Anticoagulant protein S – new insights on interactions and functions

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Summary

Protein S is a critical regulator of coagulation that functions as a cofactor for the activated protein C (APC) and tissue factor pathway inhibitor (TFPI) pathways. It also has direct anticoagulant functions, inhibiting the intrinsic tenase and prothrombinase complexes. Through these functions, protein S regulates coagulation during both its initiation and its propagation phases. The importance of protein S in haemostatic regulation is apparent from the strong association between protein S deficiencies and increased risk for venous thrombosis. This is most likely because both APC and TFPIα are inefficient anticoagulants in the absence of any cofactors. The detailed molecular mechanisms involved in protein S cofactor functions remain to be fully clarified. However, recent advances in the field have greatly improved our understanding of these functions. Evidence suggest that protein S anticoagulant properties often depend on the presence of synergistic cofactors and the formation of multicomponent complexes on negatively charged phospholipid surfaces. Their high affinity binding to negatively charged phospholipids helps bringing the anticoagulant proteins to the membranes, resulting in efficient and targeted regulation of coagulation. In this review, we provide an update on protein S and how it functions as a critical haemostatic regulator.

Key words: anticoagulant, APC, factor V, protein S, TFPI

Introduction

Protein S was first isolated and characterised in 1977 and since then it has been suggested to have important functions in a range of processes [1]. These include participation in regulation of coagulation, cell proliferation, apoptosis, and regulation of inflammation, atherosclerosis and vasculogenesis [2, 3]. For many years though, it was mainly considered important as a cofactor in the activated protein C (APC) pathway, where it enhances the ability of APC to inactivate coagulation factors (F) Va and VIIIa (**Figure 1**) [4, 5]. However, protein S was relatively soon discovered to also have important APC independent anticoagulant functions through directly inhibiting the intrinsic tenase and prothrombinase complexes [6-8]. This was followed by the discovery of protein S functioning as a cofactor in the tissue factor pathway inhibitor (TFPI) pathway, in which it enhances both the inhibition of FXa generation by TF-FVIIa and the direct inhibition of FXa activity by TFPIα [9, 10]. Both APC and TFPIα are highly dependent on protein S for efficient regulation of coagulation. The clinical importance of protein S is evident by the increased thrombotic risk associated with all types of its deficiency [11]. Homozygous mutations leading to severe protein S deficiency are rare but extreme examples which are associated with life-threatening purpura fulminans [2]. The pathological importance of protein S deficiency has also been shown in mouse models where complete deletion of the protein S gene results in death in utero from coagulopathy and haemorrhages [12, 13]. Furthermore, platelet protein S was recently shown to regulate fibrin deposition in a growing thrombus at low but not at high shear stress *in vivo*, suggesting that platelet protein S limits venous but not arterial thrombus growth [14]. More than 40 years after protein S was first described, we are still learning how it functions and its multiple roles as an anticoagulant protein. In this review we will describe the anticoagulant roles of protein S, and provide an update on the molecular mechanisms involved.

Protein S – the protein

Protein S is a 73kDa vitamin K-dependent glycoprotein, circulating at ~20-25 μg/ml (300-350nM) with approximately 2.5% of circulating protein S being stored in α-granules of platelets [3, 14]. It consists of an N-terminal Gla domain, a thrombin sensitive region (TSR), four EGF-like domains and a sex hormone-binding globulin (SHBG)-like region which is

constituted of two laminin G-type (LG) domains (**Figure 2**). Most of the domains are involved in various protein-protein interactions, all of which are critical for the various roles of protein S. One particularly important aspect for the anticoagulant properties of protein S is its ability to bind to negatively charged phospholipids membranes. The interaction with membrane surfaces is mediated by the Gla-domain which contains 11 Y-carboxy-glutamic acid residues (Gla) [15]. The TSR region of protein S is a loop, located between the Gla-domain and EGF1, and owes its name to its susceptibility to thrombin and FXa cleavage [3]. Unlike the Gla-domain, the TSR is not directly involved in phospholipid binding. However, it has been shown to stabilise the structure of the Gla-domain and to increase its ability to bind Ca^{2+} and therefore also the affinity of protein S for negatively charged membranes [16]. While it is known that proteolysis of the TSR leads to a reduction in the APC cofactor function [17], the importance of its structural integrity for the TFPI cofactor function of protein S has yet to be defined. Due to the roles of protein S Gla and TSR domains in the binding of protein S to phospholipid membranes, they are essential for its overall functions. Many of the protein-protein interactions which are involved in the more specific anticoagulant roles of protein S are located in its other domains and are described in the following sections.

Protein S and C4BP

Approximately 60% of plasma protein S circulates as part of a non-covalent high-affinity complex with C4b-binding protein (C4BP). It is mainly the free proportion of protein S (~40%) that is considered to have full anticoagulant properties [18, 19]. The protein S binding site is localized in the β-chain of C4BP and the two proteins circulate in a 1:1 molar ratio with an estimated K_d of ~0.1nM [20]. Protein S is known to interact with specific residues in the most N-terminal complement control protein domain (CCP1) of the β-chain, with also some contribution of CCP2 [21-23]. Several studies have addressed the C4BP interaction site in protein S. While the specific residues involved are not known in detail, potential regions have been suggested. We know that the interaction site is located within the SHBG-like region, with both LG1 and LG2 contributing to the interaction (**Figure 2**) [24-28]. While we still do not know the function of the protein S-C4BP complex, it has been suggested that protein S may play a role in regulation of the complement system by causing the recruitment of C4BP to negatively

charged phospholipids present on the surface of apoptotic cells, activated platelets and microparticles [29, 30].

APC cofactor function

APC is a serine protease that down-regulates thrombin generation in the propagation phase of coagulation. This is achieved through inactivation of FVa and FVIIIa, two important cofactors in the prothrombinase and intrinsic tenase complexes, respectively [31]. Protein S acts as a cofactor for both APC-mediated proteolytic reactions and is essential for efficient APC dependent regulation of coagulation [4, 32].

FV consists of six domains; A1-A2-B-A3-C1-C2 and is circulating at ~20 nM [33]. Like protein S, FV is present in plasma as well as in platelet alpha granules and, unlike the plasma levels, several studies have suggested a correlation between the levels of protein S and FV in platelets [34]. When FV is activated by thrombin, the whole B-domain is excised, leading to exposure of the FXa interaction site and the formation of the prothrombinase complex [35]. APC inactivates FVa by proteolytic cleavages at three sites in its A2 domain, Arg306, Arg506 and Arg679, with the Arg306 and Arg506 cleavages being considered the most physiologically relevant. The cleavages at Arg306 and Arg506 have both been extensively studied by multiple groups [36-40]. It is now well known that FVa is inactivated by rapid cleavage at Arg506, followed by slow cleavage at Arg306. However, the cleavages not only differ in rate, but also in magnitude of protein S enhancement. Protein S increases the rate of proteolysis at Arg306 by 20-30 fold compared to a relatively modest (1-5 fold) enhancement of Arg506 cleavage [39, 41]. Despite detailed knowledge of how protein S influences the APC-mediated FVa cleavage rates, the molecular mechanisms of how rate enhancements are achieved is only partially understood. This poor understanding is mainly due to the phospholipid dependence of all interactions involved in the pathway. There are currently several hypotheses for protein S enhancement of the efficiency of APC. It has been suggested that binding of protein S to APC triggers an allosteric conformational change, which relocates the active site of APC closer to the phospholipid membrane and therefore also into closer proximity to the Arg306 cleavage site in FVa [42]. It has also been proposed that protein S removes the ability of FXa to protect FVa from APC-mediated inactivation [41, 43]. However, in contrast to FXa,

prothrombin protects FVa against APC-mediated proteolysis also in the presence of protein S. In fact, the protective effect was stronger in its presence than in its absence [44]. Combined, these studies suggest a complex mechanism, where protein S may help overcome the ability of FXa to protect FVa from proteolysis only once the local prothrombin levels are reduced, such as during ongoing coagulation. One widely accepted hypothesis is that protein S increases APC affinity to negatively charged phospholipid surfaces. This was initially shown by Walker *et al.,* who demonstrated that APC binds to phospholipid surfaces with 10-fold higher affinity in the presence of protein S than in its absence [32]. This was followed by a study by Smirnov *et al.* who reported that APC binding to phospholipids was enhanced more efficiently by the presence of both FVa and protein S, than by protein S alone [45]. Building on these studies, we recently attempted to elucidate the nature of the phospholipid-dependent interactions among APC, protein S and FVa. Using a flow cytometry approach and the use of fluorescently labelled/inactive APC, our study confirmed that FVa is involved in the recruitment of APC to the phospholipid surfaces [46]. While protein S enhanced the binding of APC to negatively charged membranes by a modest ~2-fold, the affinity was enhanced >14-fold in the presence of both protein S and FVa (**Figure 3**). FVa, in the absence of protein S, did not enhance APC binding to the membranes, pointing to synergistic enhancement by protein S and FVa. This latter study demonstrated directly the formation of an APC-protein S-FVa inactivation complex. It also emphasised that the enhancement by protein S alone is too small to account for the full enhancement by protein S observed in functional assays, and illustrated how FVa, therefore, plays a central role in the formation of its own inactivation complex.

It is well known that C4BP-bound protein S is an inefficient cofactor of APC [18, 19]. According to Maurissen *et al.*, C4BP-bound protein S enhanced APC-mediated FVa proteolysis at Arg306 ~10-fold (compared to 20-30 fold by free protein S), while it inhibited the Arg506 cleavage by 3-4 fold. Together, this results in an overall 6-8 fold reduction in enhancement of APC-mediated inactivation of FVa by C4BP-bound compared to free protein S [19]. This could be explained by the results presented in our study, which showed that C4BP binding reduced the ability of protein S to be incorporated into the tri-molecular FVa inactivation complex. The SHBG-like region, which interacts with C4BP, has previously been suggested to be important for the enhancement of APC and to contain an interaction site for FVa (**Figure 2**) [47-49]. It is therefore possible that C4BP blocks a protein S-FVa interaction site.

Over the years, many studies have attempted to determine the various interaction sites involved in the APC cofactor function of protein S. These have concluded that the membrane proximal domains (Gla-TSR-EGF1-EGF2) in protein S are involved in its APC cofactor function (**Figure 2**) [15, 50-52] . These domains have also been suggested to be responsible for a direct interaction with APC [53, 54]. Similarly, APC Gla domain residues Asp36, Leu38 and Ala39, have been shown to be critical for its ability to be enhanced by protein S [55]. Previous studies from our lab have identified protein S residues Gla36 and Asp95 in the protein S Gla and EGF1 domains, respectively, to be important for the ability of protein S to enhance APC (**Figure 2**) [50, 51]. Our recent study showed that this is due to its involvement in the formation of the complex with APC and FVa. Furthermore, our results suggested that both residues may be involved in the interaction with FVa. This is supported by a paper published by Heeb *et al*. who identified protein S residues 37-50 as a potential binding site for FVa [56].

FVIII shares the same domain organisation as FV (A1-A2-B-A3-C1-C2), and like FV it also loses its B-domain upon activation by thrombin. However, unlike FV, the FVIII B domain is not responsible for maintaining its procofactor state. Instead, cleavage at Arg372 is necessary for FVIII to gain cofactor activity [57]. FVIIIa inactivation is achieved by APC proteolytic cleavage at Arg336 and Arg562 within A2. Since FVIIIa can be inactivated by spontaneous dissociation of the A2 domain, APC-mediated inactivation is believed to be physiologically relevant mainly for FVIIIa in the tenase complex [58, 59]. As mentioned before, protein S functions as a cofactor for APC-mediated inactivation of FVIIIa [5]. This enhancement is relatively moderate (1.5 fold), compared to that of FVa inactivation [60]. However, FV, in its procofactor form, has been shown to act as a synergistic cofactor, together with protein S, for APC-mediated inactivation of FVIIIa [60, 61]. Together, these two cofactors enhance FVIIIa proteolysis by APC ~11-fold, with cleavage at Arg 336 being stimulated 3-5 fold and Arg562 cleavage by 7-8 fold [60]. This cofactor function of FV was shown to be stimulated by APC-mediated cleavage at Arg506 [62]. In contrast to APC-mediated FVa inactivation, where protein S Gla-EGF2 domains have been shown to be of most importance, the SHBG-like region of protein S appears crucial for expression of its synergistic APC cofactor activity in FVIIIa inactivation, acting together with FV [48]. This is of particular interest since a FV interaction site has been identified within this region [49]. However, the molecular mechanisms involved in protein S enhancement of FVIIIa inactivation remains poorly defined. It has been suggested that the

roles of protein S in FVIIIa and in FVa inactivation differ [59]. Whether protein S and FV play similar roles in the APC-mediated inactivation of FVIIIa to that of FVa inactivation still remains to be investigated.

The most common genetic risk factor for venous thrombosis among caucasians is FV Leiden, which is caused by a single point mutation, resulting in substitution of Arg506 to a glutamine [31, 63]. A recently described FV mutation is FV Nara [64]. FV Nara is also caused by a point mutation, but this one is located within the FV C1 domain (FV W1920R). This rare mutation is also thought to cause increased risk of venous thrombosis. When it was first described [64], the authors showed that FVa Nara was less efficient in enhancing prothrombin activation by FXa compared to wild type FVa. However, while the procoagulant functions of FVa Nara were decreased, the inactivation of FV/FVa by APC was even more so. Specifically, Arg306 cleavage was severely reduced. In a recent follow-up study [46], we showed that this was in part due to reduced affinity towards negatively charged phospholipid membranes. Using detailed biochemical characterisation our results also suggested that the FV Nara mutation resulted in reduced binding to protein S and, as a consequence, also reduced its ability to be incorporated into the FVa inactivation complex, together with APC and protein S.

TFPI cofactor function

Protein S also functions as a cofactor in the TFPI pathway, regulating of the extrinsic pathway of coagulation. TFPI inhibits FXa directly as well as the activation of FIX and FX through the formation of a quaternary complex involving TF/FVIIa/FXa/TFPI. It is produced as two major isoforms, TFPIα and TFPIβ. The majority is bound to the endothelium (TFPIβ) whereas the most often studied isoform circulates in a soluble form, either freely or packaged inside platelets and released once platelets are activated (TFPIα) [65, 66]. TFPIα consists of an acidic amino-terminal polypeptide, followed by three tandem Kunitz-type domains (Kunitz domains 1, 2 and 3) and a basic carboxy (C)-terminal tail. In TFPIβ the Kunitz domain 3 and basic C-terminus of TFPIα are replaced with a glycosylphosphatidylinositol (GPI)-anchor addition sequence, which localizes TFPIβ to the cell surface [65]. Protein S functions as a cofactor for TFPIα, both that present in plasma and that released by platelets. In contrast, it has no enhancing effect on TFPIβ [9, 67]. Through kinetic characterisation it has been shown that

protein S enhances the direct inhibition of FXa activity by TFPIα by approximately 4-10 fold [9, 68, 69]. This is essential, because out of the 1.4-2.5nM total TFPI in plasma, only ~10-20% is the full-length TFPIα form with anticoagulant activity [34, 65, 70]. Through its cofactor function, protein S therefore reduces the inhibition constant of TFPIα for FXa to below the TFPIα plasma concentration, which enables it to bind its substrate. Whether or not protein S also functions as a cofactor for TFPIα in the inhibition by TF-FVIIa has been more difficult to determine [9, 71]. However, recently Peraramelli *et al*. established that this is the case and showed that protein S enhances the inhibition of TF-FVIIa mediated FXa generation, as well as the FXa dependent inhibition of FIXa generation, by ~9-fold [10].

The plasma concentration of TFPIα is associated with that of protein S [34]. Individuals with quantitative protein S deficiency also have reduced circulating TFPIα levels [72]. This association has been shown to be due to TFPIα and protein S interacting in circulation [72], which also suggests that the two proteins interact with high affinity. However, the dissociation constant for the TFPIα-protein S complex has been estimated to ~1μM in direct binding assays [68, 72-74]. The cause of the discrepancy between these observations is not clear, but it is possible that another factor may be involved in the interaction in circulation, which increases their affinity in solution.

For many years, the role of TFPIα Kunitz domain 3, remained unclear. However, once the TFPI cofactor function of protein S was discovered it only took a few years before it was established that this domain contains the interaction site for protein S [75]. Through our own work and that of Ndonwi and colleagues we now know that protein S interacts with TFPIα Kunitz domain 3 residues Arg199, Glu226, Glu234 and Arg237, with Arg199 and Glu226 being most important [68, 75]. Following on from these findings, we showed that the complementary interaction site for TFPIα is located within the LG1 domain of the SHBG-like region of protein S (**Figure 2**) [74, 76]. The same domains are therefore involved in both TFPIα and C4BP interaction. As a consequence, C4BP-bound protein S shows limited ability to function as a cofactor for TFPIα [74]. Whether the reduced cofactor function is due to both proteins sharing the same interaction site, or whether C4BP is sterically hindering TFPIα binding is currently not known.

From these studies, it is evident that a direct TFPIα-protein S interaction is needed for the enhanced inhibition of FXa to occur. However, the molecular mechanism underlying the TFPI cofactor function of protein S is still not clarified. TFPIα inhibits and interacts with FXa in a two-step process. This occurs through an initial fast interaction, forming a TFPIα-FXa encounter complex, followed by a slow isomerisation and the formation of the tight inhibitory complex [77]. Protein S enhances TFPIα mainly in the formation of the encounter complex [9, 68]. In contrast to TFPIβ, which is bound to the endothelial surface through its GPI-anchor, TFPIα only binds to phospholipid membranes with very low affinity via its C-terminal tail [78, 79]. Wood *et al*. generated a mutant TFPI, containing all 3 Kunitz domains (K1K2K3) whilst being cell surface-bound through a GPI-anchor. Like TFPIβ, this mutant was not enhanced by protein S [80]. These results suggest that protein S is not needed as a cofactor for cell-surface associated TFPI in the inhibition of FXa, independent on whether it contains the protein S interaction site or not. Together, these results strongly suggest that the role of protein S is to bring TFPIα into proximity of the active site of the protease domain of FXa on the activated phospholipid surface, thereby decreasing the concentration of TFPIα needed for efficient inhibition of FXa (**Figure 4**) [9, 67, 68, 73, 74].

TFPI cofactor function and FV

In recent years it has become clear that protein S is not the only cofactor for TFPIα. Factor V has been shown, by us and others, to play a role within the TFPI pathway [69, 81-83]. As mentioned above, FV is a coagulation factor with both pro- and anticoagulant functions. In its activated form (FVa) it functions as a cofactor for FXa by enhancing the activation of prothrombin to thrombin by 5 orders of magnitude [33]. In its procofactor form, FV enhances the APC-mediated inactivation of FVIIIa by functioning as a cofactor together with protein S [60, 61, 84]. Despite its important role in the prothrombinase complex, FV deficiency in humans is associated with only mild bleeding. This paradox is explained by a concomitant reduction of TFPIα levels [85]. The link between FV and TFPIα levels has been suggested to be caused by a direct interaction in circulation, leading to reduced clearance of TFPIα [83]. Due to the direct binding of TFPIα to FV, the functional role of FV in the TFPIα-mediated inhibition of FXa has been investigated by us and others [69, 81-83, 86]. It is now evident that protein S

and FV function are synergistic cofactors for TFPIα, leading to an overall ~12-fold enhancement [69]. In addition to inhibiting FXa, protein S and FV were also able to synergistically enhance the inhibition of prothrombinase formation by TFPIα. The synergistic cofactor function was completely dependent upon the TFPIα-protein S interaction [69]. TFPIα has also been shown to inhibit the prothrombinase complex through a direct interaction with partially activated FV, thereby slowing down the initiation phase of coagulation [81]. Exactly what role protein S plays in this process still remains to be fully determined.

It has recently been found that differences in splicing of the FV transcript results in lower molecular weight isoforms of FV in circulation, so called FV-short. To date, two FV-short isoforms have been described, one that is upregulated in individuals suffering from East Texas bleeding disorder, and the very similar FV-short Amsterdam [83, 87]. Both are caused by mutations in the FV gene which transforms a weak into a strong splice site, resulting in increased abundance of the alternatively spliced transcript, FV-short [83]. In FV, two regions within the intact B-domain, called the acidic and basic regions, are interacting with high affinity and stabilises FV in a procofactor state [35]. In FV-short a large portion of the FV B-domain is absent, resulting in the acidic region being constitutively exposed in circulation. This is of particular importance since the acidic region, when exposed, binds the TFPIα C-terminus with high affinity (pM range) [81, 83]. As a result, in East Texas bleeding disorder the upregulation of FV-short leads to a ~10-fold increase in the level of TFPIα in plasma which is the indirect cause of the bleeding phenotype. An intriguing finding in this study is that FV-short also is present in plasma from healthy individuals [83]. It is present at approximately the same concentration as full-length TFPIα. It has therefore been suggested that FV-short may function as a carrier for TFPIα, regulating its plasma levels and half-life by reducing the rate of filtration through the kidneys [3]. However, due to lack of specific quantification methods for FV-short, this has not been formally demonstrated. How this new finding relates to the previous suggestions that protein S binds TFPIα in circulation is currently uncertain [72]. Whether TFPIα can interact with both FV-short and protein S simultaneously, and whether FV-short may increase the affinity of TFPIα towards protein S, still remains to be investigated.

Similar to our findings for FV described above, Dahlbäck *et al*. recently reported a synergistic cofactor function by FV-short and protein S for TFPIα [83, 88]. It appears as if the mechanism behind both cofactor functions are similar, albeit at least 100-fold more efficient for FV-short

than for FV [88]. However, the precise fold enhancement FV-short and protein S has on the affinity of TFPIα-FXa (K_i) remains to be determined. Once again, the synergistic cofactor function appears to be dependent upon a TFPIα-protein S interaction, since the lack of protein S SHBG-like region completely reversed the cofactor function [89]. It is therefore evident that the main effect of FV (and also suggested for FV-short) is to enhance the initial TFPIα-FXa encounter complex formation. Considering that TFPIα and FV/FV-short appear to be present as a complex in circulation, this is plausible. It is therefore likely that protein S and FV/FV-short act as synergistic cofactors, increasing the affinity of the TFPIα-FXa interaction by localising TFPIα in close proximity to FXa on the phospholipid membrane (**Figure 4**).

APC and TFPIα independent anticoagulant roles of protein S

In addition to being a cofactor for APC and TFPIα, protein S has been shown to directly inhibit prothrombinase and intrinsic tenase activities. These functions are independent of APC and TFPIα and are caused by direct interactions with FXa and FVa (prothrombinase) [6-8] as well as FIXa and FVIIIa (intrinsic tenase) [90, 91]. While some of these inhibitory effects have been attributed to multimerisation of protein S [92], these APC and TFPIα independent effects are still observed after this has been adjusted for. In contrast to the APC and TFPI cofactor functions of protein S, the APC and TFPIα independent anticoagulant functions of protein S were not affected by C4BP binding to protein S. The protein S-C4BP complex was shown to directly inhibit prothrombinase activity as efficiently as free protein S and has also been suggested to prolong clotting time even more efficiently than free protein S [8, 93].

The N-terminal domains of protein S have been suggested to be most important for the inhibition of prothrombinase, with residues in the Gla domain being involved in the interaction with FVa [56] and the TSR region being involved in the interaction with FXa [8]. In the direct inhibition of prothrombinase and FXa, retention of Zn^{2+} has been shown to be essential [94]. The inhibition of the intrinsic tenase by protein S has been suggested to be due to protein S directly impairing the assembly of the complex [91]. Recent work from Plautz *et al*. has investigated the protein S-FIXa interaction. Using immunoprecipitation they found that the interaction occurs in plasma and involves FIXa residues Lys126, Lys132 and Arg170 in its heparin-binding exosite [95]. Using a haemophilia B mouse model, they also showed that mice

infused with a variant FIXa, unable to interact with protein S, showed increased rate of thrombus formation compared to mice infused with WT FIXa. Together, these results suggest that protein S may maintain normal haemostasis by inhibiting FIXa [95].

Protein S in haemostatic regulation

With its multiple roles, protein S is a key regulator of coagulation. As mentioned above, deficiencies are strongly associated with DVT, highlighting its essential roles for maintaining a haemostatic balance. In a recent paper by Calzavarini *et al*., the authors specifically studied the functional importance of platelet protein S. To do this, they generated mice lacking protein S expression in platelets [14]. Using various thrombosis models, such as the $FeCl_3$ model, the authors showed that platelet protein S is regulating the fibrin deposition in the growing thrombus at low shear stress, such as in vena cava, but not at high shear, such as in the carotid artery. Furthermore, the authors showed that this effect was, at least in part, mediated through its APC and TFPI cofactor functions. Whether or not also the APC and TFPIα independent anticoagulant roles of protein S, such as FIXa binding and intrinsic tenase inhibition, contributes to these effects by platelet protein S is still not known. However, these results strongly suggest that not only protein S in circulation, but that also platelet protein S plays a critical role for maintaining a balanced haemostasis.

Due to its strong association with DVT, the role and therapeutic potential of protein S in haemophilia has been studied. Several therapies targeting TFPI are currently investigated in clinical trials [96]. However, targeting protein S may have advantages over TFPI, in that it has broader functional roles. Like TFPI, a therapy targeting protein S would be effective in the treatment of both haemophilia A and B. In a recent study by Prince *et al*., the authors showed that the lethal phenotype observed in protein S deficient mice can be rescued by a concomitant FVIII deficiency [97]. This suggests that the lack of anticoagulant regulation by protein S can be counterbalanced by a deficiency of FVIII to maintain a balanced haemostasis during embryonic development. Furthermore, genetically targeting protein S improved the bleeding tendency of FVIII deficient mice and protected them from hemarthrosis, a common bleeding complication among haemophilia patients [97]. This is of particular importance since the authors detected both protein S and TFPIα in synovial tissue from in these mice as well as

individuals with haemophilia A and B. Ellery *et al.* recently measured the concentrations of TFPIα, protein S and FV in platelets and in plasma of individuals with haemophilia. They showed that platelet TFPIα and FV levels were increased (21 and 50%, respectively) compared to healthy blood donors, while only minor differences were seen in plasma. In contrast, protein S levels were reduced both in plasma (33%) and in platelets (26%) [98]. Prince and colleagues showed that blocking protein S anticoagulant functions in plasma of individuals with haemophilia normalised their thrombin generation *in vitro* [97]. Together, these findings suggests that the reduction in protein S levels helps partially rebalance their coagulation. They also suggest that targeting protein S could promote haemostasis in individuals with haemophilia.

Conclusions and future perspectives

The clinically important role of protein S as an anticoagulant protein is well established. We know that it is essential as a cofactor for two of our anticoagulant pathways by enhancing APC and TFPIα functions. There is a clear pattern emerging with synergistic enhancement by protein S and various forms of FV playing roles in both the anticoagulant pathways (**Figures 3** and **4**). This is the case for FVa and protein S in the enhancement of APC binding to phospholipid membranes in the inactivation of FVa (**Figure 3**) [32, 45, 46], in the enhancement of FVIIIa inactivation by APC [5, 61] as well as for the synergistic enhancement of TFPIα in the direct inhibition of FXa by protein S together with both FV and FV-short (**Figure 4**) [69, 88, 89]. An essential feature of these anticoagulant pathways is the requirement for localisation to activated membrane surfaces. To facilitate this, multi-protein complexes form in both pathways, with mutually interacting proteins.

The recent discovery of FV-short adds intriguing complexity to the TFPI pathway. Understanding how FV-short modulates the multiple anticoagulant roles of protein S is a current challenge. Similarly, the specific roles of plasma and platelet protein S as regulators of thrombin generation within and outside the platelet thrombus still remains to be determined. Overall, further detailed molecular account of the many interactions involved in the various protein S functions. They could also provide the basis for understanding how

dynamic and flow conditions determine the functional and clinical effectiveness of protein S as a regulator of haemostasis.

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Figure 1

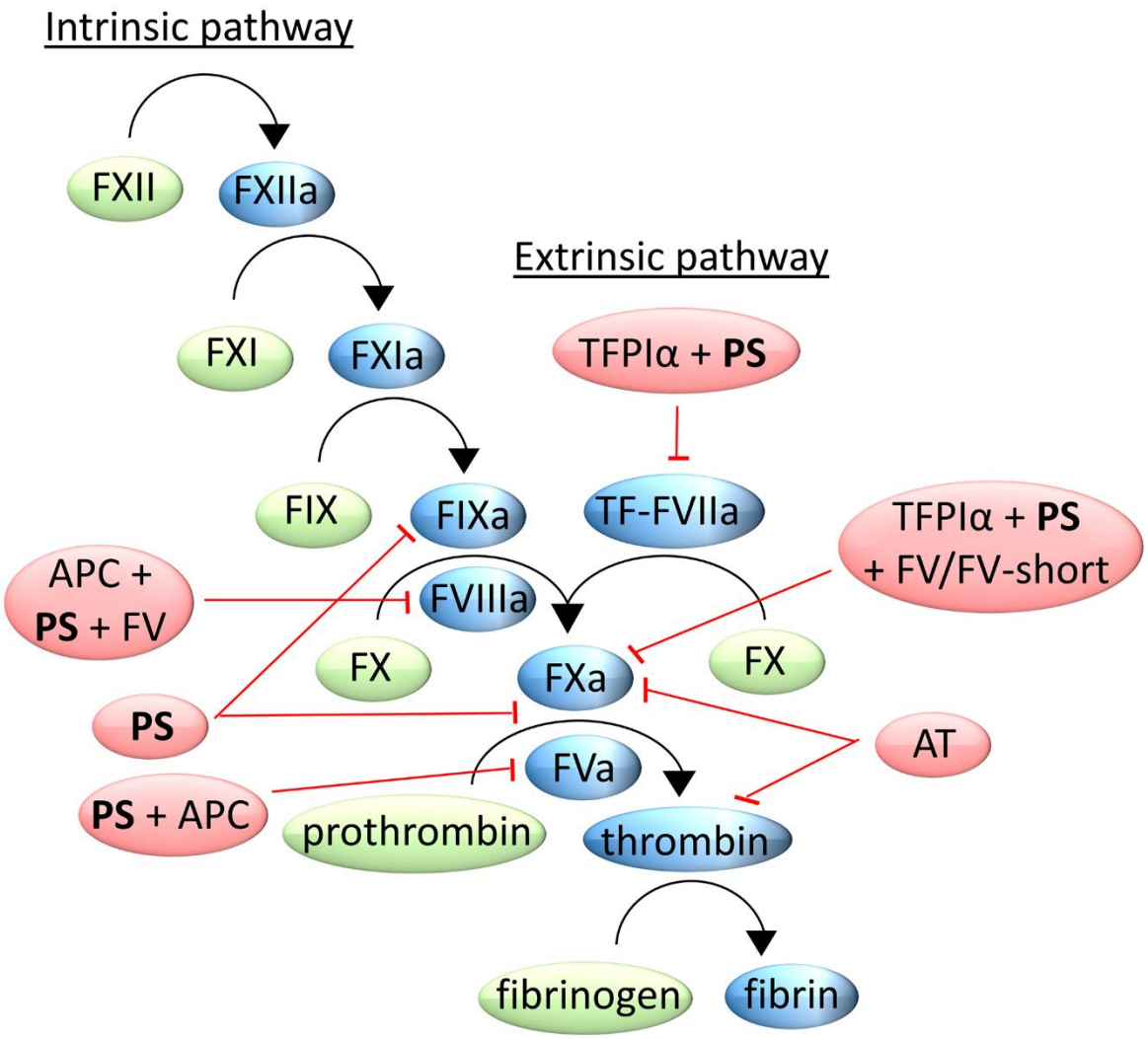

Figure 1. The multiple roles of protein S in the regulation of coagulation. Coagulation is regulated at different levels by three main anticoagulant pathways, namely the TFPI, APC and antithrombin (AT) pathways. Protein S (PS) is a critical multi-function anticoagulant regulator, acting as a cofactor for TFPIα and APC, in addition to having TFPIα and APC independent roles. Together, these functions allow protein S to regulate coagulation both during the initiation phase of the extrinsic pathway (TFPI cofactor function) as well as during the propagation phase (APC cofactor function and TFPIα and APC independent activities).

Figure 2

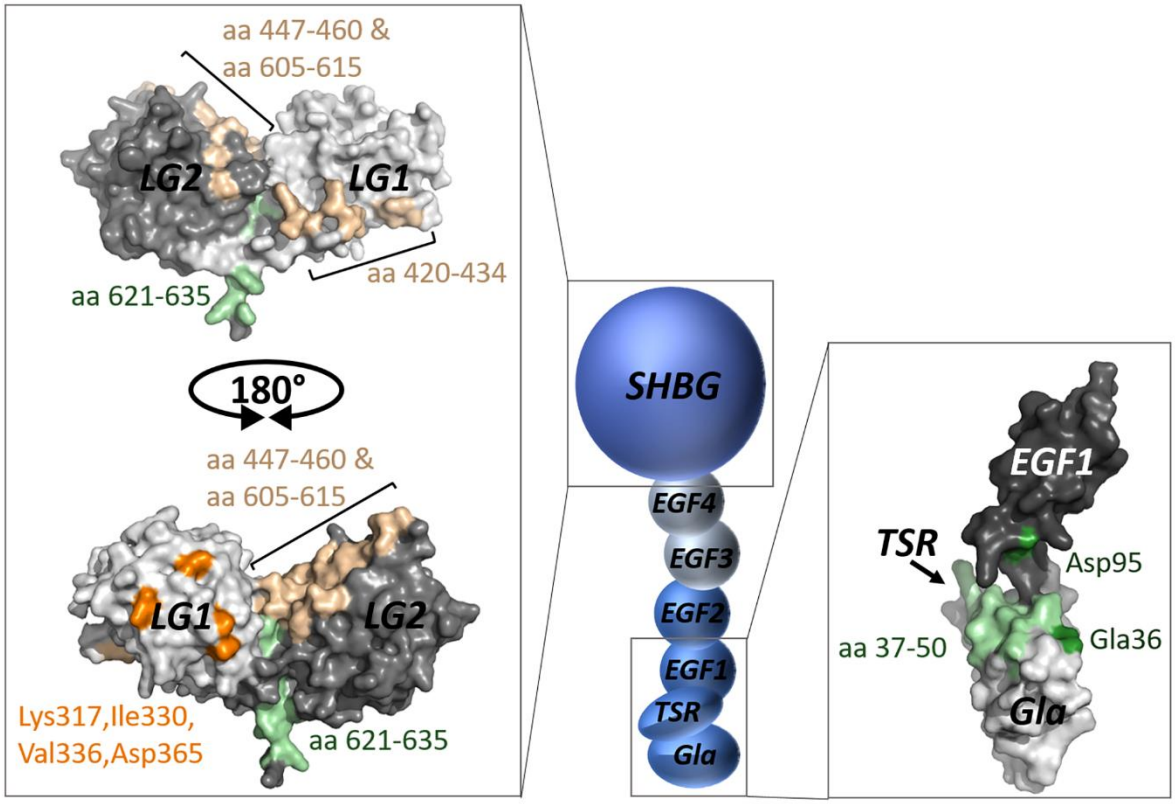

Figure 2. Protein-protein interaction sites of protein S involved in the APC and TFPI anticoagulant pathways. Interaction sites cluster around the protein S N-terminal Gla-TSR-EGF1-EGF2 domains and the C-terminal SHBG-like region (shown in blue in the cartoon model of protein S). The Gla-TSR-EGF1-EGF2 are known to be essential for the APC cofactor function of protein S and have also been suggested to contain the APC interaction site [16, 52, 53, 99]. Protein S Gla36 and Asp95 (dark green) are both needed for APC cofactor function and we recently hypothesised that they may be part of a FVa interaction site [46, 50, 51]. This is in agreement with a previously published study, identifying Gla-TSR residues 37-50 (light green) being involved in FVa binding [56]. The EGF2 domain has also been shown to be essential for the APC cofactor function of protein S, suggesting its involvement in important protein-protein interactions [52]. There is currently no model of EGF2 published. The TSR has been shown to be involved in the direct interaction with FXa [6, 8]. The SHBG-like region appears to be a critical region for various protein-protein interactions with protein S. The C4BP binding has been shown to involve both LG1 and LG2 domains, and more specifically amino acids (aa) 420-434, 447-460 and 605-615 (beige) [24-28]. These are partly overlapping with a suggested FVa interaction site, involving residues 621-635 (light green) [49]. More recently we have identified LG1 as being the domain responsible for TFPIα interaction [74], with contributions of amino acid residues K317, I330, V336 and D365 (orange) [76]. The models are adapted from Giri *et al*. and Villoutreix *et al.* [16, 100].

Figure 3

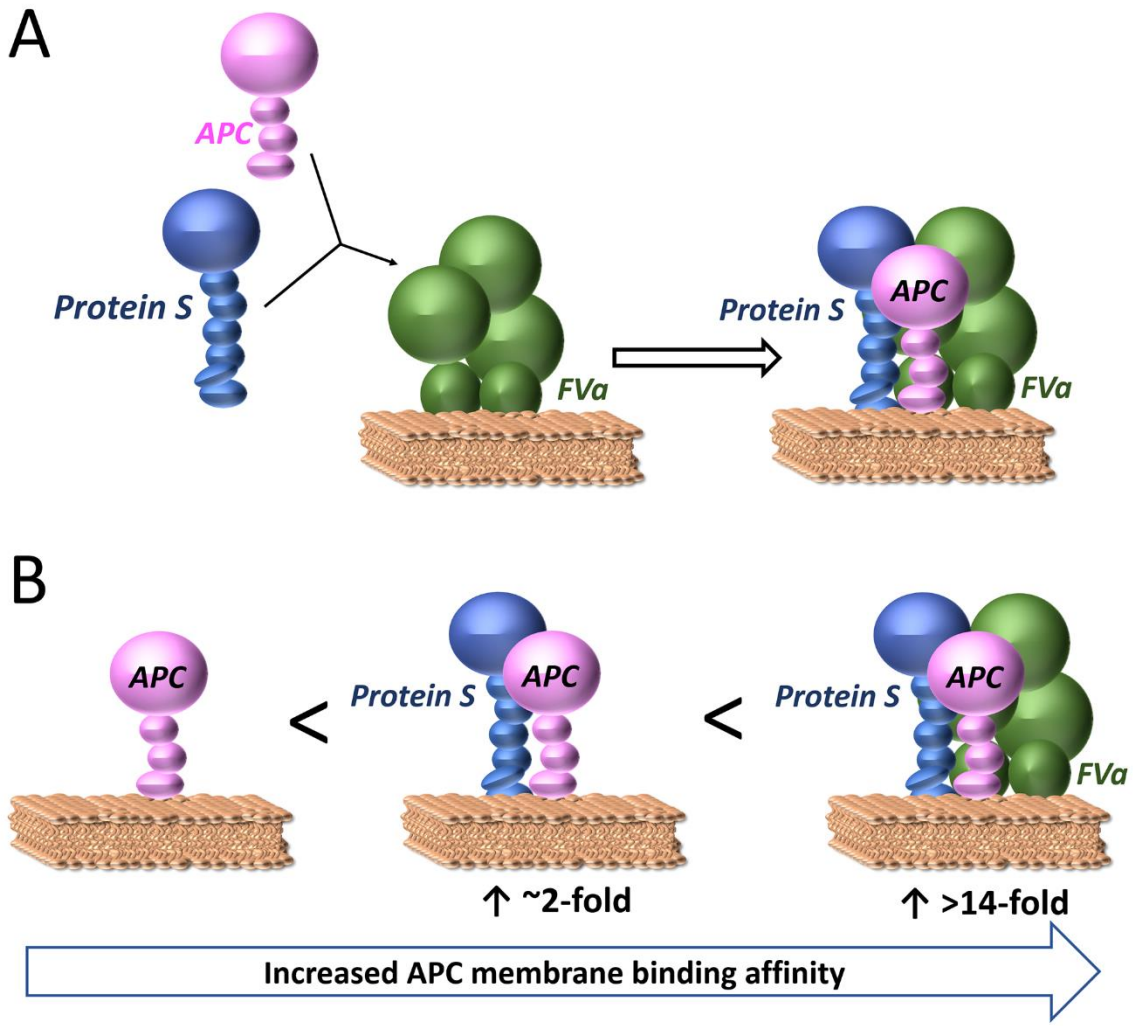

Figure 3. Molecular mechanism involved in the APC cofactor function of protein S for inactivation of FVa. (A) Several studies have shown that protein S and FVa enhance APC binding to a negatively charged phospholipid membrane, where it forms a FVa inactivation complex [32, 45, 46]. (B) We recently showed that protein S enhances the APC affinity for phospholipid membranes by ~2-fold while protein S and FVa synergistically enhances the APC-phospholipid binding by >14-fold [46].

Figure 4

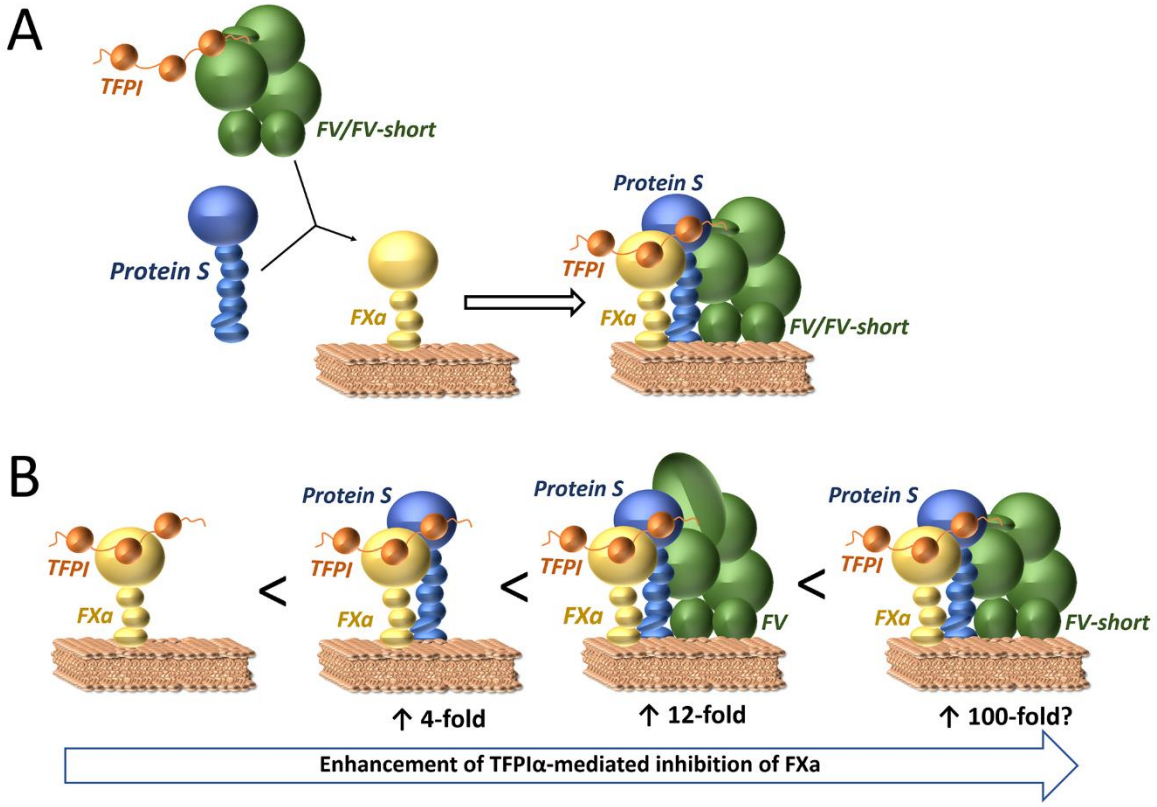

Figure 4. Molecular mechanism involved in the TFPI cofactor function of protein S for the direct inhibition of FXa. (A) Several studies have suggested that protein S functions as a cofactor for TFPIα by bringing it to the negatively charged phospholipid membrane, where it increases the binding and inhibition of TFPIα to FXa [9, 74, 89]. Recent studies have shown that TFPIα circulates as a complex with FV and FV-short in circulation and that protein S can function as a synergistic cofactor, together with FV/FV-short, in the inhibition of FXa [69, 86, 88]. We have shown that protein S alone as well as protein S in complex with FV does this by enhancing the initial encounter complex binding between TFPIα and FXa [69]. It is therefore likely that a similar mechanism is involved in the enhancement by protein S and FV/FV-short. (B) When comparing the enhancement of TFPIα-mediated inhibition of FXa by protein S alone to that of protein S together with FV, we showed that protein S enhanced the inhibition ~4-fold, compared to ~12-fold by protein S and FV together [69]. FV-short has been suggested to function as a much more efficient synergistic cofactor together with protein S for TFPIα than FV. The results suggested a more than 100-fold enhancement. However the kinetic constants (K_i) still remains to be determined at physiological concentrations [88].