BRIEF REPORT

Protein S K196E mutation reduces its cofactor activity for APC but not for TFPI

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Abstract
Background: Protein S (PS) is an anticoagulant molecule that functions as a cofactor for activated protein C (APC) in the inactivation of activated coagulation factors Va (FVa) and VIIIa. It also serves as a cofactor for tissue factor pathway inhibitor (TFPI) in the efficient inhibition of factor Xa (FXa). The Lys196-to-Glu (K196E, Tokushima) mutation in the EGF-2 domain of PS is a genetic risk factor for venous thromboembolism (VTE) in the Japanese population.

Objectives: To investigate the molecular basis of the thrombophilic phenotype of Japanese patients carrying the PS K196E mutation.

Methods: We expressed recombinant human PS wild-type (PS-K) and K196E-mutant (PS-E) in CHO cells, and purified them by Ni2+-affinity and anion exchange column chromatography. We investigated the anticoagulant functions of PS-K and PS-E by measuring APC cofactor activity, TFPI cofactor activity, affinity for the β chain of complement component C4b-binding protein (C4BP), and cleavage by thrombin.

Results: PS-E had approximately 40% APC cofactor activity compared with PS-K in a clotting-based assay and a FVa inactivation assay. The TFPI cofactor activity of PS-E in the FXa inactivation assay was equivalent to that of PS-K in the absence and presence of coagulation factor V. The strengths of PS-E and PS-K binding to the β chain of C4BP were comparable, and both were equally cleaved by thrombin.

Conclusions: The PS K196E mutation increases the risk of VTE because of reduced APC cofactor activity but does not alter various other properties, including the TFPI cofactor activity.

KEYWORDS
coagulation factor V, genetic variation, protein C, protein S, tissue factor pathway inhibitor, venous thromboembolism

Essentials
- Protein S (PS) K196E is the most common mutation found in type II PS deficiency in Japan.
- The mutation reduced PS cofactor activity for APC but not for TFPI.
- The mutation did not affect binding to the C4BP β chain or cleavage by thrombin.
- PS K196E may increase the thrombotic risk solely because of the reduced APC cofactor activity.
1 | INTRODUCTION

Protein S (PS) acts as a cofactor for activated protein C (APC) and tissue factor pathway inhibitor (TFPI). PS enhances the APC-mediated inactivation of coagulation factors Va (FVa) and VIIIa, and also the TFPI-mediated inhibition of coagulation factor Xa (FXa).1–4 Furthermore, the TFPI cofactor activity of PS was enhanced in the presence of coagulation factor V (FV).3 PS deficiency is a risk factor for developing venous thromboembolism (VTE). PS is composed of an N-terminal vitamin K-dependent Gla domain, a thrombin-sensitive region (TSR), four epidermal growth factor (EGF)-like domains, and a sex hormone binding globulin (SHBG)-like domain. Approximately 60% of circulating PS binds to the β chain of complement component C4b-binding protein (C4BP). The PS-C4BP complex is still a cofactor for APC that enhances APC-mediated proteolysis at Arg306 of FVa more than 10-fold, but inhibits the proteolysis at Arg306 of FVa 3–4-fold.3 In plasma, the APC cofactor activity of PS is inhibited by proteolytic cleavage in the TSR.1,2

We and others previously identified a missense mutation (c.586A>G, p.K196E, formerly known as PS Tokushima or K155E in the mature protein numbering) in the PROS1 gene as a risk factor for VTE, with odds ratios between 3.74 and 8.56.5–7 The frequency of the mutant E-allele in the Japanese general population is approximately 0.009.8,9 This mutation is present only in the Japanese population.10–13 PS K196E knock-in mice had decreased APC cofactor activity in plasma and were more susceptible to deep vein thrombosis compared with wild-type (WT) mice.16 Two studies analyzed the function of recombinant PS K196E mutant (PS-E) and WT (PS-K) expressed in mammalian cells.17,18 One showed that PS-E failed to interact with APC and had no APC cofactor activity.17 In contrast, the other demonstrated that the APC cofactor activity of PS-E decreased to 58% of that of PS-K.18 The discrepancy between these studies may be partly due to differences in preparation methods of the recombinant proteins: the former used partially purified recombinant proteins expressed in baby hamster kidney cells, and the latter used conditioned media containing recombinant proteins expressed in human embryo kidney 293 cells. In addition, these studies did not investigate the TFPI cofactor activity of PS-E.

The aim of this study was to reevaluate and further elucidate the molecular basis of the thrombophilic phenotype of the PS-E mutant. We highly purified recombinant human PS-K and PS-E expressed by Chinese hamster ovary (CHO) cells and analyzed their anticoagulant functions. Our data indicate that the PS K196E mutation impairs the protein’s function as an APC cofactor but not as a TFPI cofactor.

2 | MATERIALS AND METHODS

2.1 | PS expression, purification, and quantification

Human PS-K or PS-E (Ala42–Ser676), with a C-terminal tobacco etch virus (TEV) protease cleavage site followed by tandem His-tag sequences at the C terminus, was stably expressed in CHO Lec 3.2.8.1 cells.19 PS-E was constructed through site-directed mutagenesis and verified by DNA sequencing. The cells expressing PS-K or PS-E were cultured in α-MEM containing 5% fetal bovine serum and 10 μg mL−1 vitamin K1. All the concentrations in the Materials and Methods section are indicated as final concentrations. The 24-hour culture medium was collected, and 1 mmol L−1 phenylmethylsulfonyl fluoride was added. The medium was supplemented with 5 mmol L−1 imidazole and purified by Ni2+-affinity chromatography (Nacalai Tesque, Kyoto, Japan). The His-tagged PS molecules were eluted with 20 mmol L−1 Tris-HCl, 300 mmol L−1 NaCl, and 300 mmol L−1 imidazole. To remove the His-tag, the eluates were incubated with TEV proteinase, dialyzed against a buffer (TBS: 50 mmol L−1 Tris-HCl, 150 mmol L−1 NaCl, pH 7.4), and then loaded onto a Ni2+-affinity column. Next, to prepare properly γ-carboxylated PS, the flow-through sample was supplemented with 5 mmol L−1 EDTA, loaded onto a Q Sepharose Fast Flow column (GE Healthcare, Uppsala, Sweden), and eluted with the buffer containing 5 mmol L−1 CaCl2.20 Then, the fractions of interest were pooled and dialyzed against a buffer.

FIGURE 1 APC cofactor activity of PS in a clotting based assay (A) and a FVa inactivation assay (B). PS-E showed significantly less efficient cofactor activity than PS-K. Means with error bars (± standard deviation) of three experiments are shown. (inset) Purified PS-K and PS-E proteins were stained with Coomassie Brilliant Blue after SDS-PAGE under reducing (+) and nonreducing (−) conditions. DTT, dithiothreitol.
(50 mmol L\(^{-1}\) Tris-HCl, 100 mmol L\(^{-1}\) NaCl, pH 7.4). The purity of PS was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Figure 1A inset), and concentrations were determined by an in-house sandwich ELISA. Standards for the ELISA were prepared using purified human PS (Haematologic Technologies, Essex Junction, VT).

2.2 | Phospholipid vesicle preparation

Synthetic phospholipids (Avanti Polar Lipids, Alabaster, AL) 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in chloroform were blended, and chloroform was evaporated under a nitrogen stream. The phospholipids were resuspended in TBS and mixed vigorously for 1 hour with shaking. Phospholipid vesicles were prepared by extrusion using an Avanti Mini-Extruder (Avanti Polar Lipids).

2.3 | Clotting based assay of APC cofactor activity

APC cofactor activity of PS (PS-K or PS-E) was determined using a clotting assay (Statclot Protein S; Diagnostica Stago, Asnieres, France) in accordance with the accompanying protocol using Start 4 Hemostasis Analyzer (Diagnostica Stago). PS (0-13.5 nmol L\(^{-1}\)) was incubated with PS-depleted plasma, FVa, and APC for 4 minutes. Clotting was initiated by addition of CaCl\(_2\), and clotting time was measured. The APC cofactor activity was evaluated based on the slope of a linear increase in clotting time.

2.4 | PS-dependent APC-mediated FVa inactivation assay

PS (0-100 nmol L\(^{-1}\)) was incubated with 0.3 nmol L\(^{-1}\) human APC, 25 μmol L\(^{-1}\) phospholipid vesicles (DOPS/DOPC/DOPE, 10:70:20), and 50 nmol L\(^{-1}\) human FVa (Haematologic Technologies) in

**FIGURE 2** TFPI cofactor activity of PS in a FXa inhibition assay. Representative data of three experiments in the absence (A) and presence (B) of FV are shown. The remaining FXa activity in the absence (C) and presence (D) of FV was plotted against PS concentration. Means with error bars (± standard deviation) of three experiments are shown. PS-K and PS-E equally enhanced TFPI-mediated inactivation of FXa. The apparent counteraction of FXa inhibition (B, D) is due to the concentration of FXa (0.5 nmol L\(^{-1}\)) over that of TFPI (0.25 nmol L\(^{-1}\))
2.5 | PS-dependent TFPI-mediated FXa inhibition assay

PS (0-320 nmol L⁻¹) and 1 nmol L⁻¹ human TFPI were mixed with 10 µmol L⁻¹ phospholipid vesicles (DOPS/DOPC/DOPE, 20:60:20), 5 mmol L⁻¹ CaCl₂, and 400 µmol L⁻¹ S-2765 (Chromogenix). Alternatively, PS (0-320 nmol L⁻¹), 0.25 nmol L⁻¹ human TFPI, and 30 nmol L⁻¹ FV (Haematologic Technologies) were mixed with 25 µmol L⁻¹ phospholipid vesicles (DOPS/DOPC/DOPE, 20:60:20), 5 mmol L⁻¹ CaCl₂, and 400 µmol L⁻¹ S-2765. The reaction was started by addition of 0.5 µmol L⁻¹ FXa, and the remaining FXa activity was measured by the cleavage of the chromogenic substrate S-2765 at 405 nm for 30 minutes at 1-minute intervals using the Multiskan Ascent microplate reader. The remaining FXa activity obtained in the absence of PS was considered as 100%.

FIGURE 3  Binding of PS to the β chain of C4BP. PS-E and PS-K bound equally to the β chain of C4BP. Means with error bars (± standard deviation) of three experiments are shown

2.6 | Binding of PS to C4BP

We prepared and purified a recombinant fragment (Glu²¹-Ser¹³⁶, corresponding to complement control protein domains 1 and 2) of human C4BP β chain using a Brevibacillus Expression system II (Takara Bio, Kusatsu, Japan). The concentrations of the recombinant protein were determined by the absorbance at 280 nm assuming an E₂₈₀^¹%= 10.0. The 96-well plates were coated with 2 µg mL⁻¹ C4BP β chain in 50 mmol L⁻¹ carbonate-bicarbonate buffer at 4°C overnight. The wells were blocked with TBS containing 1% BSA for 1 hour at room temperature. PS (0-75 nmol L⁻¹) was incubated in the wells for 2 hours, and bound PS was detected using a horseradish peroxidase-conjugated anti-PS antibody (Affinity Biologicals, Ancaster, Canada). The wells were washed with TBS containing 2 mmol L⁻¹ CaCl₂, 0.1% BSA, and 0.05% Tween 20 between each step. After addition of 3,3′,5,5′-tetramethylbenzidine, color development was measured by absorbance at 450 nm against a reference wavelength of 650 nm using the Multiskan Ascent microplate reader. The apparent dissociation constant (K_diss) was determined by nonlinear regression analysis for one site binding using Graph Pad Prism software (GraphPad Software, San Diego, CA).

2.7 | Cleavage of PS by α-thrombin

PS (560 nmol L⁻¹) was incubated at 37°C for 0-120 minutes with 2 nmol L⁻¹ human α-thrombin (Haematologic Technologies) in TBS supplemented with 5 mmol L⁻¹ EDTA (pH 7.5) and the reaction was stopped by adding SDS gel-loading buffer. The samples were electrophoresed on SDS-PAGE gels under reducing conditions and stained with Coomassie Brilliant Blue.

3 | RESULTS AND DISCUSSION

In this study, we first examined whether the PS K196E mutation affects the protein’s APC cofactor activity by performing a clotting-based assay and a FXa inactivation assay. In the clotting-based assay, PS-E showed 41.4% ± 3.1% APC cofactor activity compared with PS-K (Figure 1A), which is in agreement with a previous report¹⁸ and a study on recombinant mouse PS-E.¹⁶ In the FXa inactivation assay, an approximately twofold concentration of PS-E, as compared to PS-K, was required to achieve 50% inactivation of FXa activity (PS-K

FIGURE 4  Time course of PS cleavage by thrombin. The samples were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. PS-K and PS-E were equally cleaved by thrombin
11.1 ± 1.2 nmol L⁻¹, PS-E 24.5 ± 1.1 nmol L⁻¹; Figure 1B). Thus, PS-E had a reduced ability to enhance APC-mediated inactivation of FVa. Under the experimental conditions, neither PS-K nor PS-E affected the FVa activity in the absence of APC (data not shown). APC binding regions of PS are located in the TSR, EGF-1, and EGF-2 domains. Based on modeling of the tertiary structure of the EGF-2 domain of PS, Lys196 is on the surface of the molecule, where it likely interacts with APC. The functional importance of Lys196 was previously reported by an Ala replacement study, where the K196A variant showed modestly reduced APC cofactor activity. We have previously obtained monoclonal antibodies specific to the PS-E in the native form, indicating the surface presentation of the Lys196 residue.

We next examined whether the PS K196E mutation affects its cofactor activity for TFPI in FXa inhibition. The regression curves for TFPI/PS-dependent FXa inhibition were well fitted by the equation for biphasic inhibition in the absence of FV (Figure 2A). The biphasic inhibition was not observed in the presence of FV (Figure 2B), probably because of the experimental conditions with excess FXa over TFPI. In both the absence and presence of FV, PS-K and PS-E efficiently and equally enhanced TFPI-mediated inhibition of FXa (Figure 2C, D), indicating that the K196E mutation, located in the EGF-2 domain, does not affect TFPI cofactor activity. This is consistent with the finding that the C-terminal SHBG-like domain of PS mediates binding with the C-terminal basic region of TFPI.

The APC cofactor activity of PS is reduced by its binding to C4BP and by the proteolytic cleavage in the TSR by thrombin. In the present study, PS-K and PS-E showed equal binding to the j chain of C4BP (Kd app PS-K, 8.2 ± 1.1 nmol L⁻¹; PS-E, 8.2 ± 0.8 nmol L⁻¹; Figure 3). This is consistent with the observation that the SHBG-like domain of PS interacts with the j chain of C4BP and that the plasma free PS level of a homozygous E-allele carrier is within normal limits. Meanwhile, the TSR of PS is involved in the interaction with APC and the GlA domain for phospholipid binding, and the cleavage of the TSR by thrombin results in the reduction of APC cofactor activity. We demonstrated that PS-K and PS-E were equally cleaved by thrombin (Figure 4). Taken together, these data show that the K196E mutation does not affect the loss of function of PS caused by its binding to C4BP or by thrombin-dependent cleavage.

In conclusion, the present study reveals that the K196E mutation impairs the molecular function of PS as an APC cofactor but not as a TFPI cofactor. The impaired APC cofactor activity of the PS K196E mutant causes an increased risk of VTE in E-allele carriers.

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RELATIONSHIP DISCLOSURE

T. Miyata reports a grant from Takeda Science Foundation. T. Miyata, K. Maruyama, and M. Akiyama have a patent pending for the anti-mutant protein S antibody, its derivatives, and detection methods and kits to detect mutant protein S using those antibodies.

AUTHOR CONTRIBUTIONS

K. Maruyama performed most of the experiments and wrote the manuscript. M. Akiyama constructed the plasmid vectors for generating PS-K, PS-E, and C4BP j chain, and wrote the manuscript. T. Miyata and K. Kokame conceived this study and wrote the manuscript. All authors approved the final version of the manuscript.

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