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A theoretical model for the Gla-TSR-EGF-1 region of the anticoagulant cofactor protein S: From biostructural pathology to species-specific cofactor activity

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Summary

Protein S (PS), which functions as a species-specific anticoagulant cofactor to activated protein C (APC), is a mosaic protein that interacts with the phospholipid membrane via its γ-carboxyglutamate-rich (Gla) module. This module is followed by the thrombin-sensitive region (TSR), sensitive to thrombin cleavage, four epidermal growth factor (EGF)-like modules and a last region referred to as the sex hormone binding globulin (SHBG) domain. Of these, the TSR and the first EGF-like regions have been shown to be important for the species-specific interaction with APC. Difficulties in crystallising PS have so far hindered its study at the atomic level. Here, we report theoretical models for the Gla and EGF-1 modules of human PS constructed using prothrombin and factor X experimental structures. The TSR was built interactively. Analysis of the model linked with the large body of biochemical literature on PS and related proteins leads to suggestions that (i) the TSR stabilises the calcium-loaded Gla module through hydrophobic and ionic interactions and its conformation depends on the presence of the Gla module; (ii) the TSR does not form a calcium binding site but is protected from thrombin cleavage in the calcium-loaded form owing to short secondary structure elements and close contact with the Gla module; (iii) the PS missense mutations in this region are consistent with the structural data, except in one case which needs further investigation; and (iv) the two PS 'faces' involving regions of residues Arg^{49}–Gln^{52}–Lys^{97} (TSR-EGF-1) and Thr^{103}–Pro^{106} (EGF-1) may be involved in species-specific interactions with APC as they are richer in nonconservative substitution when comparing human and bovine protein S. This preliminary model helps to plan future experiments and the resulting data will be used to further validate and optimise the present structure.

Introduction

Human protein S is a 75 kDa (635 residues), nonenzymatic vitamin-K-dependent glycoprotein synthesised primarily by liver cells [1,2]. In human plasma, it functions as a cofactor to activated protein C (APC) in the degradation of coagulation factors Va and VIIIa [3,4] in the presence of phospholipids and calcium ions. Recently, it was suggested that both protein S (PS) and intact factor V act as synergistic cofactors to APC [5]. The anticoagulant activity of PS is clearly demonstrated by the higher incidence of recurrent venous thromboembolism in individuals deficient in PS [6–8]. PS has a much stronger affinity for negatively charged phospholipids than APC, and presumably increases the affinity of the latter for this type of membrane through the formation of a 1:1 complex [3,9]. In addition, PS has an APC-independent anticoagulant function [10–12]. Interestingly, PS seems to have other roles than anticoagulation. These include mitogenic action to cultured aortic smooth muscle cells [13] and an unknown action in brain tissues [14]. Receptors for PS and a PS homologue, growth arrest-specific factor 6 (GAS6), have just been presented [15,16], but in these the physiological roles of PS remain to be understood.

The primary structure of human PS (hPS) is known [17]. Two homologous genes for hPS have been found

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and map close to the centromer of chromosome 3. They are referred to as protein Sα, the active gene, and protein Sβ, a pseudogene [18]. PS is a mosaic protein composed of multiple domains or modules. Starting from the N-terminus, it contains a γ-carboxyglutamic acid (Gla) module (residues 1–46) ending by the so-called aromatic stack region (residues 37–46), the thrombin-sensitive region (TSR, residues 47–72), four epidermal growth factor (EGF)-like modules (residues 76–242) and a sex hormone binding globulin (SHBG)-like module (residues 243–635). The Gla module binds calcium ions and negatively charged phospholipids. The human TSR can be cleaved by thrombin or other proteases after Arg residues 49 and 70 and possibly 60, but the Gla domain remains connected to the first EGF via a disulphide bridge. TSR integrity is essential for APC cofactor activity [19,20]. The N-terminal EGF-like module contains an *erythro*-β-hydroxyaspartic acid (Hya) and each of the other three EGFs contain an *erythro*-β-hydroxyasparagine (Hyn) [21]. Each of the three Hyn-containing EGF-like modules have a high-affinity calcium binding site [22]. The functional role of the Hyn and Hya residues is unknown with regard to protein C (PC) since substitution of these residues by an Asp or Asn does not modify APC cofactor activity [23]. Human PS displays three potential N-glycosylation sites in the SHBG-like module [17,24].

In human plasma 60–70% of the PS is noncovalently associated to C4b-binding protein (C4BP), a regulator of the classical complement pathway [4] and acute-phase reactant [25]. Upon complex formation PS loses its APC cofactor activity [4], but retains its ability to bind negatively charged phospholipids. C4BP is a spider-like multimeric protein [26] composed of six or seven α-chains, with or without a β-chain. The α-chains are involved in the binding of C4b while the remaining core of C4BP can interact with the serum amyloid P component, a protein present in all types of amyloid deposits, including those of Alzheimer's disease [27]. Eighty per cent of the circulating C4BP contains the β-chain, which is the only binding site for PS [28–32]. On the PS molecule, the C4BP binding site involves only the SHBG-like module [33–36].

The anticoagulant activity of APC is species specific and this seems to be the result of species-specific interaction between APC and PS [37]. Bovine PS can support both human and bovine APC, but human PS functions only with human APC. The main structural determinants in the PS molecule responsible for species specificity most likely involve PS TSR and EGF-1 regions [37].

Association between familial PS deficiency and thrombosis has been reported [6–8,38–40]. Three types of PS deficiency were initially reported. Type I (quantitative) manifests itself as decreased plasma antigen levels of both free and PS-C4BP complex. Type IIa (qualitative, also called type III) is characterised by a normal concentration of total PS with low free PS levels, whereas type IIb (qualitative, also called type II) implies normal free PS level but low cofactor activity (functional defect) [41,42]. It seems, however, that type I and type IIa (type III) deficiencies are phenotypic variants of the same genetic disease [43]. When a single point mutation leads to a type I deficiency, it is generally expected that the substitution either (i) disturbs a structurally important salt bridge or hydrogen bond; or (ii) introduces a charge in the hydrophobic core; or (iii) replaces a short side chain in the tightly packed core interior by a large one; or (iv) creates a cavity in the structure. A type II deficiency is, in general, consistent with a solvent-accessible residue and/or a residue in an active site or binding area, which suggests that the replaced residue is not critical for stability or folding. Recently, several substitutions in the PS gene responsible for polymorphism, type I and II deficiencies, were described [44–46] and the ones in the Gla-TSR-EGF-1 region can now be investigated from a structural standpoint.

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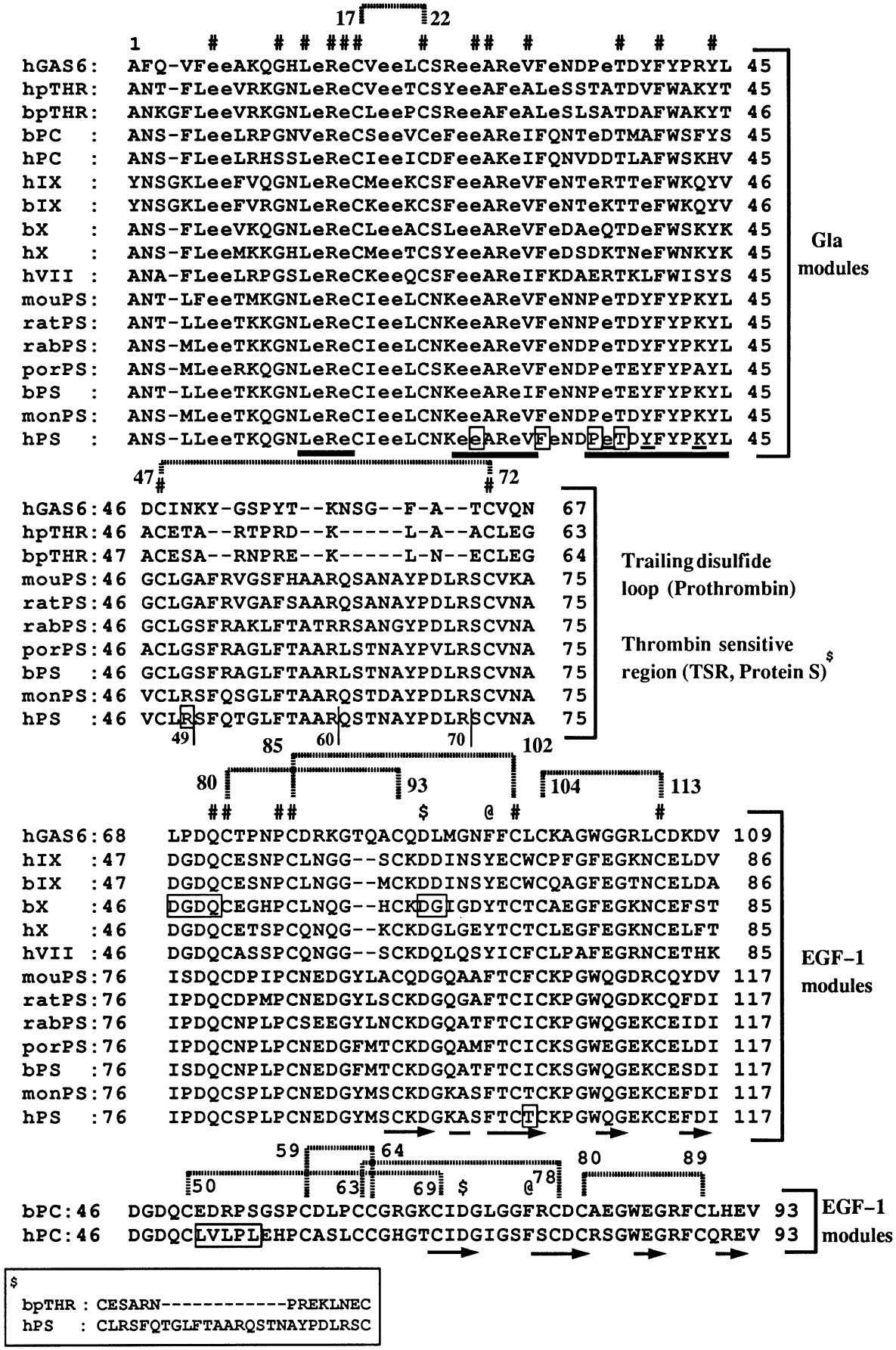
Fig. 1. Sequence alignment of the Gla-EGF-1 regions. The sequences of human protein S (hPS), monkey PS (monPS), bovine PS (bPS), porcine PS (porPS), rabbit PS (rabPS), rat PS (ratPS), mouse PS (mouPS), human factor VII (hVII), human and bovine factor X (hX, bX), human and bovine factor IX (hIX, bIX), human and bovine protein C (hPC, bPC), human and bovine prothrombin (hpTHR, bpTHR) and human growth arrest-specific factor 6 (hGAS6) were aligned (see text). The sequence alignment in the TSR has to be taken with caution (see the Materials and Methods section) and the one used initially for the hPS modelling is noted in the inset. The # symbol above the sequences depicts residues buried from the solvent in the hPS model, computed with the method of Lee and Richards [104]. No information is reported for the TSR since its structure is still speculative. The dashed lines indicate the disulphide bridging pattern in PS and in the first EGF of protein C (PC EGF-1 has an extra disulphide). The Cys residues of hPS are numbered while those of PC are shown for the first EGF. Beneath the sequences, black arrows represent the β-strands in the calcium-loaded EGF-1 NMR structure of bX, while the solid black lines indicate the α-helices in the calcium-loaded X-ray structure of bpTHR (in the trailing disulphide loop, the bpTHR helical segment involves REKLNECL). The secondary structure elements of bX EGF-1 are mentioned for the PC sequences although no experimental evidences are yet available for this module structure. The small black boxes in the bX EGF-1 sequence indicate residues involved in calcium binding, while such a box in the hPC EGF-1 sequence indicates residues that may, in part, play a role in species-specific interaction. In the PS sequence, the boxes refer to residues involved in type I and II mutations or polymorphism (see text). The lower case e indicates Gla residues (some Gla residues were assigned by sequence comparison and Glu^{35} in hVII was not modified according to Ref. 81). The $ symbol denotes *erythro*-β-hydroxyaspartic acid, not present in all proteins (e.g. factor VII [105] while in many proteins the hydroxylation is only partial). The @ symbol indicates an aromatic residue (indirectly) important for calcium binding. The | symbol in the TSR represents the possible thrombin cleavage sites (Arg^{49}, Arg^{60} and Arg^{70}). Underlined residues in the hPS sequence are some residues expected to be involved in the Gla-EGF-1 intermodule interaction.


Gla modules
Trailing disulfide loop (Prothrombin)
Thrombin sensitive region (TSR, Protein S)$
EGF-1 modules
EGF-1 modules
$
bpTHR : CESARN----------PREKLNEC
hPS : CLRSFQTGLFTAARQSTNAYPDLRSC

PS lack of solubility has so far hindered its structural study by X-ray crystallography or NMR. We decided therefore to investigate PS with some of the tools used in computational biochemistry [47,48]. A growing literature has demonstrated the strength of protein modelling in helping to elucidate structure–function questions and plan experiments [49–56].

In this paper we report the modelling of the Gla-TSR-EGF-1 region of hPS. This preliminary model can be built since the Gla and EGF folds are extremely well conserved [57–65]. The model is used to map PS point mutations (which in turn help to validate the model structure), to investigate the possible mechanisms involved in the TSR degradation and to probe the possible regions involved in the PC-PS specific interaction.

Materials and Methods

Modelling the N-terminal region of hPS

The sequence alignment used for this modelling study is shown in Fig. 1. Sequences were obtained from the Swiss-Prot database [66]. Several glutamic acid residues in the Gla modules are mentioned as Gla residues by sequence comparison, while in some cases no experimental evidences have yet been reported to validate this hypothesis. This applies also to the EGF-1 Hya residue.

hPS Gla module

The X-ray structure of the calcium-loaded bovine prothrombin (bpTHR, Fig. 2A) [61] (PDB entry 2PF2 [67]) was used as a template to build the hPS Gla module. The sequence identity between these two domains is 50%. A single deletion was effected by computationally removing bpTHR Gly^{4}. The PS Gla module was then locally refined by energy minimisation. Side chains were modelled using the programs InsightII and Biopolymer [68]. When the residues differed, χ angles were copied as appropriate and the remaining part of the side chains was built in an extended conformation. Side-chain orientations were then optimised interactively, if needed, using a rotamer library [69]. The seven bpTHR X-ray structure calcium ions were kept. It is possible to assign hPS Gla residue 36 to bpTHR Ser^{36} instead of Ala^{37} as described here. Such a change would assume the sequence alignment of Ref. 59. Both solutions seem acceptable from a structural standpoint (see the Results section).

hPS TSR

Interactive investigation of different models on the graphic computer, recent advances in secondary structure prediction [70–72], threading [73] as well as a comparison of the TSR sequences with a structural database [74] were used. Residues L48RSFTGLFTAAR60 could adopt an α(or distorted)-helical conformation with a 'loop' around residues Q61STNAY66 followed by an α-helical segment P67DLRS71 antiparallel to the previous one (Fig. 2B). Other possibilities have, however, to be considered (see the Results section). The TSR was built manually with the trailing disulphide loop of prothrombin and the above structural predictions as a starting point. The sequence alignment used is given in the inset of Fig. 1. This alignment does not introduce any gap or insertion in the bpTHR trailing disulphide loop α-helical segment, even if other possibilities remain. The TSR alignment in Fig. 1 takes into account GAS6, but it is not possible to introduce the structural information for PS and GAS6 at the same time. The Cys^{47}–Cys^{72} disulphide bridge of hPS was built using the corresponding residues of bpTHR (Fig. 1) and the hPS Gla module's C-terminal helix was extended up to residue Arg^{60}. Residues Pro^{67}–Ser^{71} were then assumed to be α-helical, in analogy with the bpTHR trailing disulphide loop [61]. After 'docking' the hPS EGF-1 module onto the Gla domain, the TSR was briefly energy minimised followed by a 3 ps room temperature molecular dynamics (MD) simulation and brief energy minimisation. This last structure was then submitted to a 100 ps MD simulation at 300 K. From this trajectory, coordinates were sampled every ps and energy minimised. This procedure allows partial energetic comparison along the trajectory. However, significant effort would be required to validate the TSR model further (e.g. the use of several starting conformations and the addition of solvent). All MD simulations were performed in vacuo with the Gla and EGF-1 model structures held fixed using Discover [68] on a Silicon Graphics Indigo2 R10000 workstation.

Orientation of the Gla and EGF-1 modules

The initial orientation between the hPS Gla and EGF-1 modules was assumed to be similar to the one observed for the calcium-free NMR structure of blood coagulation factor X (bX) [59]. Further changes were then introduced interactively. Several alternatives can be expected with regard to the intermodule orientation, but for the sake of simplicity only one structure is discussed here (Fig. 2B).

hPS EGF-1 module

The EGF-1 module of hPS was built using the energy-minimised calcium-loaded NMR structure of the first EGF module of bX [58]. The sequence identity between the hPS and bX EGF-1 modules is 49%. Since the hPS EGF-1 may lack calcium binding [63], a model was built also from the apo form. The high sequence identity between hPS EGF-1 and bX EGF-1 and the lesser need for deletions and insertions favour our template choice over known non-calcium binding EGF structures [64,65]. Side-chain substitutions were introduced interactively. The few unfavourable contacts were removed using a rotamer library, as above. The insertion in the loop region 89–92

of hPS was generated using the random tweak algorithm [75] and refined by brief energy minimisation.

Results

Critical evaluation of the model and structural analysis

hPS Gla module

Bovine prothrombin [61] is structurally characterised by three α-helices, an ω-loop-like region that interacts with the phospholipid membrane, a disulphide loop (Cys^{18}–Cys^{23}) that interacts with an aromatic cluster (Phe^{41}, Trp^{42}, Tyr^{45}) surrounded by several hydrophobic residues (Leu^{14}, Leu^{19}, Leu^{32} and Leu^{62}) and finally by 10 Gla residues which chelate a total of seven (or possibly more) calcium ions (Fig. 2A). The sequence of the hPS Gla module is extensively similar to that of bpTHR (Fig. 1).

The hPS amino terminus forms three ion pair interactions with Gla residues 16, 20 and 26 as in the bpTHR X-ray structure. Prothrombin Gly^4 is solvent accessible and deletion of this residue in the PS model is unproblematic. The replacement of bpTHR Val^9 by Thr^8 in hPS is interesting since most Gla modules have a more hydrophobic residue there (Val, Leu, Met or Phe). This area, with the hydrophobic prothrombin residues 5 and 6 or bovine factor X residues Phe^4, Leu^5 and Val^8, is known to be critical for membrane binding [59].

The two positive charges prothrombin Arg^{10} and Arg^{16} are conserved in the model structure (hPS Lys^9 and Arg^{15}). As seen in Fig. 1, positive residues are very conserved in this part of the molecule. In bpTHR these two residues interact by van der Waals, hydrophobic and aromatic forces and may also play a role in the interaction with the membrane phosphate groups. The strictly conserved bpTHR Arg^{16} is part of a hydrogen bond network (mainly with surrounding water molecules), has ionic interactions with the side chain of Gla residue 17 and with the backbone carbonyl of Lys^{11} and may, for this reason, be a key structural residue.

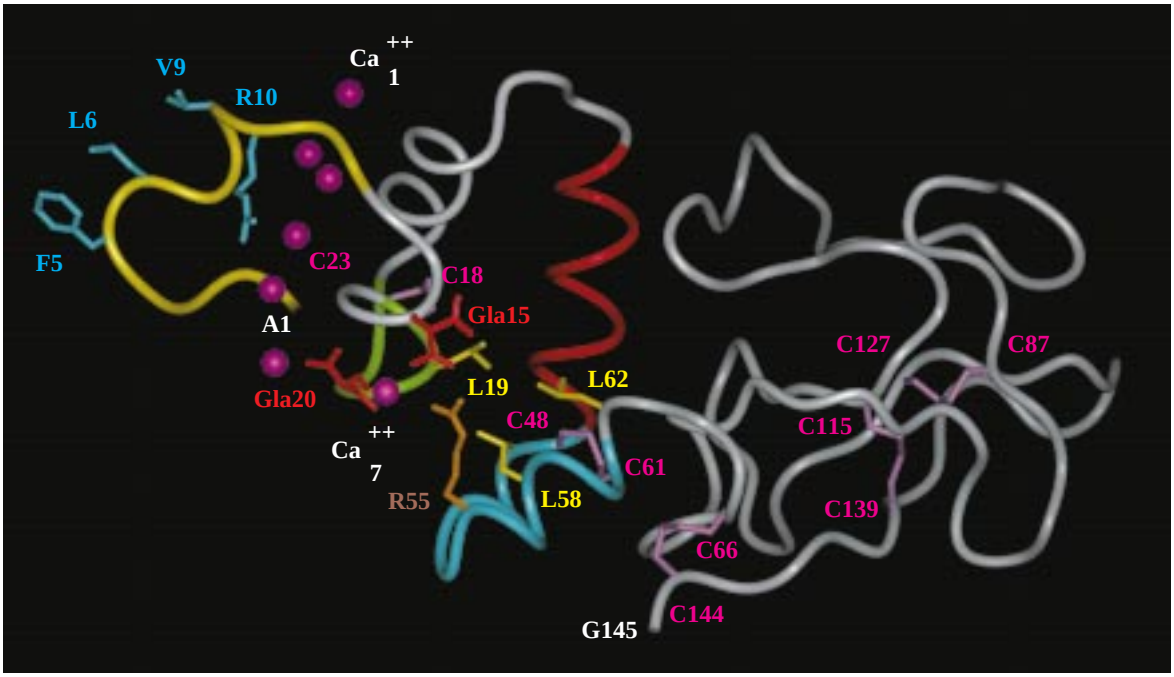
Substitution of the solvent-accessible prothrombin Lys^{11} by Gln^{10} in hPS was straightforward. Proline residues are most often solvent exposed in proteins. The solvent-accessible bpTHR Pro^{22} seems to undergo a trans/cis isomerisation upon the addition of calcium [76], but is nevertheless easily replaced by Leu^{21} in hPS. The role of prolines is difficult to evaluate although they often contribute to protein stability through favourable entropic effects [77,78]. PS Leu^{21} is conserved (Figs. 1–2B) and forms together with the nearby Leu^{48} and Phe^{51} a small hydrophobic cluster possibly important to the TSR and Gla structures. Replacement of prothrombin Phe^{29} by Arg^{28} in hPS is acceptable since the residue is solvent accessible and no steric conflict is created upon its substitution. hPS Arg^{28} forms a salt bridge with Asp^{34}, an acidic residue at the beginning of an α-helix (Fig. 1), and very likely interacts in a similar fashion with Gla residue 25. hPS Asp^{34} corresponds to bpTHR Leu^{35}. This substitution is a spatially correlated mutation and useful for the validation of the model. Interestingly, this salt bridge may be conserved in human GAS6 but altered in some other PS species (Asp^{34} is often changed to an Asn). In very close vicinity, hPS Lys^{24} forms a salt bridge with Asp^{38}. This interaction in hPS would correspond to the salt bridge involving bpTHR Arg^{25} and Asp^{39}. bpTHR Ser^{36} is changed to Pro^{35} in hPS. This seems appropriate to initiate [79] the next helical segment. Ala^{37} in bpTHR is replaced by an extra Gla residue in hPS. The sequence alignment of Ref. 59 would entail a locally different conformation, but in both cases hPS Gla residue 36 remains fully solvent accessible. The hPS Arg^{28}–Asp^{34} interaction, the absence of deletion in the Gla module last helical segment and the Pro^{35} as first or second residue of this helix support the sequence alignment given here. Moreover, Gla residue 36 is well positioned to be hydrogen bonded to the Asn^{33} side chain. Tyr^{39} points towards the solvent and not into the aromatic stack. Replacement of bpTHR Ala^{43} by the conserved Pro^{42} in the PS sequences is interesting and may induce a kink [79] in the C-terminal region of this helix or even terminate it. However, this helix most likely continues as in bpTHR, at least in the calcium-loaded form. The Pro^{42} induced kink seems to make the terminal part of this helix 'wrap' around the Gla module (and not in the direction opposite to the Gla module), thus bringing the TSR into close contact with the membrane binding domain. However, the bend cannot be too pronounced due to the aromatic stack (Tyr^{44} packs against Phe^{40} and the nearby Cys^{17}–Cys^{22} disulphide). Proline cis/trans isomerisation may locally modify the protein conformation, but this problem is difficult to address with present molecular modelling tools.

hPS TSR

The trailing disulphide loop of bpTHR consists of an extended chain and two turns of α-helix (R55 to L62) (Fig. 2A). The main interactions between the Gla module and the loop involve Gla residues 15 and 20, and Leu^{19} and Arg^{55}, with Arg^{55} being salt bridged with the two Gla residues and with the Leu^{19} carbonyl atom. Further, the side chain of Arg^{52} seems to interact with Glu^{49} carboxylates. A small hydrophobic core is formed, mainly from the interaction between the side chains of residues Leu^{19}, Leu^{58}, Leu^{62} and Cys^{48}–Cys^{61}. This hydrophobic cluster is in contact with the aromatic stack. The trailing disulphide loop may contribute to the stabilisation of the Gla domain, but its own folding is certainly critically influenced by the presence of the Gla module. A few additional interactions come from the kringle-1 module.

In the case of calcium-loaded hPS, the TSR appears to be stabilised by the following interactions: the side chain of hPS Leu^{48} interacts with the side chains of Tyr^{44}, poss-

A



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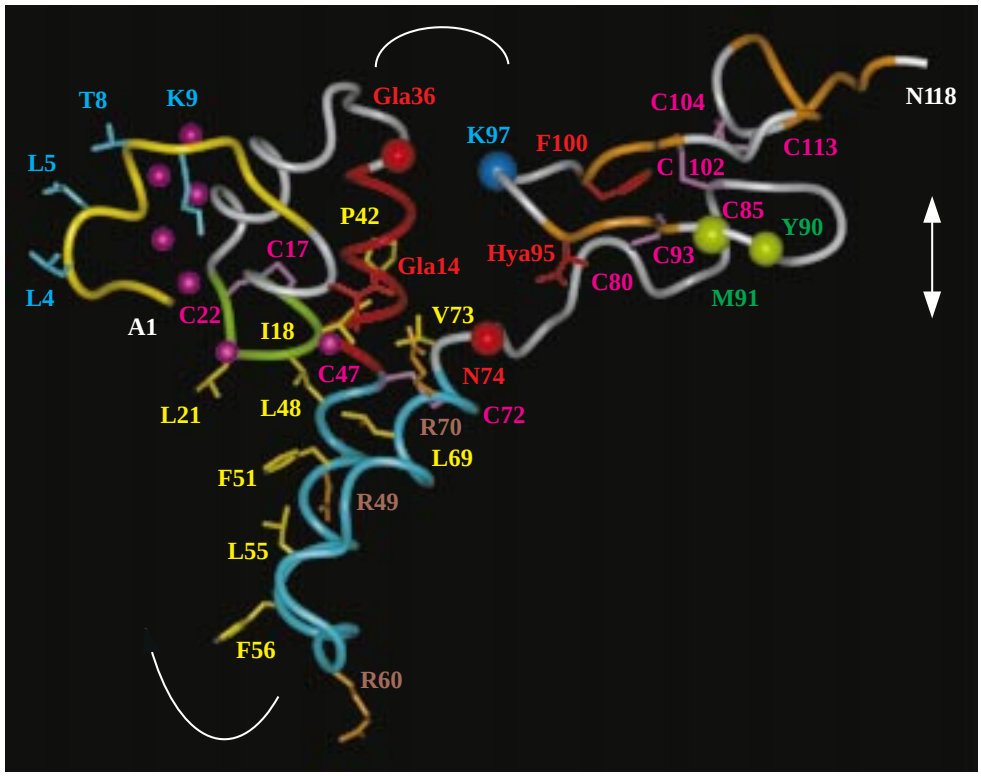

Fig. 2. Ribbon diagrams of bpTHR and hPS. (A) bpTHR fragment 1 X-ray structure. The Gla module's ω-loop (residues 1–12) is shown in yellow, the disulphide loop (C18–C23) in green and the helix forming part of the aromatic stack in red. The trailing disulphide loop (C48–C61) is highlighted in blue and the remaining part of the structure (including the kringle-1 domain) is coloured grey. Some suggested residues involved in phospholipid binding are in blue (F5, L6, V9, R10). Calcium ions are magenta and disulphides pink. Among the many side chains discussed in the text, only the essential ones are shown in order to simplify the figure. The colours have been chosen to help in the reading of the figure but are not related to the one used for the ribbon trace. (B) hPS Gla-TSR-EGF-1 theoretical model. The Gla module (residues 1–46), with the ω-loop (yellow), the disulphide loop (green) and the aromatic stack (red), is represented in an orientation similar to the one used for Fig. 2A. The TSR (C47–C72) follows (blue), further continued by the first EGF module. The suggested EGF-1's β-sheets are coloured light brown. Calcium ions are magenta and disulphides pink. The side chains or C^{α} atoms of some residues (see text) are shown as stick or CPK models, with different colours from the backbone. The white curved lines indicate flexibility and/or undefined angles between the modules. The vertical arrow highlights the fact that the EGF-1 may be located more towards the lower right part of the figure.

ibly with Phe[51] and Leu[21] which together with Ile[18], Leu[55], Ala[65], Tyr[66], Leu[69], Val[73] and the Cys[47]–Cys[72] disulphide forms a small hydrophobic core. The hPS Arg[49] side chain is fully solvent exposed and may be hydrogen bonded to Gln[52]. This interaction would match the bpTHR Glu[49]–Arg[52] pair. The hydrophobic cluster, particularly around Leu[21] and Phe[51], is probably the driving force that produces the close contact between the TSR and the Gla domain. Motion of this nature was observed in our preliminary MD simulation, suggesting that a more compact structure, when compared to the model presented (Fig. 2B), may be possible.

hPS Arg[60] is fully solvent accessible. The last α-helix in the Gla module may be stabilised in its N-terminal part by negatively charged residues (generally conserved there, Fig. 1). This 'capping' is mainly performed by Gla residue 32 and Asp[34], while Arg[49] or Arg[60] could stabilise the C-terminus. hPS Tyr[66] may play a role in the binding of calcium ions close to Gla residues 14 and 19. The peptide geometry of hPS Pro[67] was considered to be trans. This proline would be the starting point of the proposed second helical segment. hPS Asp[68] is solvent accessible and lacks obvious interactions with surrounding residues. The hPS Arg[70] guanidinium group appears to stabilise the TSR by forming a salt bridge with Gla residue 14 and possibly residue 19, as in bpTHR (bpTHR Arg[55] with Gla residues 15 and 20). hPS Val[73], in the linker to the first EGF, is in hydrophobic contact with Ile[18] (conserved in all PS sequences), immediately below the Gla residue 14–Arg[70] salt bridge.

Our reported TSR model displays charged and polar residues at the surface while hydrophobic ones are more shielded from the solvent and/or interact with each other. Threading experiments support the model further. Many protein segments able to accept the TSR sequence are helical (either one helix or helix–turn–helix). However, no obvious templates for PS were identified in the structural database used and interactive modelling was deemed more appropriate.

During the MD simulation, the TSR first hypothetical helical segment was partially disrupted and ended at Ser[50]. Pro[65] to Val[73] kept an overall helical structure, with backbone angles close to a 3_{10} conformation. The side chain of Tyr[66] entered an aromatic interaction with Arg[70] (π-electrons). Analysis of the TSR structure along the simulation does not suggest the possibility of a calcium ion binding site there. Moreover, only one negatively charged residue, Asp[68], tends to be conserved in the sequences (Fig. 1).

Orientation of the hPS Gla and EGF-1 modules

In the NMR structure of calcium-free factor X, a major interaction between the Gla and EGF-1 modules

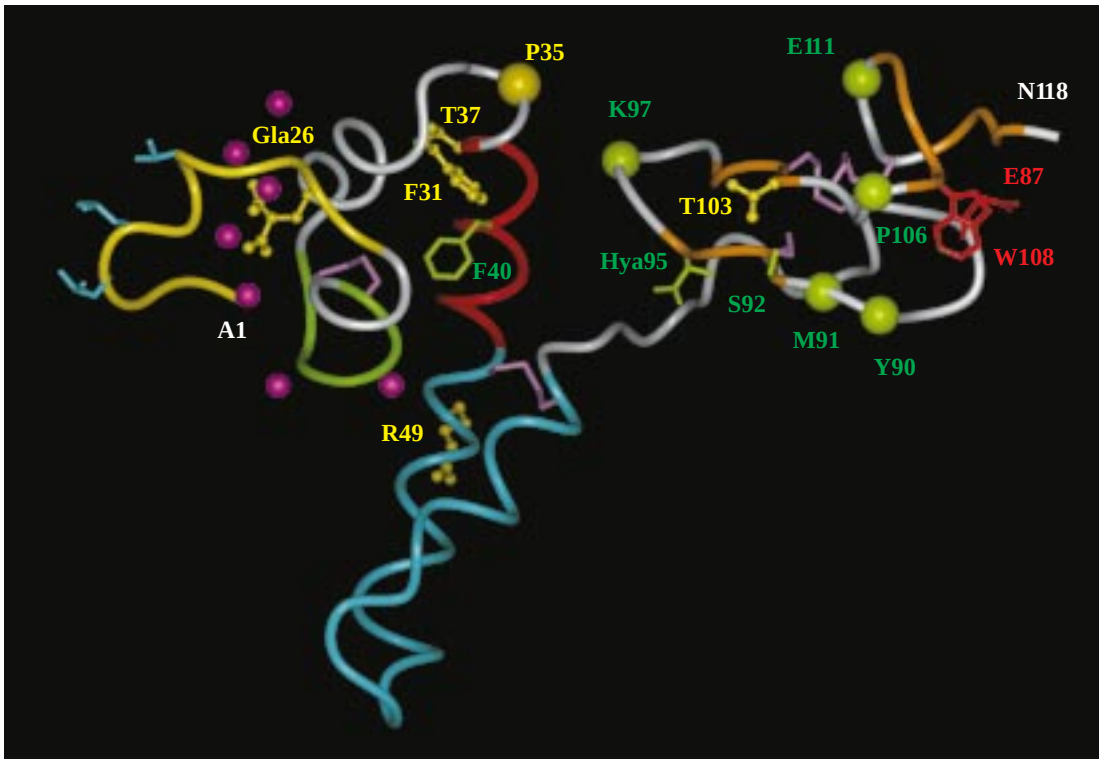

Fig. 3. Biostructural pathology and species-specific cofactor activity. Ribbon representation of the hPS model from the same direction as in Fig. 2B. Residues involved in type I or II deficiency are shown in yellow (see the Discussion section). The C^{α} atom of Pro[35] is displayed as a yellow CPK sphere; this indicates a polymorphism. Some other residues of interest are highlighted (i.e. PC-PS interaction, PS EGF-1-EGF-2 interaction, see text).

involves Phe[40] and Ile[65] [59]. Also Tyr[44], Leu[18] and Gla residue 14 are close to Ile[65]. For various reasons (protein–protein interaction, membrane binding, blood flow), it is difficult to determine the intermodule orientation in vivo, but also because the conformation most likely changes on calcium binding. Hydrophobic residues at the tip of the major EGF-1 β-sheet [58] suggest that this area is involved in the Gla-EGF contact. The most probable arrangement appears to be that Lys[97] (tip of the major hPS EGF-1 β-sheet) makes contact with the Gla module via Gla residue 36. Upon calcium/membrane binding, the relative module orientation may change to expose Lys[97] for macromolecular interaction.

hPS EGF-1 module

Calcium binding EGF modules are structurally characterised by one major (strands 1–2) and one minor (strands 3–4) twisted β-sheet (Fig. 2B), while the major β-sheet often has a short additional strand in non-calcium binding EGF modules. The disulphide bonding pattern is generally 1–3, 2–4, 5–6 [80]. Blood proteins often have a Hya or Hyn residue that participates in calcium binding. Such interaction stabilises the EGF-1 N-terminal region in factor X [58]. Polar and negatively charged residues contribute to the binding of calcium. In bX, the backbone carbonyls of Gly[47] and Gly[64] as well as the carboxylate/carboxyamide side chains of Gln[49] and Asp[48], Hya residue 63 and possibly Asp[46] are calcium ligands. The situation is similar in the crystal structures of human factor IX EGF-1 [62] and of the complex tissue factor-factor VIIa [81]. On the assumption that hPS EGF-1 binds calcium, the more favourable Ca^{2+} 6 to 8 coordination may involve Asp[78], Hya residue 95 (carboxyl oxygen), Gln[79] and the backbone carbonyl of Gly[96]. The 'missing' aspartate (corresponding to bX Asp[46]) and carbonyl group (corresponding to bX Gly[47]) are conceivably replaced by Gla module residues and/or other proteins or water molecules. The carbonyl or side chain of Asn[74] may also form part of the calcium binding site. The generally conserved aromatic residue, hPS Phe[100], known to play an indirect role in calcium binding [62], is present and interacts with Pro[84], near the Cys[80]–Cys[93] disulphide. The N^{ζ} atom of Lys[43] is about 5–6 Å from the calcium binding site, but with another angle between the modules this residue would have no specific or little interaction with the EGF-1. In the model, Gla residue 36 does not contribute to the EGF-1 calcium binding site, as noted in recent X-ray structures [81,82]. The above analysis suggests that the entire Gla-TSR-EGF-1 structural unit is needed for the investigation of the EGF-1 calcium binding site.

Several charged and aromatic/hydrophobic residues are noted in the C-terminal part of the hPS EGF-1 module and are conserved in PS sequences. In hPS, the cluster involves Glu[87], Asp[88], Tyr[90], Met[91], Lys[105], Pro[106], Trp[108], Gln[109], Glu[114] and Phe[115]. Protein S Glu[87] probably corresponds to factor IX Asn[58] [62,63] which may be involved in an interaction with the following EGF, while hPS Trp[108] (Fig. 3) is homologous to human fibrillin-1 (module 32) Tyr[2157] also shown to be involved in an EGF_n-EGF_{n+1} contact [63]. Some of the above hPS EGF-1 residues probably interact with the next EGF and/or macromolecules. A calcium binding site could be shared by residues coming from the first (like hPS Glu[87]) and second EGF modules (Hyn residue 136 area). The insertion of Tyr[90] and Met[91] is interesting since it exposes aromatic-hydrophobic residues that may be important for intermolecular interaction (see below). These two residues are very conserved in the PS sequences (YM, FM or YL).

It is important to note that the presented PS EGF-1 model has been built using a calcium-loaded template. Some structural differences are noted when a calcium-free structure is used, but these affect only the EGF calcium binding sphere.

Discussion

Molecular modelling

In spite of local modifications, the structure of the EGF and Gla modules has been shown to be highly conserved. Equivalent residues in the core of the PS Gla and EGF-1 modules were conserved in relation to the template structure. No charged residues were buried in the model structure or were found uncompensated, in agreement with experimentally determined structures [83]. Moreover, no severe steric clashes were noted during the modelling process. The above findings suggest that the structure of the two PS modules is reasonable, while the TSR and the orientation of EGF-1 relative to the Gla module are more tentative. The trailing disulphide loop of prothrombin seems a good initial template for GAS6 (unpublished data). This suggests that it may be appropriate also for PS since PS and GAS6 are homologous with about 44% amino acid identity for the entire protein [15]. Nature has probably conserved the overall structural characteristic of this loop during evolution.

The present model fits into an approximate box of $39 \times 50 \times 65$ Å (1 Å = 0.1 nm). The distance between the ω-loop and the C-terminal area of EGF-1 is 65 Å, that between the first residue of the last helix of the Gla module and the 'turn' in the TSR is 50 Å and the width of this presumably 'heart-shaped' part of the PS molecule is about 39 Å.

Biostructural pathology

Hereditary PS deficiency has been associated with both venous and arterial thrombosis. Recently, several point mutations have been reported [44–46] and the ones located within the Gla-TSR-EGF-1 region can be investigated (Fig. 3). Analysis of the effects of mutations in

terms of their clinical phenotypes generally suffers from a lack of structural data, which would allow a more rational interpretation. The study of diseases at the atomic level has been referred to as biostructural pathology [84]. This type of work helps to understand the molecular mechanisms involved in the disease process and, in some cases, to clarify the potential effect of mutations [49,50, 62,84]. Moreover, consistency between these naturally occurring point mutations and a structural model offers a means to assess the quality of the three-dimensional structure.

Gla residue 26 to Ala (Gla module, type I)

Gla residue 26 is buried in the core of the Gla module (Fig. 1). It interacts with calcium ions, presumably with water molecules, and with Ala^{1}, Gla residues 6, 7, 16, 25 and 29 and the Cys^{17}–Cys^{22} disulphide. This residue certainly plays a major role in the module's thermodynamic stability. Gla residue 26 and also Gla residues 7, 16 and 29 have been shown to be important by mutagenesis studies of hPC and prothrombin [85,86].

Phe^{31} to Cys (Gla module, type I)

Phe^{31} interacts with the aromatic stack region and is not accessible to the solvent (Fig. 1). Its side chain has van der Waals contact mainly with Leu^{13}, Gla residue 36 and Phe^{40}. Aromatic interactions play a role in protein stability since they contribute some 4 kJ/mol to the stability for a pair of solvent-exposed Tyr [87]. The extensive aromatic/hydrophobic packing around Phe^{31} implies a significant thermodynamic contribution to protein stability. In addition, a Cys at position 31 may produce misfolding through disulphide bridge formation with Cys^{17} or Cys^{72} and/or unfavourable interaction with the nearby Gla residue 36. The required tight packing of this area would in any case be disrupted.

Pro^{35} to Leu (Gla module, polymorphism)

This replacement is located at the beginning of the last Gla module helical segment. Such a location is acceptable for proline residues. In PS and GAS6, this Pro is either preceded by an Asp or Asn. This is often the case in helical regions and there a proline trans isomer is favoured [79]. Leucine residues have a high helix propensity, such that the mutation is acceptable although not reciprocal when compared to the other sequences. In fact, most related proteins (Fig. 1) have a polar or negatively charged residue there.

Thr^{37} to Met (Gla module, type I)

Thr^{37} is close to Phe^{31}, buried and strictly conserved (Fig. 1). There is no room for a methionine side chain and significant steric clashes seem to result, mainly with the backbone atoms of Arg^{28}. In consequence, the mutation would significantly disrupt the aromatic stack region, known to be important for enhancing the Ca^{2+}-dependent properties of the Gla modules [88,89].

Arg^{49} to His (TSR, type I)

Arg^{49} seems to be hydrogen bonded to the side chain of Gln^{52}. This Arg is solvent accessible in the model and its location is reasonable for a charged residue. The Arg to His substitution is evolutionary closer [90] than its natural replacement by a Gly in several PS sequences. Its replacement by a Leu (site-directed mutagenesis), a nonconservative change, does not induce any noticeable folding problem [91]. These suggest that Arg^{49} is probably not involved in any structurally important buried salt bridge and that it is solvent accessible. In the model Arg^{49} is part of an α-helical segment. A His there may react with the thiol group of the nearby Cys^{47} or Cys^{72} and/or disturb the formation of this putative helical segment. With the available data, the model does not offer any explanation as to why the mutation would produce a type I deficiency. Further experimental and theoretical investigations are needed to evaluate the exact role of this residue [45].

Thr^{103} to Asn (EGF-1, type II or IIb)

Thr^{103} is solvent accessible and an Asn can be accommodated there. Nearby are the side chains of Thr^{101}, Met^{91} and Ser^{92}. In the model the main interaction of the Thr^{103} side chain is with the hydroxyl of Ser^{92}, immediately preceding the Cys^{80}–Cys^{93} disulphide. The side chains of Lys^{94} and Lys^{105} are also near Thr^{103}. A Thr to Asn substitution may produce a hydrogen bond with Lys^{94}. It is interesting to note that PS residues Thr^{103} and Ser^{92} correspond to murine EGF Asn^{32} and Val^{19}, respectively [65], with Asn^{32} having some interactions with the side chain of Val^{19}. This mutation may therefore play a role in PS cofactor activity.

The thrombin-sensitive region

In a calcium-free conformation, the TSR is sensitive to thrombin and other protease cleavage. In order to be cleaved, the TSR will have to undergo conformational changes. In the absence of calcium, the factor X Gla module assumes a different conformation and the Gla-EGF-1 intermodule angle is modified [92]. In PS, without calcium, hydrophobic contacts between the TSR and the Gla domain (e.g. Phe^{51}–Leu^{21} area), hydrogen bonds within the TSR, and the putative salt bridge (Arg^{70}–Gla residue 14) would be partially lost. In the calcium-free form, Gla residue 20 clashes with Phe^{51} and seems to push the TSR away from the Gla module. Thus, prior to or upon interaction with the proteases, the TSR would adopt a more 'random' conformation and finally match the canonical P3-P'3 structure of serine protease substrates/inhibitors [93]. The removal of the TSR will then disturb the

PS cofactor activity with APC since this part of the molecule is involved therein.

After cleavage, PS shows a reduced affinity for Ca^{2+} and phospholipid membrane [94,95]. In a calcium-loaded form, PS residues Leu^{55} and Phe^{56} lie almost in the same plane as residues 4 and 5, suggesting that this part of the TSR could contact the phospholipid membrane (Fig. 2B). The cleavage of the TSR would then disturb the interaction with the membrane. Here, four points have to be stressed: (i) The trailing disulphide loop of prothrombin does not seem involved in a contact with the membrane [96] while of course much shorter than the TSR (Fig. 2). (ii) Data obtained with antibody HPS 67 [97], expected to bind to or near the TSR, would support the fact that the TSR does not interact directly with the phospholipids. (iii) If the TSR hydrophobic residues were involved in the interaction with the membrane, removal of the loop by thrombin would certainly lead to a PS having very reduced membrane binding activity that probably could not be restored through the addition of calcium. (iv) In addition, PS has a high affinity for negatively charged phospholipids, strongly suggesting that hydrophobic interactions are not the key ones. Thus, for thermodynamic reasons, all or most of the TSR hydrophobic residues probably make contact with the Gla module.

The TSR cleavage is likely to remove an important anchoring region contributing to the folding of the Gla module. The addition of calcium ions up to a certain concentration could counterbalance this instability. Interestingly, the TSR does not seem to form a calcium binding site in itself due to the lack of negatively charged residues.

Overall orientation between the PS Gla module and the membrane surface

The Gla module of vitamin-K-dependent proteins is involved in phospholipid binding. Some critical residues have been suggested: Phe^{5}, Leu^{6} and Val^{9} in bpTHR, Phe^{4}, Leu^{5} and Val^{8} in hpTHR, Leu^{5} in PC, Phe^{4}, Leu^{5}, Val^{8} and Lys^{9} in bovine X [98,99]. Li et al. [99] suggest that a pair of hydrophobic residues could be more strongly integrated in the membrane, e.g. PC Phe^{4} and Leu^{5}, while in other proteins, the hydrophobic pair 8 and 9, e.g. factor IX pair 9–10, inserts in the cell surface. In PS, the first pair (residues 4–5) is always hydrophobic (Leu/Met/Phe), whereas the second (residues 8–9) is more hydrophilic (Thr, Arg, Lys, only mouse has a Met there). The first pair should then be crucial for the insertion, while the second pair may interact with the membrane phosphate groups (Fig. 2B). Involvement of this small hydrophobic patch in membrane binding has, however, to be taken with caution [100]. The model structure suggests that the interaction of the PS Gla module with the membrane may be different from the one currently accepted for other vitamin-K-dependent blood coagulation proteins. In order to accommodate the TSR, the area of PS residues 4–5, 8–9, 28 and 34–36 may contact the membrane. This would lead to a slightly tilted orientation between PS and the membrane plane with respect to the proposed orientation shown in Ref. 99.

PC-PS interaction

Rhesus monkey PS can function as cofactor for human APC, while porcine and rabbit PS can be cofactor of bovine APC [101]. Bovine PS can support human and bovine APC, but human PS functions only with human APC. Only the human and bovine cases are well documented and are discussed here. In the bovine and human species, the EGF-1 and TSR have been shown to be essential for the PS-APC species-specific interaction [37, 97] while the PS SHBG-like domain does not seem involved [102]. The very high sequence identity between the PS molecules suggests that few residues may play a key role in the specificity [37,103].

We have identified two surface accessible clusters, rich in nonconservative replacements between the human and bovine sequences, most likely responsible for the species-specific interaction. The first involves hPS Leu^{45}, Val^{46}, Arg^{49}, Gln^{52}, Ile^{76}, Pro^{77}, Lys^{97} and the putative EGF-1 calcium binding site region, while the second comprises Met^{91}, Thr^{101}, Thr^{103}, Pro^{106}, Gln^{109}, Glu^{111} and Phe^{115}. Since substitution of Thr^{103} produces a type II deficiency [45], the second cluster is likely to be important. Site-directed mutagenesis experiments will probe further these two sites.

Conclusions

The Gla and EGF-1 modules of PS are structurally similar to those of related blood coagulation factors. The orientation between Gla and EGF-1 is still tentative while at present impossible to determine in vivo. The basic conformation of the TSR is likely to follow the one of prothrombin, based upon comparison with GAS6. The TSR structural model presented here is consistent with the available clinical and biochemical data. Forty-one point mutations in this region of PS have been developed in our laboratory by Drs. Xuhua He and Lei Shen, and are under examination with respect to the structural model. The results of these experiments will help to refine further the present model.

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