Amino acid residues in the laminin G domains of protein S involved in tissue factor pathway inhibitor interaction

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Running title: Protein S SHBG in the enhancement of TFPI

Word count: 4931

Abstract word count: 248

References: 49

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Financial support:

This work was supported by the Swedish Research Council (grant 71430), grants from the Swedish Heart and Lung Foundation, Söderberg's foundation, the Alfred Österlund's Foundation, research funds from the University Hospital in Malmö and the British Heart Foundation (FS/12/60/29874).

ABSTRACT

Protein S functions as a cofactor for tissue factor pathway inhibitor (TFPI) and activated protein C (APC). The sex hormone binding globulin (SHBG)-like region of protein S, consisting of two laminin G-like domains (LG1 and LG2), contains the binding site for C4b-binding protein (C4BP) and TFPI. Furthermore, the LG-domains are essential for the TFPI-cofactor function and for expression of full APC-cofactor function. The aim of the current study was to localise functionally important interaction sites in the protein S LG-domains using amino acid substitutions. Four protein S variants were created in which clusters of surface-exposed amino acid residues within the LG-domains were substituted. All variants bound normally to C4BP and were fully functional as cofactors for APC in plasma and in pure component assays. Two variants, SHBG2 (E612A, I614A, F265A, V393A, H453A), involving residues from both LG-domains, and SHBG3 (K317A, I330A, V336A, D365A) where residues in LG1 were substituted, showed 50-60% reduction in enhancement of TFPI in FXa inhibition assays. For SHBG3 the decreased TFPI cofactor function was confirmed in plasma based thrombin generation assays. Both SHBG variants bound to TFPI with decreased affinity in surface plasmon resonance experiments. The TFPI Kunitz 3 domain is known to contain the interaction site for protein S. Using *in silico* analysis and protein docking exercises, preliminary models of the protein S SHBG/TFPI Kunitz domain 3 complex were created. Based on a combination of experimental and *in silico* data we propose a binding site for TFPI on protein S, involving both LG-domains.

Key words: protein S, tissue factor pathway inhibitor, C4b binding protein, sex hormone binding globulin-like region, laminin G-like domain

INTRODUCTION

Protein S is a vitamin K-dependent protein, well known for its activated protein C (APC) cofactor function and ability to enhance inactivation of coagulation factors (F) Va and VIIIa in the propagation and termination phase of coagulation. Protein S is also functioning as a cofactor for tissue factor pathway inhibitor (TFPI) in the direct inhibition of FXa and TF/FVIIa during the initiation of coagulation (1, 2). In human plasma, 30-40% of the circulating protein S is free and fully active as an APC and TFPI cofactor, the remaining 60-70% being bound to the complement regulatory protein C4b-binding protein (C4BP) (3).

Protein S shares several structural features with other coagulation proteins. The N-terminal Gla-domain, containing 11 γ -carboxylated glutamic acid (Gla) residues, binds calcium and negatively charged phospholipids. It is followed by the thrombin sensitive region (TSR) and four epidermal growth factor (EGF)-like domains. The Gla-, TSR- and EGF-domains have been identified as being important for the APC-cofactor function of protein S (4-7). The C-terminal half of protein S, which is homologous to sex hormone binding globulin (SHBG), is composed of two laminin G-like domains, LG1 and LG2, and contains three N-linked glycosylation sites (8, 9). While the Gla-domain is essential for all anticoagulant functions of protein S through the binding to negatively charged phospholipids, the SHBG-like region has been identified to be of great importance for the majority of the protein S functions, including binding to C4BP (10-16) and regulation of coagulation through APC and TFPI (17, 18). There are also data suggesting that the protein S interaction with tyrosine kinase receptors of the TAM-family (Tyro3, Axl, Mer) is located within the LG-domains (19).

C4BP is composed of 6-7 identical α -chains, and one unique β -chain. Protein S binds to the β -chain with high affinity ($K_d < 1$ nM), the complex forming already intracellularly during synthesis of C4BP and protein S (20). The protein S binding site on C4BP has been localized to the N-terminal part of the C4BP β -chain (21-23). The interaction site in protein S for C4BP resides completely within the two LG-domains (24) and several regions of the LG-domains have been proposed to be involved in this binding (10-16). However, despite numerous attempts, the detailed binding site has not yet been identified.

The importance of the LG-domains for the APC cofactor function has been investigated at a number of occasions using domain substitutions or deletion (17, 19, 25). The direct role of the LG-domains is still not clear, however, both are needed for full anticoagulant activity. Interestingly, the LG-domains have been suggested to bind FV and FVa (26). This proposed binding is consistent with some of the effects seen when studying the APC cofactor function, in particular a pronounced increase in APC-cofactor function in the degradation of FVIIIa, where FV and protein S are functioning as synergistic cofactors (17).

Protein S is known to directly interact with TFPI, and more specifically with Glu226 and Arg199 within TFPI Kunitz domain 3 (27, 28). Furthermore, it has been shown that this interaction is essential for protein S to function as a cofactor for TFPI. Recently, it was also discovered that the SHBG-like region, and in particular the LG1 domain, is important for the TFPI interaction (18). However, further details of the TFPI interaction site in protein S remains to be determined.

In an attempt to clarify the role of the LG-domains in the diverse functions of protein S, we have created four recombinant protein S variants in which clusters of 3-5 amino acids in LG1, LG2 or both LG-domains were substituted for alanine. The choice of point mutations was based on previous work, aiming at finding the C4BP and FV interaction sites (10-16, 26). The four variants were tested for C4BP and TFPI binding and for APC- and TFPI cofactor functions. While all four protein S variants bound to C4BP with the same affinity as wild-type (WT) protein S and were fully functional as cofactors for APC, two variants showed reduced TFPI cofactor function and binding. Based on a combination of experimental data and *in silico* analysis, we propose possible binding sites for TFPI on protein S.

MATERIALS AND METHODS

Material

Protein S-free C4BP was purified (29) and APC was activated from in house purified protein C as described (30-32). Recombinant FV R506Q/R679Q was expressed and quantified as described (33), bovine FX (34) and human prothrombin were purified from plasma (35). Human α -thrombin was prepared from purified prothrombin (36). Phospholipids were purchased from Avanti Polar Lipids and include the natural phospholipids phosphatidylserine (PS; brain extract), phosphatidylethanolamine (PE; egg extract) and phosphatidylcholine (PC; egg extract) and the synthetic phospholipids 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-sn-glycero-3-phosphoserine (DOPS), and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Phospholipid vesicles were prepared by extrusion technique (37).

Structural analysis

The protein S SHBG 3D model (9, 38) was first investigated interactively using Pymol in order to select possible protein/protein interaction (PPI) regions that could be mutated (e.g., regions rich in solvent exposed charged amino acids and/or hydrophobic/aromatic areas). Previously reported putative binding sites for C4BP and FV (10-16, 24, 26) were also

mapped onto the predicted protein S model. In addition, prediction of protein/protein interface regions was carried out with MetaPPI (39). This tool combines several PPI prediction engines (ConSurf, InterProSurf, PPI-PRED, SPPIDER) to predict consensus PPI site regions. Residues predicted to take part in the different PPI regions were further analysed ~~interactively in regards to the structure in~~ and mapped onto the SHBG 3D model structure. Residues whose amino acid substitutions ~~that~~ were considered likely to cause ~~destabilisation of the~~ protein misfolding were excluded from the clusters. A total of four PPI regions were chosen to be studied further through the generation of the four composite variants (SHBG1-4; see Mutagenesis section). The solution structure of TFPI Kunitz domain 3 (PDB file 1IRH) (40) was also analysed and prediction of protein/protein interaction sites were performed with MetaPPI. Docking of protein S and the TFPI Kunitz domain 3 was carried out with the online server pyDockWEB (41).

Mutagenesis

Clusters of amino acids in the two LG-like domains were mutated to alanine to create four protein S mutants SHBG1 (N490A, D583A, F586A), SHBG2 (F265A, V393A, H453A, E612A, I614A), SHBG3 (K317A, I330A, V336A, D365A) and SHBG4 (H453A, D455A, V459A, H466A, V606A). The mutagenesis was performed using QuikChange mutagenesis kit (Stratagene), a WT protein S/pcDNA3 expression vector (42) and mutagenic oligonucleotide primers (Eurofins MWG Operon). The mutagenesis was confirmed by sequence analysis using BigDye terminator v3.1 (Applied Biosystems).

Protein S expression, purification and characterisation

Protein S, WT and variants, were stably expressed in human embryonic kidney cells (HEK293) in the presence of vitamin K (10 µg/ml Konakion Novum). Recombinant protein S was purified as previously described (4, 43) and purity and calcium-binding properties were analysed using electrophoretic methods. The concentration of purified protein S was determined with a previously described *in house* ELISA (4, 44). The concentrations were confirmed by quantitative amino acid analysis performed by DTU – Biosys, EPC, Danish Technology University, Copenhagen, Denmark.

TFPI expression, purification, and characterisation

TFPI was expressed, purified, and quantified as previously described (27). Briefly described, TFPI was transiently expressed in HEK293T cells. Full-length TFPI was purified from harvested culture media using heparin Sepharose FF chromatography followed by affinity chromatography using an anti-TFPI Kunitz domain 1 antibody (Sanquin). Apart from TFPI,

no other proteins were detected using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and TFPI migrated as a single band with an apparent molecular weight of 41 kDa. Two *in-house* ELISAs were used to quantify total and full-length TFPI (27). The purified recombinant TFPI used for this study was judged to be 92% in its intact full-length form.

Immunoassays

Analysis of protein S binding to immobilized C4BP or polyclonal anti-protein S (*in house*) was performed essentially as described (44). Briefly, 96-well plates were coated with 5 µg/ml of protein S-free C4BP or 10 µg/ml anti-protein S (7909) in carbonate buffer followed by incubation of purified protein S in 0.15M NaCl, 20mM Tris, pH 7.4 (TBS) supplemented with 0.2% bovine serum albumin (BSA, Sigma-Aldrich) and 2mM CaCl₂ overnight, 4°C. Bound protein S was detected with biotinylated monoclonal anti-protein S (MK54), 2 µg/ml for 2 hours at room temperature, followed by HRP-conjugated streptavidin/biotin complexes for 30 minutes and development by addition of OPD. The reaction was terminated with 0.5M H₂SO₄ and the absorbance was read at 492 nm.

For the analysis of protein S binding to FVa, 96-well Maxisorp plates (Nunc) were coated with 5 µg/ml FVa (HTI) or fish gelatine, as a negative control, in carbonate buffer pH9.6 in 37°C for 1 hour. Washing steps were performed in triplicate with 250 µl of TBS supplemented with 5mM CaCl₂ (TBSCa) and 0.1% Tween 20 between each step. The plates were quenched with 5% fish gelatine in TBSCa for 3 hours at room temperature. Protein S was titrated (0-1000nM) in 5% fish gelatine and incubated for 1.5 hours at room temperature. Bound protein S was detected using biotinylated monoclonal anti-protein S (15nM, HTI), followed by HRP-conjugated streptavidin/biotin complexes, both diluted in TBSCa with 0.5% fish gelatine and incubated for 1 hour at room temperature. Also higher concentrations of the detection antibody was added giving similar results. The assay was developed by addition of OPD, terminated with H₂SO₄ and the absorbance was read at 492nm.

The data from all binding curves were fitted using nonlinear regression for the equation $Y=B_{\max} \times X/(K_d+X)$, where Y is the absorbance; B_{max}, the maximal binding; X, protein S concentration, and K_d is the concentration of ligand required to reach half maximal binding. This equation was used, assuming that the free ligand was in surplus over bound ligand.

Activated partial thromboplastin time (APTT)

Protein S depleted plasma (50 μ l) was added to 50 μ l TriniClot (Trinity Biotech) reaction mix and incubated for 270 seconds. Protein S (10 μ l) and APC (10 μ l) were added at 270 seconds and 280 seconds, respectively. Coagulation was initiated by addition of 50 μ l, 25mM CaCl_2 and the time to clot was measured in Amelung-Coagulometer KC4A. Before addition of CaCl_2 the APC and protein S concentrations were 5nM and 0-75nM, respectively. Prolongation of the time to clot was analysed as a function of protein S concentration.

APC and TFPI cofactor function in thrombin-generation assays determined by CAT

Calibrated automated thrombography (CAT) was performed in protein S depleted plasma (Enzyme Research Laboratories) using a Fluoroscan Ascent FL plate reader (Thermo Labsystem) and Thrombinoscope software (Synapse BV), essentially as described previously (4, 5, 18, 27). The protein S-depleted plasma was also deficient in protein S-C4BP complexes, which allowed specific studies of TFPI and APC cofactor functions without influence of effects caused by altered C4BP binding, as protein S-C4BP complexes have previously been shown to have limited TFPI- and APC-cofactor functions (1, 18, 45). In the CAT assay, protein S (0-120nM) was added to plasma (80 μ l/well) in the presence or absence of 9nM of APC (Haematologic Technologies Inc.) or 1nM TFPI. Thrombin generation was initiated with 1pM tissue factor (Dade Innovin), 50 μ M phospholipid vesicles (20:20:60; DOPS/DOPE/DOPC), and 16.6mM CaCl_2 in a total volume of 120 μ l. The amount of thrombin formed was monitored using 0.42mM of a thrombin-sensitive fluorogenic substrate, Z-Gly-Gly-Arg-AMC (Bachem). To inhibit contact activation, corn trypsin inhibitor (Enzyme Research Laboratories) was added (65 μ g/ml plasma). All given concentrations are final.

FVa-degradation assay

Recombinant FV R506Q/R679Q (1.3nM) in presence of 42 μ M phospholipid vesicles (10:20:70; PS/PE/PC) was activated with 0.5 U/ml of thrombin (10 minutes, 37°C), and the reaction was stopped by addition of 1 U/ml hirudin (Pentapharm). A degradation mix (50 μ l), containing 25 μ M phospholipid vesicles (10:20:70; PS/PE/PC), 0.25nM APC, 0.8nM FVa and 0-40nM protein S was incubated for 10 minutes at 37°C to allow APC-mediated degradation of FVa. To stop the reaction, the mix was diluted 1/10 in ice cold 25mM Hepes, pH 7.7, 150mM NaCl, 5mM CaCl_2 supplemented with 0.5% BSA. To evaluate the remaining FVa-activity, a prothrombinase assay was performed as described (46). The final concentrations in the prothrombinase assay were 50 μ M phospholipid vesicles (10:90; PS/PC), 5nM FXa, 0.5 μ M prothrombin and 16pM FVa/FVi.

FVIIIa-degradation assay

An activation mix containing FVIII (1 U/mL; Octapharma) and FIXa (8.75 nM; Haematologic Technologies Inc.) and phospholipid vesicles (10:20:70; PS/PE/PC; 87.5 μ M) was treated with 0.1 U/ml thrombin (37°C, 3 minutes) to activate FVIII. The reaction was stopped with 0.3 U/ml hirudin. The activation mix was diluted with FIXa (8.75nM) and phospholipid vesicles (10:20:70; PS/PE/PC; 87.5 μ M) to obtain 350 mU/ml FVIIIa. Reagents were added to the activated FVIII creating a reaction mix containing FVIIIa (200mU/ml), FIXa (5nM), APC (2.5nM), FV (plasma purified as described (47); 2.5nM), protein S (0-50nM) and phospholipid vesicles (10:20:70; PS/PE/PC; 50 μ M) (total volume 105 of μ l). After incubation (2.5 minutes in 37°C), 20 μ l bovine FX (final concentration 0.5 μ M) was added and incubated for 3 minutes. The reaction was stopped by diluting 1/16 in ice-cold 50mM Tris-HCl, 100mM NaCl, 20mM EDTA, 1% polyethylene glycol 6000, pH 7.9. The remaining activity of FVIIIa was determined by the amount of formed FXa, which was assessed by kinetic measurement of the conversion of the chromogenic substrate S-2765 (Chromogenix).

FXa inhibition assay

The inhibition of FXa (0.5nM; Enzyme Research Laboratories) activity by TFPI was monitored by the cleavage of the chromogenic substrate S-2765 (Chromogenix). This was measured in the presence or absence of 2nM TFPI and protein S (0-160nM), 25 μ M phospholipid vesicles (20:20:60; DOPS/DOPE/DOPC) and 5mM CaCl₂, as described previously (18, 27, 28). To estimate the concentration of protein S required to reach 50% of the maximal potentiation of TFPI-mediated inhibition of FXa (EC₅₀), the initial velocity (V_0) of the S-2765 cleavage was plotted against the protein S concentration. The V_0 was determined using nonlinear regression as previously described (1, 27). The EC₅₀ was determined by a one-phase exponential decay nonlinear curve fit, as previously described (18, 27, 28).

Binding of protein S to TFPI using surface plasmon resonance

Binding of protein S to TFPI was measured using surface plasmon resonance (SPR) as described previously (18, 27, 28). Briefly, purified recombinant TFPI was immobilized on a CM5 chip (GE Healthcare) using amine coupling according to the manufacturer's instructions. The immobilisation levels were around 2500 response units. The measurements were carried out at 25°C at a flow rate of 30 μ l/minute. The flow cells were perfused with protein S (0-1000nM) in running buffer (5mM CaCl₂ in TBS with 0.005%

Tween20) for 450 seconds. The dissociation of protein S into running buffer was followed for 2000 seconds. The chip was regenerated by perfusion with 1M NaCl in running buffer. Binding was expressed as RU after correction for the reference flow cell, coupled only with ethanolamine.

RESULTS

Design and expression of protein S variants

In an attempt to identify functionally important sites in the SHBG-like region we decided to explore regions including, or being close to, the putative C4BP and FV binding sites (Fig. 1A) (10-16, 26). The molecular surface was analysed for hydrophobic and aromatic residues (Ala, Val, Ile, Leu, Trp, Phe, Tyr) shown in yellow in Figure 1B, as these tend to be present in many PPI sites. In addition, we investigated charged residues, generally known to play a role in PPI, surrounding these hydrophobic/aromatic areas. This information was taken into account when designing our protein S variants (Fig. 1C). In addition, *in silico* predictions of PPI sites were also considered in the design process. Thus, our strategy was to integrate available knowledge to create a small number of variants, allowing us to probe predicted key protein/protein interaction areas of the protein S SHBG region. Four protein S variants (SHBG1-4) were created, in which clusters of 3-5 amino acids were substituted. In SHBG1 and SHBG4, the substitutions were located in LG2, while SHBG3 contain residue substitutions in LG1. Amino acids in both LG-like domains were substituted in SHBG2. The quality and purity of the purified protein S variants were examined by different electrophoretic methods. The purified proteins appeared as single bands, the variants migrating like WT protein S on SDS-PAGE with an apparent molecular weight of 73kDa. On native PAGE, the protein variants migrated as homogenous bands without apparent multimers (results not shown). The proteins were also analysed by native agarose gel electrophoresis in the presence of calcium or EDTA to test their calcium binding ability. The mobility of all variants was noticeably slower in presence of calcium than in EDTA, demonstrating that the protein S variants bound calcium similarly to WT protein S (results not shown). We compared the affinities of all protein S variants for polyclonal anti-protein S antibodies in a solid-phase assay. All variants bound to the antibodies with similar affinity, suggesting that the amino acid substitutions did not lead to any major conformational change (Table 1).

Binding of protein S variants to C4b-binding protein

The choice of amino acid substitutions in the four protein S variants was based on previous work identifying possible interaction sites for C4BP (10-16). Binding of our protein S variants to C4BP was analysed in a solid-phase assay (Table 1). WT protein S and variants bound equally well to C4BP, the estimated K_D ranging between 0.7-0.9nM (Table 1) suggesting that the substituted residues are not directly involved in the protein S/C4BP interaction.

Evaluation of APC cofactor function and binding to FVa of protein S variants

Thrombin generation using CAT, APTT-based assays, as well as FVa and FVIIIa inactivation assays, were used to study APC cofactor function of the protein S variants. All protein S variants were fully functional as cofactors for APC in all four functional assays (Fig. 2). In fact, SHBG1 and SHBG4 showed slightly higher APC cofactor function than WT protein S in CAT. This difference is however not significant and could not be detected in any of the other assays suggesting that the substituted residues are not important for the enhancement of APC by protein S. The binding of the protein S variants to FVa was also studied using a solid-phase assay. As the binding did not reach saturation due to the low affinity of the interactions we could not calculate binding constants. However, all variants were found to bind to FVa in a similar manner at WT protein S, SHBG3 possibly binding slightly better than the others. (Fig. 3).

Evaluation of TFPI cofactor function of protein S variants in plasma

The protein S enhancement of TFPI was evaluated in protein S depleted plasma by CAT. Protein S was added to plasma in the presence or absence of TFPI. While protein S did not show any inhibition of thrombin generation in the absence of TFPI, it increased TFPI-mediated inhibition of thrombin generation in a dose dependent manner, as previously reported (1, 18, 27). All protein S variants functioned as cofactors for TFPI, however while SHBG1 and SHBG4 enhanced TFPI equally well as WT (Fig. 4A, B and E), TFPI cofactor function of SHBG2 and SHBG3 was moderately decreased (Fig. 4C, D). The reduction in lag time for SHBG3 was statistically significant compared to WT protein S at 100nM (Fig. 4F).

Evaluation of protein S enhancement of TFPI-mediated FXa inhibition

To evaluate whether the moderately decreased TFPI cofactor function of SHBG2 and SHBG3 could be seen also in the direct inhibition of FXa, we used FXa inhibition assays with purified reagents to kinetically evaluate the enhancement of TFPI in its inhibition of FXa. Progress curves of FXa inhibition by TFPI and increasing concentrations of protein S show that all four protein S variants increased the efficiency of TFPI (Fig. 5). FXa and TFPI are known to interact in a 2-step process where an immediate encounter complex is formed,

followed by a slow isomerization into a final tight complex. Protein S is known to mainly enhance the initial interaction. The initial rates of S-2765 hydrolysis by FXa in the presence of TFPI was plotted against protein S concentration, allowing EC_{50} values for the protein S enhancement to be derived (Fig. 5F and Table 1). Similarly to the results seen in the plasma-based assays, SHBG3 shows reduced enhancement of TFPI with an approximately 2.6-fold increase in EC_{50} , as compared to WT protein S. A similar (~2.2-fold) increase was also seen for SHBG2. In addition, a mild, although not significant, decrease was seen for SHBG4.

Evaluation of binding of protein S to TFPI by SPR

Our functional assays suggest that the residues substituted in SHBG2 and SHBG3 are needed for full TFPI cofactor function. To investigate whether the decrease in TFPI cofactor function is caused by decreased binding to TFPI we performed SPR. Increasing concentrations of protein S were perfused over immobilized TFPI. As previously described, WT protein S bound to TFPI in a dose dependent manner (Fig. 6). Since protein S binding did not reach equilibrium, in agreement with previous publications, no affinity for the protein S/TFPI interaction could be derived (18, 27, 28, 48). Nevertheless, SHBG2 showed decreased binding to TFPI compared to WT protein S (Fig. 6C and F). In addition, while the maximum response units after 450 seconds association for WT protein S and SHBG3 are comparable, the association rate is clearly decreased for SHBG3 (Fig. 6D). While the shape of the binding curves do not allow full kinetic evaluation of the binding, preliminary analysis of association rates gave a ~50% reduction in association rate of SHBG3 compared to WT. These results suggest that residues substituted in SHBG2 and SHBG3 are required for efficient interaction with TFPI.

Creation of a theoretical model of protein S SHBG/TFPI Kunitz domain 3

Combining our experimental data for SHBG2 and SHBG3 with the TFPI Glu226 and Arg199, two amino acid residues known to be directly involved in the protein S interaction, we have attempted to propose theoretical models of the protein S SHBG/TFPI Kunitz domain 3 complex. This was done through protein docking computations using the homology model of the protein S SHBG-like region (9) and the solution structure of the TFPI Kunitz 3 domain (Figure 7A) (40). Using the pyDockWEB server, we generated the 100 best, possible models of the protein complex using a new scoring algorithm recently implemented in pyDock (41). While the scoring of these models is based on estimated binding energy, in numerous cases, these score values are not sufficient to discriminate a likely binding pose from a wrong orientation of similar value because of several limitations in the scoring functions. As such, the protocols that give the best solutions incorporate experimental information (e.g.,

mutation data) into the process and involve either biasing the docking by exploring only regions/residues of the molecular surface that are experimentally known to play a role, or exploring the entire surface and then using post-docking filtering where only the models that are compatible with the experimental information are selected for further analysis. In this study, we used the second approach, i.e. exploring the entire surface and selecting the complexes that are in agreement with the mutagenesis data. Thus, we investigated the 100 models interactively and selected those that brought TFPI Glu226 (and/or Arg199) in direct contact with protein S areas in proximity of the SHBG3 substitutions. Two poses were found compatible with our experimental data, pose A and pose B (Fig. 7B-C). In pose A (Fig. 7B), the TFPI Kunitz domain 3 Glu226 was predicted to bind to the side chain of residue Lys 317 (mutated to Ala in SHBG3) ~~Ile318 (close to position 317 that was substituted for Ala in SHBG3)~~ through an electrostatic interaction, while the nearby TFPI Trp188 bound to protein S Val336 (mutated to Ala in SHBG3) through hydrophobic interactions. TFPI Arg199 was not in direct contact with protein S but was predicted to bind to LG1 residues Glu394 and Glu396 (both located close to the V393A substitution in SHBG2) through long-range electrostatic interactions (around 10 Å). In this orientation, minor contacts were predicted between TFPI and SHBG2/SHBG4 residues. In pose B (Fig. 7C), TFPI interacted directly with protein S Lys317 and Ile330, both substituted in SHBG3. In this orientation TFPI could also interact with residues substituted in SHBG2. In addition, TFPI Glu226 was predicted to bind to protein S Lys399 or Arg314, both being in close proximity to Ile330, (substituted in SHBG3) through electrostatic interactions. However, in this orientation, TFPI Arg199 pointed away from the LG domains of protein S. At this stage, it is not possible to further refine the models of the protein S SHBG and TFPI Kunitz domain 3 interaction. Both models should be considered as working hypotheses, built by taking the presently available experimental data and the modelling data into account.

DISCUSSION

Several functionally important PPI sites in protein S have been located in the two LG-like domains that constitute the SHBG-like region. Considerable efforts have been made to further detail the sites for the multiple interactions that the protein S LG-like domains are involved in. Peptide studies and site directed mutagenesis have yielded important information but our knowledge on the binding interactions is still incomplete. In this study, we created four protein S variants with clusters of amino acid residues in the SHBG-like region substituted for alanine. The selection of the clusters was based on a combination of *in silico* analysis and available experimental results (10-16, 26). According to previous experiences in probing the surface of many coagulation proteins, the effects of single substitutions to alanine are rarely strong enough to perturb a PPI, unless one directly hits the hotspot

residues. The substituted residues (none of them ~~which~~ have previously been associated with protein S deficiency) were selected to minimise the risk of disrupting the 3D structure of the protein. Good expression levels (1.1-1.8mg/L expression media) and retention of most functional properties argue in favour of correct folding of the protein variants. Functional characterization of the protein variants demonstrated that two were impaired in TFPI interaction, whereas all four variants had normal APC-cofactor activity and interaction with C4BP. Based on the present results, and the demonstration of Glu226 and Arg199 within Kunitz domain 3 as being essential for the protein S/TFPI interaction (27, 28), structural models for the TFPI-protein S interaction were created.

TFPI is on its own a poor inhibitor of coagulation and protein S enhances the TFPI-mediated inhibition of FXa ~10-fold and brings the inhibitory constant below the plasma concentration of TFPI (1). The binding of protein S to negatively charged phospholipid surfaces is believed to be an important mechanism for its functioning as TFPI cofactor and it is suggested that protein S enhances the immediate interaction between TFPI and FXa by bringing TFPI to the proximity of FXa on the phospholipid membrane (18, 49). Recently, the protein S LG1 domain was shown to be important for the interaction with TFPI (18). In this study, we have, through the creation and characterisation of composite protein S variants, identified residues within both LG domains to be involved in the TFPI interaction. Two of the variants, SHBG2 and SHBG3, demonstrated moderate reduction in TFPI cofactor function, most likely resulting from decreased affinity of the TFPI/protein S interaction. The substitutions in SHBG3 are located within LG1, while the substitutions in SHBG2 are located within both LG1 and LG2 (Fig. 1C). It is interesting to note that most residues substituted in SHBG2 and SHBG3 are well conserved between species, which is consistent with what is ~~previously~~ known about residues involved in protein-protein interactions. His454 was predicted to take part in two PPIs and was subsequently substituted in both SHBG2 and SHBG4. However, as SHBG4 interacts normally with TFPI, it is unlikely that this residue plays a major role in the interaction. The SHBG2 and SHBG3 variants demonstrated a relatively modest decrease in affinity for TFPI, suggesting that although we have identified residues that are involved in the TFPI/protein S interaction, key residues still remain to be discovered.

The C4BP binding site on protein S is still an intriguing enigma. Previous studies have suggested that both LG-like domains are important for the binding (9, 24) and in an effort to further detail the interaction, we substituted residues situated close to positions previously suggested to be involved in binding to C4BP (10-16). Somewhat surprising all four protein S variants bound C4BP with the same affinity as WT. However, it takes many amino acid residues (~12) to form a high affinity protein-protein interaction surface. It is possible that the residues substituted in the SHBG variants take part in, or are close to, an interaction site for

C4BP but that they only marginally contribute to the free energy of binding and therefore show limited or no effect on the affinity. Protein S bound to C4BP has reduced cofactor function for TFPI (1, 18). In addition, while both TFPI and C4BP bind to protein S in plasma, it was suggested by Castoldi *et al.* that the two proteins bind to different populations of protein S (48). Our present results do not contribute insights into the question of whether or not TFPI and C4BP bind to the same or overlapping surfaces on protein S or whether the binding of the large C4BP molecule sterically hinders TFPI binding.

The APC cofactor function of the four protein S variants was studied in plasma and in assays with pure component. All four variants were fully functional as APC cofactors in both settings. All protein S variants also bound to FVa similarly to WT protein S. Taken together, these data suggests that the substituted residues were of little importance for the protein/protein interactions involved in this function of protein S.

In conclusion, we have targeted potential PPI sites in the SHBG-like region of protein S by clustered mutagenesis in four recombinant protein S variants. Two of the protein S variants (SHBG2 and SHBG3) showed decreased binding and enhancement of TFPI, thereby confirming the importance of the LG1 domain in the TFPI cofactor function of TFPI. Combining experimental and *in silico* data we propose possible models of the protein S SHBG/TFPI Kunitz domain 3 complex.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Research Council (grant 71430), grants from the Swedish Heart and Lung Foundation, Söderberg's foundation, the Alfred Österlund's Foundation, research funds from the University Hospital in Malmö and the British Heart Foundation (FS/12/60/29874).

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Table 1. Enhancement of TFPI-mediated inhibition of FXa by protein S and binding of protein S to C4BP and polyclonal anti-protein S antibodies.

	WT	SHBG1	SHBG2	SHBG3	SHBG4
EC₅₀ for FXa inhibition	23.3±8.3	29.6±14.3	51.4±17.0**	60.6±22.8**	40.7±22.0
C4BP	0.76±0.16	0.70±0.03	0.92±0.08	0.83±0.05	0.59±0.12
Anti-protein S	0.64±0.14	0.48±0.04	0.46±0.03	0.51±0.06	0.45±0.09

EC₅₀/K_{d app} values obtained from 6 and 3 independent experiments, respectively, are given in nanomolar and are expressed as mean±SD.

**p<0.01 as compared to WT protein S using Mann-Whitney test.

EXTRA TABLE

What is known on this topic:

- Protein S functions as a cofactor for TFPI by enhancing the TFPI-mediated inhibition of FXa ~10-fold.
- A direct interaction between protein S and TFPI is needed for protein S to function as a cofactor for TFPI.
- The TFPI/protein S interaction is dependent on TFPI Glu226 and Arg199 and the protein S SHBG-like region where specifically the LG1 domain has been shown to be important.

What this paper adds:

- Using composite protein S variants we have found residues within the SHBG-like region that are needed for full TFPI cofactor function.
- Through our protein S variants we confirm the importance of the SHBG-like region, and more specifically the LG1 domain, in the direct interaction with TFPI.
- Using *in silico* analysis combined with experimental data we propose models of the protein S SHBG/TFPI Kunitz domain 3 complex, increasing our understanding of an interaction of major importance for the TFPI pathway.

FIGURE LEGENDS

Figure 1. Molecular models of the SHBG-like region demonstrating the mutagenesis strategy. A) Cartoon representation of the predicted SHBG-like region of protein S (9) with LG1 and LG2 domains shown in magenta and blue, respectively. Peptides suggested to be important for the binding of C4BP are shown in green, while a postulated binding site for FV is shown in red. Glycosylation sites (N-linked) are labelled in orange. B) The solid surface model of the SHBG region shown in the same orientation as in A, as well as in a 180° rotation (back view), using the same colour code as in A. In addition, solvent exposed hydrophobic and aromatic residues, regions potentially important for protein/protein interactions (PPI) are shown in yellow. C) Location of the amino acid substitutions in the four protein S variants. The orientation is the same as in A. D) Predicted consensus PPI site using MetaPPI analysis shown as transparent green area. This region partly overlaps with SHBG2, 3, and 4. For both C and D, the LG1 and LG2 domains are shown in magenta and blue and the mutated zones are highlighted as follow: SHBG1 in red, SHBG2 in yellow, SHBG3 in blue and SHBG4 in orange.

Figure 2. Enhancement of APC by protein S variants. A) Thrombin generation was measured in protein S depleted plasma supplemented with 1pM TF, 50µM phospholipids, 9nM APC and 0-120nM of protein S variants. A dose-dependent decrease in maximum thrombin generation (peak height) could be seen after addition of increasing concentrations of protein S. Addition of APC in the absence of protein S did not inhibit thrombin generation (result not shown). B) The time to clot was measured using activated partial thromboplastin time. Protein S depleted plasma was mixed with TriniClot reaction mix followed by addition of 0-75nM protein S and 5nM APC. The time to clot was measured in Amelung-Coagulometer KC4A and the prolongation of the time to clot was plotted as a function of protein S concentration. C) Protein S enhancement of APC-mediated cleavage of FVa at Arg306 was measured in a FVa inactivation assay. Protein S (0-40nM) was incubated with 0.25nM APC, 0.8nM FVa R506Q/R679Q and 25µM phospholipids for 10 minutes. Remaining FVa activity was analysed in a prothrombinase assay. D) Protein S enhancement of APC in the inactivation of FVIIIa was measured using a FVIIIa inactivation assay. Protein S (0-50nM) was incubated with 200 mU/mL FVIIIa, 5nM FIXa, 2.5nM APC, 2.5nM FV and 50µM phospholipids for 2.5 minutes. The remaining FVIIIa activity was measured in a Xase assay. Data are presented as mean±SD; n=3.

Figure 3. Binding of protein S variants to FVa. FVa (5µg/ml) was coated in 96-well plates and incubated with protein S WT, SHBG1, SHBG2, SHBG3 and SHBG4 (0-1000nM). WT protein S binding to wells coated with 5µg/ml fish gelatine was used as a negative control.

Bound protein S was detected using biotinylated monoclonal anti-protein S (15nM), followed by HRP labelled streptavidin-biotin. The plates were developed by addition of OPD, stopped by H₂SO₄ and read at A492. The data were normalised for the maximum binding of WT protein S in each experiment and are presented as mean±SEM, n=3.

Figure 4. Enhancement of TFPI-mediated inhibition of thrombin generation by WT protein S and variants. Thrombin generation was measured in protein S depleted plasma supplemented with 1pM TF, 50µM phospholipids and 0-100nM of protein S in the presence of absence of 1nM TFPI. The enhancement by WT protein S (A) was compared to that of SHBG1 (B), SHBG2 (C), SHBG3 (D) and SHBG4 (E). Results from a representative experiment are shown (n=3). The lag times for thrombin generation in the presence of TFPI and increasing concentration of WT protein S and protein S variants are plotted against protein S concentration. The results are presented as mean±SD; n=3. **p<0.01 for SHBG3 as compared to WT protein S using Mann-Whitney tests.

Figure 5. Enhancement of TFPI by WT protein S and variants in the direct inhibition of FXa. Cleavage of 200µM S-2765 by 0.5nM FXa was monitored in real time at 405nm in the presence of 25µM phospholipids, 2nM TFPI and 0-160nM WT protein S (A), SHBG1 (B), SHBG2 (C), SHBG3 (D), SHBG4 (E). Results from a representative experiment are shown (n=6). F) The v_0 (initial velocity of substrate conversion; relative to the v_0 in the absence of protein S) was calculated and plotted against the protein S concentrations for WT protein S and protein S variants. Data are presented as mean±SD; n=6. **p<0.01 in EC₅₀ compared to WT protein S using Mann-Whitney tests.

Figure 6. Binding of WT protein S and protein S variants to TFPI studied by SPR. A CM5 chip was coupled with TFPI to 2500 resonance units (RU). The flow cell was perfused with increasing concentrations (0-1000nM) of WT protein S (A), SHBG1 (B), SHBG2 (C), SHBG3 (D) or SHBG4 (E). Results from a representative experiment are shown (n=2). F) The maximum RU (450 seconds of association) for WT protein S and variant binding was plotted against the protein S concentration. Data are given as mean±SEM; n=2.

Figure 7. Proposed models of the interaction between the protein S SHBG-like region and TFPI Kunitz domain 3. A) The NMR structure of TFPI Kunitz domain 3 is shown as a cartoon (left) and solid surface (right). The consensus region shown in red is predicted to be critical for protein/protein interaction using the MetaPPI online prediction tool. This region gave the highest predicted scores, but other areas can also be of importance. Glu226 and Arg199, two residues shown to be involved in the interaction with protein S are labelled in blue (27, 28). B and C) Two suggested models (pose A and pose B) of the protein S SHBG/TFPI Kunitz domain 3 complex. The LG1 and LG2 domains are shown in magenta

and blue, respectively and the TFPI Kunitz domain 3 is shown in green, either as surface or cartoon presentation. The substituted residues in the protein S variants are marked with deviant colours.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7