FXase assays were performed with these PS preparations in the presence and absence of neutralizing antibodies against tissue factor pathway inhibitor (TFPI), to ensure that the PS preparations did not contain sufficient TFPI to affect activity assays (11). Note that PS potency was similar in the presence and absence of PL in Fig. 1D, in keeping with our previous report (10).

Controls were performed in Fig. 1C–D experiments to ensure that no PL was present. Phospholipase A2 had no effect on prothrombinase activity and no effect on PS inhibition in the absence of PL. Activity of the phospholipase was confirmed in prothrombinase mixtures that contained PL, where prothrombinase activity was greatly diminished by phospholipase (data not shown).

Effect of mAb S4 on PS binding to FVa and narrowing the epitope

We previously showed that PS can bind to immobilized FVa in the absence of PL (9), therefore we tested the ability of mAb S4 to block this interaction. MAb S4 efficiently blocked PS binding to immobilized FVa, while nonimmune mouse IgG and two different anti-PS mAbs had no effect (Fig. 2). The IC₅₀ for mAb S4 blockage of binding was ~28 nM of antibody, while a cross-competing Ca²⁺-dependent mAb, S17 was less efficient at blocking binding (IC₅₀ ~90 nM, data not shown). MAb S17 is very similar to mAb S4; it also blocks the direct anticoagulant activity of PS, although somewhat less efficiently.

Since mAb S4 is Ca²⁺-dependent, the antibody's epitope might be located in the N-terminal region of PS, near the γ -carboxyglutamate (Gla) domain that mediates Ca²⁺-dependent binding of vitamin K-dependent proteins to negatively charged phospholipids. Therefore the ability of several mAbs to bind chymotrypsin-cleaved PS on immunoblots was tested, since chymotrypsin cleaves PS after the Gla domain at Phe40. MAb S4 and the cross-competing mAb 17 failed to recognize PS treated with chymotrypsin, while another mAb against PS (SC6) did recognize chymotrypsin-cleaved PS, which migrated faster on non-reduced SDS-PAGE (Fig. 3A). Sequencing revealed a new N-terminus at Tyr41 in chymotrypsin-cleaved PS. PS cleaved at Arg49 in the TSR by thrombin was also not recognized by mAbS4 (data not shown). Thus, cleavage at Phe40 or at Arg 49 disrupts the PS epitope for mAbS4.

MAB S4 bound to the chemically synthesized Gla domain coupled to the thrombin-sensitive region (Gla-TSR), residues 1–72 (Fig. 3B). MAB S4 also bound to a number of immobilized 14-mer or 15-mer peptides representing residues in the Gla-TSR region. Binding to peptides representing residues 37–50 and 54–67 was detected, thus narrowing the epitope to residues 37–67.

Ability of peptide PSP37 (representing PS residues 37–50) to mimic PS inhibition of FVa-dependent prothrombinase activity

Of the 14-mer peptides that were tested for binding of mAb S4, only PSP37 (TDYFYPKYLVCLRS, residues 37–50) exhibited FVa-dependent inhibition of prothrombinase activity (Fig. 4A). Peptides PSP30 (residues 30–43) and PSP48 (residues 48–61), with sequences overlapping that of PSP37 did not inhibit prothrombinase. A peptide with D-amino acids in the sequence of PSP37 also failed to significantly inhibit prothrombinase (Fig. 4A). PSP37 was inhibitory when it was preincubated with FVa and PL prior to addition of the remaining prothrombinase components and measurement of thrombin generation ($IC_{50} = 11 \mu M$). If PSP37 was preincubated with FXa and PL, and prothrombinase was performed without FVa, there was no inhibition up to $80 \mu M$ peptide, and modest inhibition at $160 \mu M$ peptide (Fig. 4A). Thus, the inhibitory activity of PSP37 was FVa-dependent, and residues 37–50 of PS likely contain a binding site for FVa. Confirming that suggestion, FVa, but not FXa, bound to immobilized PSP37, as detected with rabbit anti-FV antibodies and chromogenic substrate for FXa, respectively (Fig. 4B). FVa did not bind to a control peptide and PSP37 did not block binding of FXa to immobilized FVa (data not shown). However, PSP37 did block Ca^{2+} -dependent binding of FVa to immobilized PS (Fig. 4C), with a half maximal effect at $15 \mu M$, similar to the IC_{50} for PSP37 inhibition of prothrombinase. Also, inhibition of prothrombinase by PSP37 was diminished when FVa concentration was increased from $50 pM$ to $1400 pM$, but was not diminished when the concentration of FXa was increased from 1 to $9 nM$ (data not shown).

PSP37 was anticoagulant in a FVa-based plasma clotting assay. Prolongation of clotting time in this assay, which has been used to measure APC cofactor activity of mouse PS, was linear with PS concentration (22). PSP37 displayed a dose-dependent linear increase in clot time in PSdP in the absence of APC, indicating an APC-independent anticoagulant effect (Table 1). Prolongation of clot time by APC in PSdP was less than in normal plasma, as expected. Prolongation of clot time in PSdP was approximately additive with APC and PSP37 in combination. Thus, PSP37 was not sufficient to serve as a cofactor for APC.

Kinetic analysis of inhibition of prothrombinase by PSP37

PSP37 essentially displayed a pattern of noncompetitive inhibition of prothrombinase (Fig. 5), similar to that displayed by PS (9). K_m was altered ~2-fold, calculated as $-1/x$ intercept on a Lineweaver-Burke plot. V_{max} decreased as peptide concentration increased, with 9.8-fold decrease and 90% inhibition at $40 \mu M$ peptide. In other terms, k_{cat}/K_m decreased 21-fold, from $2.46 \times 10^6 s^{-1} M^{-1}$ without peptide to $1.16 \times 10^5 s^{-1} M^{-1}$ with $40 \mu M$ peptide.

Discussion

Monoclonal antibodies and synthetic peptides are useful tools to interrogate protein sequences for their roles in protein-protein interactions. Here we found that in purified systems that lacked PL, mAb S4 blocked the ability of PS to inhibit prothrombinase only when FVa was present, suggesting that mAb S4 either blocked PS interaction with FVa, or blocked PS binding to a FVa binding site on FXa. The first possibility is likely, because mAb S4 inhibited binding of PS to FVa in the absence of PL or of FXa, and because a peptide that represented part of the mAb S4 epitope (PSP37) bound directly to FVa, but not to FXa, in the absence of PL. Furthermore, PSP37 did not block binding of FXa to FVa.

Ca²⁺-dependent mAb S4 interfered with PS binding to PL as well as to FVa, since mAb S4 blocked binding of PS to immobilized PL in experiments not shown, and in prothrombinase assays with saturating PL, blockage of PS inhibition of prothrombinase by PS was weakly dependent on FVa, suggesting that PS binding to PL was partially blocked. The epitope for mAb S4 differs from that of two other Ca²⁺-dependent mAbs, S1 and S5 (mAb S5 data not shown). Neither mAb S1 nor mAb S5 blocked PS inhibition of prothrombinase in the absence or presence of PL, although mAb S5 did block binding of PS to PL. There have been a number of reports of Gla domain functions that are independent of PL binding (26). MAb S4 did not appear to significantly interact with the Gla domain of other vitamin K-dependent proteins, since it shortened the FXa-1-stage clotting time in normal plasma, but had no effect in PSdP.

The epitope for mAb S4 was disrupted or dissociated by cleavage after Phe40 or Arg49 in PS, thus the epitope must be in this general region. MAb S4 bound to several synthetic 14-mer peptides in this region, especially peptides representing residues 37–50 (pre-TSR) and 54–67 (TSR), further narrowing the epitope. Only peptide PSP37, residues 37–50, mimicked the ability of PS to inhibit prothrombinase in a FVa-dependent manner. The peptide had little activity when FVa was absent, and exhibited the most potent activity when it was preincubated with FVa prior to prothrombinase assays. Thus, residues 37–50 appear to define a FVa binding site on PS.

Peptide PSP37 altered the *k*_{cat}/*K*_m of prothrombinase by up to 17-fold, and essentially exhibited noncompetitive inhibition. It is uncertain if the *K*_m for prothrombin was significantly altered by PSP37, since reports of the *K*_m in the absence of any inhibitor range from 0.1–0.3 μM (9), within the range for conditions with and without the peptide in these studies. PS itself alters the *K*_m by 2-fold and the *V*_{max} by 2.2-fold at 60% inhibition (9), while PSP37 altered the *V*_{max} by 3.2-fold at 69% inhibition. Although the kinetic parameters are similar for PS and PSP37, it must be considered that PS inhibits FXa as well as FVa (10), while PSP37 does not inhibit FXa in the absence of FVa.

In studying the region of PS represented by PSP37, we recently found that conserved residue Thr37 PS can be phosphorylated by platelet kinases in a manner that enhances the APC cofactor activity of PS (27). In other studies, mutation of Thr37 to Met was associated with low expression of rPS, 3.6-fold decreased APC cofactor activity, and diminished affinity for phospholipids (PL)(28). The T37M mutation was reported as associated with low or

borderline PS antigen in a compound heterozygous patient who experienced recurrent deep vein thrombosis (29;30). Expression of recombinant T37M PS was about 32% below normal level, while the second mutation in this patient (G11D) had a more deleterious effect on PS function when expressed in recombinant PS. Mutation at adjacent residue Glu36 ablated APC cofactor activity (31), and Arg49 is a site where thrombin cleaves and inactivates APC, possibly in part because Arg 49 participates in electrostatic interactions with APC and species specificity of PS (32–34). Different mutations at Arg49 had different phenotypes (35). A patient with the mutation D38Y experienced DVT and mild pulmonary embolism during early pregnancy (28). Expression and activity of recombinant D38Y PS was markedly low. Thus, the PS region of residues 37–38 in PSP37 has been implicated as important for PS function and expression. Many other PS mutations have been described, usually in association with venous thrombosis (32;36). In most cases, it was not determined whether the mutations affected the direct anticoagulant activity of PS, the APC cofactor activity of PS, or the ability of FV to act as a synergistic cofactor to PS during APC inactivation of FVIIIa or FVa (37;38). It is possible that the region of residues 37–50 is important for more than one of these activities, since they all involve PS interaction with FVa. The most prevalent mutation that is associated with venous thrombosis and low PS activity in the Japanese population (1 in 55–70 cases) is K155E (39). Recombinant K155E PS exhibits defective binding to FXa and to APC, but normal binding to FVa, distinguishing it from the region of the mAb S4 epitope and the PSP37 peptide.

Previous studies defined a binding site for FVa near the C-terminus of PS, residues 621–635 (19). A peptide representing these residues inhibited prothrombinase activity only in the presence of FVa, and inhibited PS binding to FVa. The 621–635 peptide appeared to compete with FXa for a binding site on FVa. This binding site on PS is seemingly distant from the residue 37–50 region identified here. However, FVa is a large molecule and two binding sites on PS are possible. There is no crystal structure of PS, so it is uncertain how far apart these two sites are in three dimensions. A site on FVa has been implicated as a binding site for PS, overlapping with a binding site for FXa (40). This may explain why FXa protects FVa from inactivation by APC, while PS overcomes that protection (41). The FXa-competing PS site could be the 621–635 site, but is likely to differ from the 37–50 site, since the PSP37 peptide did not block FXa binding to FVa.

At this time it is difficult to determine whether PS interaction with FVa at the binding site that was identified here has a role in the APC cofactor activity of PS, because this cannot be tested in the absence of PL, and mAb S4 also interferes with PL binding. However, it is suggested that PS inhibition of FVa plays a role in the direct anticoagulant activity of plasma PS, since mAb S4 shortened the FXa-1-stage clotting time in normal plasma, but not in PSdP. It also blocked the prolongation of clotting time in PSdP reconstituted with purified PS, suggesting that the Zn^{2+} -containing PS used in these studies is similar to the PS in plasma, as previously reported (42).

PS is emerging as a multifunctional anticoagulant that can serve as a cofactor to APC, and that can directly inhibit FVa, FXa, and tissue factor. Definition of key sites of molecular interaction on PS aids in understanding these various functions. This study reveals a detailed mechanism by which PS can exert its direct anticoagulant activity, and in combination with

other studies of this region, this study suggests a possible lead for a small molecule compounds that might be useful for antithrombotic therapy.

Acknowledgments

We thank Dr. Fabian Stavenuiter for technical help and assistance with figures. We also thank Dr. Richard Houghten for peptide synthesis, and Jim Roberts and Ben Gutierrez for generation of monoclonal antibodies.

Financial support: This work was supported in part by National Institutes of Health grants RO1 HL088375 (MJH) and RO1 HL21544 (JHG).

References

1. Burstyn-Cohen T, Heeb MJ, Lemke G. Lack of Protein S in mice causes embryonic lethal coagulopathy and vascular dysgenesis. *J Clin Invest.* 2009; 119:2942–53. [PubMed: 19729839]
2. Saller F, Brisset AC, Tchaikovski SN, Azevedo M, et al. Generation and phenotypic analysis of protein S-deficient mice. *Blood.* 2009; 114:2307–14. [PubMed: 19567881]
3. Mahasandana C, Suvatte V, Marlar RA, et al. Neonatal purpura fulminans associated with homozygous protein S deficiency (Letter). *Lancet.* 1990; 335:61–2. [PubMed: 1967377]
4. Schwarz HP, Fischer M, Hopmeier P, et al. Plasma protein S deficiency in familial thrombotic disease. *Blood.* 1984; 64:1297–300. [PubMed: 6238642]
5. Mahmoodi BK, Brouwer JL, Veeger NJ, et al. Hereditary deficiency of protein C or protein S confers increased risk of arterial thromboembolic events at a young age: results from a large family cohort study. *Circulation.* 2008; 118:1659–67. [PubMed: 18824642]
6. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. *N Engl J Med.* 1984; 311:1525–8. [PubMed: 6239102]
7. Walker FJ. Protein S and the regulation of activated protein C. *Semin Thromb Hemost.* 1984; 10:131–8. [PubMed: 6234659]
8. Griffin JH, Zlokovic BV, Mosnier LO. Protein C anticoagulant and cytoprotective pathways. *Int J Hematol.* 2012; 95:333–45. [PubMed: 22477541]
9. Heeb MJ, Mesters RM, Tans G, et al. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. *J Biol Chem.* 1993; 268:2872–7. [PubMed: 8428962]
10. Heeb MJ, Rosing J, Bakker HM, et al. Protein S binds to and inhibits factor Xa. *Proc Natl Acad Sci USA.* 1994; 91:2728–32. [PubMed: 8146182]
11. Fernandes NL, Mosnier LO, Tonnu L, et al. Zinc-containing protein S inhibits extrinsic factor X activating complex independently of tissue factor pathway inhibitor. *Journal of Thrombosis and Haemostasis.* 2010; 8:1976–85. [PubMed: 20492471]
12. Hackeng TM, van't Veer C, Meijers JCM, et al. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. *J Biol Chem.* 1994; 269:21051–8. [PubMed: 8063724]
13. Schwarz HP, Heeb MJ, Wencel-Drake JD, et al. Identification and characterization of protein S in human platelets. *Blood.* 1985; 66:1452–5. [PubMed: 2933098]
14. Stavenuiter F, Davis NF, Duan E, et al. Platelet protein S directly inhibits procoagulant activity on platelets and microparticles. *Thromb Haemost.* 2012; 109
15. Heeb MJ, Marzec U, Gruber A, et al. Antithrombotic activity of protein S infused without activated protein C in a baboon thrombosis model. *Thromb Haemost.* 2012; 107:690–8. [PubMed: 22370911]
16. Hackeng T, Sere KM, Tans G, et al. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. *Proc Natl Acad Sci U S A.* 2006; 103:3106–11. [PubMed: 16488980]
17. Yegneswaran S, Hackeng TM, Dawson PE, et al. The thrombin-sensitive region of protein S mediates phospholipid-dependent interaction with factor Xa. *J Biol Chem.* 2008; 283:33046–52. [PubMed: 18784085]

18. Stenberg Y, Muranyi A, Steen C, et al. EGF-like module pair 3–4 in vitamin K-dependent protein S: modulation of calcium affinity of module 4 by module 3, and interaction with factor X. *J Mol Biol.* 1999; 293:653–65. [PubMed: 10543957]
19. Heeb MJ, Kojima Y, Tans G, et al. C-terminal residues 621–635 of protein S are essential for binding to factor Va. *J Biol Chem.* 1999; 274:36187–92. [PubMed: 10593904]
20. Heeb MJ, Prashun D, Griffin JH, et al. Plasma protein S contains zinc essential for efficient activated protein C-independent anticoagulant activity and binding to factor Xa, but not for efficient binding to tissue factor pathway inhibitor. *FASEB J.* 2009; 23:2244–53. [PubMed: 19244162]
21. Tans G, Rosing J, Thomassen MC, et al. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. *Blood.* 1991; 77:2641–8. [PubMed: 2043766]
22. Fernandez JA*, Heeb MJ*, Xu X, et al. Species-specific anticoagulant and mitogenic activities of murine protein S. *Haematologica.* 2009; 94:1721–31. *equal contributions. [PubMed: 19815836]
23. Heeb MJ, Koenen RR, Fernandez JA, et al. Direct anticoagulant activity of protein S-C4b binding protein complex in Heerlen heterozygotes and normals. *J Thromb Haemost.* 2004; 2:1766–73. [PubMed: 15456488]
24. Heeb MJ, Griffin JH. Activated Protein C-dependent and -independent Anticoagulant Activities of Protein S have Different Structural Requirements. *Blood Cells Mol Dis.* 2002; 29:190–9. [PubMed: 12490286]
25. Rosing J, Tans G, Govers-Riemslog JWP, et al. The role of phospholipids and factor Va in the prothrombinase complex. *J Biol Chem.* 1980; 255:274–83. [PubMed: 7350159]
26. Saller F, Villoutreix BO, Amelot A, et al. The gamma-carboxyglutamic acid domain of anticoagulant protein S is involved in activated protein C cofactor activity, independently of phospholipid binding. *Blood.* 2005; 105:122–30. [PubMed: 15308562]
27. Stavenuiter F, Gale AJ, Heeb MJ. Phosphorylation of protein S by platelet kinases enhances its activated protein C cofactor activity. *FASEB J.* 2013; 27 in press.
28. Rezende SM, Lane DA, Mille-Baker B, et al. Protein S Gla-domain mutations causing impaired Ca(2+)-induced phospholipid binding and severe functional protein S deficiency. *Blood.* 2002; 100:2812–9. [PubMed: 12351389]
29. Ikejiri M, Tsuji A, Wada H, et al. Analysis three abnormal Protein S genes in a patient with pulmonary embolism. *Thromb Res.* 2010; 125:529–32. [PubMed: 20181378]
30. Neki R, Fujita T, Kokame K, et al. Genetic analysis of patients with deep vein thrombosis during pregnancy and postpartum. *Int J Hematol.* 2011; 94:150–5. [PubMed: 21811774]
31. Ahnstrom J, Andersson HM, Canis K, et al. Activated protein C cofactor function of protein S: a novel role for a gamma-carboxyglutamic acid residue. *Blood.* 2011; 117:6685–93. [PubMed: 21508412]
32. Garcia de FP, Fuentes-Prior P, Hurtado B, et al. Molecular basis of protein S deficiency. *Thromb Haemost.* 2007; 98:543–56. [PubMed: 17849042]
33. Villoutreix BO, Teleman O, Dahlback B. A theoretical model for the Gla-TSR-EGF-1 region of the anticoagulant cofactor protein S: from biostructural pathology to species-specific cofactor activity. *J Comput Aided Mol Des.* 1997; 11:293–304. [PubMed: 9263856]
34. He X, Shen L, Villoutreix BO, et al. Amino acid residues in thrombin-sensitive region and first epidermal growth factor domain of vitamin K-dependent protein S determining specificity of the activated protein C cofactor function. *J Biol Chem.* 1998; 273:27449–58. [PubMed: 9765274]
35. Giri TK, de Frutos PG, Yamazaki T, et al. In vitro characterisation of two naturally occurring mutations in the thrombin-sensitive region of anticoagulant protein S. *Thromb Haemost.* 1999; 82:1627–33. [PubMed: 10613646]
36. Gandrille S, Borgel D, Ireland H, et al. Protein S deficiency: a database of mutations. For the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost.* 1997; 77:1201–14. [PubMed: 9241758]
37. Shen L, Dahlbäck B. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. *J Biol Chem.* 1994; 269:18735–8. [PubMed: 8034625]

38. Cramer TJ, Griffin JH, Gale AJ. Factor V is an anticoagulant cofactor for activated protein C during inactivation of factor Va. *Pathophysiol Haemost Thromb*. 2010; 37:17–23. [PubMed: 20501981]
39. Hayashi T, Nishioka J, Suzuki K. Molecular mechanism of the dysfunction of protein S (Tokushima) (Lys155-->Glu) for the regulation of the blood coagulation system. *Biochim Biophys Acta*. 1995; 1272:159–67. [PubMed: 8541347]
40. Heeb MJ, Kojima Y, Hackeng TM, et al. Binding sites for blood coagulation factor Xa and protein S involving residues 493–506 in factor Va. *Protein Sci*. 1996; 5:1883–9. [PubMed: 8880912]
41. Solymoss S, Tucker MM, Tracy PB. Kinetics of inactivation of membrane-bound factor Va by activated protein C: Protein S modulates factor Xa protection. *J Biol Chem*. 1988; 263:14884–90. [PubMed: 2971660]
42. Heeb MJ, Radtke K-P, Fernandez JA, et al. Plasma contains protein S monomers and multimers with similar direct anticoagulant activity. *J Thromb Haemostasis*. 2006; 4:2215–22. [PubMed: 16824189]

Figure 1. Dose-dependent anticoagulant activity of PS in the absence of exogenous PL

A) Prolongation of FXa-1-stage clotting times by two different PS preparations (PS and PS2) reconstituted in PSdP. A standard curve of varying FXa in the absence of PS was used to convert the prolongation of clot time due to PS to % FXa activity. 100% FXa activity was 19 pM FXa. IC₅₀'s were 160 and 96 nM PS, respectively. The base clotting time in absence of PS was 98 sec. Maximal prolongation of clotting time by PS was 95 sec. Assays are described under Materials and methods. **B)** Neutralization of the anticoagulant activity of two different PS preparations by mAb S4 in the FXa-1-stage clotting assay. **C)** Inhibition of prothrombinase activity in purified component assays by two different PS preparations in the presence of FVa and in the absence of PL. Doses of PS for 50% inhibition (IC₅₀) were 200 and 65 nM, respectively. 100% prothrombinase activity averaged 16 pM thrombin formed/min. PS was preincubated for 15 min with FXa and FVa prior to addition of prothrombin and measurement of thrombin generated. Details are described under Materials and methods. **D)** Neutralization of the anticoagulant activity of 160 nM PS or 96 nM PS2 in purified component prothrombinase assays by mAb S4 in the absence and presence of PL. MAb S1 was used as a control Ca²⁺-dependent mAb that did not neutralize PS inhibition of prothrombinase. PS was preincubated with or without 3.2 μM mAb S4 or S1 for 30 min prior to addition of FVa and FXa without or with optimal PL for an additional 15 min prior to addition of prothrombin and measurement of thrombin generation. 100% prothrombinase activity averaged 14.8 nM thrombin formed/min in the presence of PL, and 0.016 nM thrombin formed/min in the absence of PL. All findings were confirmed in separate experiments.

Figure 2. Dose-dependent inhibition of PS binding to immobilized FVa in the absence of PL by mAb S4

FVa (15 nM) was coated to the wells of a microtiter plate and the wells were blocked. PS (67 nM) was preincubated without and with various antibodies prior to incubation in the wells. After washing, bound PS was detected with goat polyclonal anti-PS antibodies. Noncoated, blocked wells served as controls whose values were subtracted from values for the coated wells. MAb S3 is a non-competing Ca^{2+} -dependent mAb against PS; MIgG is nonimmune mouse IgG. Results of two different experiments were combined.

Figure 3. Defining the epitope for mAb S4

A) Recognition of chymotrypsin-cleaved PS by various antibodies on nonreduced immunoblots. SC6 is a non- Ca^{2+} -dependent monoclonal antibody that does not neutralize the anticoagulant activity of PS. PS was used at 50 ng/lane on 7% SDS-PAGE; antibodies were used at 25 nM. Findings were confirmed in other immunoblots, and PS cleaved in the TSR by thrombin had a similar recognition pattern by the mAbs. **B)** Binding of mAb S4 to immobilized peptides. Each 14-mer peptide representing sequences in PS is designated by the first amino acid residue in the sequence and was coated at 20 μ M to the wells of microtiter plates. Gla-TSR designates a synthetic oligopeptide comprising residues 1–72 of PS, with correct disulfide bonds introduced. After blocking, mAb S4 (26 nM) was incubated in the wells and bound mAb S4 was detected with anti-mouse IgG. Values for noncoated, blocked wells were subtracted from values for coated wells. Data from two experiments performed on different days were combined.

Figure 4. Inhibition of FVa prothrombinase activity by PSP37, representing PS residues 37–50
A) Peptides at the indicated concentrations were preincubated 10 min with FVa/FXa/PL, except where PSP37 was preincubated 10 min with FXa/PL only (no FVa, squares). Prothrombin was then added and thrombin generation was measured. Data for PSP37 (open circles) and for a D-amino acid sequence of PSP37 (closed circles) with FVa preincubation were accumulated over several days. 100% prothrombinase activity was 7.2 nM thrombin formed/min when FVa was included, and 0.38 nM thrombin formed/min when FVa was omitted. **B)** Binding of FVa, but not FXa, to immobilized PSP37. PSP37 was coated to microtiter plates at 30 μM and wells were blocked. FVa or FXa was bound at 23°C for 40 min in HBS-0.5% BSA containing 5 mM CaCl₂. After washing, bound FVa was detected as described under Materials and methods. Bound FXa was detected with chromogenic FXa substrate. The K_d app for FVa binding was 14 nM. **C)** Blockage of FVa binding to immobilized PS by peptide PSP37. FVa (2.5 μg/ml) was preincubated with and without varying concentrations of PSP37 for 30 min, then a measured volume of the mixtures was transferred to a plate coated with immobilized PS (4 μg/ml) for 30 min. Bound FVa was detected (see Methods). Nonspecific binding of FVa to uncoated wells was subtracted from specific binding. All results were confirmed in other experiments.

Figure 5. Inhibition of prothrombinase by PSP37

Lineweaver-Burke plot of prothrombinase activity in presence of FVa and in the absence and presence of several concentrations of peptide PSP37. Assay conditions are described under Materials and methods.

Table 1

Effect of peptide PSP37 and APC in a FVa-based clotting assay

Plasma	PSP37	APC	Prolongation
PSdP	1 μ M	-	21 (sec)
PSdP	2 μ M	-	47
PSdP	4 μ M	-	68
PSdP	5 μ M	-	96 $\pm$ 1
PSdP	-	3 nM	76 $\pm$ 3
PSdP	5 μ M	3 nM	190 $\pm$ 23
Normal	-	3 nM	257 $\pm$ 7

PSdP: PS-depleted plasma. Base clot time: 269 $\pm$ 4 sec. See Methods.

Table 2

What is known about this topic?

- Protein S binds to both FVa ($K_{d,app} \sim 33$ nM) and FXa ($K_{d,app} \sim 18$ nM).
- Protein S inhibits prothrombinase activity in the absence of activated protein C.
- Protein S prolongs clotting times in normal and protein S-deficient plasma.

What does this article add?

- Protein S inhibits prothrombinase even in the absence of phospholipids.
 - A monoclonal antibody that neutralizes protein S inhibition of prothrombinase in the absence or presence of phospholipids has an epitope between protein S residues 37–67.
 - A peptide representing protein S residues 37–50 inhibits FVa-dependent prothrombinase activity and binds to FVa.
 - Peptide 37–50 appears to represent a FVa binding site on protein S that contributes to its anticoagulant activity.
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