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The SHBG-like region of protein S is crucial for factor V-dependent APC-cofactor function

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Abstract Activated protein C (APC) regulates blood coagulation by degrading factor Va (FVa) and factor VIIIa (FVIIIa). Protein S is a cofactor to APC in the FVa degradation, whereas FVIIIa degradation is potentiated by the synergistic APC-cofactor activity of protein S and factor V (FV). To elucidate the importance of the sex-hormone-binding globulin (SHBG)-like region in protein S for expression of anticoagulant activity, a recombinant protein S/Gas6 chimera was constructed. It comprised the amino-terminal half of protein S and the SHBG-like region of Gas6, a structurally similar protein having no known anticoagulant properties. The protein S/Gas6 chimera expressed 40–50% APC-cofactor activity in plasma as compared to wild-type protein S. In the degradation of FVa by APC, the protein S/Gas6 chimera was only slightly less efficient than wild-type protein S. In contrast, the protein S/Gas6 chimera expressed no FV-dependent APC-cofactor activity in a FVIIIa-degradation system. This demonstrates the SHBG-like region to be important for expression of APC-cofactor activity of protein S and suggests that the SHBG-like region of protein S interacts with FV during the APC-mediated inactivation of FVIIIa.

Key words: Protein S; Factor V; Chimera; SHBG-like region; APC-cofactor function; Hemostasis

1. Introduction

The protein C anticoagulant pathway is essential for maintaining the hemostatic balance, as illustrated by the association between venous thrombosis and genetic defects affecting the pathway [1,2]. Protein C is activated by thrombin bound to thrombomodulin on the surface of vascular endothelium, and activated protein C (APC) inhibits coagulation by cleaving and inactivating phospholipid-bound activated factor V (FVa) and factor VIII (FVIIIa). In the degradation of FVa, APC efficiently cleaves FVa at position Arg-506, whereas FVa cleavage at Arg-306 requires the presence of protein S, which functions as an APC cofactor [3]. Cleavage of FVa at Arg-506 results in partial loss of FVa activity, but full FVa inhibition requires cleavage at both Arg-506 and Arg-306. The APC-mediated degradation of FVIIIa appears to be more complex than that of FVa. APC alone is inefficient in inhibiting the activity of the FVIIIa-FIXa (factor IXa) complex. Protein S increases the efficiency of APC only slightly, and full inactivation of FVIIIa requires the presence of yet another protein cofactor, namely factor V (FV) [4–6]. FV has been found to potentiate the APC-mediated inhibition of FVIIIa both in plasma and in purified systems. Full expression of anticoagulant activity of FV is dependent on the presence of protein S and the two proteins appear to function as synergistic APC cofactors [4–6].

Human protein S is a 635 amino acid long multidomain protein, composed of a γ-carboxyglutamic acid-containing (Gla) domain, a thrombin-sensitive region (TSR), four epidermal growth factor (EGF)-like domains and a region which is homologous to sex-hormone-binding globulin (SHBG) [7]. The Gla domain is required for binding of protein S to phospholipid membranes. Studies using specific monoclonal antibodies [8], human-bovine chimeras [9], or recombinant EGF-like domains [10] have demonstrated the TSR and EGF1 to be important for expression of the APC-cofactor function. This is consistent with the observation that a protein S deletion mutant, 'mini-protein S' lacking the whole SHBG-like region, is able to function as an APC cofactor [11]. However, mini-protein S did not demonstrate full APC-cofactor activity in a plasma system, possibly indicating the SHBG-like region to be required for full APC-cofactor function [11].

To elucidate the importance of the SHBG-like region of protein S for expression of APC-cofactor activity, a recombinant protein S/Gas6 chimeric protein was expressed and used in a series of functional assays. Gas6 (the product of growth-arrest-specific gene 6, *gas6* [12]) is homologous to protein S, sharing 44% amino acid identity and similar modular composition [13]. Gas6 is a ligand for the Axl subfamily of tyrosine kinase receptors [14] and affects cell growth, mitogenic activity and apoptosis [15,16]. While protein S has been suggested to stimulate some of these receptors [17–19], Gas6 is not known to play any role in anticoagulation. We now wish to report that the SHBG-like region of protein S is crucial for the FV-dependent APC-cofactor function expressed during inactivation of FVIIIa.

2. Materials and methods

2.1. Materials

Phospholipid vesicles composed of 25% (w/w) phosphatidylserine, 37.5% (w/w) phosphatidylcholine and 37.5% (w/w) phosphatidylethanolamine were prepared as described [20]. Simplastin Excel (thromboplastin reagent) and FV-deficient (immuno-depleted) plasma were purchased from Organon Teknika and Biopool, respectively. Human protein C was purified from plasma and activated as described [21]. Protein S, FV, FVa, and a-thrombin (all of human origin) were purchased from Haematologic Technologies Inc. Human FVIII (Octonative M) was from Pharmacia and hirudin from Sigma. Purified bovine factors X and IXa, chromogenic substrate S-2222 and the Coatest APC Resistance kit were kindly provided by Chromogenix. Protein

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Abbreviations: APC, activated protein C; EGF, epidermal growth factor; FV, coagulation factor V; FVa, activated factor V; FVIII, coagulation factor VIII; FVIIIa, activated factor VIII; FIXa, activated factor IX; Gla, γ-carboxyglutamic acid; SHBG, sex-hormone-binding globulin; TBS, Tris-buffered saline; TSR, thrombin-sensitive region

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S-depleted plasma was prepared as previously described [22], but with monoclonal antibody HPS54 [8], coupled to Affigel-10 (Bio-Rad), replacing the immobilized polyclonal anti-protein S antibodies. The concentration of FVIII in the protein S-deficient plasma was adjusted to 1 IU/ml using Octonative M.

2.2. Recombinant proteins

The full-length human protein S cDNA [7] was subcloned into the *Bam*HI site of the expression vector pcDNA3 (Invitrogen). The protein S/Gas6 chimera was obtained by recombinant PCR techniques [23] using protein S and Gas6 (a kind gift from Dr. Claudio Schneider) cDNAs as templates. Two PCRs were initially performed using the primer combinations A+B and C+D. Primer A, 5′-CGG GAT CCA TGA GGG TCC TGG GTG G-3′ (*Bam*HI site underlined), corresponds to the 5′-part of protein S, whereas primer B, 5′-GCA CGG CAA GAT GTC CTC ACA ACT CTT CTG-3′ (Gas6 sequence underlined) is an antisense hybrid between protein S and Gas6 sequences. Primers C, 5′-CAG AAG AGT TGT GAG GAC ATC TTG CCG TGC-3′ (protein S sequence underlined) is complementary to primer B. Primer D, 5′-CGG GAT CCT CGG ACA GAG ACT GAG AA-3′ (*Bam*HI site underlined) corresponds to the antisense strand of the 3′-part of Gas6. The human protein S sequence ends at nucleotide 995 [24], corresponding to Glu-242 of mature protein S, whereas the Gas6 starts at nucleotide 969 [13], corresponding to Asp-231 of the mature Gas6. The two PCR products were then used as templates together with primers A and D to create the protein S/Gas chimera. After *Bam*HI cleavage the construct was subcloned in the pcDNA3 vector and the nucleotide sequence was confirmed by dideoxy sequencing, using a sequencing kit from Perkin-Elmer. The full-length cDNA of human Gas6 was subcloned into the *Eco*RI site of pcDNA3. Human kidney 293 cells were stably transfected with the three vectors, containing protein S, Gas6 or the protein S/Gas6 chimeras, using Lipofectin (Life Technologies). The neomycin analogue G418 (400 μg/ml) was used for selection of transfected cells. Colonies of resistant cells giving the highest expression level, according to immuno blotting, were chosen for further expression. A mock transfection was carried out in parallel, using pcDNA3 without an insert. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal calf serum, 3.5 mM glutamine, 44 IU penicillin, 44 μM streptomycin and 10 μg/ml vitamin K1. The medium was to changed to Optimem Glutamax (Gibco) supplemented with 10 μg/ml vitamin K1, 72 h prior to collection. The recombinant protein S and the protein S/Gas6 chimera were each purified from 2 l of culture medium using techniques described previously [9]. The total amount of each protein obtained was around 3 mg and the exact concentration was determined from the amino acid composition after 24 h of hydrolysis in 6 M HCl in vacuo.

2.3. Electrophoretic and blotting techniques

Proteins subjected to 10% SDS-PAGE were either detected by Coomassie brilliant blue R-250 or transferred to polyvinylidene difluoride membrane (Millipore) for Western blotting. After transfer, membranes were quenched with 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, containing 3% (v/v) fish gelatin and 0.1% (v/v) Tween 20, and then incubated with the different antibodies diluted in the same buffer. The membranes were rinsed with buffer without fish gelatin and then incubated with anti-immunoglobulins (DAKO) conjugated with horseradish peroxidase. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) and the films were analyzed using a Personal densitometer SI (Molecular Dynamics). Polyclonal antibodies against the 25 C-terminal amino acids of human Gas6 were obtained in rabbits using a technique previously described for a receptor-derived peptide [18]. The Ca^{2+}-dependent monoclonal antibody HPS21, against the Gla domain of unreduced human protein S, has previously been characterized [8].

2.4. APC-cofactor activity expressed by protein S or the protein S/Gas6 chimera in a plasma-APTT system

In a modified APTT test [25], protein S-deficient plasma (50 μl) was mixed with recombinant protein S or the protein S/Gas6 chimera (10 μl). The cofactors were diluted in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 1% ovalbumin. The APTT reagent (50 μl of the APTT reagent present in the Coatest APC Resistance kit) was added and the mixture incubated at 37° for 180 s. The reaction was started by the addition of APC (approximately 6 nM) in 25 mM $CaCl_2$ (50 μl of the APC/$CaCl_2$ mixture present in the Coatest APC Resistance kit) and the clotting time recorded.

2.5. APC-cofactor activity expressed by protein S or the protein S/Gas6 chimera in FVa degradation

FVa (30 nM), phospholipids (20 μM) and protein S or the protein S/Gas6 chimera were incubated with human APC (0.3 nM) at 37°C in TBS containing 0.05% ovalbumin and 2 mM Ca^{2+}. Aliquots were removed at intervals, diluted 1:10 in ice-cold TBS and assayed for FVa-procoagulant activity using FV-deficient plasma. In this assay, FV-deficient plasma (50 μl) was incubated with the diluted samples (50 μl) at 37°C for 200 s. Simplastin Excel (100 μl) was added and the clotting time recorded. The FVa activity was calculated from a standard curve constructed with known concentrations of purified FVa.

2.6. APC-cofactor activity expressed by protein S or the protein S/Gas6 chimera in FVIIIa degradation

The FVIIIa-inhibition assay described by Shen and Dahlbäck [4] is used with minor modifications. In the present assay, FVIII (0.05 IU/ml), FIXa (0.025 IU/ml) and phospholipids (20 μM) were incubated (in TBS containing 0.2% ovalbumin and 2 mM Ca^{2+}) with thrombin for 3 min. FVIII activation was stopped by the addition of hirudin and the mixture was put on ice. In the FVIIIa-inhibition step, APC (4.8 nM) was added to the FVIII-FIXa mixture together with the indicated concentrations of protein S, or the protein S/Gas6 chimera, and FV. At the time stated in each experiment, bovine factor X was added and 6 min later, the generated FXa was measured by 10 min hydrolysis using S-2222. The amount of FXa that was generated corresponded linearly with the FVIIIa activity.

2.7. Statistical analysis

All the results are presented as mean values ± S.E.M. of three independent experiments performed in duplicate.

3. Results

3.1. Characterization of the protein S/Gas6 chimera

Human recombinant protein S and the protein S/Gas6 chimera (Fig. 1A) were expressed in 293 cells. The fold of the chimeric protein appears to be correct since the protein activates members of the Axl subfamily of tyrosine kinase receptors equally well as intact Gas6 (data not shown). The same observations with a similar chimera have been reported by Mark et al. [26]. From SDS-PAGE analysis (Fig. 1B), the apparent molecular weight of the protein S/Gas6 chimera (75 kDa) was found to be slightly lower than that of recombinant or plasma-derived protein S (79 kDa). This difference is explained by the number of *N*-linked glycosylation sites present in the SHBG-like region of protein S and Gas6 (three in protein S and one in Gas6). As expected, the protein S/Gas6 chimera reacted with the calcium-dependent monoclonal antibody HPS21, which is directed against an epitope in the unreduced protein S Gla domain (Fig. 1C). This antibody did not react with Gas6. In contrast, both Gas6 and the protein S/Gas6 chimera were recognized by a polyclonal antibody against the carboxy-terminal portion of human Gas6, an antibody which did not react with protein S (Fig. 1D).

3.2. APC-cofactor activity expressed by the protein S/Gas6 chimera in an APTT system

The ability of recombinant protein S or the protein S/Gas6 chimera to function as cofactors to APC in protein S-deficient plasma was tested using an APTT-based coagulation assay. Recombinant protein S yielded a dose-dependent prolongation of clotting time. The protein S/Gas6 chimera was also able to prolong the clotting time, but less efficiently than protein S (Fig. 2). The protein S/Gas6 chimera expressed ap-

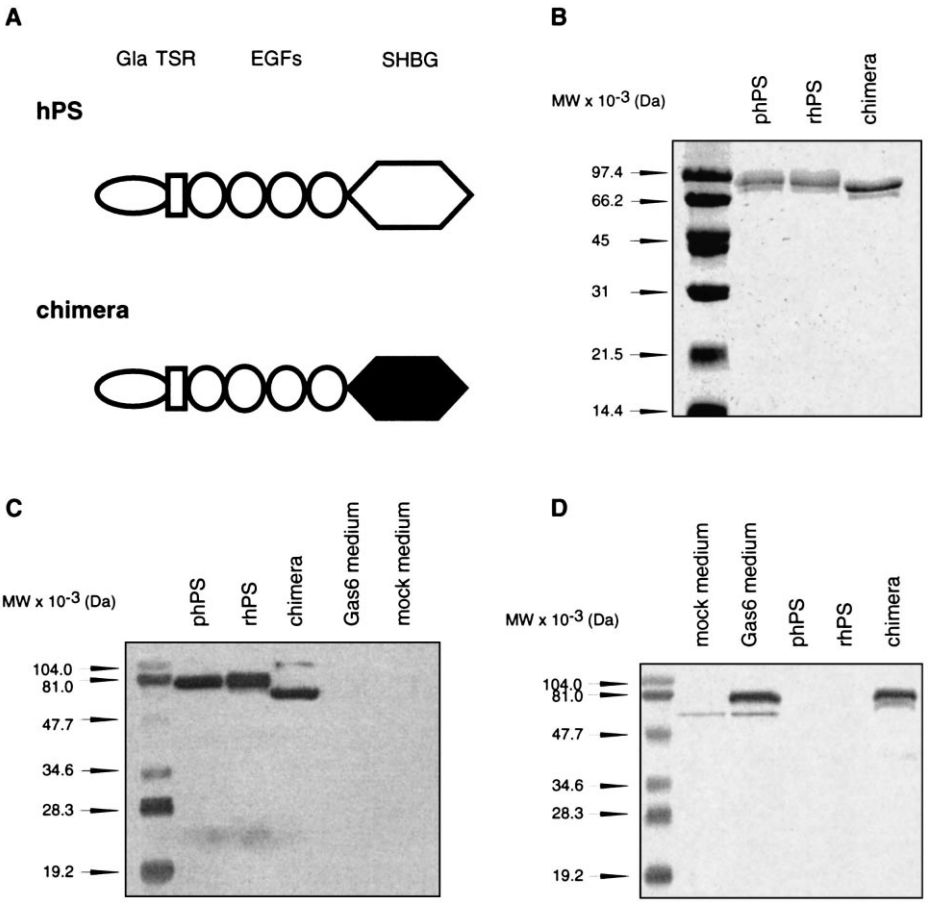

Fig. 1. Characterization of recombinant protein S and the protein S/Gas6 chimera. A: Schematic models of the two recombinant proteins, demonstrating the multiple domains of protein S (in white) or Gas6 (in black). B: Coomassie-stained SDS-polyacrylamide gel of (phPS), plasma purified human protein S; (rhPS), recombinant human protein S; and (chimera), protein S/Gas6 chimera (2 μg of protein is added in each lane). C: Western blots using antibodies against the Gla domain of human protein S (HPS21) or (D) for the last 25 amino acids of human Gas6. Conditioned media of cells expressing human Gas6 or from mock-transfected cells were used as controls to test the specificity of the antibodies. Abbreviations are as in B. The samples analysed in C were unreduced while those in B and D were treated with reduction agent.

proximately 40–50% APC-cofactor activity as compared to recombinant protein S.

3.3. *APC-cofactor activity of the protein S/Gas6 chimera in FVa degradation*

Inactivation of FVa by APC in the presence of physiological concentration of either protein S or the protein S/Gas6 chimera was followed as a function of time (Fig. 3). APC alone, at an enzyme to substrate ratio of 1:100, yielded fast inhibition of FVa activity, and after 2.5 min of incubation, less than 50% of the original FVa activity remained. The fast initial loss of activity was followed by a phase with slower loss of FVa activity, and even after 15 min incubation, approximately 20% FVa activity remained. This biphasic inhibition curve, which is typical for APC-mediated inhibition of FVa in the absence of protein S, was recently explained by a two-step inhibition mechanism. The initial fast APC-mediated cleavage at Arg-506 yields partial loss of FVa activity, whereas the slow cleavage at Arg-306 results in complete loss of activity [3]. Protein S is known to enhance the cleavage at Arg-306 [27,28]. Both the recombinant protein S and the protein S/Gas6 chimera enhanced the second phase of the FVa inactivation, i.e. the cleavage at Arg-306 (Fig. 3). This was demon-

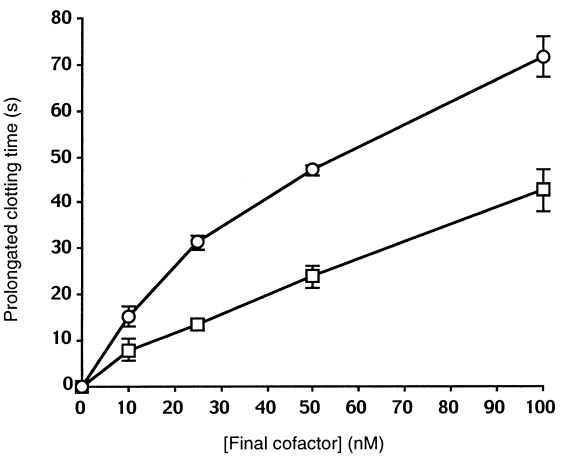

Fig. 2. APC-cofactor activity of protein S or the protein S/Gas6 chimera in an APTT-based assay. Recombinant human protein S (○) or the protein S/Gas6 chimera (□) were incubated with protein S-deficient plasma and APTT reagent for 180 s before the addition of APC/$CaCl_2$. The prolongation of clotting time is expressed as function of the concentration of protein S or the protein S/Gas6 chimera in the final solution. Without added APC cofactors, the clotting time in the absence of APC was 50 s and in its presence, 88 s.

strated by the faster appearance of a 306 cleavage product detected by Western blotting (data not shown).

3.4. The protein S/Gas6 chimera expresses no FV-dependent APC-cofactor activity in the degradation of FVIIIa

Approximately 90% of initial FVIIIa activity remained after 2.5 min inactivation of the FVIIIa-FIXa mixture with APC alone. This is consistent with results on record, demonstrating FVIIIa to be a poor substrate for APC alone [4,29,30]. The addition of protein S or the protein S/Gas6 chimera resulted in only a small further loss of FVIIIa activity (Fig. 4A). However, the combined addition of protein S and FV resulted in efficient APC-dependent inhibition of FVIIIa activity (Fig. 4B,C), demonstrating the synergistic cofactor activity of protein S and FV. In contrast, in the presence of the protein S/Gas6 chimera, FV did not stimulate APC-dependent inhibition of FVIIIa, suggesting the SHBG-like region of protein S to be crucial for expression of FV-dependent APC-cofactor function.

4. Discussion

The anticoagulant function of protein S is essential for control of hemostasis, as illustrated by the life-threatening risk of thrombosis associated with homozygous protein S deficiency [2]. Despite its clinical and physiological relevance, the molecular mechanism of the protein S function is incompletely understood. A puzzling observation has been that protein S seems more active as APC cofactor in plasma, than in FVa- and FVIIIa-degradation systems using purified components [29,31]. A possible explanation for this discrepancy is the existence of other plasma components affecting the protein S activity. One such component is FV, which has been found to enhance APC-mediated inhibition of the FVIIIa-FIXa complex on the surface of phospholipids [4,30]. The APC-cofactor activity of FV is particularly pronounced in the presence of protein S, suggesting a synergistic APC-cofactor activity of protein S and FV to be important for the inhibition of FVIIIa [4–6]. It has been proposed that the APC-cofactor activity of FV is mainly expressed after APC cleavage of intact FV [29]. In contrast, cleavage of FV by thrombin results in the loss of its APC-cofactor activity [4]. Recently, using recombinant FV and site-directed mutagenesis, we demonstrated the APC-cofactor activity of FV to be lost as a result of thrombin-mediated cleavage of FV at Arg-1545, whereas thrombin-mediated cleavages of FV at Arg-709 or Arg-1018 had no effect on the APC-cofactor activity [32]. Moreover, we found the last 60 amino acid residues of the B-domain of FV to play an important part in the expression of the APC-cofactor activity of FV.

It is of interest to compare the functional properties of the protein S/Gas6 chimera with those of mini-protein S, a deletion mutant of protein S lacking the whole SHBG-like region

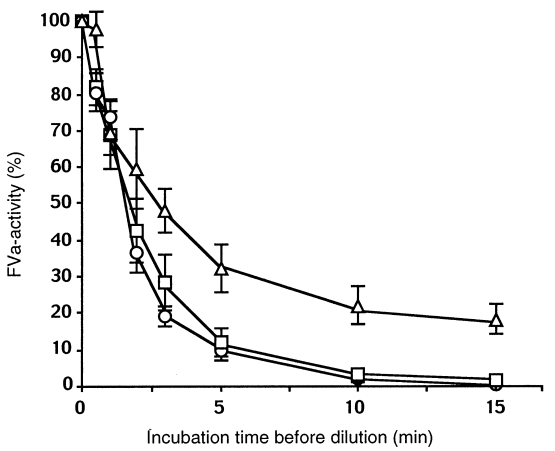

Fig. 3. APC-mediated inactivation of purified FVa in the presence of protein S or the protein S/Gas6 chimera. The inhibition of FVa (30 nM) by APC (0.3 nM) alone (△), in the presence of 120 nM of either protein S (◯) or the protein S/Gas6 chimera (□) was followed for various times. The FVa activity was measured by a clotting assay described in Section 2.

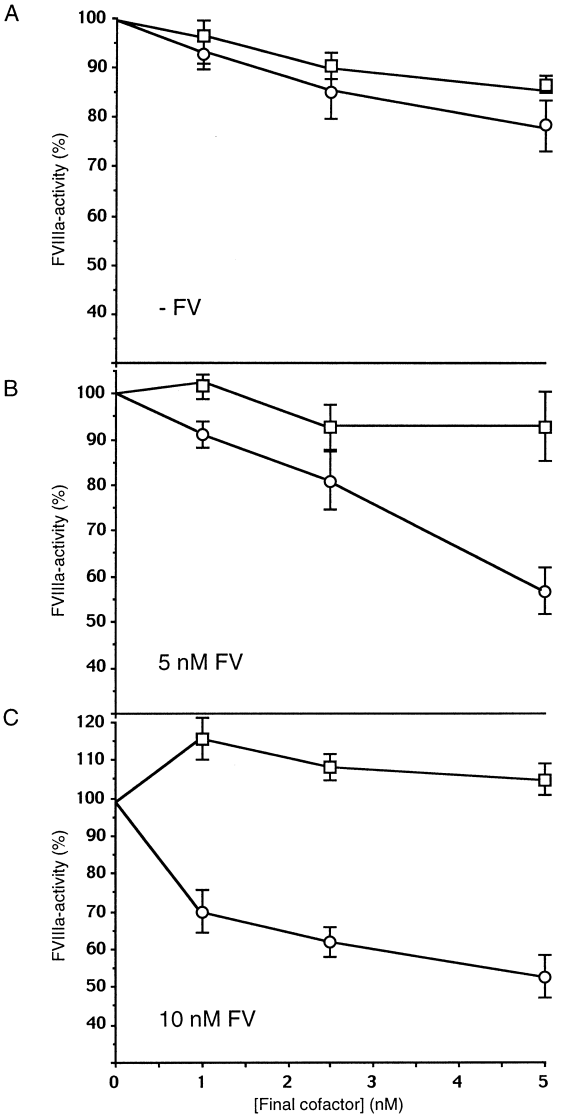

Fig. 4. The effects of protein S and the protein S/Gas6 chimera on APC-mediated FVIIIa degradation. Incubation was performed either in the absence or in the presence of FV, as FV works in synergy with protein S as cofactor in the degradation of FVIIIa. FVIIIa was degraded for 2.5 min with 4.8 nM APC and different concentrations of either human recombinant protein S (◯), or the protein S/Gas6 chimera (□). A: No FV added to the inactivation mixture. B: 5 nM FV added. C: 10 nM FV added. The FVIIIa activity was related to the FVIIIa activity expressed in an inactivation mixture with APC (with or without FV) and without protein S or the protein S/Gas6 chimera.

[11]. Although mini-protein S had low APC-cofactor activity in a plasma-based assay, its activity could not be distinguished from that of wild-type protein S in the degradation of FVa and FVIIIa, using purified components. In this context it is of importance to note that FV was not included in the FVIIIa-degradation system used by van Wijnen et al. [11]. Therefore, conclusions related to the importance of the SHBG-like region for the synergistic APC-cofactor activities of protein S and FV could not be drawn.

The low APC-cofactor activity of the protein S/Gas6 chimera and mini-protein S in plasma, is most likely explained by a lack of APC-cofactor synergy between either of them and FV. Thus, substitution of the SHBG-like region of protein S by that of Gas6 completely abolished the anticoagulant effect of FV in the degradation of FVIIIa. This implies that protein S/Gas6 chimera did not interact properly with FV and suggests the existence of a specific binding site for FV in the SHBG-like region of protein S. It has previously been shown that protein S can bind directly to immobilized FVa (in microtiter plates), and with less affinity to FV [33–35]. It is possible that interactions between protein S and FVa/FV are important both for the degradation of FVa and FVIIIa. An interaction between FVa and protein S may be involved in FVa degradation, whereas an interaction between protein S and FV is important for FVIIIa degradation. In our present investigation, the FVa degradation was less affected than the FVIIIa degradation by the SHBG exchange between protein S and Gas6. Therefore, the now proposed binding site in the SHBG-like region of protein S is likely specific for FV and not for fully activated FVa, which is cleaved at Arg1545 and therefore has no APC-cofactor activity [32].

In conclusion, we demonstrate the SHBG-like region of protein S to play an important part in the expression of full APC-cofactor activity. Thus, substitution of the SHBG-like region of protein S by that of Gas6 resulted in total loss of the synergistic APC-mediated anticoagulant effect of protein S and FV, in the degradation of FVIIIa. This suggests the existence of a specific interaction between FV and the SHBG-like region of protein S to be important for efficient degradation of FVIIIa.

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