Coagulation, Inflammation and Apoptosis: Different Roles for Protein S and The Protein S-C4b Binding Protein Complex

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Abstract

Protein S (PS) has an established role as an important cofactor to activated protein C (APC) in the degradation of coagulation cofactors Va and VIIIa. This anticoagulant role is evident from the consequences of its deficiency, when there is an increased risk of venous thromboembolism. In human plasma, PS circulates ~40% as free PS (FPS) and ~60% in complex with C4b-binding protein (C4BP). Formation of this complex results in loss of PS cofactor function and C4BP can then modulate the anticoagulant activity of APC. It had long been predicted that the complex could act as a bridge between coagulation and inflammation due to the involvement of C4BP in regulating complement activation. This prediction was recently supported by the demonstration of binding of the PS-C4BP complex to apoptotic cells. This review aims to summarise recent findings on the structure and functions of PS, the basis and importance of its deficiency, its interaction with C4BP and the possible physiological and pathological importance of the PS-C4BP interaction.

Protein S

PS is a vitamin K-dependent plasma glycoprotein with a molecular weight (MW) of ~70 kDa. In humans, PS is mainly synthesised by hepatocytes, but also by megakaryocytes, endothelial cells, Leydig cells of testis, osteoblasts and vascular smooth muscle cells. PS normally circulates in human plasma at a concentration of ~25 mg/L (0.30 µM). After post-translational modifications, including γ-carboxylation of glutamic acid residues, disulphide bond formation, β-hydroxylation of asparagine and aspartic acid residues and N-linked glycosylation, both the pre- and pro-peptides (for signal peptidase and carboxylase recognition, respectively) are removed by proteolytic processing. Mature PS has a modular structure consisting of a Gla domain (residues 1 to 46) containing a short aromatic stack (residues 38 to 46), a region sensitive to cleavage by thrombin and factor Xa (TSR; residues 47 to 75), four epidermal growth factor (EGF)-like domains (EGF1-4, residues 76 to 242) and a sex hormone binding globulin-like region, containing two laminin G-type repeats (LGR; residues 243 to 635) (Figure 1). To date, PS seems refractory to crystallisation attempts and the 3D structure has not yet been reported.

Human PS is expressed from the gene PROS that is located near the centromere of chromosome 3 (region 3q11.2). Recently, PROS appeared for the first time in the Ensembl human genome browser (http://www.ensembl.org/). The genomic sequence currently encompasses exons 1 to 12, although a gap still remains that presumably includes exons 13 to 15 and downstream sequences. The pseudogene (PROSP) is present at 3p21-cen, although the full genomic sequence is not currently available in public databases. The coding regions of the two gene sequences (PROS and PROSP) exhibit 97% sequence similarity. The cis-acting regulatory elements that govern expression of PROS are still poorly defined. This may be due to the presence of additional exon(s) 5′ to that
Figure 1. Distribution of PROS mutations associated with quantitative (A) and qualitative (B) PS deficiency.

The domain structure of PS is shown at the top of the figure, and the colour coding indicates the corresponding exon encoding each protein domain. The numbers inside squares, diamonds, triangles and circles denote the numbers of different missense, frameshift, nonsense and splice site mutations, respectively, for each PS domain. A. Mutations causing quantitative PS deficiency are distributed throughout the PROS. Data source available from heterozygous, homozygous and compound heterozygous states compiled by and from 19 new mutations recently published after a Medline search (key words: protein S; mutations) between Jul/1999 and Sep/2002. B. Mutations causing qualitative PS deficiency tend to locate in the Gla and EGF-like domains of the PROS. Abbreviations: AS, aromatic stack; TSR, thrombin-sensitive region; EGF, epidermal growth factor-like domain; LGR; laminin-G type repeat. currently considered exon 1. This might explain the lack of reports describing promoter activity of the 5’ flanking sequence, since the gene sequence was published more than a decade ago. The recently available genomic sequence information for both human and mouse PROS may lead to progress in this area.

**APC-dependent anticoagulant functions of PS**

The biological function of PS thought to be of primary importance is its ability to enhance APC-dependent proteolytic inactivation of factors Va and VIIIa, which are respectively the cofactors in the prothrombinase and tenase complexes of the coagulation cascade. In this way, PS plays an important
role in regulating thrombin generation, and therefore controlling procoagulant activity. Crucial to the
APC-dependent functions of PS is the high affinity interaction between PS and negatively charged
phospholipid surfaces, conferred by calcium-induced folding of the amino-terminal Gla domain.\(^9,10\)
The importance of the Gla domain to PS function is illustrated by the presence of mutations
associated with qualitative (functional) PS deficiency within this domain (Figure 1B). These mutations
are predicted\(^8\), or have been shown\(^11\), to prevent the proper folding of the Gla domain in the
presence of calcium ions. With a \(K_d\) in the nanomolar range, PS binding to these surfaces (such as
those exposed on activated platelets, damaged endothelial cells and apoptotic cells, see below)
seems to be highly efficient. It should be noted, however, that the affinity varies somewhat with the
composition of the phospholipids present in the membrane (see below). Moreover, a recent report has
questioned the uniformity of high affinity binding of PS to phospholipids, as it showed that different PS
isoforms within purified preparations of PS had distinct affinities. Of these, only a multimeric form (that
accounted for less than 5% of the total PS) displayed high affinity binding (\(K_d < 1\) nM), while the
remaining 95% bound to phospholipid with a low affinity (\(K_d \sim 250\) nM).\(^12\)

To date, no direct interaction between PS and APC has been clearly demonstrated in the fluid phase,
whereas they can form a 1:1 stoichiometric complex on negatively charged lipid surfaces.\(^13\)
Formation of the complex increases the affinity of APC for membranes, and is thought to be one of
the mechanisms by which PS enhances APC activity. When factor Va is inactivated by APC in the
absence of PS, the reaction is biphasic, due to differences in the rate constants for cleavage at factor Va
Arg506 and Arg306. The most rapid cleavage occurs at Arg506, resulting in a molecule with intermediate
activity. In contrast, cleavage at Arg306, resulting in dissociation of the A2 domain of factor Va and
complete loss of factor Va activity, is slow\(^15,16\) (Figure 2A). In the presence of PS, APC-dependent
cleavage of Arg306 is enhanced \(\sim 20\)-fold, and is subsequently equivalent to the rate of Arg506 cleavage,
leading to a much more rapid (and monophasic) loss of factor Va activity\(^17,18\). Recently, PS was shown
to also enhance the rate of Arg506 cleavage 5-fold.\(^19\) This molecular selectivity induced by PS may be,
in part, brought about by relocation of the active site of APC to an optimal position for the cleavage of
factor Va at Arg306. This seems to occur through a rotational and/or translational movement of the
APC protease domain towards the membrane surface\(^20\) (Figure 2A).

APC-dependent inactivation of factor VIIIa in the absence of PS occurs by similar mechanisms. The
APC cleavage sites on factor VIIIa are Arg562 and Arg336\(^21,22\) (Figure 2B). Addition of PS has no
dramatic influence on the rate of factor VIIIa inactivation\(^23\), although PS cofactor function is altered
when factor VIII(a) is present in the tenase complex\(^23,24\). Effective enhancement of APC-dependent
factor VIIIa inactivation by PS is now known to require the presence of intact factor V, due to functions
associated with the B domain\(^21,25,27\). When factor V is activated by thrombin, cofactor activity is
lost, corresponding to cleavage at Arg1545 and removal of the carboxyterminal part of the B domain.
Figure 2. Schematic representation of APC and APC/PS-dependent inactivation of factors Va and VIIIa.

A. Factor V is converted into factor Va by thrombin or factor Xa by cleavages at Arg709, Arg1018 and Arg1545. This results in the loss of the B domain and formation of the factor Va heterodimer consisting of a heavy (A1-A2) and a light (A3-C1-C2) chain, coupled by a single calcium ion (Ca), tightly bound between the A1 and A3 domains. The C2 subunit of the light chain binds to negatively charged phospholipid membranes. Factor Va is inactivated by activated protein C (APC) cleavage at three sites in the heavy chain; Arg306, Arg506 and Arg679. However, the pattern of cleavage depends on whether protein S (PS) is absent (top) or present (bottom). Only cleavage at Arg306, which bisects the
A1 and A2 domain, results in complete loss of factor Va activity. In the absence of PS, rapid initial cleavage at Arg506 occurs resulting in a molecule with intermediate activity. This cleavage is slow and seems to be required for the optimum exposure of Arg306 (red box). The presence of PS increases the affinity of APC for the membrane surface (arrows) and seems to relocate the active site of APC for preferential cleavage at Arg306 (the distance of closest approach is indicated). This augments the rate of Arg306 cleavage ~ 20-fold, and rapidly inactivates factor Va. 

**B.** Factor VIII is activated by thrombin through cleavage at Arg372 (which bisects the contiguous A1-A2 domains), Arg740 and Arg1689 (which liberates the B region). Factor VIIIa is a heterotrimer consisted of A1 and A2 domains and the light chain (A3-C1-C2). The A2 associates with the A1 domain via weak electrostatic interactions (dotted line), with rapid dissociation at low concentrations. APC inactivates factor VIIIa by a similar mechanism to factor Va, although the APC cleavage sites are Arg336 and Arg562. Complete inactivation of factor VIIIa is correlated with cleavage at Arg562. In the absence of PS (top), Arg336 is cleaved at a higher rate. In the presence of PS and fragments derived from the 'B' region of factor V, cleavage at Arg562 is accelerated and factor VIIIa is inactivated more rapidly.

27. Instead, the anticoagulant cofactor function of factor V is correlated to cleavage of intact factor V at Arg506 by APC 27. However, in normal physiological haemostasis, it has been suggested that inactivation of factor Va by the protein C pathway is much more effective in regulating thrombin formation than the inactivation of factor VIIIa 22.

The composition of membrane phospholipids influences the binding affinity of PS and modulates its anticoagulant activity. The presence of phosphatidylserine has long been known to be crucial for the protein C pathway either in the presence or absence of PS 28. Phosphatidylethanolamine 11,29, high density lipoprotein (HDL) 30 and glucosylceramide 31 all also seem to specifically augment PS cofactor function. Many studies have examined the influence of phospholipid composition on PS activity in model systems using synthetic phospholipids. Some have claimed that addition of glucosylceramide 31 and phosphatidylethanolamine 19 can enhance PS-dependent APC cleavage at both Arg506 and Arg306. However, further studies are still required to resolve the complex issue of the relationship between synthetic phospholipids utilised for *in vitro* experimentation and physiological sites of activity. In addition, such experiments are usually initiated by the addition of calcium ions, and may be influenced by the reported polymerisation of PS in the absence of these ions 32.

Recently, much effort has been made on the understanding of the role of each domain of PS on its activity. Studies using epitope mapping with monoclonal antibodies and site-directed mutagenesis have supported the existence of functionally crucial interactions between the TSR and EGF1 of PS with APC 33-35. Both approaches show that residues within these domains are crucial for the species dependence of this interaction 34. In addition, cleavage of the TSR of PS by thrombin (at Arg49 and
Arg70) or factor Xa (at Arg60) causes loss of APC cofactor function, as does removal of this domain from an engineered variant. Inhibition studies using *in vitro* expressed PS EGF-like domains have supported the role of EGF1 in APC binding. Chemically synthesised EGF1 can also induce a translocation of the APC active site relative to the phospholipid surface similarly to intact PS. Furthermore, several naturally occurring missense mutations affecting EGF1 have been described in association with qualitative PS deficiency. These are Thr103Asn and +5 G>A intron e, which results in skipping of either exon 5 or exons 5 and 6 (which code for EGFs 1 and 2, respectively). Indeed, a 3D structural model of the Gla, TSR and EGF1 of PS suggests that the Gla domain interacts with the membrane, leaving the latter two domains available to interact with membrane-bound APC.

The other EGF-like domains also seem to be involved in APC cofactor function of PS. A recent report has suggested a role for EGF2, since APC cofactor function was lost upon its replacement or deletion. This suggestion is supported by the association of qualitative PS deficiency with naturally-occurring mutations in EGF2 [Lys155Glu and Cys134Phe]. EGF4 of PS has also been suggested to be important to keep EGF1 in optimal alignment for interaction with APC, although a recombinant EGF4 alone has failed to exhibit APC cofactor activity in a clotting assay. The contribution of EGF4 to function is supported by the association of classic qualitative PS deficiency with the splice site mutation -2 G>A intron g, which causes deletion of the first two residues of EGF4 (Ile-Asp203-204). The contribution of EGF4 may be related to calcium binding. The carboxyterminal portion of PS is also involved in APC cofactor function, as factor Va binding site has recently been identified in the 2nd LGR. It is possible that this region could also interact with factor V during APC-mediated inactivation of factor VIIIa.

**The APC-independent anticoagulant functions of PS**

PS exhibits APC-independent anticoagulant activities *in vitro* by directly inhibiting both the prothrombinase and tenase complexes. The mechanisms for these actions are likely to be competition for protein and/or phospholipid binding sites and reversal of the protective effect that factor Xa exhibits in factor Va inactivation. PS has also been shown to accelerate APC mediated neutralisation of PAI-1 and therefore increase fibrin clot lysis. Furthermore, PS is reported to inhibit the activation of thrombin activatable fibrinolysis inhibitor, suggesting a potential role in regulating fibrinolysis at the early stages of clot formation. Although all these properties have been proposed as functions of PS, their relevance in physiological anticoagulation remains to be demonstrated.
Non-anticoagulant functions of PS

PS has been shown to behave as a weak mitogen and to associate with the surface of vascular cells \(^{61}\). It has been suggested that the mechanistic basis for mitogen activity is via the activation of Tyro3/Axl family of receptor tyrosine kinases, although the physiological relevance of these interactions is not yet clear. Recently, however, interest in them has been renewed because of a demonstrated \textit{in vivo} neuroprotective effect of PS. PS infused in a murine model of ischaemic stroke decreased motor neurological deficit, infarction and oedema volumes. These beneficial effects may have been partly mediated by anticoagulant mechanisms, but also by a direct neuronal protective effect \(^{62}\).

C4b-Binding Protein

C4BP is an important cofactor to the serine protease factor I (FI) in the degradation of C4b in the classic complement pathway \(^{63,64}\). C4b degradation in turn inhibits the formation of the C4b2a complex (C3 convertase) and accelerates its decay \(^{65}\), a key step in producing an inflammatory response \(^{66}\). C4BP is also a cofactor to FI in the cleavage of C3b. In humans, the physiological relevance of C4BP on C3b inactivation appears to be less than that of C4b inactivation, as factor H is known to be the main inhibitor of C3b in the fluid phase \(^{67}\). Furthermore, a 1,000-fold molar excess of C4BP over factor H is required for inactivation of C3b on the cell surface \(^{68}\). A recent report has indicated that C4BP is an ineffective cofactor for C4b degradation when it is present on the cell surface \(^{69}\), suggesting that it must play an early role before C4b is deposited on there. This could be important in avoiding deposition of inflammatory complexes on tissues and maintaining tolerance for self-antigens, thereby providing protection against inflammation and autoimmunity.

C4BP in human plasma (~200 mg/L) exists in several forms, which are formed by different combinations of its constituent \(\alpha\) and \(\beta\) chains \(^{70}\). C4BP contains either six or seven \(\alpha\) chains (expressed from \textit{C4BPA}) and either one or no \(\beta\) chain (expressed from \textit{C4BPB}), resulting in a spider-like structure held together by disulphide bonds (Figure 3). The \(\alpha\) and \(\beta\) chains of C4BP are composed of, respectively, eight and three 60 residue domains known as Sushi domains, short consensus repeats, or complement control protein (CCP) domains \(^{71}\). The \(\beta\) chain of C4BP contains the PS binding site and it therefore binds only to \(\beta\) chain-containing isoforms (known as C4BP\(\beta^+\)) \(^{72,74}\) (see below). In contrast, the \(\alpha\) chains of C4BP bind to several ligands (Figure 3). The binding site for C4b is contained in CCP1-3 of each of the \(\alpha\) chains, peripheral to the central core \(^{75}\). The interaction is ionic.
Several ligands bind C4BP, most of them to the α chains. It should be noted that while the binding sites are shown on a single chain, these will be replicated on all the other α chains. C4b and LRP bind to CCP1-3. C3b binds to either CCP1-4 or CCP1-5 (see text). The pathogens S. pyogenes, B. pertussis bind to CCP1-2 and E.coli OmpA to CCP3. Different coat proteins of N. gonorrhoeae bind to different regions of C4BP; pili binds to CCP1-2, whereas Por IA and Por IB bind to CCP1. SAP binds to the central core of the C4BP. In contrast, PS binds to CCP1 of the single β chain present in C4BPβ+. Abbreviations: PS, protein S; LRP, low density lipoprotein receptor-related protein; SAP, serum amyloid protein. This figure was prepared based on different sources, particularly 76.

in nature and mediated by a cluster of positively charged amino acids (Arg39, Lys63, Arg64 and His67) located in the interface between CCP1 and CCP2 of the α chain 76,77. The binding site for C3b has been mapped to CCP1-468 and CCP1-567 of the α chains (Figure 3).

Recently, there have been many reports concerning the binding of C4BP to pathogenic bacteria. Indeed, C4BP (mainly via its α chains) can be captured by many bacterial pathogens and the type of interaction can be either ionic (B.pertussis, N. gonorrhoeae pili and Por IB) or hydrophobic (S. pyogenes M protein, E.coli OmpA and N. gonorrhoeae Por IB) 76 (Figure 3). The physiological relevance of this interaction is not yet clear, but the binding to C4BP has been correlated with resistance to phagocytosis of S. pyogenes 78. If this is also the case for other pathogens, C4BP might have a major role in the phagocytosis process. Serum amyloid protein (SAP) component also binds to C4BP in the central core that links the 7 or 8 chains (Figure 3) and binding impairs its FI cofactor.
activity. SAP binds to macrophage receptors, neutrophils, immune complexes and seem to be involved in the opsonization process. Recently, it has been shown that a low density lipoprotein receptor-related protein (LRP) binds to CCP1-3 and mediates the catabolism of C4BP.

**PS binding to C4BPβ**

In human plasma, ~60% (~15mg/L) of total PS (TPS) circulates in complex with C4BP, whereas ~40% (~10mg/L) circulates in the free form. PS and C4BPβ form a 1:1 stoichiometric complex of high affinity (Kₐ ~0.1-0.6 nM), which is enhanced by calcium ions. PS binds close to the core of C4BP, to a large solvent-exposed hydrophobic patch in CCP1 (Figure 3) that involves residues Ile16, Val18, Val31 and Ile33, with secondary effects from Leu38 and Val39. Lys41 and Lys42, located close to the hydrophobic patch, contribute slightly to the interaction. CCP2 seems not to contribute to the binding, as previously thought, but is instead involved in the orientation and stabilisation of CCP1.

To date, the binding site for C4BP on PS has not been fully defined, but it does appear to be contained in the two carboxyterminal LGRs. There is experimental evidence to suggest that three different regions in these repeats are involved in the interaction. These include PS residues 420-434, 447-460 and 605-614. All of these three suggested sequences are predicted to be solvent-exposed, therefore all could potentially play a role in the interaction. Site directed mutagenesis of the 420-434 region has suggested a role for Lys423, Gln427 and Lys429. In contrast, site directed mutagenesis of residues in the 605-614 region do not generally support the suggested role from peptide inhibition studies, which can be themselves controversial. More recently, the region containing the residues 447-460, originally detected in phage display experiments, has been narrowed to residues 453-460, by an alanine scanning strategy. The role of calcium ions in increasing the affinity of PS and C4BPβ might be related to a predicted calcium binding pocket located in the first LGR of PS involving Asp292, Asp406 and Glu294. Indeed, a recombinant molecule consisting of both PS LGRs was recently shown to bind calcium ions.

**Functions of the PS-C4BPβ complex**

In normal human plasma, ~80% of C4BP is present as the C4BPβ form and this is almost entirely found in complex with PS, due to the very high affinity of the interaction. The binding of PS to C4BPβ abolishes the function of PS as a cofactor for APC in factor Va degradation. Furthermore, although complex formation does not appear to inhibit the mild PS enhancement of APC-dependent
inactivation of factor VIIIa, it does inhibit the PS/factor V synergistic effect. For these reasons, only the FPS fraction in plasma is generally considered to have active anticoagulant function. However, it should be noted that some of the APC-independent anticoagulant functions of PS are not inhibited by the PS-C4BPβ+ complex. For example, direct inhibition of the prothrombinase complex is not modulated by C4BPβ+, probably due to unaltered binding to prothrombin and factor Xa. Indeed, inhibition of the tenase complex is enhanced by the presence of C4BP, due to increased affinity of PS for factor VIII.

It has been proposed that the concentration of circulating FPS is determined by the concentration of C4BPβ+ and that this corresponds to the molar excess of PS over C4BPβ+, due to the high affinity of the interaction. C4BPβ+ expression is therefore tightly regulated to ensure near constant functional levels of FPS for adequate anticoagulation during physiological and pathological states. C4BP is an acute phase protein and its level in blood may increase up to 400% during the inflammatory response. However, this rise in level is largely due to an increase in the C4BPα form, as the concentration of the PS-C4BP complex is little altered.

Until recently, the physiological role of the PS-C4BP complex had remained uncertain. Some years ago, it was suggested that PS could act as a bridge between coagulation and inflammation, by localising C4BP to negatively-charged phospholipids and controlling complement proliferation at sites where the coagulation system was activated. Indeed, PS has been shown to enhance the binding of C4BP to neutrophil surface in the presence of calcium. PS in complex with C4BPβ+ retains the ability to bind to membrane surfaces, but not to platelet-derived microparticles. Two very recent reports have supported these early suggestions, and have proposed an important link between PS, the complement system and apoptosis (see below). It seems important that early complement activation occurs in order to promote prompt clearance of apoptotic cells by phagocytic macrophages. However, if the later cascade is activated, unwanted inflammation may occur around the apoptotic cell(s). Like coagulation, it seems that complement activation must be strictly localised and regulated for normal physiological function. If this regulation is disrupted, it is likely that the immune system will be exposed to intracellular components, which could result in excessive inflammation and autoimmunity. One example of this is the association of C1q deficiency with systemic lupus erythematosus (SLE). C1q is involved in the clearance and processing of self-antigens contained in surface blebs originated from apoptotic cells. The development of SLE could be related to impairment on clearance of self-antigens, which will chronically stimulate an autoimmune response.

One of the earliest stages of apoptosis is now known to be exposure of negatively charged phosphatidylserine on the outer leaflet of the plasma membrane, providing a potential site for PS and PS-C4BPβ+ complex binding. Using Jurkat cells as a model for anti-Fas antibody mediated...
induction of apoptosis, PS has been found to bind to apoptotic cells in a dose dependent and saturable manner (K_d ~200nM) \(^{104}\). PS binding, dependent upon its Gla domain, recruited C4BP specifically to the surface of apoptotic, but not live cells. C4BP on its own was found to contain no intrinsic ability to bind apoptotic cells. The cell surface PS-C4BP\(\beta^+\) complex was found to be able to bind to C4b, even in the presence of serum (containing SAP, which is a modulator of the C4b-C4BP interaction), strongly suggesting that the complex is biologically relevant. An independent experiment used BL-41 cells in a model of etoposide induced apoptosis in an \textit{in vitro} model of macrophage phagocytosis, utilising dual colour fluorescence labelling. PS was shown to be the factor responsible for the observed ~4-fold increase in phagocytosis of apoptotic cells associated with serum \(^{105}\). The binding of C4BP\(\beta^+\) to PS was not reported in this manuscript, and the observations were postulated to be due to tyrosine kinase stimulatory activity of PS \(^{106}\). However, complement proteins are also known to be important for phagocytosis of apoptotic cells \(^{105}\), and it can be speculated that the PS-C4BP\(\beta^+\) complex somehow acts to signal macrophage mediated phagocytosis of the apoptosing cell. A direct link between C4BP binding and the phagocytic process has not yet been demonstrated and this suggestion may be undermined somewhat by the observed resistance to phagocytosis found in pathogenic bacteria to which C4BP is bound \(^{78}\).

Subsequent to these studies, the above mentioned binding of PS to neutrophils has been shown to be apoptosis dependent, i.e. PS binds only to the apoptotic subpopulation of neutrophils via the exposed negatively charged phospholipid \(^{107}\). In addition, PS has been shown to reduce the number of cortical neurons in which apoptosis is induced after hypoxia/reoxygenation injury by ~70% \(^{62}\). It seems likely that PS would bind to these cells during apoptosis, via the same mechanism, although this was not examined directly.

**PS deficiency: what role for C4BP?**

PS deficiency can be either hereditary or acquired. Hereditary PS deficiency is a rare but serious autosomal dominant disorder, the manifestations of which are similar to those of the other coagulation inhibitors \(^{108}\). Individuals with heterozygous PS deficiency most commonly present with deep venous thrombosis (DVT), pulmonary embolism (PE) and superficial thrombophlebitis, the latter been observed in about 50% of patients with the deficiency. These manifestations account for ~90% of all thrombotic events and can require prolonged anticoagulation [for comprehensive reviews see \(^{109,110}\)]. Homozygous and compound heterozygous PS deficiencies are rarely reported \(^{111,112}\) and both are usually associated with severe \textit{purpura fulminans} in the neonatal period \(^{113}\). The prevalence of PS deficiency in the general population is uncertain, but is known to be low \(^{114}\). A study of 3,788 Scottish blood donors found PS deficiency to have a prevalence of between 0.03-0.13% \(^{115}\). In
familial VT, PS deficiency has been found in 2-10% of patients, the precise prevalence varying with the selection criteria 114,116,117.

An unresolved issue about PS deficiency concerns its precise associated risk of VT. The lifetime risk of developing thrombosis has been reported to vary between 5 to 11.5-fold in carriers of PS deficiency when compared with non-carrying family members 118-120. However, in a population-based, case-control study, PS deficiency was not found to be associated with increased risk 121,122, possibly due to the very low prevalence of the deficiency in this particular population. It has been suggested that the thrombotic risk associated with PS deficiency may vary according to underlying genetic defect 120,123. If this is the case, the risk will vary for different families carrying different mutations and this could possibly explain the clinical heterogeneity between families with PS deficiency. Indeed, preliminary data already suggest this to be the case 124. Several concomitant factors, either genetic or acquired, can increase the risk for thrombosis in PS-deficient individuals. These include oral contraceptives 125, the postpartum period (probably associated with reduced plasma PS levels in this period) 126, and the factor V Leiden 127-129 and prothrombin G20210A 130 mutations.

**Diagnosis, classification and molecular basis of hereditary PS deficiency**

There are technical and genetic uncertainties in the diagnosis of hereditary PS deficiency. The presence of both FPS and the PS-C4BPβ+ complex complicates antigenic and functional determination of PS. PS levels in plasma are subject to biological, physiological and pathological influences (see Table 1). There is a large overlap between PS levels in normal and in heterozygous individuals that fluctuate over time. Also, traditional assays for FPS are generally imprecise 131. Recently, a novel assay utilising the high affinity interaction between PS and C4BP (IL Test™ free PS), has proved to be precise and reliable 132. A recent report has described falsely high values for FPS at elevated assay temperatures, possibly due to a temperature-dependent increase in the dissociation of the PS-C4BPβ+ complex in PS-deficient samples 133. A detailed discussion of the diagnosis of PS deficiency has recently been presented elsewhere 134-135.

Unlike other coagulation factor deficiency states which are generally classified as type I (quantitative) and type II (qualitative), a second quantitative category, type III, was included to distinguish deficiencies with normal or reduced TPS levels (see below). It was recently proposed that types I and III deficiencies be grouped together as quantitative PS deficiency, since both present with low FPS plasma levels 8. Here, for practical reasons, we present the different quantitative phenotypes 8 according to the classification of Bertina, proposed at the International Society of Thrombosis and Haemostasis (ISTH) Subcommittee meeting in 1991.
Table 1. Biological factors influencing plasma PS levels

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<th>Biological factor</th>
<th>Parameter</th>
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<tr>
<td>Gender</td>
<td>Men have higher FPS and TPS than women</td>
<td>136-138</td>
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<tr>
<td>Age</td>
<td>Neonates have low TPS but high FPS (and PS activity) due to decreased levels of C4BP; elderly have higher TPS levels</td>
<td>137,139,140</td>
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<tr>
<td>Hormonal state</td>
<td>PS levels are decreased during pregnancy (particularly during weeks 18-28 of pregnancy) and in women taking oral contraceptives, in particular, desogestrel-containing preparations</td>
<td>136,141-143</td>
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<tr>
<td>Shared environment</td>
<td>This is predicted to have a strong effect on FPS levels</td>
<td>144</td>
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Mutations within PROS that are associated with PS deficiency are collected and published in an online database by the ISTH 145. This last version of this database lists 131 different detrimental mutations along with 30, apparently neutral, polymorphisms within PROS (Figure 1). According to the database, 95% of patients had a quantitative (type I or type III) and 5% had a qualitative (type II) deficiency. The vast majority of PS deficient individuals carry one PROS defect that effects a single nucleotide within the gene. However, eight individuals who are homozygous or compound heterozygous have been reported and three have been found to carry a large gene deletion (two unique events). Haplotype analysis of mutations in PROS has revealed that many of the families with the same mutation share a founder effect 146.

Type I and type III protein S deficiency

Type I and type III PS deficiencies both exhibit reduced FPS levels in plasma. Whilst TPS levels are reduced in type I, they are within the normal range in type III. Until relatively recently, the genetic basis for these deficiency subgroups had been uncertain. One possibility was that type III deficiency is caused by mutations leading to increased affinity of the resultant PS molecules for C4BPβ+ with consequent reduced levels of FPS. This notion appeared to be supported by observations that mutations within exons 12, 13 and 14 (encoding the LGRs that contain the C4BPβ+ binding site) seemed to be more prevalent in type III PS deficiency 147. A common sequence abnormality, Ser460Pro (commonly known as PS Heerlen), has been reported to have this effect 148, although this had been refuted by subsequent studies 149,150. Furthermore, no mutations in C4BPB could be identified in patients with PS Heerlen to explain the increased affinity 151. PS Heerlen occurs within
the consensus sequence for N-linked glycosylation of Asn458 and does cause loss of this modification
150,152. PS Heerlen is found in ~0.5% of the normal population and has been reported to be in
linkage dysequilibrium with type III PS deficiency 153. It now seems clear that heterozygous carriers of
the Heerlen variant have lower FPS levels when compared with their normal relatives 148,149. This
mild quantitative deficiency does not seem to be due to reduced secretion/synthesis since a
recombinant PS Heerlen was synthesised and secreted similarly to the wild type 149. An alternative
explanation relies on reduced half-life of the Heerlen variant in the circulation 149,154, as suggested
by decreased heat stability of this variant in vitro 149.

Type I and type III deficiencies may arise more commonly from acquired biological influences. It has
been suggested that they are phenotypic variants of the same genetic disorder, as both phenotypes
were found to coexist in the same families 123,148,155-157. In one of these families, deficient
individuals were subsequently found to carry the same causative mutation and the change in
phenotype (type I to type III) was shown to be due to an age related increase in TPS levels 140. The
mutation present in this family (Gly295Val) was later shown to completely abrogate PS expression
from the affect allele 123. This demonstrated that the wide range of biological influences on TPS
levels (and therefore on the classification, see Table 1) are strong determinants of the PS deficiency
phenotype. Indeed, it is suggested by leading coagulation laboratories that separate normal ranges
should be established for men, pre- and postmenopausal women 135. It should however, be noted
that there are some cases where a genetic mechanism explains the familial coexistence of the
variable phenotype, via a gene dosage effect. In a PS-deficient family that carries the Arg520Gly
mutation, individuals exhibit either type I or type III PS deficiency while in the homozygous or
heterozygous state, respectively 158. This effect has also been reported with PS Heerlen 149,154.

Since the underlying genetic cause of quantitative PS deficiency is highly variable (Figure 1A),
individual PROS defects may give rise to different PS plasma levels. In general, splice site and major
structural defects have been shown to result in lower levels of FPS and TPS compared to missense
defects 120. However, the effect may be even greater than predicted by this study, as it is now known
that some missense mutations are also associated with severe secretion defects and low levels of
plasma FPS and TPS 123,159. It seems that the location, conservation and physical properties of the
substituted residue play a role in the magnitude of the defect. For instance a Val-24Glu mutation,
severely disrupting the hydrophobic region of the signal peptide of PS, is expressed at very low levels
due to drastic intracellular degradation. In contrast, the mutation Arg49Cys, located at a non-essential
residue for the folding of the aminoterminal region of PS, results only in a mild secretion defect 123. It
is interesting to note that, in two studies, the severity of the secretion defect more closely reflected the
FPS, rather than the TPS plasma levels in individuals who were heterozygous for specific mutations
123,159. This was the case even considering that FPS levels were always below the lower limit of the
normal range. The mechanism behind this observation has not been demonstrated, but it is likely to
result from the dependence of FPS levels on the molar excess of TPS over C4BPβ+. In severe PS defects, TPS levels and C4BPβ+ levels will approximate to one another and FPS levels will be severely reduced.

Taken together, currently available data suggest that C4BP exerts no direct influence in determining the phenotype (type I and type III) of inherited PS deficiency. Instead, the variable phenotype mostly arises because of acquired influences on plasma PS levels, such as biological factors (age, gender, hormonal levels) in the healthy control population used to define the normal range and the physiological status of the individual being tested. In a minority of cases, gene dosage effects of defective PROS alleles also play a role. Whether the acquired influences might be partly influenced by variations in C4BP expression remains to be determined. It is interesting to note that in a recent study, chromosomal region 1q32 (containing both C4BPA and C4BPB) was found to be in genetic linkage with normal FPS levels in plasma 160.

**Acquired PS deficiency**

Several pathological states are associated with an acquired deficiency of PS and these are also generally associated with increased thrombotic events. These include nephrotic syndrome, disseminated intravascular coagulation, liver disease, and the use of drugs such as oral anticoagulants (warfarin) and L-asparaginase 147,161. Autoimmune PS deficiency can also develop following an infection (such as chickenpox and HIV) 162-164 or in association with multiple myeloma 165. Here, skin necrosis and *purpura fulminans* are severe manifestations and begin 7-10 days after the onset of the precipitating infection. The antibodies, which are IgG subclass and are independent of the anti-phospholipid antibodies, inhibit PS activity and persist for about three months 166. Autoantibodies against PS can also be found in antiphospholipid syndrome, and these seem to be associated with increased thrombotic risk when in combination with antibodies against β2-glycoprotein I 167. Indeed, anti-PS antibodies and acquired PS deficiency are common findings in SLE 168 and a report has suggested that acquired PS deficiency may provoke a hyperinflammatory response 169.

**Future perspectives**

The recent findings establishing an important role for the PS-C4BPβ+ complex in apoptosis suggests that the clinical manifestations of PS deficiency might be more diverse than previously thought. In hereditary PS deficiency, where the severity of the PS defect is anticipated to reflect FPS rather than TPS, this might suggest a requirement to maintain appropriate levels of the PS-C4BPβ+ complex. If so, what are the consequences of reduced levels of the complex? Is there an excess of autoimmune disease and/or inflammation in the affected individuals? 105. In this context, it is interesting to note
that superficial thrombophlebitis has an inflammatory contribution that might be explained by this new function of PS. The immediate clinical consequences of homozygous PS deficiency are dramatic and may obscure underlying autoimmune or inflammatory sequelae. The new findings also predict that the acquired PS deficiency observed frequently in SLE could be an important modulator of autoimmunity in this debilitating illness. If PS levels are substantially decreased, the level of the PS-C4BPβ+ complex might also be affected, potentially reducing PS-C4BPβ+ binding to the surface of apoptotic cells. This could result in reduced efficiency of cell removal by phagocytic macrophages, and the generation (for example) of anti-DNA antibodies resulting in autoimmunity. The reported mitogenic activity of PS towards vascular smooth muscle cells 170 will also have to be re-examined in the light of the finding that PS exerts a neuroprotective effect in murine models 62. Indeed, the latter observation suggests a possible role of PS as a therapeutic agent with combined anticoagulant and cell protective effects. It is intriguing to speculate how the neuroprotective effects might be modulated by complex formation with C4BPβ+.

These newly identified roles for PS and the PS-C4BP complex have exploded the concept that only FPS is the relevant portion in human plasma. Coagulation/anticoagulation is now suggested not to occur in isolation from inflammatory and apoptotic pathways. Indeed, APC has also emerged as an important modulator of these functions, again independently of its anticoagulant role 171. It appears that we have just begun to realise the full potential of PS and C4BP to regulate a wide range of pathophysiological conditions.
References


Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex

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