Molecular basis of protein S deficiency

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Summary

Protein S deficiency (PSD) has been the most difficult to study among the classical inherited thrombophilic factors. This is in part due to the peculiar biology of protein S (PS), which has an anticoagulant role but no enzymatic activity, and because it interacts with plasma components that function in both haemostasis and inflammation. Clinically, it also has been difficult to define and standardise valuable assays to determine PS status and implication in thrombosis. Despite these drawbacks, at present heterozygous PS deficiency is well established as an autosomal dominant trait associated with an increased risk of thrombosis from data on familial and population studies. Almost two-hundred mutations have been characterised in PROS1, and approximately 30% of them have been characterised in vitro, clarifying the mechanisms leading to PSD. Furthermore, recent studies on the presence of large deletions in PROS1 have increased the number of PSD associated to PROS1 mutations. Finally, the discovery of new functions for PS, both in the anticoagulant system as well as in the interaction with cellular components through receptor tyrosine kinases, is broadening the importance of this molecule in the context of biomedicine.

Keywords

Protein S, PROS1, mutations, natural anticoagulants, thrombophilia, vitamin K

Introduction

Anticoagulant protein S (PS) was discovered in the 1970s as a new vitamin K-dependent plasma glycoprotein containing γ-carboxyglutamic residues (1). In contrast to the other plasma vitamin K-dependent proteins, PS is not a serine protease. Further studies demonstrated that PS is a cofactor of the anticoagulant enzyme activated protein C (APC) in the degradation of factor (F)Va (2). Shortly afterwards, this anticoagulant function was strongly supported by the finding of an association between PS deficiency (PSD) and venous thromboembolism in patients with familial history of thrombosis (3, 4). The primary structure of PS provided the basis for studies on structure/function relationship, which are leading to a detailed knowledge of PS biology at the molecular level. In turn, these studies are helping to better understand the mechanisms of thrombotic disease. PSD has remained relatively difficult to study in terms of its prevalence and the severity associated with it. The present article will focus on recent findings and unsolved questions on the thrombophilia caused by PSD. The reader is referred to general reviews on the biochemistry of PS or the PC anticoagulant pathway for a more thorough revision of these topics (5–8). In the present article, we have adopted Human Genome Organisation (HUGO) recommendations (http://www.hgvs.org/mutnomen/) and annotate mutations based on the start codon (ATG) or the first translated methionine. The previous amino acid numbering (8) can therefore be obtained from the present one by subtracting 41 (for residues of the mature protein) or 42 (for residues that belong to signal peptide and propeptide).

Structure of protein S

The human vitamin K-dependent proteins identified so far can be divided in four functional groups; proteins related with mineralised tissue (osteocalcin and matrix Gla protein), plasma co-
Thrombophilia and anticoagulant pathway

Figure 1: Schematic representation of domain organisation in PS and APC. The phospholipid-binding Gla domains are indicated as blue ovals, while calcium-binding and non-calcium-binding EGF domains are given as smaller light sea green and dark orange ovals, respectively. The trypsin-like serine protease (SP) moiety of APC is given as a sphere, and the LG modules of PS are shown as yellow-green ovals. Only disulphide bridges that link the ends of the thumb loop and domain LG2 to the EGF4-LG1 connector are explicitly indicated. Inactivating cleavage sites in the thumb loop are marked with black arrows (thrombin) or a red arrow (FXa).

agulation proteins, a growth arrest-specific factor (Gas6), and a group of putative membrane-bound proteins (PRGP1 to 4) of unknown function (9). These proteins are characterised by the post-translational modification of specific glutamic acid (Glu) residues to γ-carboxyglutamic acid (Gla) residues. Carboxylation is catalysed by an enzyme, γ-carboxylase, which is present in essentially all human tissues, and uses a vitamin K derivative as co-factor (9). A common feature of vitamin K-dependent proteins is their Gla-mediated interaction with Ca\(^{2+}\). Plasma vitamin K-dependent proteins and Gas6 share a structurally similar N-terminal module, the Gla domain, which is essential for binding to negatively charged phospholipid membranes in a Ca\(^{2+}\)-dependent manner (10). The interaction with phospholipids enhances the efficiency of the initial steps of coagulation and tightens their regulation through the dependence on the presence of negatively charged ("activated") membranes. Conformational changes within the Gla domain required for membrane binding are induced by Ca\(^{2+}\)-binding to Gla-residues resulting in the exposure of three conserved hydrophobic residues within a prominent Ω-loop (10). Ca\(^{2+}\) may also support membrane binding by bridging negatively charged residues and the phosphate groups of phospholipids, although more specific protein-lipid ion pairs have also been proposed (10).

The human PS precursor contains 676 amino acid residues in eight folding modules encoded by different PROS1 exons; the modular structure of the protein (Fig. 1) is demonstrated by the correspondence between the exon-intron organisation of PROS1 and structural features in the gene product. Thus, exon 1 codes for the translation start site and the signal peptide (residues 1 to 24). Exon 2 encodes the propeptide (residues 25 to 41), which contains the sequence recognised by the vitamin K-dependent γ-carboxylase. The signal peptide and the propeptide are removed by proteolytic cleavages prior to protein secretion, resulting in a mature, single-chain multi-modular protein of 635 residues and an apparent mass of 75 kDa. The Gla-domain (residues 42 to 86) is encoded by the 3' end of exon 2 and exon 3 (10). Hence, exons 2 and 3 form a functional unit including the required features for the γ-carboxylation of 11 Gla residues present in the amino-terminal Gla domain. Exon 4 codes for a disulphide-bridged thumb loop (residues 87–113), similar to those found in Gas6 and prothrombin. The thumb loop is one of the PS regions that has been implicated in interaction with APC; and removal of this peptide upon thrombin-mediated cleavages at positions 90 and 111 (arrows in Fig. 2B) abolishes anticoagulant activity of PS (11–13). For this reason, this loop is also known as the thrombin-sensitive region. In addition, the scissile peptide bond Arg101-Gln is cleaved by free, membrane-bound FXa (arrowhead in Fig. 2B), which also results in almost complete loss of cofactor activity (14). The thumb loop stabilises the structure of the Gla module and increases its affinity for Ca\(^{2+}\)-binding, but does not seem to be involved in direct contact with phospholipids (15). Exons 5 to 8 code for one EGF-like domain each, one of the most common structural modules in extracellular and transmembrane proteins. Not surprisingly, EGF modules show extremely low sequence conservation; presence of six Cys residues that in the vast majority of cases adopt the disulphide-bonding pattern (C1-C3, C2-C4, and C5-C6) represents the only conserved feature. The three C-terminal EGF-like modules in PS bind Ca\(^{2+}\) in a cooperative manner and with very high affinity (K\(_D\) values range from 10\(^{-7}\) to 10\(^{-9}\) M), which seems to be important for its anticoagulant function (16, 17). An uncommon post-translational modification is also present in these modules, as specific Asp and Asn residues are β-hydroxylated to form hydroxyaspartic acid (HyA) and hydroxyasparagine (Hyn) (10). These modifications do not seem to affect PS anticoagulant function (18).

The carboxy-terminal half of human PS (residues 284–676), encoded by exons 9 to 14 and the 5' end of exon 15, is completely different from that of other vitamin K-dependent proteins, being homologous to the globular domains of laminin A (LG modules) found in proteins of the extracellular matrix such as agrin, perlecain and merosin, as well as in the plasma proteins, PS, Gas6 and sex hormone-binding globulin (SHBG) (19). Because of the

Figure 2: Location of the different mutations related to PSD characterised to date.
A) Type and position of each mutation given in a schematic representation of the PROS1 gene, grouped according to the reported phenotype.
B) Alignments of human and mouse PS and Gas6 sequences. All disulphide bridges are explicitly shown. Cleavage sites for thrombin (12) and FXa (14) are indicated with black and red arrows, respectively, and an empty arrowhead points to the maturation site. In the LG domains, the secondary structure given below the alignment corresponds to the crystal structure of the Gas6-Axl complex (PDB code 2C5D, (27)); α-helices (cylinders) and β-strands (arrows) are colour-coded (blue and green for domains LG1 and LG2, respectively) as in ref. (27). The major C4BP-binding site is boxed, and the three N-glycosylation sites are marked.
A) Thrombophilia and anticoagulant pathway

B) Molecular basis of protein S deficiency

- Missense
- Nonsense
- Splice
- Missense/Splice
- Small frameshift insertions
- Small frameshift deletions

PROS_HUMAN
PROS_MOUSE
GASS_HUMAN
GASS_MOUSE

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latter similarity, these domains are also named SHBG-like region. Each LG domain contains an internal disulphide-bridged loop, while LG2 is bound to the EGF4-LG1 spacer by another disulphide bond. The C-terminal LG module also contains the three N-linked glycosylation sites at Asn residues 499, 509 and 530 (13). None of these sites are crucial for PS function in vitro, as demonstrated in a series of mutants lacking each Asn, although some of the mutants showed slightly increased anticoagulant activity (13). Still, it has been shown that glycosylation modulates the half-life of PS in plasma, and several mutations involving loss of glycosylation sites have been found associated with PS deficiency (8). The LG modules contain a high-affinity binding site for the complement cascade regulator C4b-binding protein (C4BP) (20–22), which is also essentially unaffected by N-linked glycosylation (13, 23).

Structural information for PS is limited to the solution NMR structure of the pair of C-terminal EGF moieties, EGF3-4 (24). Isolated Gla and EGF domains, as well as some Gla-EGF and/or EGF-EGF pairs found in coagulation factors have been intensively studied (10). In addition, tandems of EGF domains found in thrombomodulin (25), the LDL receptor and in ECM proteins such as fibrillin and Notch (26) have been solved by NMR and/or X-ray crystallography. Finally, crystal structures of LG domains from Gas6, both free (27) and bound to the extracellular region of its receptor, Axl, were recently reported (28). All these experimentally solved structures provide excellent templates for modelling the corresponding regions in the APC cofactor. Based on this wealth of structural data, 3D models of the different PS modules have been generated (15, 22, 29–31).

Role of protein S in natural anticoagulation

PS acts as cofactor of the anticoagulant protease, activated protein C (APC), which proteolytically inactivates the activated procoagulants, FVas and FVIIIa. PC is present in plasma as an inactive serine protease that is activated by thrombin in complex with the endothelial cell membrane protein, thrombomodulin (5). A specific PC receptor on endothelial cell membranes (EPCR) plays a role in the regulation of PC activation and its anticoagulant activity. Free APC is a poor anticoagulant, but its activity is highly enhanced by the presence of PS. Exploiting the fact that bovine PS does not activate human APC in spite of overall high sequence similarities of both moieties, bovine-human PS chimeras were generated to identify residues important for the interaction with human APC. The human thumb loop and the first EGF-like module were found to confer full human APC cofactor activity to these chimeras, and the residues conferring the species specificity in the interaction were deciphered (31). The importance of the thumb loop is also reflected in the fact that thrombin or FXa cleaved PS loses its APC cofactor activity (14).

Studies using specific monoclonal antibodies have shown that those directed against the thumb loop and EGF1 are the most potent inhibitors of APC cofactor activity (16). Besides, recombinant EGF modules from PS are inhibitory only when they contain EGF1 (32). On the other hand, a truncated PS lacking the LG modules showed normal APC cofactor activity in the degradation of FVas and FVIIIa in purified systems, but not in plasma. This implies that the LG domains are required for full APC-cofactor function (33). Interestingly, inactivation of FVIIIa by APC and PS is enhanced by intact FV, implicating a dual role for FV in vivo: intact FV has anticoagulant activity but is converted by thrombin into a potent procoagulant cofactor (34). A PS/Gas6 chimera, containing the N-terminal modules of human PS and the LG domains of Gas6, lacks this FV-dependent APC-cofactor function during inactivation of FVIIIa (35). This suggests that FV-PS contact sites are located in the LG domains, and that this interaction is important for the anticoagulant properties of PS as measured in a plasma clotting assay (33, 35).

The molecular mechanism by which PS enhances the activity of APC is partially understood. It has been shown that PS increases the relatively low affinity of APC for different phospholipid membranes. Using fluorescence energy transfer (FRET) techniques, it was determined that PS relocates the active site of APC with respect to the membrane, and if this relocation is accomplished by mutagenesis on APC, the need for PS cofactor function is abolished (36). Accordingly, PS mainly accelerates APC cleavage of FVa after residue Arg306, one of the three sites targeted by APC (34, 37). Another aspect of PS cofactor mechanism is its ability to displace FXa from its complex with FVa, allowing APC to cleave FVa at Arg506, a more favourable site for APC cleavage. Interestingly, the APC-PS system could use high-density lipoprotein as physiological surface for down-regulating the anticoagulant activity (38). This suggests that the finding that plasma PS is partly associated with triglyceride-rich lipoproteins but not with high density lipoproteins (39).

PS circulates in human plasma at a fairly high total concentration of 20–25 mg/l (260–330 nM) with a half life of 42 hours (40). In human and rat, but not in mouse, rabbit or bovine plasma, PS forms a very stable equimolar complex with C4b-binding protein (C4BP) (41–45). PS binding sites are found in the β-chain of the complement regulator (46), which are missing in some species while others have lost the functional C4BP β-chain gene, C4BPβ (44, 45, 47). Because only free PS possesses APC cofactor activity (48), it is not surprising that the human plasma concentrations of β-chain-containing C4BP and of PS are coordinately regulated to maintain a fairly constant concentration of free PS (49, 50).

PS also has an APC-independent anticoagulant activity through direct inhibition of the prothrombinase and possibly the Xase complexes (51), as has been observed both in plasma and in purified systems (52–54). PS not only binds phospholipids with high affinity but also the procoagulant factors Va, Xa and VIIIa. The fact that phospholipid membranes are limiting in the initial steps of coagulation suggest that all these interactions could contribute to this APC-independent anticoagulant effect (55). PS would occupy the phospholipid surface and either compete for the binding of procoagulant complexes or dismantle them through its specific interactions with FVa, FXa or FVIIIa. This could in turn facilitate APC cleavage of FVa and FVIIIa connecting both proposed anticoagulant effects of PS. Interestingly, and in contrast to APC cofactor activity, PS bound to C4BP inhibits both Xase and prothrombinase (56), and C4BP has been shown to bind to FVIII, but not FV (57). The APC-independent function of PS as anticoagulant is enhanced by the presence of low-molecular-weight heparin, allowing development of a specific plas-
ma assay (56). Recently, a direct interaction of PS with tissue factor pathway inhibitor in the inhibition of FXa has been proposed as an alternative anticoagulant mechanism (58). All these anticoagulant functions of PS could explain why, in heterozygous deficiencies associated with inherited thrombophilia, the risk for thrombosis of patients diagnosed with PC or PS deficiency is similar (59). This could be interpreted as a strict dependence of APC for its cofactor PS in vivo, or could reflect the importance of the APC-independent function of PS.

PS deficiency and risk of thromboembolism

Thrombophilia is a complex multifactorial disorder where genetic factors predispose to the development of thromboembolic events (60, 61). In inherited thrombophilia at least one genetic factor with a main effect segregates in a pedigree. Among known genetic risk factors of thrombosis, many mutations in the antithrombin, PC and PS genes have been described. Congenital PSD (OMIM: +176880) is found in 2–12% of patients with thrombosis, depending on several factors, including geographical area (62–64). The important anticoagulant role of PS is dramatically illustrated by the severity of the few homozygous or compound heterozygous cases reported (8, 65, 66). Heterozygous mutations causing PSD result in an increased frequency of thrombosis, close to 10-fold that of their healthy relatives (64, 67–69). In contrast, in population-based studies, the relative risk of thrombosis associated with low plasma PS values is only two-fold or less (64, 69). This apparent paradox, which is not common to PC or antithrombin deficiencies, suggests that the screening methods to determine PSD are not able to identify most patients that have a high risk of thrombosis, or that most PSD cases have associated a low risk of disease (64, 70–73).

Determination of the risk conferred by a certain trait depends on a reliable measurement of its prevalence in the general population and on the range of normal values used. This has been difficult in the case of PS, whose plasma concentration depends on both genetic and environmental factors, including sex, hormonal status, smoking, age, and disease (69, 74–76). Nevertheless, recent reports have calculated a prevalence of PSD of less than 0.5% and around 1.5% in European and Japanese populations, respectively (77, 78). In this context, analysis of PROS1 and identification of mutations causing PSD will offer an unequivocal diagnosis of affected and unaffected individuals. Once a particular mutation has been identified, it is generally a simple task to screen for its presence in the proband’s relatives to offer them an unambiguous diagnosis of their PS status. PROS1 gene analysis is also needed for a better understanding of the relationship between gene and structure and function, as well as for a better classification of the different types of PSD based on their molecular cause.

Diagnosis of PSD essentially relays on the results obtained from the laboratory evaluation of PS plasma concentration. One of the difficulties for devising a clinically useful assay in PSD is the presence of pools of free and C4BP-bound PS in human plasma. Realising the potential significance of these differences, specific tests for the free and total forms of PS have been developed, as well as several functional tests that measure its APC-dependent and independent functions (56, 79, 80). Based on these measurements, PSD is classified in three subtypes. Type I deficiency is characterised by a decrease in the total PS antigen and, concomitantly, of free PS. Type II or qualitative deficiency is characterised by normal antigen levels and reduced PS activity due to a dysfunctional PS in plasma, and is either relatively uncommon or not easily diagnosed. Type III deficiency, in turn, is characterised by low free PS levels while the total plasma concentration of PS is normal. The distinction between type I and type III could be of clinical importance to assess the risk of thrombophilia in a given individual, but its biological basis and significance has been controversial (73, 81). On the one hand, type I and III deficiencies are commonly found in the same kindred, and in some cases have been demonstrated to be associated with a single mutation in PROSI (64, 82–86). On the other, several studies have detected kindreds where PSD appears only as type III, frequently associated with the relatively common glycosylation variant, PS Heerlen (79, 87–89).

Mutations in PROS1 associated with PSD

Characterisation of the genetic locus coding for PS initiated the search for genetic mutations associated with PS deficiencies in individuals and families diagnosed as PSD. Two highly homologous genes about 5 Mb apart have been characterised and mapped near the centromere at 3p11.1–3q11.2. These are the active gene, PROSI (GeneID: 5627; MIM # 176880) and a transcriptionally inactive pseudogene, PROSP (GeneID: 5628). The PROSI gene spans 101 Kb of genomic DNA and contains 15 exons that are transcribed in about 3.3 Kb of mRNA. PROSP spans 34 Kb and shares 96.5% and 95.4% homology with PROSI exons and introns, respectively, but lacks exon 1 and contains several detrimental mutations.

The most common approaches used in the mutational analysis of PROSI have been PCR-amplification of all coding and intron flanking regions of the gene followed by single-strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis analysis and DNA sequencing (90, 91). Direct DNA sequencing of PROSI amplified fragments is also used at present (64). PROSI databases from the International Society on Thrombosis and Haemostasis (ref. [8] and http://www.med.unc.edu/isth/) and HGMD (Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/) list almost 200 different mutations, most of them point mutations or short deletions or insertions, which have been found associated with PSD (Table 1). Large deletions (5 cases) or insertions (1 case) account for less than 4% of the cases. Most of these mutations have been associated with quantitative type I or type III PS deficiencies and are distributed along the whole gene with no region showing a clearly higher prevalence of mutations (Fig. 2A). PROSI mutations associated with type II PSD are of the missense type and mainly affect protein domains essential for the APC cofactor function of PS (Gla to EGF4).

Although the results of the mutation screening depend very much on selection criteria, different studies have found mutations in 50–90% of PS-deficient cases with familial thrombophilia (64, 74, 86). In a series of PSD families of at least three members and at least two individuals with PSD, the PROSIT study found a PROSI variant in almost 70% of probands (64). In those cases
where no mutation is found, it is still possible that PSD is caused by a mutation in PROS1 affecting the transcription regulatory sequences at 5' of the gene, which have been characterised only recently (92–95). Moreover, there might be gene conversion events between PROS1 and PROSP. Indeed, linkage between PSD and the PROS1 locus is found in almost all families with confirmed diagnosis of PSD, suggesting the existence of as yet unidentified PROS1 mutations (96). The standard screening methods for mutations in PROS1 are not able to analyse large deletions, which are not amplified by PCR, and therefore not detected. Recently, the use of haplotypes in segregation analysis allowed identification of large deletions in three out of eight families with PSD, which had been previously studied without identifying any PROS1 mutation. The authors concluded that large gene deletions are relatively common in PSD deficiency (97).

Some of the PROS1 mutations/variants found in PSD patients are likely not to be the cause of thrombotic disorders. This is usually suggested by absence of co-segregation of the mutation and PSD in the pedigree, and/or because both analysis of their potential structural effect and characterisation in vitro of the protein S variants indicates that some PROS1 variants detected in PSD individuals behave in vitro as neutral polymorphisms (30, 64, 82, 84, 98).

The mutations identified in PROS1 can be grouped in different types according to their expression in mRNA and recombinant protein analyses (Table 1). The clearest situation corresponds to those mutations in PROS1 leading to a deleterious allele, such as nonsense, frameshift or splice-site mutations that result in significant sequence alterations, and which often introduce premature termination codons (PTC). PROS1 mRNA analysis of some of these mutations (99–101) indicates that most of them cause PSD through a nonsense mediated mRNA decay (NMD) mechanism that prevents the synthesis of potentially toxic proteins (102). NMD in PROS1 happens preferentially when the stop codon is introduced at least 50 bp upstream of an exon-exon junction (Hurtado et al., manuscript in preparation). Another cause of deleterious PROS1 alleles are missense mutations that give very low to undetectable levels of the mutated protein, as determined in in-vitro experiments. In these cases, the amino acid substitution results in a structurally unstable or misfolded protein that is not secreted to the extracellular medium (64, 82, 84, 103). Heterozygous carriers of such alleles will present PSD, most frequently of type I, but not exceptionally of type III due to the intrinsic variability in PS concentration (85, 86, 104). In these cases it is expected that the mutation would be clearly associated with both PSD (whichever type) and thrombotic tendency. To prove this association and calculate the risk associated with a specific mutation requires large families with an isolated cause of PSD and a properly established medical record. Until now only one such case has been studied, confirming the hypothesis advanced above (68).

A second situation is found in familiar deficiencies where a missense mutation is found in PROS1, but this leads to a relatively milder effect on PS expression (64, 84). In these cases, a partial deficiency could correlate with low levels of PS and, possibly, a more frequent type III phenotype, as it has been observed for mutation p.Arg561Gly (86). PS from the mutant allele would contribute to the plasma pool and could have altered functional properties. If this is the case, these mutations could be considered a combined quantitative/qualitative deficiency (64). To test this possibility, a careful characterisation of naturally occurring PS mutants should be performed, a task that has been undertaken during the last years for a selected group of mutations (30, 64, 82, 84, 105–107).

PSD kindreds that are always diagnosed as type III have been studied by several groups, frequently finding no mutation in PROS1 or even no linkage with PROS1 (86, 87). Frequently, a rare and apparently neutral polymorphism in exon 13 of PROS1 leading to a PS variant known as PS Heerlen (p.Ser501Pro) is the only abnormality detected in type III deficient probands, al-

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<th>Mutation type</th>
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<td>Large insertions</td>
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**Table 1: Mutations identified in PROS1 according to PSD phenotype.**

<sup>a</sup> I: Type I PSD; II: type II PS; III: type III PS; I/III, pedigrees in which the same mutation cosegregates with both, type I and type III PS; nc, unclassified PSD.

<sup>b</sup> Percentage of each mutation type with respect to the total number of different mutations identified in each PSD phenotype. %Percentage the total number of different mutations identified in each PSD phenotype with respect to the total number of mutations identified.
though this trait does not always cosegregate with PSD in the family (87, 88, 108). Still, it is striking to find PS Heerlen in a much larger proportion of individuals with type III deficiency than expected from the population prevalence (74, 89, 109). Type III kindreds could have a second genetic trait affecting PS concentration, the most likely candidate being the C4BPB gene, known to affect plasma PS concentration (50, 110, 111). Until now this trait remains elusive, as C4BPB has been shown not to cosegregate with PSD in the cases studied (87, 112). From all these results it follows that while quantitative type I PSD is essentially due to PROSI allelic heterogeneity, the molecular basis of type III PSD remains to be solved, as its causative effect on thrombosis is unclear (73, 81).

A last group of mutations on PROSI linked to PSD are those leading to normal levels of a protein that lacks anticoagulant activity (qualitative or type II deficiency). The most dramatic example of such a case is a mutation on the donor splice site of intron 5, leading to exon 5 skipping. This protein lacks EGF1 and is found in plasma of carriers but lacks anticoagulant activity (113). Another example of type II mutation is PS p.Ile244_Asp245del caused by the introduction of a cryptic splice site in intron 7 (114). Compared with PC and antithrombin, very few mutations leading to qualitative deficiency have been found in PROSI, while most of the initially reported cases were actually due to APC resistance (79).

In the following, we catalogue missense mutations detected in PROSI (see Fig. 2) and briefly discuss their likely implications on cofactor structure and function. For simplicity, mutations will be grouped according to the affected structural domain (22).

**Signal peptide**

The hydrophobic nature of this peptide is compromised by mutations at positions 15 (91) and 18 (82), which replace a hydrophobic residue by a polar one (His and Glu, respectively). These substitutions within the more apolar, N-terminal end of the α-helical signal peptide are likely to compromise its interactions with the secretory machinery and/or impair correct membrane translocation, with concomitant defects in protein secretion. Indeed, mutation p.Leu15His was identified in a proposita with type I PSD (91), and recombinant PS p.Val18Glu was expressed at low to undetectable levels (82).

**Propeptide**

Substitutions at the P1 and P2 positions of the PS maturation cleavage site, p.Arg41His and p.Arg40Leu, respectively, result in type II PSD and are associated with thrombotic disorders (90). Given the narrow specificity of subtilisin-like protein convertases for positively charged Arg/Lys residues at positions P4, P2 and P1 (115), both mutations would impair maturation of the full-length cofactor. The consequence of retained propeptide appears to be indirect, and at least in case of PS p.Arg40Leu leads to abnormal carboxylation of Gla domain residues (90).

**Gla domain**

Several mutations compromise overall structure and membrane-binding capability of this module. Mutation p.Phe72Cys (90) replaces a strictly conserved aromatic residue, also found in most Gla domains from coagulation factors, by a smaller Cys residue. This would create a destabilising cavity, resulting in a protein more prone to degradation. Additional structural consequences of this mutation are also conceivable. For instance, we note that the disulphide-bonded pair Cys58-Cys63 is strictly conserved in PS and Gas6 from different species (Fig. 2), and thus contributes to structural integrity. It is conceivable that Cys72 either engages in intermolecular linkages or forms aberrant disulphide bridges with Cys58 or Cys63 to generate "scrambled" PS variants. Because the Sy atom of Cys72 is located at least 7 Å away from those of Cys58/Cys63 in a properly folded Gla domain, formation of these aberrant linkages would necessarily be accompanied by gross structural modifications.

Another mutation within the Gla domain, p.Glu67Ala has been reported in several apparently unrelated kindreds with type I PSD (90, 116, 117), and eliminates one of the residues that are modified to Gla. Given the co-operative nature of Ca²⁺-induced folding of Gla domains (10), loss of the Ca²⁺-binding ligand, Gla67, could have profound effects on overall domain structure and would result in a reduced interaction with phospholipid membranes. Indeed, PS p.Glu67Ala completely lacks cofactor activity (64), which is at least partly attributable to its missing phospholipid-binding ability (117).

Two other deleterious mutations identified in the Gla domain introduce Asp residues at positions where PS/Gas6 possess the strictly conserved small residues Gly52 (118) and Ala68 (8). Mutation p.Gly52Asp would locate a negatively charged side chain close to a Gla residue(s), most probably Gla70, with potentially destabilising effects. Indeed, mutant PS p.Gly52Asp was normally produced but possessed 15-fold reduced anticoagulant activity than wild type, which correlated with a five-fold decrease in affinity for anionic phospholipid vesicles (118). Even more dramatic rearrangements must be expected from mutation p.Ala68Asp, which would press a charged side chain into the hydrophobic/aromatic core of the domain. Another strictly conserved residue, Pro76, was recently found to be mutated in a proband with type I PSD (117). In this case, however, a negatively charged, exposed side chain is found in Gla domains from several coagulation factors, and thus a leucine side chain can be accommodated at position 76 without affecting the overall Gla domain structure. Accordingly, mutant PS p.Pro76Leu is efficiently secreted and possesses wild-type cofactor activity (64). On the other hand, essentially preserved domain structure but impaired Ca²⁺-induced phospholipid binding might explain the deleterious effect of reversal-of-charge mutant p.Lys50Glu, which would disturb the conformation of nearby Gla47, whether modified itself to a Gla residue or not. Indeed, this mutation was identified in a patient with normal levels of total PS but reduced cofactor activity (116).

Finally, replacement of the strictly conserved Thr78 by a Met leads to about one-third lower expression levels than wild-type PS (90, 118). The bulkier Met side chain is likely to clash with main chain atoms of Arg69 (29) and/or the nearby cluster of conserved aromatic residues, the so-called aromatic stack (118). In turn, these alterations in the overall domain structure might account for the slight decreases in affinity for phospholipid vesicles (1.5-fold) and FVa-inactivating activity (3.6-fold). Composite p.Asp52Gly and p.Thr78Met missense defects results in recurrent thrombosis (118), highlighting the relevance of Gla-
mediated positioning of PS on phospholipid membranes for its cofactor activity.

**Thumb loop**

Four of the mutations that map to this region: p.Arg90His (90), p.Arg90Cys (82), p.Arg101Cys (119, 120), and p.Arg111Ser (121) eliminate cleavage sites for thrombin/FXa. Both Arg90 and Glu93 contribute to species-specific interactions with APC (31), and mutations to either His or Cys are associated with different PSD phenotypes. While p.Arg90His appears as a neutral PS variant (30, 90), p.Arg90Cys, whose recombinant form is partially secreted, is associated with type I-III PSD and a mild thrombotic phenotype (82). In addition, mutant PS p.Arg101Cys is normally produced and binds to phospholipid vesicles with the same affinity as wild type, but essentially lacks cofactor activity due to an impaired interaction with APC (120). We also note that replacement of the nearby, conserved Gly95 by an Arg did not produce any definite abnormality leading to PSD (107), while mutation p.Gly95Glu was identified in a heterozygous proband with low levels of PS and low cofactor activity (116). Altogether, these findings suggest formation of an electropositive surface patch on PS, contributed essentially by the thumb loop (Arg90, Glu93, Arg101, Arg111), which interacts with a negatively charged exosite on the APC moiety. However, experiments with recombinant variants suggest that the impact of isolated mutations might be low, except for FVIIIa inactivation (30). PS mutants p.Arg90Cys and p.Arg101Cys could form either multimers or scrambled forms, with concomitant masking or loss of the native conformation for the APC-binding thumb loop. Elimination of the disulphide bond Cys88-Cys113 in mutant p.Cys88Tyr (64) would severely compromise stability and affect the relative orientation between Glu and EGF1 domains. This substitution impairs protein expression and also abolishes binding to phospholipid membranes, explaining its lack of cofactor activity (117), and the associated severe phenotype (64), also in line with the proposed role of this loop in stabilising the Ca$^{2+}$-loaded conformation of the Glu domain (29). Finally, variant p.Thr98Ser is associated with marginally low PS concentration and did not segregate with PSD. PS mutant p.Thr98Ser is efficiently secreted (82), confirming that it represents a rare neutral allelic variant (91). Position 98 is not conserved in PS from different species.

**EGF domains**

Most missense mutations identified within the four tandem EGF modules of PS affect overall domain stability, and Cys residues are replaced in half of them. Most residues introduced in place of a Cys possess aromatic side chains as in p.Cys121Tyr (106, 122), p.Cys154Phe (HGMD database), p.Cys175Phe (64, 123), p.Cys186Tyr (72, 116, 124), and p.Cys265Trp (90), or the isosteric serine as in p.Cys228Ser (HGMD database), p.Cys241Ser (116), and p.Cys267Ser (64). Irrespective of the specific replacement, absence of a stabilising disulphide bridge in these mutants would likely compromise to different extents the overall 3D structure of the affected EGF module and favour protein mis-merisation and/or formation of scrambled variants, with concomitant defects in protein stability and impaired secretion, as has been verified experimentally (72, 90, 106, 117). Of particular note, recombinant PS p.Cys175Phe lacks APC-cofactor activity, although it binds normally to phospholipids, in line with the role of domain EGF1 in APC-binding (31, 113). Interestingly, the p.Cys267Ser mutation in EGF4 also results in an inactive form, although the mutated protein retains phospholipid-binding ability (117). This finding might point to a role of the more C-terminal EGF moiety in folding of the whole tandem (see also below). Two mutants in EGFs introduce a cystine instead of well conserved aromatic residues, resulting in large, potentially destabilising cavities (125). Possibly because the cavity formed is smaller in PS p.Tyr266Cys, this mutant retains partial cofactor activity (64, 117), while EGF1 mutant PS p.Trp149Cys results in a more severe phenotype (119).

Other common mutations affect high-affinity calcium binding sites in EGF modules 2, 3 and 4. Mutant p.Asn258Ser (126), which lacks a residue that is normally β-hydroxylated and forms part of the Ca$^{2+}$-binding shell in EGF4, has been thoroughly investigated. Secretion of the mutant protein is impaired by about 70%, but it possesses essentially normal APC anticoagulant activity (82, 127). Surprisingly, the p.Asn258Ser mutation impairs not only Ca$^{2+}$-binding to its "own" EGF domain, but also affects the conformation of EGF1 (127). It follows that binding of calcium to EGF4 of PS is important for maintaining the overall structure of PS, and in particular that of the sequentially distant EGF1. This finding, in turn, suggests a quaternary arrangement that locates the two terminal EGF modules in close proximity. However, SAXS analysis showed a dominant, extended conformation for EGF1-4, suggesting counterproductive interaction(s) between EGF2 and EGF4 if EGF3 is in a bent conformation (24). These authors speculate that the ability of EGF3 to adopt different conformations may be of functional significance in protein-protein interactions involving PS. A similar structural effect to mutant p.Asn258Ser could be expected in three further mutations replacing negatively charged residues that are either known or predicted Ca$^{2+}$-ligands: p.Glu204Gly (64), p.Asp243Asn (128), and p.Asp157Gly (121). In a similar manner, ion binding would be compromised in EGF4 mutation p.Asp245Gly (90), as residues immediately preceding and following this Asp residue are Ca$^{2+}$-ligands (24). At least for the p.Glu204Gly mutation, the protein retains partial cofactor activity and normal phospholipid binding capability (64, 117). Another suggestion of inter-EGF cooperativity is EGF4 mutant p.Glu249Lys (90), which would disrupt a salt bridge formed with Lys208 from EGF3 (24), destabilising the bent conformation of this EGF pair.

Two less deleterious mutations have been reported in EGF domains. Arg233 is conservatively replaced by a Lys in a probably neutral variant (8); inspection of the EGF3-4 structure reveals that the Lys side chain can be accommodated without sterical clashes. Mutation p.Asp129Gly would not seem to be neutral, as it replaces a well-conserved residue (Lys in Gas6). Indeed, secretion of recombinant PS p.Asp129Gly was impaired, although the mutant retains full cofactor and phospholipid binding capacities (64, 117, 123). A few structurally neutral mutations have been reported in EGF modules, which are nevertheless associated with functionally defective molecules (type II PSD). For example, mutation p.Thr144Asn (90) affects a position without apparent structural constrains, as the topologically equivalent...
residues in EGF modules from PS/Gas6 range from Ala to Tyr (Fig. 2). Therefore, this mutation is unlikely to alter EGF1 structure. Nevertheless, functional characterisation of recombinant PS p.Thr144Asn revealed that it possesses about half of the wild-type cofactor activity (105), pointing to an important interaction site that probably also includes the exposed side chain of Lys138 (31). Another mutant, p.Lys196Glu (107, 129, 130) is a known polymorphism in the Japanese population (5, Tokushima), and represents a genetic risk factor for deep vein thrombosis in Japanese patients (131). In APC cofactor assays, the specific anticoagulant activity of PS p.Lys196Glu represents only 58% of that of wild-type PS (132). Altogether, these findings support the notion that the EGF1-2 tandem contributes important binding epitopes for APC.

**LG domains**

LG domains essentially consist of two tightly packed β-sheets; in contrast to Gla and EGF domains they do not appear to require metal binding and/or disulfide bond formation to adopt their native conformations. About 50% of all reported mutations within the LG pair involve either loss or gain of residues with unique physicochemical properties (Cys, Pro and Gly), highlighting the relevance of its structural integrity. Similar to EGF domains, all cysteine residues except Cys288 (Fig. 2) are found mutated in patients suffering from different thrombotic disorders: p.Cys449Ser (116), p.Cys475Arg (121), p.Cys568Tyr (121), p.Cys639Phe (133), p.Cys639Tyr (64), and p.Cys666Arg (72, 134). Loss of the Cys288-Cys568 bridge would appear to be particularly deleterious, as it directly affects the orientation of N-terminal modules relative to LG2, which is required for full cofactor activity (135). Introduction of Cys residues at non-natural positions has also been reported in LG domains. In particular, association of p.Gly482Cys and, more clearly, p.Tyr485Cys with PSD (86) has been confirmed by low recombinant-protein secretion (84). Further, mutant PS Nagoya p.Arg515Cys affects one of the best-conserved residues in LG modules (103). The mutation is found in type I PSD, although the structural basis for the strict requirement of a basic side chain at this position is not clear. Mutation p.Tyr636Cys also results in type I PSD; the mutant protein is intracellularly degraded indicating severe misfolding (107). Finally, expression of the recently reported mutation p.Arg316Cys is only moderately impaired, but the mutant protein is significantly less efficient APC activator than wild-type cofactor (106). Considering that the Arg316 side chain is well exposed, these findings substantiate an anticoagulant role for domain LG1, in particular for the synergism between PS and FV in APC-dependent degradation of FVIIa (35). One binding site for FVα has been mapped to the C-terminal tail of PS (136), and both stretches together could be critical for simultaneous positioning of FV(a) and APC in macromolecular complexes (see also below).

A number of mutations within the LG modules of PS either replace or introduce the amino acid Pro. Pro replacement has been detected in mutants p.Pro416Gln (106) and p.Pro667Leu (91). The former introduces a Gln at a position that is not strictly conserved and without structural constraints, and accordingly has only minor effects on secretion of an otherwise functional variant (106). Expression of recombinant PS p.Pro667Leu was only modestly low, ~50% of wild type indicating minor alterations of the overall fold (84). It can be assumed that this mutation affects the correct orientation of the preceding cysteine residue, thus compromising formation of the Cys639-Cys666 disulphide bond (91). Pro is introduced in several mutants instead of PS/Gas6 residues that are strictly preserved [positions 300 (134), 446 (121), 515 (123), 525 (157), and 664 (82)] or conservatively replaced in different species [p.Ser324Pro (116), p.Leu339Pro (128), as well as p.Leu351Pro and p.Ala616Pro (99)]. Most of the mutated residues are hydrophobic and either found within β-strands (Fig. 2), or otherwise buried in the core as Leu446 and His664 (27). It is therefore probable that these substitutions affect protein folding and secretion. Indeed, expression experiments have shown very low to undetectable expression levels for mutants PS p.Leu446Pro, PS p.Arg515Pro and PS p.His664Pro (64, 82).

Both LG modules of PS contribute to cofactor association with C4BP (20), with major contributions from the exposed loop His494-Ser501 (21). Residues Val500 and Ser501 appear to be particularly relevant in this regard, as deletion of this dipeptide completely abolished binding of C4BP. As mentioned before, the relatively common PS p.Ser501Pro mutation, termed PS Heerlen, is the only sequence abnormality detected in several type III deficient probands (86, 88, 89, 109). This mutation is found within the protruding 494–501 loop, and its most notable consequence is loss of the N-glycosylation site at Asn499 (88). However, absence of this carbohydrate moiety by itself does not affect APC cofactor function or C4BP binding, and seems to have only limited structural effects according to modelling experiments (23, 138, 139). While some effects on PS function could be attributed to this mutation (23, 140), the most important effect of p.Ser501Pro is likely a reduction in protein stability (23), leading to a decreased half-life in serum (40). This is also in agreement with the finding that total and free plasma levels of PS are significantly lower in p.Ser501Pro heterozygotes than in Ser501 homozygotes (23, 89, 108). Similar considerations apply to the less common mutant p.Ser501Ala (121). Another mutation within the C4BP-binding loop, p.Asp496Tyr, has been reported to result in impaired expression of about 50% of wild-type PS (106). The Asp496 carboxylate is exposed fully solvent, and thus the reduced expression levels might point to the interaction of this region with a chaperone during protein expression. Finally, we also note that the side chain of Lys612 is located close to this protruding loop, explaining the reduced affinity of porcine PS for C4BP, as it possesses a glutamate at this position (141).

Mutations that result in either loss or gain of a Gly residue are also relatively common in LG modules. Interestingly, several of the replacements that more severely affect secretion introduce a charged Asp at positions 336, 521, or 638 (64). In addition, the fairly common mutation p.Gly336Val (68, 83) also leads to intracellular protein degradation and thus largely impaired secretion (82). Although not yet characterised, it is conceivable that mutant PS p.Gly336Ser (8) results in a similar expression defect. These three glycines are located at the N-terminal end of a β-strand, and introduction of other side chains at these positions would result in severely clashes with core residues (e.g. with Leu446 for mutations at position 336). Intermediate expression levels have been reported for mutant p.Gly357Arg, and the mutant protein
possesses normal APC-cofactor activity (64, 117). This residue is found in a tight turn between two β-strands, and the basic Arg side chain is certainly tolerated at this position. Finally, replacements of Gly381 for both Asp (116) and Val (8) have been reported. Although this residue is found in an extended conformation it does not form part of a regular β-strand, and bulkier side chains appear to be accommodated without steric clashes. Therefore, the observed type II PSD phenotype indicates that it belongs or is located close to an important binding epitope, perhaps for FV(a). Introduction of Gly residues has been identified at positions Val508 (8) and Arg561 (86). The former affects a position where a buried aliphatic side chain is always found in PS/Gas6, explaining the severe phenotype associated with this mutation. By contrast, the side chain of Arg561 is exposed, and loss of a salt bridge with Asp549 would seem to have less dramatic consequences. Indeed, mutation p.Arg561Gly only modestly affected protein expression (42% of wild type), and conservative replacements by Lys/His or even by a glutamate resulted in almost normal PS expression levels (84).

Several mutants have been described in which a strictly or well-conserved, usually completely buried hydrophobic side chain is replaced by a basic Arg/Lys [p.Leu575Arg (121), p.Met611Lys (64), p.Leu622Arg (72)]. In a particularly interesting case, the strictly conserved Trp at position 383 is replaced by an Arg (125). As the indole moiety of Trp383 is sandwiched between the side chains of Arg330 and Arg451, this mutation would affect the overall domain conformation. Curiously, the Arg330-Trp383-Arg451 stacking resembles the Trp-Arg-Trp triplet conserved in C domains of FV and FVIII, where also disruption of this sandwich structure severely affects overall domain folding (142). Replacements of buried hydrophobic residues usually found within regular β-strands by other polar moieties cluster in domain LG2: p.Leu526Ser (99, 125), p.Leu584Gln (99), p.Met611Thr (124, 126), p.Met640Thr (91) and p.Ile644Ser (143), and are likely to result in gross structural defects.

Two Thr→Ala substitutions have been identified in domain LG2, at positions 532 (121) and 588 (HGMD database). The former mutation eliminates the most C-terminal N-glycosylation site in PS (see Fig. 2). Although carbohydrate moieties attached to the cofactor are not critical for its activity (13), N-glycosylation is usually important for secretion of properly folded proteins, perhaps explaining the reduced PS levels measured in the carrier of this mutation. The side chain of Thr588 is exposed fully solvent, and the Thr→Ala mutation would seem to be perfectly tolerated, also in line with the presence of a glycine at this position in Gas6. Residue Ser665 is rather buried at the interface between both LG moieties, and its replacement by a bulkier leucine will certainly result in important clashes with residues Asp447 (LG1) and Arg662 (LG2), perhaps leading to improper interdomain orientation. Further, two reversal-of-charge mutants have been reported in domain LG1, p.Lys364Glu (121) and p.Glu390Lys (125). Both mutations affect strictly or conservatively replaced residues and result in type I PSD. The side chain of Lys364 is partially buried and surrounded by the carbonyl oxygens of Asp345, Glu437 and Leu438. More dramatically, the side chain of Glu390 is completely buried in the protein core, hydrogen-bonded to the main chain nitrogen atoms

Although apparently less deleterious, mutations Asp333Asn (HGMD database) and p.Asp376Asn (90) result in type I PSD. The carboxylate groups of the former residue is buried in the protein core where it accepts hydrogen bonds from the main chain nitrogen atoms of Ser334 and Asp447, and is thus critical for holding neighbouring β-strands in place. An asparagine side chain at this position could not accept both H-bonds, thus disrupting the 3D structure. By contrast, the side chain of Asp376 is fully solvent exposed. Furthermore, the isosteric asparagine is found in mouse and rat PS, and lysine occupies this position in the bovine cofactor. These observations point to an important role for this side chain in species-specific interactions, thus provoking reassessment of its co-segregation with type I PSD (90).

Finally, a number of probable neutral changes or polymorphisms have been identified within LG modules. Mutations p.Val510Met (121), p.Ile559Met (84, 91) and p.Ile562Leu (64, 123) conservatively replace aliphatic side chains. Leu562 is found for instance in the mouse cofactor, and is also conserved in Gas6 from different species, while position 559 is occupied by alanine or valine in these other proteins. Accordingly, the mutant proteins PS p.Ile559Met and PS p.Ile562Leu could be efficiently expressed (64, 84). Further, substitution of Thr630 by the isosteric isoleucine did not produce any definite abnormality leading to a low plasma PS activity (107), also in line with its fairly exposed location within a long loop.

Mutation p.Asn583His replaces a polar residue within a poorly conserved exposed loop; glycine or threonine are found at this position in the mouse and rat cofactors or in Gas6, respectively (see Fig. 2); accordingly, PS p.Asn583His was expressed at wild-type levels in COS-1 cells (64). By contrast, the apparently conservative replacement p.Arg355His leads to a severe expression defect (106, 122). The guanidinium group of Arg355 is partially buried and donates hydrogen bonds to consecutive main chain carbonyls of Lys440 and Pro441, thus contributing to stabilise the conformation of this long loop, and also forms a salt bridge with the carboxylate of Glu360.

Multifactorial interactions in PSD

In the previous section we have considered PSD as an isolated trait, but our concept of thrombophilia is changing to consider it as a multigenic disease. In this paradigm more than one genetic trait would add to the probability of thrombosis to manifest. This manifestation would also imply the interaction with environmental factors. This is clearly illustrated by the highly variable penetrance of thrombotic symptoms; the thrombotic frequency found in individuals with combined defects is much higher than in individuals with single gene defects (144). Large studies screening known genetic risk factors are clarifying the picture in terms of frequency of combined compared to single risk factors and in terms of conferred risk (145). It is possible that certain genetic traits, which are not risk factors by themselves, increase the risk of thrombosis in PSD. Plasma concentration of PS influences of the phenotype of certain thrombophilic factors, such as the c.20210G/A mutation in prothrombin (146). This also
seems to be the case for SERPINE1 (PAI1) – 4G/5G and F12 –4C/T promoter polymorphisms, which by themselves are only weakly associated with thrombophilia (147, 148). Interestingly, the occurrence of pulmonary embolism was significantly higher in PS-deficient individuals of the – 675 4G/4G genotype (148). Another good example of this concept is the finding of a link between the PC anticoagulant pathway and antiphospholipid antibodies; recent studies have implicated PS in the thrombotic mechanism of antiphospholipid antibodies (149, 150) and oral contraceptives (151).

**Novel functions of protein S**

In contrast to the fairly strict liver specificity of most vitamin K-dependent coagulation genes, PROS1 is transcribed to comparable levels in several other organs, including brain, lung, uterus, ovaries, male gonads and bone. This broader pattern of expression suggested other roles of PS besides regulation of coagulation. As part of the PC system, PS has an important effect on the inflammatory response in vivo, which has been demonstrated to be of relevance in several models of disease, including septic shock and stroke (152–154). Furthermore, several lines of evidence suggest that PS could have anti-inflammatory properties (42, 155–157).

PS similarity to Gαs6 also suggests similar functions for the two proteins outside the coagulation cascade, as has been reviewed elsewhere (157). Gαs6 is the ligand of the TAM family of receptor tyrosine kinases, composed by three structurally related receptors, Tyro3, Axl, and MerTK. The role of PS as a ligand of these receptors remains to be fully elucidated, although recent findings suggest a clear involvement of PS in phagocytosis by retinal pigment epithelium (158). Indeed, PS has been identified as the serum factor that increases phagocytosis of apoptotic cells by human macrophages (159). Certainly, PS acts as a mitogen for cultured smooth muscle cells in vitro, a function which could be related to its interaction with the Axl family of receptors (160). The availability of an animal model of PSD would be crucial in understanding these roles of PS beyond anticoagulation.

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### References


Thrombophilia and anticoagulant pathway