Implication of protein S thrombin-sensitive region with membrane binding via conformational changes in the γ -carboxyglutamic acid-rich domain

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In the vitamin K-dependent protein family, only protein S (PS) contains a thrombin-sensitive region (TSR), located between the domain containing the γ -carboxyglutamic acid and the first epidermal growth factor-like domain. To better define the role of TSR in the PS molecule, we expressed a recombinant human PS (rHPS) and its analogue lacking TSR (rTSR-less), and prepared factor Xa- and thrombin-cleaved rHPS. A peptide reproducing TSR (TSR-peptide) was also synthesized in an attempt to obtain direct evidence of the domain involvement in PS anticoagulant activity. In a coagulation assay, both rTSR-less and factor Xa-cleaved PS were devoid of activated protein C cofactor activity. The TSR-peptide did not inhibit rHPS activity, showing that TSR must be embedded in the native protein to promote interaction with activated protein C. The binding of rHPS to

activated platelets and to phospholipid vesicles was not modified after factor Xa- or thrombin-mediated TSR cleavage, whereas the binding of rTSR-less was markedly reduced. This suggested a role for TSR in conferring to PS a strong affinity for phospholipid membranes. TSR-peptide did not directly bind to activated platelets or compete with rHPS for phospholipid binding. The results of the present study show that TSR may not interact directly with membranes, but probably constrains the γ -carboxyglutamic acid-rich domain in a conformation allowing optimal interaction with phospholipids.

Key words: coagulation, coagulation inhibitor, Gla domain, phospholipid, structure/function relationship.

INTRODUCTION

The importance of protein S (PS) as a natural anticoagulant is shown by the occurrence of severe thrombotic complications in infants with a homozygous hereditary deficiency and by the mild thrombotic tendency in heterozygous subjects [1]. PS acts as a non-enzymic cofactor of activated protein C (APC), a serine-protease inactivating activated factor V (FVa) and activated factor VIII (FVIIIa; reviewed in [2]). APC inactivates FVa in a biphasic reaction, with rapid cleavage at Arg-506 followed by slower cleavage at Arg-306 [3]. In a purified system, PS has a moderate effect on APC anticoagulant activity, with only a 2-fold enhancement of FVa inactivation [4]. However, PS stimulates the second phase of FVa inactivation by a 20-fold enhancement of cleavage at Arg-306 [5]. Besides APC cofactor activity, PS has APC-independent anticoagulant activity. By binding to FVa or to activated factor X (FXa) [6], PS may directly inhibit prothrombin activation. Moreover, direct interaction of PS with FVIII/FVIIIa in the presence of phospholipids could preclude FX activation [7].

PS is a single-chain 635-amino acid glycoprotein with an N-terminal part homologous to that of other vitamin K-dependent proteins [8], and a large C-terminal domain homologous to the sex-hormone-binding protein (SHBG) [9]. This SHBG-like domain mediates the binding of PS to a complement-regulator component, C4b-binding protein (C4BP), which results in inhibition of APC cofactor activity. In plasma, 60% of PS is complexed with C4BP with only the remaining 40% free to act as a cofactor [10]. The N-terminal part of PS has a domain rich

in γ -carboxyglutamic acid (Gla domain; amino acids 1–46) and four epidermal growth factor (EGF)-like domains [8]. Compared with other vitamin K-dependent coagulation proteins, the Gla domain and the first EGF domain (EGF1) of PS are separated by an additional segment of 29 residues, containing a disulphide bond initially named the thrombin-sensitive region (TSR).

Thrombin inactivates PS by cleaving the TSR at two sites (Arg-49 and Arg-70) [11], this enzymic reaction occurring only in the absence of Ca^{2+} ions. Thrombin-cleaved PS is no longer able to promote APC anticoagulant activity [12,13], suggesting that the TSR of PS interacts directly or indirectly with APC. FXa was shown to cleave the TSR after Arg-60 in the presence of Ca^{2+} ions and phospholipids [14], and the resulting PS form could not enhance the inactivation of FVIIIa by APC. Under physiological conditions, 15% of circulating PS is cleaved within the TSR domain after Arg-60 [15]. Human PS stimulates human but not bovine APC, and several reports have shown that both the TSR and EGF1 domains account for this species specificity [16–18]. The involvement of the EGF1 domain of PS with the APC cofactor activity is supported by the absence of APC cofactor activity of a natural mutant lacking the EGF1 domain in a patient with thrombosis [19], and by the observation that the chemically synthesized EGF1 domain inhibits APC cofactor activity [20]. Direct evidence of TSR involvement has never been obtained, however.

Besides an uncleaved TSR and the first EGF domain, the interaction of PS with APC requires the binding of both proteins to membranes exposing negatively charged phospholipids. The anchorage of PS to activated platelets [21], endothelial cells [4] or

Abbreviations used: PS, protein S; TSR, thrombin-sensitive region; Gla domain, γ -carboxyglutamic acid-rich domain; EGF, epidermal growth factor; SHBG, sex-hormone-binding globulin; PC, protein C; APC, activated PC; rHPS, recombinant human PS; rTSR-less, rHPS lacking the TSR; TSR-peptide, disulphide-bond peptide reproducing TSR; FVa, activated factor V; FVIIIa, activated factor VIII; FXa, activated factor X; mAb, monoclonal antibody; PE-GAM, phycoerythrin-conjugated goat F(ab')₂ anti-mouse IgG; PE, phycoerythrin; C4BP, C4b-binding protein; RVV, Russell's viper venom; Fmoc, N-(9-fluorenyl)methoxycarbonyl; TFA, trifluoroacetic acid.

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platelet microparticles [22] is a prerequisite for PS biological activity [23]. As with all vitamin K-dependent proteins, Ca^{2+} binding to the Gla domain is critical for PS binding to phospholipids [24]. PS cleavage by thrombin has been reported to reduce Gla domain Ca^{2+} affinity [12,25,26], raising the possibility that cleaving the TSR loop impairs PS binding to membranes. However, it seems that even after cleavage, PS remains able to interact with phospholipids [14,22,24,27].

To clarify the role of TSR in PS binding to membranes, we expressed a recombinant human PS from which the TSR region had been deleted (rTSR-less), and compared its ability to interact with activated platelets or phospholipid vesicles with that of a recombinant human PS (rHPS), and with that of FXa- and thrombin-cleaved PS. We also synthesized a disulphide-bond peptide reproducing TSR (TSR-peptide) to test the direct effect of this domain.

EXPERIMENTAL

Materials

An antibody directed against the C4BP α chain was provided kindly by Dr B. Dahlbäck (Lund University, Malmö, Sweden). Monoclonal antibodies (mAbs) recognizing PS (mAb 9F9G9, mAb 23A10B6 and mAb 20F10B6) and protein C (PC; mAb 2E2A8D2 and mAb 10D1A3D11) were provided kindly by bioMérieux Laboratories (Marcy l'Etoile, France) and α -thrombin was prepared as described in [28]. Restriction endonucleases *Pml*I and *Cla*I were from New England Biolabs (Beverly, MA, U.S.A.). *Pfu* DNA polymerase was from Promega (Marcy l'Etoile, France). Hygromycin B was from Gibco BRL (Gaithersburg, MD, U.S.A.). Vitamin K1 was from Roche (Neuilly-sur-Seine, France). The HEK-293 cell line was from the European Collection of Animal Cell Cultures (E.C.A.C.C., no. 85120602). *Escherichia coli* strains JM110 and DH5 α F' were from Stratagene (Ozyme, Montigny-le-Bretonneux, France). PS-deficient plasma and Russell's viper venom (RVV) were from Diagnostica Stago (Asnières, France). Chromogenic substrate S2765 was from Chromogenix (Mölnådal, Sweden) and hirudin was provided kindly by Dr G. F. Pay (Ciba Geigy, Basel, Switzerland). Phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, QAE-Sephadex and A23187 were from Sigma (St Louis, MO, U.S.A.). Polyclonal peroxidase-conjugated antibodies against PS or PC were from Dako (Trappes, France), metrizamide was from the Aldrich Chemical Company (Milwaukee, WI, U.S.A.), PVDF membranes were from Millipore (Bedford, MA, U.S.A.) and CNBr was from Pharmacia (Orsay, France). Phycoerythrin-conjugated goat F(ab')₂ anti-mouse IgG (PE-GAM) was from Coultronics France SA (Margency, France), and streptavidin-phycoerythrin (PE) was from Amersham Pharmacia Biotech (Little Chalfont, Bucks, U.K.). All other reagents were of the highest grade available.

Site-directed mutagenesis

Full-length human PS cDNA cloned into the pS-hd2 plasmid, which was a generous gift from Dr B. W. Grinnell (Lilly Research Laboratories, Eli Lilly Company, Indianapolis, IN, U.S.A.), was used as the template to delete the TSR (amino acids 47–75). Two PCRs were performed using the primer combinations A + B and C + D. Primer A, 5'-GGTAGCGGTCGTTGTCCACTAG-3', is located within pS-hd2, upstream of the PS cDNA, whereas primer D, 5'-GACACCATATTCATAGACCATT-3', corresponds to a sequence encoding part of the SHBG domain (amino acids 339–346). In primer B, 5'-CTGGTCTGGAATAACTA-AGTATTTTGGATAAAAAATAAT-3', and primer C, 5'-AT-

ACTTAGTTATTCCAGACCAGTGTAGTCCTCTGC-3', sequences in bold are complementary to the part of the cDNA that encodes the first EGF domain and sequences in italics are complementary to the section of cDNA that encodes the Gla domain. The sequence underlined in primer B is complementary to the sequence underlined in primer C. The two PCR products were then used as templates, together with primers A and D, to create the fragment lacking TSR. PCR products generated by mutagenesis were digested with *Pml*I and *Cla*I and ligated into the expression vector pS-hd2 from which the corresponding fragment was removed. *E. coli* strains JM110 and DH5 α F' were used for routine transformation and plasmid preparation.

Cell lines, transfection and selection

HEK-293 cells were transfected using calcium phosphate DNA precipitates as described previously [29]. After transfection (2 days), drug selection was applied by adding 0.1 mg/ml hygromycin. High-level rHPS or rTSR-less producer clones were isolated and mass production was achieved by maintaining cells at confluence in medium containing 5 μ g/ml vitamin K1 in roller bottles.

Purification of rHPS and rTSR-less

Both recombinant proteins were immunopurified from 2 l of conditioned medium as described in [29]. The protein concentration was determined by monitoring the absorbance at 280 nm using an $\epsilon_{1\text{cm}}^{1\%}$ value of 9.5 and a molecular mass of 78 000 Da [30]. γ -Carboxyglutamic acid analysis was performed by Dr C. Vermeer (Department of Biochemistry, University of Limburg, Maastricht, The Netherlands) according to the method of Kuwada and Katayama [31].

Purification and activation of plasma PC

Vitamin K-dependent proteins were concentrated by BaCl precipitation. PC was then immunopurified using Sepharose-immobilized anti-PC mAb 2E2A8D2 coupled to CNBr-Sepharose 4B. This purification procedure was followed by anion-exchange chromatography (Mono Q) using a CaCl_2 gradient to elute PC. PC was activated by contact with insolubilized Protac in 50 mM Tris/HCl/50 mM NaCl/5 mM EDTA pH 7.5, for 8 h. Finally, APC was concentrated by anion-exchange chromatography on a Mono Q column, and active-site titration was performed using H-D-Phe-Pro-Arg-chloromethylketone (PPACK) [32].

Digestion of human PS by α -thrombin and FXa

rHPS and rTSR-less (700 nM) were incubated at 37 °C for 2 h with α -thrombin (15 nM) in Tris-buffered saline (50 mM Tris/150 mM NaCl; TBS)/5 mM EDTA/0.2 % poly(ethylene glycol), pH 7.5, and the reaction was stopped by adding hirudin (20 nM).

FXa-cleaved PS was prepared as described previously by Long et al. [14], by incubation of rHPS (700 nM) and FXa (30 nM) in the presence of phospholipid vesicles in TBS/2 mM CaCl_2 /0.2 % poly(ethylene glycol), pH 7.5, for 3 h at 37 °C. To remove FXa and phospholipids from FXa-cleaved rHPS, further purification was performed by affinity chromatography (mAb 23A10B6) and anion-exchange chromatography on a Mono Q column. We checked that no residual FXa activity was detected using an FXa-specific chromogenic substrate, S2765. The terminal amino acid sequences of the cleavage products were determined by Edman degradation (Institut Pasteur, Paris, France) after SDS/PAGE and subsequent electroblotting on to PDVF membranes.

Peptide synthesis

The peptide of the TSR (residues 45–74) was synthesized on an Applied Biosystem ABI 431A automated peptide synthesizer with ABI small-scale Fmoc [N-(9-fluorenyl)methoxycarbonyl] chemistry on an HMP (*p*-hydroxymethylphenoxy) resin, using the DCC/HOBt (1,3-dicyclohexylcarbodiimide/1-hydroxybenzotriazole) coupling method and 20% piperidine in NMP (*N*-methylpyrrolidone) for Fmoc deprotection. Fastidious deprotection of Fmoc groups and therefore incomplete coupling happened from residue Thr-53 while using ordinary stepwise synthesis. These diminished deprotection signals are indicative of 'difficult sequences', caused by inter- and intra-chain hydrogen bonding in the peptide backbone, forming β -sheets or other secondary structures [33]. An efficient solution is provided by peptide backbone amide protection, and the Hmb (2-hydroxy-4-methoxybenzyl) group has proven suitable for such protection [34]. Thus the linear peptide was then synthesized by introducing Gly-54 in the form of Fmoc-(Fmoc-Hmb)Gly-OH. After deprotection of the Fmoc groups, the amino acid Fmoc-*O*-*t*-butyl-threonine was coupled in the active form of anhydride. When all the residues had been coupled and the last Fmoc group removed, the peptidyl resin was dried and cleaved with a mixture of trifluoroacetic acid (TFA)/water/tri-isopropylsilane/1,2-ethanedithiol (39:1:1:1, by vol.) and purified by semi-preparative HPLC on a Vydac C₈ column (5 μ m, 10 mm $\times$ 250 mm) with a linear gradient of solvent B from 20% to 60% (where solvent A is water containing 0.1% TFA and solvent B is 70% aqueous acetonitrile solution containing 0.09% TFA) at a flow rate of 2 ml/min. After lyophilization, the identity of the peptide was checked by electrospray MS (*m/z*, 3331.3). As this peptide had low solubility in aqueous buffered solution, Cys-47–Cys-72 disulphide-bond formation was performed in a solution of TFA containing 10% DMSO and 1% anisole [35]. After evaporation, the cyclized peptide (TSR-peptide) was precipitated with cold ether and purified by HPLC. Finally, the peptide identity was checked by electrospray MS (*MS* = 3330.4).

Clotting assays

Clotting assays were performed at 37 °C in 96-well plates as described by Smirnov et al. [36], with some modifications. Clotting was initiated at the level of FXa activation, using RVV. All reagents were diluted in TBS, pH 7.5, containing 1 mg/ml gelatin. PS-deficient plasma (20 μ l) was incubated for 2 min at 37 °C with 10 μ l of phospholipid vesicles containing phosphatidylethanolamine (final concentration, 10 μ g/ml), 10 μ l of 0.1 μ g/ml RVV, 10 μ l of APC (final concentration, 30 nM) and 10 μ l of PS at the concentrations indicated in Figure 4 (see below). Clotting was initiated by addition of 25 μ l of 20 mM CaCl₂. The clotting time was determined as the time to reach a 10% increase in the initial absorbance at 405 nm. For each point we expressed the result with the following ratio: clotting time with APC and PS/clotting time with APC only.

mAb-binding assays

Apparent K_d values for mAb–PS interaction were determined as described previously [29]. Briefly, increasing concentrations of PS were incubated in plates coated with various purified mAbs, and bound PS was detected with an anti-PS polyclonal peroxidase-conjugated antibody. The apparent dissociation constants (K_d) of PS for mAbs were estimated by non-linear regression analysis of the A_{490} value as a function of the PS concentration, using an equation derived from the Hill–Langmuir equation [37].

Phospholipid-vesicle-binding assay

Extruded phospholipid vesicles containing either 20% phosphatidylserine and 80% phosphatidylcholine, or 20% phosphatidylserine, 20% phosphatidylethanolamine and 60% phosphatidylcholine were prepared as described in [38]. Briefly, dried phospholipid mixtures were resuspended in 50 mM Tris/HCl/100 mM NaCl, pH 7.4 (1 mg/ml), vortexed for 10 min and passed 10 times through a 100 nm Nucleopore polycarbonate filter. These vesicles were used immediately or stored under argon at –20 °C. Phospholipid binding assays were performed as described previously [29]. Increasing concentrations of PS or PC were incubated in plates coated with phospholipid vesicles, and bound protein was detected with an anti-PS or an anti-PC polyclonal peroxidase-conjugated antibody.

C4BP-binding assay

The method described by Dahlbäck [39] allowed us to purify the isoform of C4BP containing the β chain. The apparent K_d for PS–C4BP interaction was determined as described previously [29]. Briefly, microtitre plates were coated with an antibody recognizing the α chain of C4BP. C4BP was allowed to bind to the antibody, which coated the plates. Increasing concentrations of PS were then incubated for 2 h and C4BP-bound PS was detected with a polyclonal peroxidase-conjugated antibody.

PAGE and immunoblot analysis

rHPS, rTSR-less and cleaved rHPS were subjected to SDS/PAGE (10% gel). For Western-blot analysis, SDS/PAGE was performed on a 10–18% gradient polyacrylamide gel under reducing conditions. After electrophoretic transfer of proteins from the gel to a nitrocellulose membrane, the membrane was blocked for 1 h in 5% (w/v) dried skimmed milk in TBS, pH 7.4, and incubated with mAb 9F9G9, followed by peroxidase-conjugated rabbit anti-goat IgG. An enhanced chemiluminescence system was used for signal detection.

Evaluation of PS and PC binding to activated platelets by flow cytometry

Blood was anticoagulated with ACD-C (130 mM citric acid/120 mM trisodium citrate/100 mM glucose) in a ratio of 1:9 (v/v). Platelets were isolated as described in [40], and finally resuspended in a buffer consisting of 10 mM Hepes, 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl₂, 5 mM NaHCO₃, 10 mM glucose and 2 mM CaCl₂, pH 7.4, and adjusted to 3×10^8 cells/ml. Platelets were then incubated with the ionophore A23187 (1 μ M) or DMSO (the final DMSO concentration did not exceed 0.5%) for 2 min at 37 °C without stirring. Then 5 μ l aliquots were diluted in 25 μ l of reaction buffer containing 2 mM CaCl₂ in the presence or absence of PS or PC. After 10 min, the binding of PS or PC to platelets was detected by adding an anti-PS mAb (20F10B6) or an anti-PC mAb (10D1A3D11) for a further 10 min. As a negative control, platelets were incubated with the same concentration of irrelevant mAb. The samples were then incubated with PE-GAMs for another 10 min in the dark. After preliminary experiments, the concentrations of mAb 20F10B6 and mAb 10D1A3D11 were adjusted to 1 μ g/ml and PE-GAM diluted 1:100, which gave the optimal specific fluorescence intensity. For TSR-peptide experiments, increasing biotinylated labelled peptide concentrations (up to 100 μ M) were incubated for 10 min with platelets. The binding of the peptide to platelets was detected by adding PE-conjugated streptavidin for another 10 min in the dark. We diluted the labelled samples 10-fold in

reaction buffer, and immediately analysed them on a Becton Dickinson FACSsort flow cytometer (San Jose, CA, U.S.A.). The light scatter and fluorescence channels were set at logarithmic gain. In each sample 10000 particles were acquired. In each experiment a sample was incubated with a PE-labelled platelet-specific mAb (PE-anti-GPIIb from Caltag) to confirm that more than 95% of analysed particles were platelets or platelet-derived microparticles.

RESULTS

Characterization of rHPS, rTSR-less, FXa-cleaved PS and thrombin-cleaved PS

HEK-293 cells were transfected with expression plasmids containing cDNAs coding for either rHPS or rTSR-less. PS antigen levels detected in the culture medium of clones transfected with each construct ranged from 1 to 5 mg/l. Recombinant proteins were purified as described in the Experimental section. During the last step, pseudo-affinity chromatography on a Mono Q column, rHPS and rTSR-less were eluted with a Ca^{2+} ion concentration similar to that required to elute plasma PS. rTSR-less γ -carboxylated glutamic acid residue content was evaluated as 10 ± 1 mol of γ -carboxylglutamic acid/mol of protein, a value similar to that of plasma PS [41]. rHPS and rTSR-less were analysed by SDS/PAGE and migrated as a single band in the absence of a reducing agent. Under reducing conditions (Figure 1), rHPS migrated as a single band with an apparent molecular mass of 80 kDa. Incubation of rHPS with thrombin or FXa resulted in complete conversion into a 74 kDa form. rTSR-less migrated as a single band at 78 kDa and, as expected, there was no detectable difference between rTSR-less and thrombin-treated rTSR-less. rHPS had only one detectable N-terminal sequence of Ala-Asn-Ser-Leu-Leu, identical with the N-terminus of uncleaved plasma PS reported by DiScipio et al. [30], confirming that the batch of recombinant PS used in the present study was almost devoid of cleaved PS. After reduction, sequence analysis of FXa-cleaved rHPS yielded one N-terminal sequence (Gln-Ser-Thr-Asn), attesting that cleavage occurs after Arg-60, whereas sequence analysis of thrombin-cleaved rHPS yielded two N-terminal sequences (Ser-Phe-Gln-Thr and Ser-Cys-Val-Asn),

Figure 1 Analysis of PS by SDS/PAGE under reducing conditions

SDS/PAGE (10% gel) under reducing conditions: lane 1, rHPS; lane 2, rHPS after incubation with thrombin; lane 3, rHPS after incubation with FXa; lane 4, rTSR-less; lane 5, rTSR-less after incubation with thrombin.

Figure 2 Binding of PS to mAb 9F9G9

mAb 9F9G9 was coated as described in the Experimental section. rHPS (■), FXa-cleaved rHPS (▽) and rTSR-less (▲) were added to wells in the presence of 2 mM Ca^{2+} and bound protein was detected with a polyclonal peroxidase-conjugated antibody. The curves are representative of three different experiments, each performed in duplicate (means $\pm$ S.D.). The S.D. values are not visible because of their small size. The solid curve was obtained by non-linear regression analysis [37], yielding an apparent K_d value of 0.88 nM for rTSR-less. Apparent K_d values for rHPS (119 nM) and FXa-cleaved rHPS (164 nM) were obtained using concentrations up to 450 nM (results not shown).

pointing to cleavage after both Arg-49 and Arg-70. Besides its ability to be cleaved by FXa and thrombin, PS is characterized by its strong affinity for C4BP. We used a solid-phase assay to compare the affinity of rTSR-less with that of rHPS for insolubilized C4BP. The binding curves were similar and yielded apparent K_d values of 8.5 ± 1.7 nM and 14.5 ± 4.1 nM for rHPS and rTSR-less, respectively.

Role of TSR in PS conformation mapped with mAbs

Nineteen different murine mAbs, produced by immunization towards human plasma PS, were used in ELISA to compare the epitope exposed in rHPS with those exposed in rTSR-less. All mAbs recognized both proteins, suggesting correct overall folding of PS in the absence of TSR. However, one of them, mAb 9F9G9, bound rTSR-less and rHPS with different affinities. As shown in Figure 2, in the presence of Ca^{2+} , rTSR-less bound to the mAb with a more than 100-fold increased affinity compared with rHPS, with respective mean apparent K_d values of 0.88 ± 0.05 nM and 119 ± 9.6 nM. FXa-cleaved rHPS bound to the mAb with an apparent K_d of 164 ± 9 nM in the presence of Ca^{2+} , suggesting that cleavage of the Arg-60-Gln-61 peptide bond did not modify the epitope conformation. To identify the PS domain encompassing the epitope, we performed immunoblot experiments. rHPS, FXa-cleaved rHPS and rTSR-less were submitted to SDS/PAGE under reducing conditions, then transferred to nitrocellulose to detect immunoreactivity with mAb 9F9G9. As shown in Figure 3, rHPS was recognized by the mAb, indicating that the epitope was continuous although partly masked in the folded protein. FXa-cleaved PS yielded a 74 kDa heavy chain (comprising the C-moiety of the TSR, four EGFs and the SHBG domain) that was not recognized by the mAb, whereas the light chain was recognized, indicating a location within the fragment encompassing residues 1–60. rTSR-less was fully recognized, which allowed us to exclude amino acids 47–75 from the epitope, which is therefore located within the Gla domain (amino acids 1–46).

Figure 3 Immunoblot for PS using mAb 9F9G9

rHPS (lane 1), FXa-cleaved rHPS (lane 2) and rTSR-less (lane 3) were subjected to SDS/PAGE (10–18% gel) under reducing conditions, then transferred on to a nitrocellulose membrane and incubated with mAb 9F9G9.

Figure 4 Comparison of the APC cofactor activity of rHPS, FXa-cleaved rHPS and rTSR-less in a plasma clotting test

Increasing concentrations of rHPS (■), FXa-cleaved rHPS (▽) or rTSR-less (▲) were incubated with APC (30 nM) in the presence of phospholipid vesicles, RVV and PS-deficient plasma. APC cofactor activity of the different PS proteins tested was evaluated by determination of the following ratio: clotting time with APC and PS/clotting time with APC only. Means $\pm$ S.D. from three separate experiments are shown. For some points the S.D. values are not visible because of their small size.

PS APC cofactor activity

We compared the effect of rTSR-less and FXa-cleaved rHPS with that of rHPS as APC cofactors, in a clotting assay. In this assay, clotting times in the presence of 0–48 nM APC ranged between 35 and 250 s. As depicted in Figure 4, rHPS dose-dependently prolonged the clotting time in the presence of 30 nM

Figure 5 Evaluation of PS binding to ionophore A23187-activated platelets by means of flow cytometry

Ionophore-activated platelets were incubated with concentrations ranging from 25 to 250 nM rHPS (■), rTSR-less (▲), thrombin-cleaved rHPS (○), FXa-cleaved rHPS (▽) or PC (□). Because of technical limitations, concentrations higher than 250 nM could not be tested. Protein binding was detected by successive addition of anti-PS mAb or anti-PC mAb and PE-GAMs. The results are means $\pm$ S.D. from at least three separate experiments. For some points the S.D. values are not visible because of their small size.

APC, whereas in the absence of APC, similar concentrations of rHPS had no effect (results not shown). In contrast, rTSR-less and FXa-cleaved rHPS had no APC cofactor activity. To determine whether TSR is directly involved in PS APC cofactor activity, we added to rHPS a synthetic disulphide-bond peptide corresponding to the TSR sequence (TSR-peptide), and measured APC cofactor activity. The TSR-peptide had no competitive effect on rHPS activity, as attested by the unvarying clotting times, even at molar concentrations of the peptide, of 1000 times that of rHPS. Similarly, rTSR-less did not recover APC cofactor activity in the presence a 1000-fold molar excess of TSR-peptide (results not shown).

PS binding to activated platelets and phospholipid vesicles

The lack of TSR or its cleavage may impair the binding of PS to activated platelets, a prerequisite for enhancing APC anticoagulant activity. To test this hypothesis, we compared the binding of the different forms of PS to activated platelets by means of flow cytometry. To evaluate bound PS, we used a mAb (20F10B6) directed against the SHBG domain [29], a region of PS known not to be involved in the binding to phospholipid surfaces [42]. The mAb did not bind to resting platelets, indicating that PS was not normally detectable on the platelet surface. Incubation of platelets with rHPS after activation with 1 μ M ionophore A23187 allowed PS to be readily detected on the platelet surface by flow cytometry. The fluorescence intensity increased with rHPS, FXa-cleaved rHPS or thrombin-cleaved rHPS concentrations ranging from 25 to 250 nM, whereas rTSR-less did not bind to activated platelets (Figure 5). In the absence of added rHPS, there was a minor increase in platelet fluorescence, indicating that platelet-derived PS does not make a major contribution to the observed binding (results not shown). The TSR-peptide was not able to bind to activated platelets. Indeed, incubation of different concentrations of biotinylated TSR-peptide, revealed by streptavidin-PE, gave identical mean fluorescence (mean fluorescence of negative control was 21.2, and mean fluorescence values of TSR-peptide at 1, 10 and 100 μ M were 21.3, 23.3 and 22.9, respectively). Moreover, neither the addition of the TSR-

Figure 6 Binding of PS and PC to immobilized phospholipid vesicles

Phospholipid vesicles containing 20% phosphatidylserine and 80% phosphatidylcholine were coated as described in the Experimental section. rHPS (■), thrombin-cleaved rHPS (○), FXa-cleaved rHPS (▽), rTSR-less (▲) and PC (□) were added to wells in the presence of 2 mM Ca^{2+} , and bound protein was detected with an anti-PS or anti-PC polyclonal peroxidase-conjugated antibody. The curves are representative of three different experiments, each performed in duplicate (means $\pm$ S.D.). The S.D. values are not visible because of their small size. Solid curves were obtained by non-linear regression analysis [37], yielding apparent K_d values of 6.1 nM for rHPS, 9.8 nM for FXa-cleaved rHPS and 21.1 nM for thrombin-cleaved rHPS. Apparent K_d values for rTSR-less (148 nM) and PC (158 nM) were determined using concentrations up to 950 nM (results not shown). Abs, absorbance.

peptide at a ratio of 10000:1 simultaneously with rHPS, nor a 30 min preincubation of the peptide before rHPS addition, altered the binding of rHPS. In addition, the binding of rTSR-less to activated platelets was not modified after adding the peptide (results not shown).

To compare PS with another protein that had the Gla domain tethered to the first EGF, we tested PC binding to activated platelets. Bound PC was evaluated using an mAb (10D1A3D11) directed against PC heavy chain, assuming that the mAb did not impair PC binding to phospholipids. PC binding was similar to that of rTSR-less, and thus minimal compared with rHPS or cleaved rHPS (Figure 5).

The defective binding of rTSR-less to activated platelets may reflect a reduced affinity for anionic phospholipids. To confirm this hypothesis, we prepared phosphatidylserine/phosphatidylcholine vesicles and evaluated PS binding in a solid-phase assay. The binding curves of rHPS, FXa-cleaved rHPS and thrombin-cleaved rHPS were not significantly different, with respective apparent K_d values of 6.1 ± 1.1 , 9.8 ± 1.3 and 21.1 ± 1.7 nM, whereas the apparent K_d value of rTSR-less binding to phospholipid vesicles reached 148 ± 15 nM (Figure 6). It should be noted that the latter value was similar to that observed with PC (apparent K_d , 158 ± 17 nM), confirming results obtained with activated platelets. To study the competition with rHPS, TSR-peptide was incubated for 2 h with 14 nM rHPS at a 10–1000-fold molar excess (results not shown): no inhibition of rHPS binding to phospholipids was observed.

DISCUSSION

The APC cofactor activities of rTSR-less and FXa-cleaved rHPS were evaluated using a coagulation assay described by Smirnov et al. [36]. The results of this assay showed clearly that neither FXa-cleaved rHPS nor rTSR-less was able to mimic the enhancement of APC anticoagulant activity observed with rHPS. This confirms previous observations that thrombin-cleaved PS

was unable to act as a cofactor of APC [12,13], and that FXa-cleaved PS did not enhance FVIIIa inactivation by APC [14]. However, the synthetic peptide reproducing TSR (amino acids 45–74), which was similar to a described previously peptide (amino acids 47–75) believed to adopt a native-like structure [20], did not inhibit rHPS APC cofactor activity. Studies based on the same approach suggested the involvement of the EGF1 domain in the APC–PS interaction. Indeed, a natural mutant lacking the EGF1 domain was identified in a patient with thrombosis and found to be devoid of APC cofactor activity [19]. Moreover, a peptide reproducing the PS EGF1 domain competitively inhibited PS APC cofactor activity [20] and induced a conformational change in APC [43]. Similarly, rTSR-less was unable to enhance APC cofactor activity. In contrast, TSR peptide did not inhibit rHPS interaction with APC, which could indicate that this domain does not play a crucial role. However, several observations have provided evidence for the involvement of TSR in the PS interaction with APC [16–18,44]. Therefore, the lack of competition of the synthetic domain with rHPS may indicate that a co-operation of TSR with other domain(s) in the entire protein is a prerequisite for PS interaction with APC. This is in keeping with previous mutagenesis experiments showing that the species restriction of APC cofactor activity depends on a combination of amino acid replacements in both TSR and EGF1, modification in TSR alone being insufficient to explain species specificity [18].

Since APC cofactor activity of PS requires the presence of phospholipids, it was important to compare the ability of the different forms of PS in terms of binding to phospholipid surfaces. We thus studied the binding of rHPS, rTSR-less and rHPS cleaved by thrombin or FXa on both activated platelets and synthetic phospholipid vesicles. The cleavage of TSR by thrombin or FXa did not alter the binding of PS to activated platelets. Moreover, the apparent binding affinities of FXa- and thrombin-cleaved PS to phospholipid vesicles were similar to that observed with rHPS. That FXa-cleaved PS binds normally to anionic phospholipids confirms the observations made by Long et al. [14], and suggests that no major rearrangement of the Gla domain occurs after Arg-60–Gln-61 cleavage. Until the recent finding that FXa could cleave PS after Arg-60, most studies on the binding to membrane phospholipids were performed with thrombin-cleaved PS, and yielded conflicting results. In some reports, thrombin-cleaved PS and uncleaved PS bind similarly to activated platelets [22], or phospholipids [14,24,27], whereas in others thrombin-cleaved PS was unable to bind to phospholipid vesicles [12] or to endothelial cells [4]. As confirmed in the present study, *in vitro* cleavage of PS by thrombin generates a mixture of two PS heavy chains with Ser-50 or Ser-71 at the N-terminus [11], the respective concentrations of which may depend on the experimental conditions. The present data clearly shows that whatever the cleavage site (Arg-49, Arg-60 or Arg-70), cleaved PS is still able to bind to phospholipid membranes.

In contrast, the deletion of the entire domain in rTSR-less almost completely precluded the binding of PS to activated platelets or to phospholipid vesicles, providing the first evidence that the TSR region is necessary for membrane binding. However, TSR-peptide did not bind to activated platelets, and the binding of rHPS to activated platelets or to phospholipid vesicles was not modified by the presence of the TSR-peptide, making it unlikely that TSR residues interact directly with phospholipids. Our results are consistent with previous observations that a mAb directed against TSR did not inhibit phospholipid binding of PS [44]. One possible explanation for our observation is that deleting TSR in PS may have modified the protein conformation. Since PS contains several domains believed to be assembled by exon

shuffling [45], the deletion of the entire exon encoding TSR (residues 45–75) was assumed to allow the other domains of the resultant rTSR-less protein to fold as in wild-type PS. Indeed, rTSR-less had binding affinities for C4BP and for a series of mAbs similar to those observed for rHPS, suggesting normal overall folding. However, for one of the mAbs (9F9G9), the epitope of which was located in the Gla domain, the apparent K_d for rTSR-less was 100-fold lower than that observed with rHPS. This mAb thus recognizes a cryptic epitope that becomes more accessible when TSR has been deleted, implying a close contact between TSR and the Gla domain in the wild-type protein. Such an interaction between TSR and its neighbouring domain has been predicted in molecular modelling of the N-terminal part of PS [46], and could have been disturbed by TSR deletion, modifying PS orientation on the cell surface in addition to a decrease in phospholipid binding.

When compared with the other vitamin K-dependent proteins, PS has a rather strong affinity for phospholipids [47,48], which up to now has not had a clear explanation. We observed that, when TSR was deleted, the apparent affinity of PS was in the same range as that observed for PC, a protein with the Gla domain directly tethered to the first EGF. This further suggests that the presence of intact or cleaved TSR indirectly contributes to the PS-binding affinity for phospholipids. In other proteins, such as factors IX and X, Ca^{2+} ions induce strong interactions between the Gla domain and the adjacent EGF1 domain [49,50], probably stabilizing their structure and inducing a reorientation of the two domains [51].

Taken together, our observations show clearly that TSR is necessary for the expression of the high affinity of PS for membranes. TSR may not interact directly with phospholipids, but probably constrains the Gla domain in a conformation allowing optimal interaction with phospholipids. The present study also establishes the requirement for an uncleaved TSR embedded in the PS molecule to promote APC anticoagulant activity. The cleavage of a single peptide bond in TSR after Arg-60 abolished APC cofactor activity but not the binding to phospholipids, implying a major role of the Arg-60–Gln-61 peptide bond in PS interaction with APC.

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