Protein S: new insights into its functions

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Introduction

Protein S is a vitamin K dependent plasma protein that is mostly known for its anticoagulant properties. The pathophysiological importance of protein S became clear clinically by identification of infants with complete deficiency, who suffer purpura fulminans despite treatment with anticoagulants\(^1\). Moreover, individuals with inherited heterozygous or acquired protein S deficiency are at enhanced risk of developing thrombotic diseases\(^1\). Recently, the importance of protein S was also demonstrated in murine knockouts, which failed to survive development, and in heterozygous mice which have a pro-thrombotic phenotype\(^2,3\). In addition to its role in coagulation, protein S is involved in the classical complement pathway and apoptotic clearance\(^1\). It has also been reported to stimulate vascular injury repair by acting as a mitogen towards smooth muscle cells and has been found to have a neuroprotective effect during ischaemic brain injury in mice\(^4\).

Protein S is a 75 kDa protein, constituted by an N-terminal Gla domain, a thrombin sensitive region (TSR), four epidermal growth factor (EGF) domains and a C-terminal sex hormone-binding globulin (SHBG) domain\(^5\). Protein S is mainly expressed by hepatocytes, but also by endothelial cells and megakaryocytes and circulates in plasma at a concentration of ~333 nM. The Gla domain of protein S, containing 11 \(\gamma\)-carboxylated glutamic acid residues, mediates binding to phospholipid surfaces, which is essential for protein S cofactor function. Approximately 60\% of protein S exists in a high affinity complex with C4b-binding protein (C4BP) to which it binds through its SHBG domain in a 1:1 stoichiometry, leaving approximately 133 nM of free protein S in circulation\(^5\).

The anticoagulant properties of protein S have mostly been investigated with respect to its cofactor activity towards activated protein C (APC) in the inactivation of coagulation factor Va (FVa) and FVIIIa. It has, however, been known since 1988 that protein S also has an APC-independent anticoagulant activity. Part of the APC-independent activity that was initially observed was later shown to be caused by protein S multimers, likely formed during \textit{in vitro} manipulation and purification procedures. The protein S multimers were able to compete with the tenase and prothrombinase for binding to limiting phospholipid surfaces\(^6\). However, an APC-independent activity of protein S was still observed in plasma at saturating concentrations of phospholipids. In 2006, the molecular mechanism behind this latter APC-independent anticoagulant activity of protein S was identified when it was shown that protein S can act as a cofactor for tissue factor pathway inhibitor (TFPI)\(^6\). Additional studies have suggested that there still are protein S direct effects, independent of both APC and TFPI, and that these are dependent on protein S binding to Zn\(^2\)+. However, the physiological importance of this protein S direct effect in comparison to APC and TFPI cofactor activity of protein S still needs to be elucidated.

Despite the pathophysiological importance of protein S in the inhibition of coagulation, the molecular mechanisms behind the cofactor functions for APC and TFPI are not completely understood. In the following sections, recent progress will be summarised.

APC cofactor activity of protein S

The anticoagulant activity of APC is exerted through inactivation of the coagulation cofactors FVa and FVIIIa. APC partially inactivates both FVa and FVIIIa by kinetically favoured initial cleavages at Arg506 and at Arg336, respectively. This is followed by subsequent, somewhat slower cleavages at FVa Arg306 and at FVIIIa Arg562. Purified assays and the use of FVa variants have allowed the detailed study of the protein S enhancement of APC. These have established that protein S primarily enhances cleavage of FVa by APC and that this occurs mainly by enhancement of Arg306 cleavage\(^6\). As shown decisively by calibrated automated thrombography, it is clear that APC is heavily dependent upon protein S for inhibition of thrombin generation in plasma \(\uparrow\text{Figure 1}\). Plasma based assays have also shown that protein S enhancement of APC is not dependent upon the concentration of tissue factor (TF) used for initiation of coagulation.

There are three proposed mechanisms by which protein S has been suggested to enhance APC. The first is by increasing the affinity of APC for phospholipid surfaces. The second is by inducing an allosteric conformational change in APC and the third, by overcoming the protective effect of FVa when bound to FXa\(^7\). Which of these proposed mechanisms is most important for APC cofactor activity of protein S \textit{in vitro} and \textit{in vivo} remains to be determined.

Both free protein S and protein S bound to C4BP can act as cofactors for APC. However, C4BP bound protein S is 6-8-fold less ef-
sufficient than free protein S at enhancing APC mediated FVa cleavage. Moreover, while free protein S acts in synergy with APC inactivated FV in the APC mediated inactivation of FVIIIa, protein S bound to C4BP does not. Several publications have proposed that the SHBG domain of protein S is responsible for an interaction with FVa\(^1\). This suggests that protein S may bind directly to both APC and FV(a), forming a trimolecular complex when acting as a cofactor for APC. Direct binding between protein S and APC has generally been difficult to study as their binding to each other is phospholipid dependent. As a consequence, protein S interaction with APC has mainly been studied using functional assays.

Many studies have tried to elucidate which domains and residues of APC and protein S are functionally important for the APC cofactor activity of protein S. We evaluated 13 recombinant protein C variants with substitutions in the Gla domain in an article published in 2006. We identified an APC variant, Asp36/Leu38/Ala39, which had normal anticoagulant activity in the absence of protein S but could not be enhanced by protein S, suggesting these residues might form part of a functional interaction site with protein S\(^7\). Several studies have suggested that the Gla, TSR, EGF1 and EGF2 domains of protein S contain interaction sites needed for the enhancement of APC\(^1,8\). The importance of the Gla domain of protein S for APC cofactor activity was later confirmed in a study in which a composite variant (termed protein S Face2) of residues Leu21/Asn23/Lys28/Arg28/Asp34/Tyr41/Leu45 was investigated. This protein S variant was largely devoid of APC cofactor activity\(^9\). We have recently investigated the role of the Gla, TSR, EGF1 and EGF2 domains in APC cofactor activity. In a first paper, we generated over 30 composite and single point variants of protein S within these domains. We identified a residue in EGF1, Asp95, which is critical for protein S enhancement of APC activity both in plasma based and purified assays. Importantly, the protein S variants with Asp95 substituted with either alanine or asparagine bound phospholipid surfaces and domain specific monoclonal antibodies with the same affinity as wild type (WT) protein S. This demonstrates that reduced activity is not secondary to reduced phospholipid binding or major conformational changes. We also identified the adjacent residues of Asp95, Asp78 and Gln79, as contributing to protein S enhancement of APC activity\(^10\). Interestingly, mutation of Gln79 was shortly after identified as the cause of a qualitative protein S deficiency\(^11\). Our work was further extended in a follow up study, in which we identified a residue in the protein S Gla domain, Gla36, which is also important for the APC cofactor activity\(^12\). Substituting the Gla36 residue for an alanine again did not affect the phospholipid binding of protein S\(^12\). This is of additional interest because it identified a role for a Gla residue, other than the well known function of calcium-coordination leading to phospholipid binding.

Currently, it may be premature to over speculate how the residues important for the APC cofactor activity of protein S might interact. However, it seems possible that an extended interaction surface involving multiple residues between APC and protein S exists, including both the Gla and EGF1 domain in both APC and protein S (▶Figure 2).

**TFPI cofactor activity of protein S**

TFPI is a Kunitz-type inhibitor that inhibits coagulation by a direct inhibition of FXa and FXa dependent inhibition of TF/FVIIa through

![Figure 1. APC cofactor activity of protein S in plasma.](image)

![Figure 2. Amino acid residues directly involved in the protein S cofactor function for APC. Residues identified as crucial for protein S cofactor activity of APC are shown in the protein S Gla-TSR-EGF1 model and the protein C Gla-EGF1 model, respectively.](image)
the formation of a TFPI/FXa/TF/FVIIa quaternary complex. TFPI is a modular protein, comprised of a negatively charged amino-terminal tail, three tandem Kunitz type domains and an acidic carboxy-terminal tail. The TFPI Kunitz domain 2 binds and inhibits FXa and TFPI Kunitz domain 1 binds and inhibits FVIIa in complex with TF. In 2006 it was shown that protein S could enhance the anticoagulant activity of TFPI in purified FXa inhibition assays. Protein S was also shown to enhance the anticoagulant activity of TFPI in plasma based assays, but only at low TF concentrations, when little thrombin generation occurs. The importance and extent of the protein S-TFPI system in limiting thrombin generation under these conditions can be shown by the addition of inhibitory antibodies against protein S and TFPI (Figure 3).

TFPI inhibits FXa protease activity through a direct interaction. However, the inhibitory constant (K_i) reported for the initial binding of TFPI to FXa in purified kinetic studies is higher than the plasma concentration of the active full-length form of TFPI, making TFPI on its own a poor inhibitor of FXa. Protein S efficiently enhances the initial interaction between TFPI and FXa by reducing the K_i, the TFPI concentration needed for efficient inhibition of FXa, to below the concentration of free full-length TFPI in plasma.

In addition to the direct inhibition of FXa, TFPI also inhibits the activity of the TF/FVIIa complex in a FXa dependent manner. Conflicting results regarding the involvement of protein S in the enhancement of TFPI mediated inhibition of TF/FVIIa have been reported. Initial results showed that protein S efficiently enhanc-
the protein S cofactor activity. Substitution of the Arg199 residue to a leucine resulted in a moderate decrease in the protein S enhancement of TFPI in purified FXa inhibition assays and plasma based assays \(^{(12)}\). We have recently further characterised the TFPI/protein S interaction by evaluating the importance of all charged residues in the TFPI Kunitz domain 3 by substituting the residues in clusters or individually \(^{(14)}\). Our results suggest that several residues are involved in the protein S cofactor activity for TFPI, including Asp194, Arg199, Glu226, Glu234 and Arg237 (Figure 4). However, substitution of Glu226 (TFPI E226Q) resulted in minimal protein S enhancement in a plasma based thrombin generation assay. TFPI E226A was also not enhanced by protein S in purified assays of FXa inhibition. The importance of Glu226 exceeded that of Arg199 when both residues were individually substituted for a glutamine. No binding between TFPI E226Q and protein S could be detected by SPR, indicating a direct involvement of Glu226 in the interaction with protein S \(^{(14)}\).

So far, very little is known regarding which protein S domains are involved in the interaction with TFPI. The substitution of Gla36, which is critical for the APC cofactor activity, did not alter the ability of protein S to act as a cofactor for TFPI \(^{(12)}\). Preliminary data (unpublished) also suggest that variants of protein S Asp95 and protein S Face2 are able to enhance TFPI. Taken together, these data indicate that distinct residues in protein S regulate the APC and TFPI cofactor activities. In addition to identifying the residues in protein S required for binding to TFPI, the molecular mechanisms by which protein S enhances TFPI still need to be elucidated.

**Concluding remarks**

Protein S plays crucial roles in anticoagulation, acting as a cofactor for both APC and TFPI. Through its dual cofactor functions, protein S is therefore able to inhibit coagulation both during the initiation phase of coagulation through TFPI, as well as during the propagation of coagulation by enhancing APC. Recently, the TFPI residues involved in the interaction with protein S as well as the protein S and APC residues important for the APC cofactor function of protein S have been identified. Published and preliminary data from our lab also suggests that the cofactor function for APC and TFPI are mediated through different amino acid residues in protein S. Although these findings form a foundation for understanding the cofactor functions of protein S, further work is required to fully elucidate the molecular basis of its anticoagulant mechanisms.

**Acknowledgements**

The research of the authors reported in this manuscript was funded by grants from the British Heart Foundation.

**References**