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Ile73Asn mutation in protein C introduces a new N-linked glycosylation site on the first EGF-domain of protein C and causes thrombosis

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ABSTRACT

Activated protein C exerts its anticoagulant activity by protein S-dependent inactivation of factors Va and VIIIa by limited proteolysis. We identified a venous thrombosis patient who has plasma protein C antigen level of 63% and activity levels of 44% and 23%, as monitored by chromogenic and clotting assays. Genetic analysis revealed the proband carries compound heterozygous mutations (c.344T>A, p.I73N and c.1181G>A, p.R352Q) in *PROC*. We individually expressed protein C mutations and discovered that thrombin-thrombomodulin activates both variants normally and the resulting activated protein C mutants exhibit normal amidolytic and proteolytic activities. However, while protein S-dependent catalytic activity of activated protein C-R352Q toward factor Va was normal, it was significantly impaired for activated protein C-I73N. These results suggest that the Ile to Asn substitution impairs interaction of activated protein C-I73N with protein S. This conclusion was supported by a normal anticoagulant activity for activated protein C-I73N in protein S-deficient but not in normal plasma. Further analysis revealed Ile to Asn substitution introduces a new glycosylation site on first EGF-like domain of protein C, thereby adversely affecting interaction of activated protein C with protein S. Activated protein C-R352Q only exhibited reduced activity in sub-physiological concentrations of Na^+ and Ca^{2+}, suggesting that this residue contributes to metal ion-binding affinity of the protease, with no apparent adverse effect on its function in the presence of physiological levels of metal ions. These results provide insight into the mechanism by which I73N/R352Q mutations in activated protein C cause thrombosis in proband carrying this compound heterozygous mutation.

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Introduction

Thrombin forms a complex with thrombomodulin (TM) on the endothelium to activate the vitamin K-dependent anticoagulant protein C zymogen to activated protein C (APC), thereby down-regulating thrombin generation by a feedback inhibition mechanism.[1] Activated protein C functions as an anticoagulant by degrading the procoagulant co-factors Va and VIIIa by limited proteolysis.[2] Protein C is a multi-domain glycoprotein composed of a non-catalytic light chain linked to the catalytic heavy chain by a single disulfide bond.[3,4] The light chain harbors the vitamin K-dependent N-terminal γ-carboxyglutamic acid (Gla) domain followed by two epidermal growth factor (EGF)-like domains.[4] The C-terminal catalytic heavy chain with a trypsin-like substrate specificity is preceded by an activation peptide, which is removed during the activation of protein C by the thrombin-TM

complex.[1,5] Through its multi-domain structural feature, APC can function as an allosteric enzyme and its proteolytic activity is modulated by protein and metal ion co-factors binding to different domains of the protein.[6-8] Protein S functions as a co-factor to promote the anticoagulant function of APC by binding to the light chain of the protease (primarily to Gla and EGF1 domains),[9-12] thereby stabilizing the active conformation of APC on negatively charged membrane surfaces in a topographical orientation in which the catalytic triad of the protease is aligned with scissile bonds of target co-factors Va and VIIIa for optimal cleavage.[13-15] Similarly, binding of both monovalent and divalent cations to specific sites of APC on both light and heavy chains allosterically modulates the catalytic and anticoagulant function of APC on membrane surfaces.[4,6-10]

Protein C deficiency exhibits autosomal dominant pattern of inheritance. The heterozygous deficiency of protein C increases risk of venous thromboembolism (VTE) and its homozygous deficiency is associated with purpura fulminans, which may be fatal if not treated by protein C replacement therapy.[16,17] The complete protein C deficiency in knockout mice is lethal.[18] There are two common types of protein C deficiency: type-I deficiency is characterized by both low antigen and activity levels, and type-II deficiency is characterized by only a lower activity level for APC.[19,20] Protein C database (*http://www.hgmd.cf.ac.uk/ac/gene.php?gene=PROC*) analysis suggests that the mutations are scattered on both light and heavy chains and involve all functional domains of the protein (Gla, EGF1, EGF2 and catalytic domains). In this study, we have identified a compound heterozygous protein C deficient VTE patient whose plasma antigen (PC:Ag) level is 63% of the normal and activity (PC:A) levels are 23% and 44% of the normal as monitored by both clotting and chromogenic activity assays, respectively. Genetic analysis revealed that the proband carries compound heterozygous mutations (c.344T>A, p.I73N and c.1181G>A, p.R352Q) in *PROC*, inheriting the I73N mutation from her mother and R352Q mutation from her father. The first mutation is located on the N-terminal EGF1 and the second mutation is located on the catalytic domain of protein C. We individually expressed both protein C variants in mammalian cells and characterized their properties in established coagulation assays. We demonstrate that thrombin-TM activates both protein C variants normally and anticoagulant and amidolytic activities of the R352Q variant are normal; however, the anticoagulant activity of the I73N variant has been specifically and significantly impaired in the presence of protein S. Further analysis revealed that the basis for the protein S-dependent defect is that the Ile to Asn substitution introduces a novel N-linked glycosylation site on EGF1 of protein C, thereby interfering with interaction of the APC mutant with its co-factor.

Methods

Construction, expression, and purification of recombinant protein C derivatives

Expression, purification and activation of recombinant human wild-type protein (WT) C and the Ile73 to Asn (I73N) and Arg352 to Gln (R352Q; R187Q in chymotrypsin numbering)[21] derivatives in human embryonic kidney cells have been described previously.[22-24]

A complete list and sources of reagents, together with details of the experimental methods used, are provided in the *Online Supplementary Appendix*.

Analysis of thrombin generation in plasma

Thrombin generation (TG) assay was performed with Thrombinoscope (Fluoroskan Ascent (Thermo Fisher Scientific, Waltham, MA, USA) using citrated human normal or protein C-deficient plasma reconstituted with either WT protein C or mutant protein C derivatives (60nM) as described.[25]

Anticoagulant assays

Anticoagulant activities of APC derivatives in the absence and presence of different concentrations of protein S and/or different concentrations of PC/PS vesicles were monitored both in purified and plasma-based assay systems as described.[24,25]

Endothelial cell permeability

Intracellular signaling activity of APC derivatives was evaluated in a permeability assay using EA.hy926 endothelial cells as described.[24] Cell permeability in response to thrombin [10nM for 10 minutes (min)] following treatment with APC derivatives [25nM for 3 hours (h)] was quantitated by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional cell monolayers using a modified 2-compartment chamber model as described.[24]

Statistical analysis

Data are expressed as mean±standard deviation from three or more experiments. Data were analyzed by Student *t*-test. $P<0.05$ was considered statistically significant.

Results

Case presentation

The proband is a 36-year old female (II-2) who was referred to the hematology clinic because of recurrent deep vein thrombosis (DVT) of the left lower limb (Figure 1A). Plasma levels of the proband's protein C revealed a combined type-I/type-II deficiency as evidenced by a moderately lower protein C antigen level based on the ELISA, but a significantly lower activity level based on the chromogenic assay, and an even more pronounced lower activity level based on aPTT (Figure 1C). Results of other routine coagulation and thrombophilia screening assays were normal (*data not shown*). Genetic analysis identified a compound heterozygous missense mutation (c.344T>A, p.I73N and c.1181G>A, p.R352Q) in *PROC* (Figure 1B). Further genetic analysis using blood samples from the proband's parents revealed that she has inherited I73N mutation from her mother and R352Q mutation from her father (Figure 1B). Both I73N and R352Q are novel mutations in *PROC*, though a thrombosis patient with type-II protein C deficiency carrying a R352W mutation has been reported previously.[19] To understand the molecular defect causing recurrent thrombosis in this patient, we expressed both I73N and R352Q protein C variants in mammalian cells for further characterization.

Characterization of recombinant protein C mutants

Following expression, protein C derivatives were purified by a combination of immunoaffinity and ion exchange chromatography as described.[22] Recombinant zymogens were activated by thrombin and APC deriva-

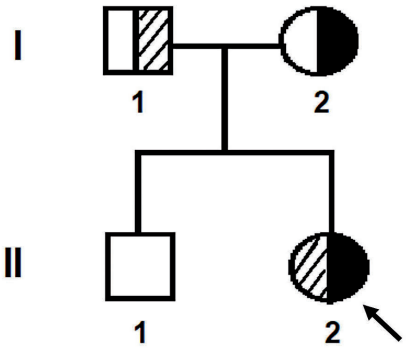
tives were separated from thrombin on the Mono Q column as described.[22] Concentrations of APC derivatives, as determined by active-site titration, were within 90-100% of those expected based on zymogen concentrations as determined from absorbance at 280 nm. SDS-PAGE analysis indicated APC derivatives have been purified to near homogeneity, with the heavy chains of both APC-I73N and APC-R352Q migrating as triple bands representing α, β and γ subforms of protein C (Figure 2A), which are glycosylation variants of the protein also observed in APC-WT.[26] Triplet bands of APC-I73N migrated as diffused bands, but slower than those observed with APC-WT and APC-R352Q (Figure 2A). SDS-PAGE analysis under reducing conditions revealed the higher molecular mass of APC-I73N under non-reducing conditions is due to its light chain, which migrated slower than light chains of other two APC derivatives (Figure 2A). As expected, heavy chains of all three APC derivatives migrated with near similar apparent molecular masses under reducing conditions (Figure 2A). Analysis of the cDNA sequence of protein C revealed that Ile73 to Asn mutation creates a potential new N-linked glycosylation (^{73}Asn-Gly-Ser^{75}) site on EGF1-domain of protein C, suggesting the glycosylation of this mutant residue is responsible for the higher molecular mass of the protein (Figure 2A). In agreement with this observation, treatment with PNGase F, which catalyzes the cleavage of N-linked oligosaccharides, eliminated the diffused nature of bands as well as differences in molecular masses observed between APC-WT and APC-I73N (Figure 2A, right). As expected, SDS-PAGE analysis of WT and I73N protein C zymogens indicated both proteins also migrate as triplet bands representing α, β and γ subforms of protein C and similar to APC derivatives, I73N zymogen migrated slower than WT protein under non-reducing conditions (Figure 2C). Analysis of zymogen derivatives under reducing conditions indicated a fraction of both proteins (a larger fraction with I73N) is not processed at the dibasic cleavage site,[27] thus migrating as single chains (Figure 2C). The single chain I73N zymogen migrated much slower than the single chain WT under reducing conditions (Figure 2C), most likely due to the additional glycan chain in the light chain of the mutant protein. Previously, we have demonstrated the single chain APC has normal amidolytic and anticoagulant activity.[27] Noting the compound heterozygous nature of protein C mutation, the relative contribution of each mutation to lower PC:Ag in the proband's plasma remains unknown.

Amidolytic activity of APC derivatives toward SpPCa is presented in Figure 2D. Kinetic analysis indicated APC-WT, APC-I73N, and APC-R352Q cleave SpPCa with similar kinetic parameters in TBS/Ca^{2+} ($K_{m(app)}$=220-240μM and k_{cat}=19-22s^{-1}), suggesting neither mutation has an adverse effect on folding and/or reactivity of the catalytic pocket.

Analysis of protein C activation

Analysis of the initial rate of protein C activation indicated relative to WT, thrombin activates both variants with similar or improved rates. Thus, in EDTA containing TBS, the rate of protein C activation by thrombin was improved 1.5- and 2-fold for I73N and R352Q, respectively (Figure 2E). However, the initial rate of activation by

A



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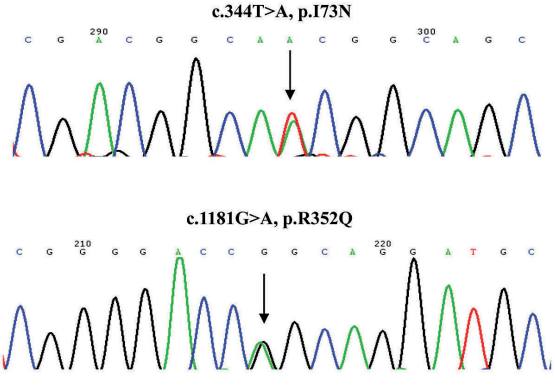


C

Pedigree	PC:A		PC:Ag (ELISA)	Mutation of *PROC*
	Clotting assay	Chromogenic assay		
I-1	70%	73%	ND	c.1181G>A,p.R352Q
I-2	42%	53%	ND	c.344T>A,p.I73N
II-2	23%	44%	63.30%	c.344T>A,p.I73N c.1181G>A,p.R352Q
Reference range	70-130%	70-140%	70-140%	

Figure 1. Genotype and phenotype analysis of the proband and her parents. (A) The pedigree of the family members of the proband (II-2). (B) Genetic analysis showing the proband carries compound heterozygous c.344T>A (p.I73N) and c.1181G>A (p.R352Q) mutations in the *PROC* gene. (C) Clinical data obtained by coagulation assays are shown for the proband and her parents. The PC:Ag data for the proband's parents were not available. ND: not determined.

thrombin-sTM in TBS/Ca^{2+} was essentially identical for all protein C derivatives (Figure 2F), further confirming a proper folding of recombinant proteins.

Anticoagulant activity

Anticoagulant activity of APC derivatives was evaluated in both the absence and presence of protein S. APC concentration-dependence of FVa degradation suggested both variants have normal anticoagulant activity toward FVa in the absence of protein S (Figure 3A). However, APC concentration-dependence of FVa degradation in the presence of protein S revealed while the anticoagulant activity of APC-R352Q is the same as APC-WT, it has been significantly impaired for APC-I73N in the presence of the cofactor (Figure 3B). Essentially identical results were obtained if anticoagulant activities of APC derivatives were evaluated by aPTT using normal and protein S-deficient plasma. Thus, all APC derivatives exhibited identical prolongation of clotting time in protein S-deficient plasma (Figure 3C); however, while the activity of APC-R352Q in the normal plasma was similar to APC-WT, it was significantly decreased for APC-I73N (Figure 3D). These results suggest APC-I73N may bind protein S with weaker affinity.

Since Ile73 is located on EGF1-domain of APC near the membrane-binding Gla-domain, both PC/PS concentration-dependence and protein S concentration-dependence of FVa inactivation by APC-WT and APC-I73N were investigated to determine the basis for the lower protein S-dependent anticoagulant activity of the mutant. PC/PS concentration-dependence of FVa inactivation in the absence of protein S was essentially identical for both APC-WT and APC-I73N, yielding $K_{d(app)}$ values of ~1.7 μM PC/PS for both proteases (*data not shown*). However, PC/PS concentration-dependence of FVa inactivation in the presence of a fixed concentration of protein S (20nM) indicated 2-fold weaker affinity of APC-I73N for phospholipids ($K_{d(app)}$ =2 μM; *vs.* APC-WT $K_{d(app)}$= 1 μM, n=3, P<0.05) (Figure 4A). A similar low dissociation constant for the APC-protein S interaction in the kinetic assay has been reported.[28] Protein S concentration-dependence in the presence of a fixed concentration of PC/PS (25 μM) indicated a similar weaker affinity for protein S by APC-I73N, suggesting the mutant interacts with protein S with weaker affinity (Figure 4B). Nevertheless, increasing the concentration of protein S did not overcome the catalytic defect of APC-I73N (Figure 4B). Since APC-R352Q exhibited similar anticoagulant activity in FVa degradation and aPTT assays (Figure 3), co-factor concentration-dependence studies were not conducted with this mutant.

Anticoagulant activities of APC-WT and APC-I73N toward FVa Leiden were also evaluated in both the absence and presence of protein S. In FVa Leiden, the APC recognition site, Arg506 is mutated to Gln; however,

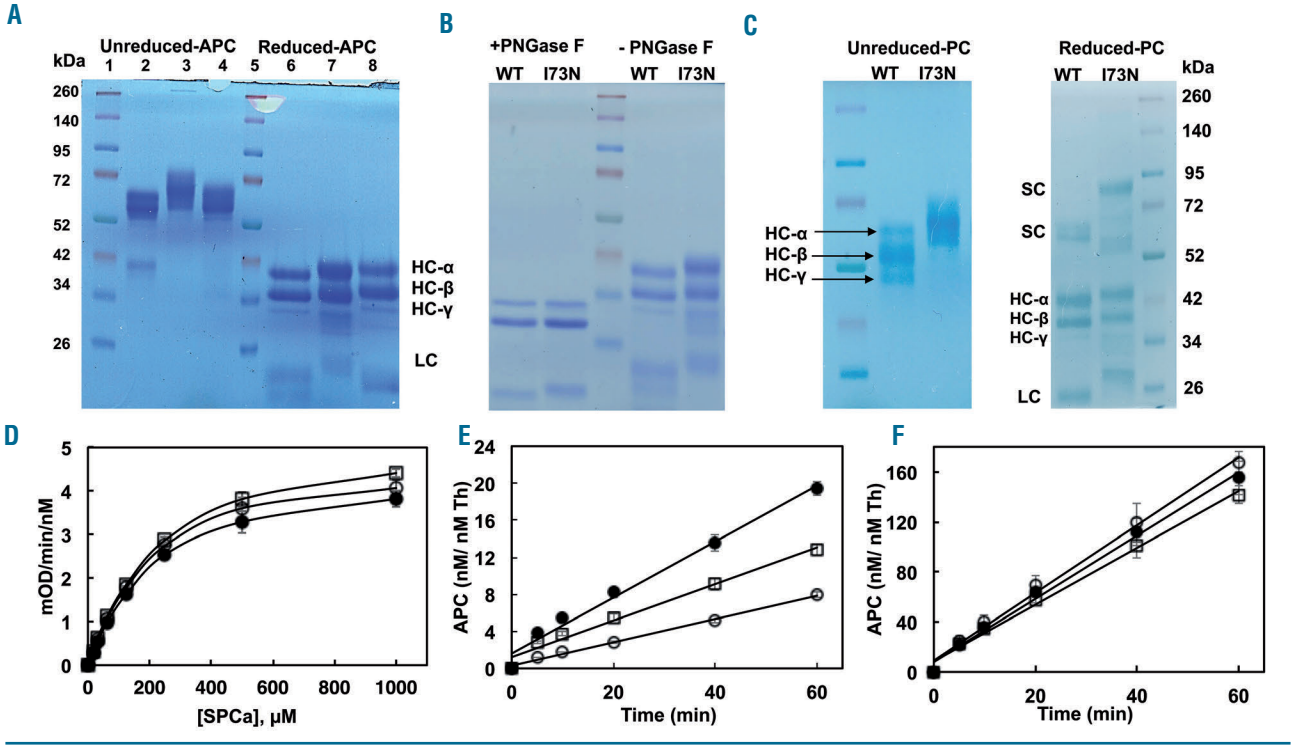


Figure 2. Characterization of recombinant activated protein C (APC) derivatives. (A) SDS-PAGE analysis of recombinant APC-wild type (WT), APC-I73N and APC-R352Q (lanes 2, 3 and 4, respectively), fractionated on a 10% gel under non-reducing and reducing conditions (lanes 6, 7 and 8, respectively). Lanes 1 and 5 represent molecular mass standards in kDa. (B) PNGase treatment of APC-WT and APC-I73N under reducing conditions fractionated on 10% gel. (C) SDS-PAGE analysis of protein C-WT, protein C-I73N fractionated on a 8.75% gel under non-reducing and reducing conditions. HC-α: heavy chain α; HC-β: heavy chain β; HC-γ: heavy chain γ; LC: light chain; SC: single chain. (D) Amidolytic activity of APC-WT (○), APC-I73N (●), and APC-R352Q (□) (5 nM each) toward the chromogenic substrate SpPCa was monitored as described in "Methods". (E) Time course of activation protein C-WT (○), protein C-I73N (●), and protein C-R352Q (□) (1 μM each) by thrombin (10 nM) was monitored in TBS buffer lacking Ca^{2+}. (F) The same as (E) except that the activation of protein C derivatives by the thrombin-sTM complex (1 nM thrombin and 50 nM sTM) was monitored in TBS/Ca^{2+}. At indicated time intervals, the activity of thrombin was inhibited by antithrombin and the rate of APC generation was determined by an amidolytic activity as described in "Methods". Data are derived from at least three independent measurements (±standard deviation). The solid lines in (D) are computer fits of data to the Michaelis-Menten equation, and those in (E) and (F) fits of data to a linear equation. min: minutes.

Arg306 site is intact.[29,30] Results indicated both APC-WT and APC-I73N inactivate FVa Leiden with similar rates in the absence of protein S (Figure 4C). However, the anticoagulant activity of APC-I73N toward FVa Leiden was significantly impaired in the presence of protein S (Figure 4D). These results suggest the co-factor function of protein S in promoting the catalytic efficiency of APC-I73N toward Arg306 has been impaired.

Anti-inflammatory signaling activity

Anti-inflammatory signaling activity of APC derivatives was evaluated in thrombin-mediated permeability assay as described.[24] Results suggest both APC-I73N and APC-R352Q exhibit normal signaling activities in the permeability assay, suggesting neither mutation adversely affects the anti-inflammatory function of APC (Figure 5A).

Interaction of APC-R352Q with Na^+

Arg352 (residue 187 in chymotrypsin numbering)[21] is located on a loop near the S1 specificity pocket (Asp189).[5] This loop is allosterically linked to the Na^+-binding 225-loop in both thrombin and factor Xa,[31-33] thus contributing to affinity of Na^+ binding. To determine whether this loop in APC contributes to Na^+-binding properties of APC, the amidolytic activity of this mutant was monitored in the presence of increasing concentrations of Na^+ in both the absence and presence of Ca^{2+}. Results demonstrated the affinity of APC-R352Q for Na^+ was impaired approximately 2-fold in both the absence and presence of Ca^{2+} (Figure 5B and C). $K_{d(app)}$ values, as determined from nonlinear regression analysis of the saturable-dependence of the chromogenic substrate activity of APC-R352Q as a function of increasing concentrations of Na^+, were 35mM in the absence and 2.8mM in the presence of Ca^{2+} (n=3, $P<0.01$). The same values for APC-WT were 18.7mM in the absence and 1.4mM in the presence of Ca^{2+} (n=3, $P<0.005$). Thus, similar to other coagulation proteases, 189-loop contributes to ligation of Na^+ in the 225-loop of APC.

Thrombin generation assay

Anticoagulant activity of APC mutants was evaluated by thrombin generation assay using both normal and protein C-deficient plasma. In normal plasma, both APC-WT and APC-R352Q exhibited identical thrombin generation inhibitory profiles at concentrations of 5nM (Figure 6A) and 10nM (Figure 6B). By contrast, identical concentrations of APC-I73N showed significantly impaired activity (Figure 6A and B). Similar results were obtained when thrombin generation assay was conducted in the presence of sTM (2nM and 5nM) utilizing protein C-deficient plasma supplemented with a near physiological concentration of protein C derivatives and a tissue factor concentration of 1pM to initiate clotting. Results in the presence of both

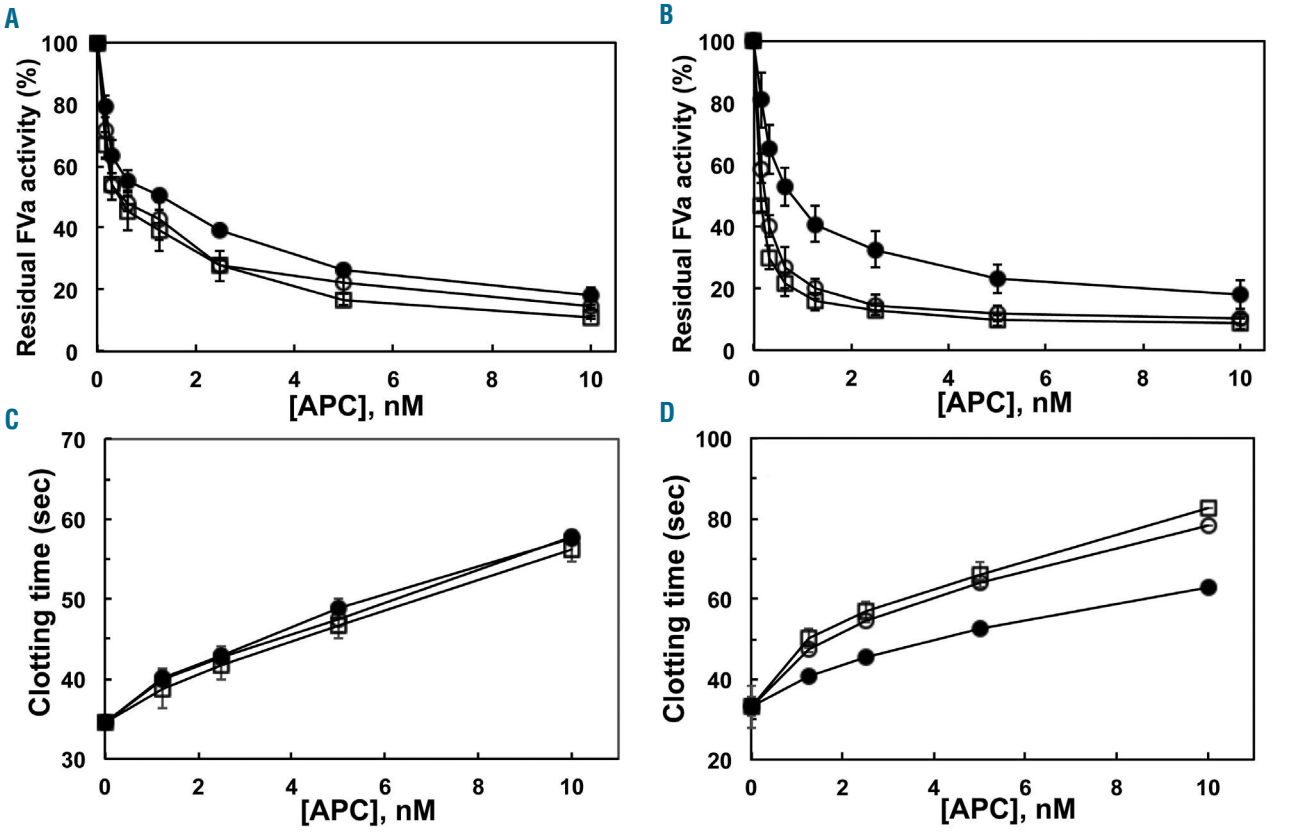


Figure 3. Assessment of the anticoagulant activity of activated protein C (APC) derivatives in the absence and presence of protein S. (A) Degradation of human FVa (2.5 nM) by increasing concentrations of APC-wild type (WT) (○), APC-I73N (●) and APC-R352Q (□) was carried out on PC/PS vesicles (25 μM) in TBS/Ca^{2+} in a 96-well assay plate. Following a 10-minute (min) incubation at room temperature, the remaining co-factor activity of FVa was determined by a prothrombinase assay (5 nM FXa and 1 μM prothrombin for 1 min) as described in "Methods". (B) The same as (A) except that the APC concentration dependence of FVa degradation was carried out in the presence of protein S (50 nM) for 1 min. (C) The anti-clotting activities of APC-WT (○), APC-I73N (●) and APC-R352Q (□) were determined in protein S-deficient plasma by an aPTT assay as a function of increasing concentrations of APC at 37 °C as described in "Methods". (D) The same as (C) except that the anti-clotting activities of the proteases were evaluated in normal plasma. sec: seconds.

concentrations of sTM confirmed the anticoagulant activity of protein C-I73N has been significantly impaired (Figure 7A and B). The lower activity of the protein C mutant is not due to its lower activation rate by thrombin-TM as demonstrated in the purified system (Figure 2F).

Discussion

We have demonstrated in this study that the I73N substitution may be responsible for the recurrent DVT in the proband who is a compound heterozygote for I73N and R352Q mutations in *PROC*. In order to decipher the molecular basis of the anticoagulant defect, we expressed each mutant separately and characterized their properties in coagulation assays. We discovered the I73N substitution introduces a potential new N-linked glycosylation site on EGF1-domain of protein C. This modification does not interfere with the activation of the mutant by thrombin-TM. However, the anticoagulant activity of APC-I73N was significantly decreased in both purified and plasma-based assays. Further analysis revealed APC-I73N exhibits weaker affinity for protein S. This conclusion is derived from the observation that APC-I73N exhibited normal activity toward FVa in the absence of protein S, but impaired activity in the presence of the co-factor. In support of this hypothesis, the anticoagulant activity of APC-I73N was normal in the protein S-deficient plasma, but reduced in the normal plasma. Furthermore, in thrombin generation assays, inhibitory activities of APC-I73N (normal plasma) and protein C-I73N (protein C-deficient plasma supplemented with sTM) were markedly decreased. In contrast to I73N, the R352Q mutation did not impair the catalytic activity of APC in any of coagulation assays, strongly indicating the molecular defect leading to recurrent DVT in the proband carrying the two mutations is primarily due to the I73N substitution. It should be noted that neither mutation adversely affected the anti-inflammatory signaling function of APC.

Activated protein C is a Na^+-binding protease and the R352Q mutation led to an approximately 2-fold lower affinity for interaction with the monovalent cation in both the absence and presence of Ca^{2+}. We have previously demonstrated an allosteric linkage between the two metal

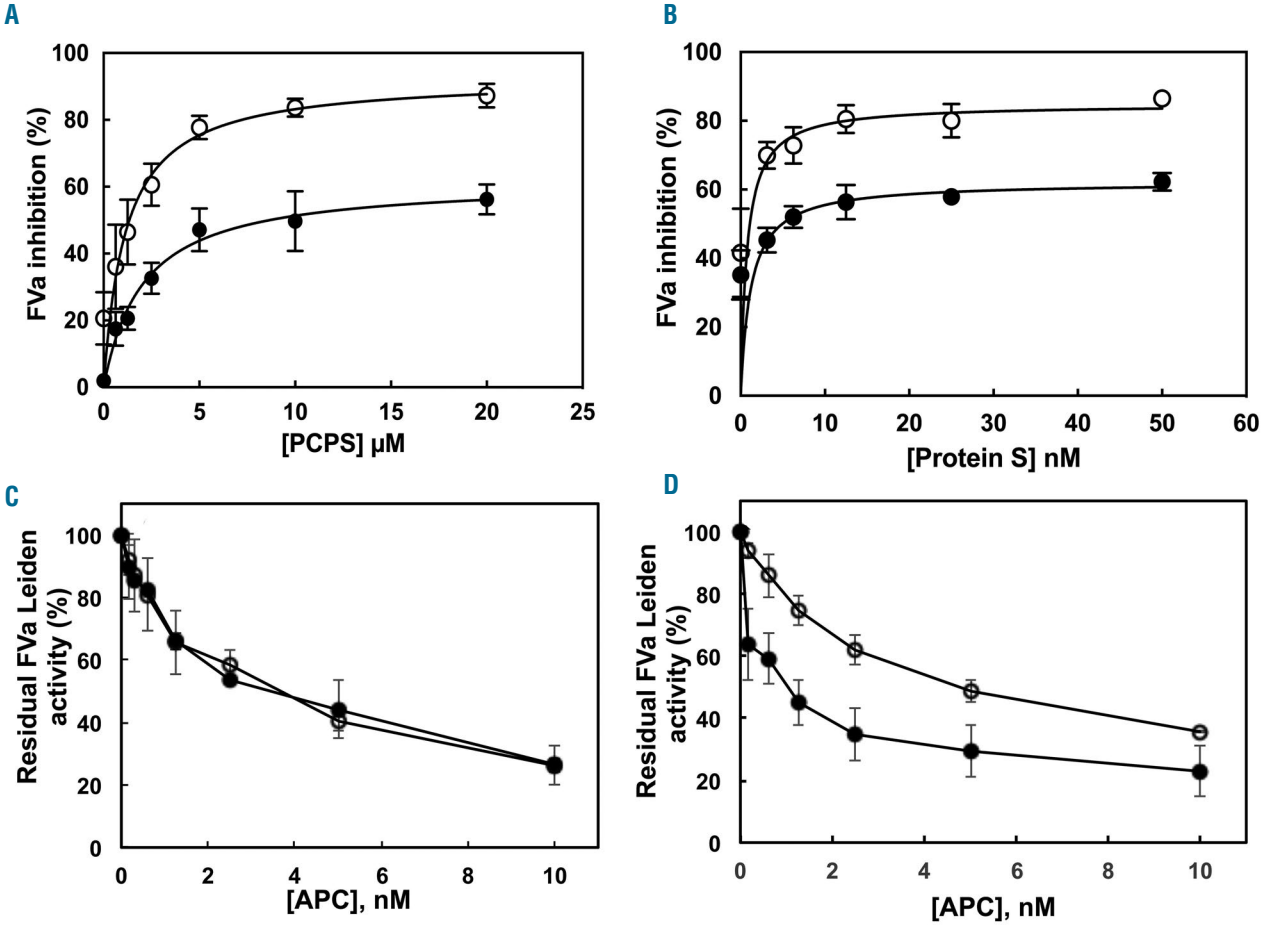


Figure 4. Assessment of the anticoagulant activity of activated protein C (APC) derivatives in the presence of increasing concentrations of PC/PS and protein S. (A) The degradation of human FVa (2.5 nM) by APC-wild type (WT) (○) and APC-I73N (●) in the presence of protein S (20 nM) was carried out as a function of increasing concentrations of PC/PS vesicles as described in "Methods". (B) The same as (A) except that FVa degradation by APC-WT (○) and APC-I73N (●) on PC/PS vesicles (25 μM) was carried out as a function of increasing concentrations of protein S. (C) The degradation of FVa Leiden (2.5 nM) by increasing concentrations of APC-WT (○) and APC-I73N (●) was carried out on PC/PS vesicles (25 μM) in TBS/Ca^{2+} in a 96-well assay plate. Following 10-minute (min) incubation at room temperature, the remaining co-factor activity of FVa Leiden was determined by a prothrombinase assay (10 nM FXa and 1 μM prothrombin for 1 min) as described in "Methods". (D) The same as (C) except that the APC concentration dependence of FVa Leiden degradation was carried out in the presence of protein S (50 nM) for 1 min.

ion binding loops, located in the C-terminal protease domain, modulates the catalytic activity of APC.[6] We showed that binding of Ca^{2+} to 70-80-loop (chymotrypsin numbering)[21] dramatically increases the affinity of Na^+ for its specific site on 225-loop of APC. A similar Ca^{2+}-dependent enhancement in Na^+ affinity for APC-R352Q was observed ($K_{d(app)}$ of 35mM and 2.8mM in the absence and presence of Ca^{2+}, respectively). These dissociation constants are approximately 2-fold higher when compared to Na^+ binding to APC-WT (Kd(app) of 18.7mM and 1.4mM in the absence and presence of Ca^{2+}, respectively). These results suggest Arg352 contributes to high-affinity interaction of Na^+ with 225-loop of APC, which has been determined to be a monovalent cation-binding loop in all coagulation proteases.[34] This residue is located near the S1 specificity site (Asp189) on an exposed surface loop (185-189-loop) immediately below 225-loop.[5] In the case of thrombin and factor Xa, mutagenesis studies have indicated that residues of 185-189-loop are critical for catalytic activity and monovalent cation-binding specificity of these proteases.[31-34] Thus, it appears that this loop makes a similar contribution to interaction of Na^+ with APC. However, noting the high physiological concentration of Na^+ in plasma and its high affinity for interaction with APC, a 2-fold lower affinity of Na^+ for APC-R352Q has no physiological relevance. The normal activity of the mutant protein C/APC in the coagulation assays is consistent with this conclusion. Thus, the basis for the type-II protein C deficiency observed with R352W mutation may be due to distortion of the active-site conformation of APC because of the large and hydrophobic nature of the Trp side-chain.[35]

The mechanism by which I73N mutation adversely affects the protein S-dependent anticoagulant function of APC is not fully understood. Similar to APC, protein S is a vitamin K-dependent protein which binds to negatively charged phospholipids. The affinity of protein S for phospholipid membranes is significantly higher than that of APC. It has been hypothesized that a co-factor function for protein S in the anticoagulant pathway is to stabilize the interaction of APC on phospholipid membranes in the vicinity of FVa/FVIIIa for optimal interaction and proteolytic degradation of these procoagulant co-factors.[9,12,28] Furthermore, there is some evidence to suggest an interaction with protein S is also associated with topographical changes in the active-site of APC, thereby aligning it with scissile bonds of co-factors on the membrane surface.[13-15] Analysis of protein S and PC/PS concentration-dependence of FVa degradation by APC indicated an approximatley 2-fold weaker affinity for the mutant with protein S on PC/PS vesicles. While there was no difference in PC/PS concentration-dependence of FVa inactivation between APC-WT and APC-I73N in the absence of protein S, the apparent dissociation constant for interaction with PC/PS in the presence of the co-factor was increased from 1 μM for WT to 2 μM for the mutant. These results

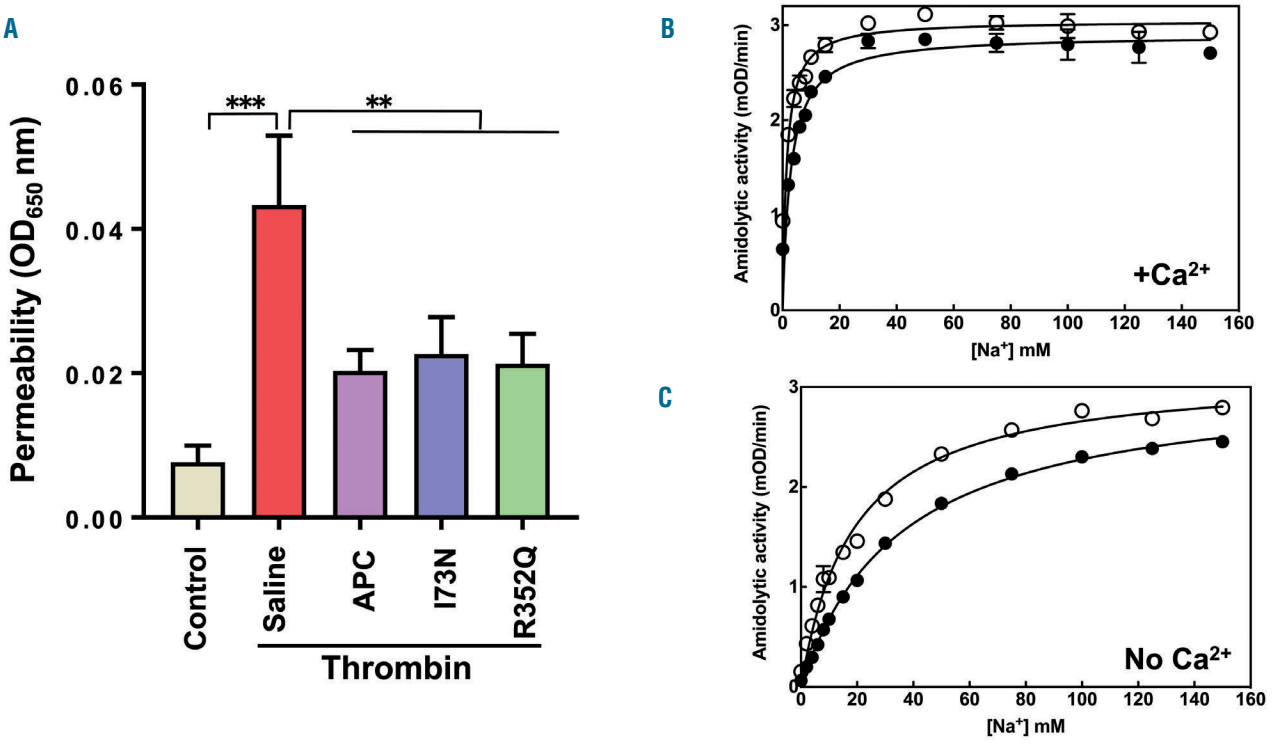
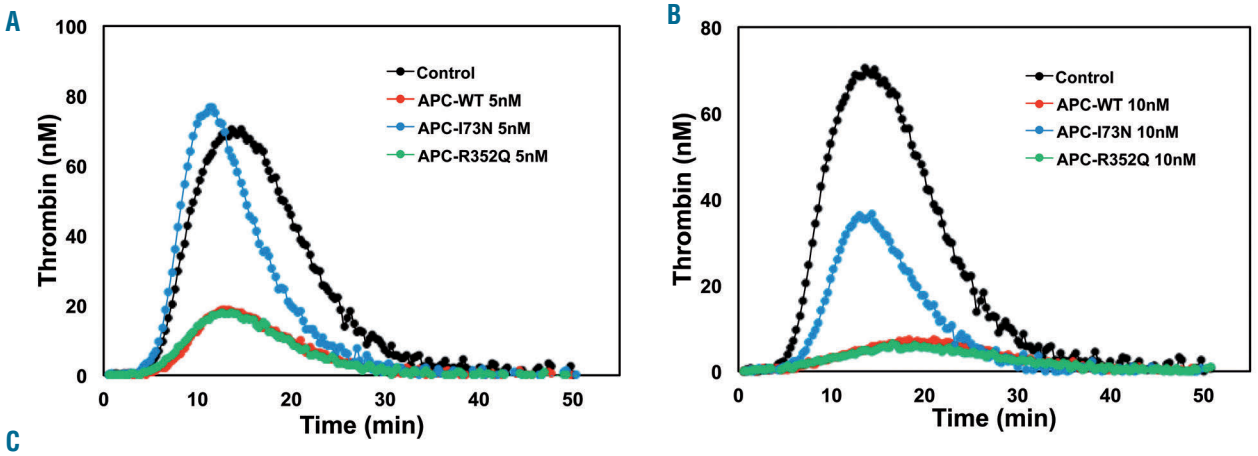


Figure 5. Analysis of the barrier-protective activity of activated protein C (APC) derivatives and assessment of the interaction of APC-R352Q with Na^+. (A) The barrier-protective signaling activity of APC derivatives was evaluated by an established permeability assay. The EA.hy926 cells were treated with saline (as a negative control) and APC derivatives (25 nM for 3 hours) followed by inducing permeability with thrombin [10 nM for 10 minutes (min)]. The barrier-protective effect of APC derivatives was quantitated by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional cell monolayers as described in "Methods". $^{**}P<0.01$ and $P^{***}<0.001$. (B) The amidolytic activity of APC-WT (○) and APC-I73N (●) toward the chromogenic substrate SpPCa was monitored in the presence of 2.5 mM $CaCl_2$ in Tris-HCl (pH, 7.4) as a function of increasing Na^+ concentrations, keeping the total ionic strength constant at 0.2 M. (C) The same as (A) except that the amidolytic activities were monitored in the absence of Ca^{2+}.

confirm the previous hypothesis that a co-factor function for protein S is to enhance the affinity of APC for negatively charged membranes. It was interesting to note that the defect in the anticoagulant function of APC-I73N toward FVa in the presence of excess protein S was not recovered, possibly suggesting that co-factor-mediated topographical changes in the active-site pocket that is required for optimal recognition of FVa scissile bond(s) by APC has also been adversely affected for mutant.

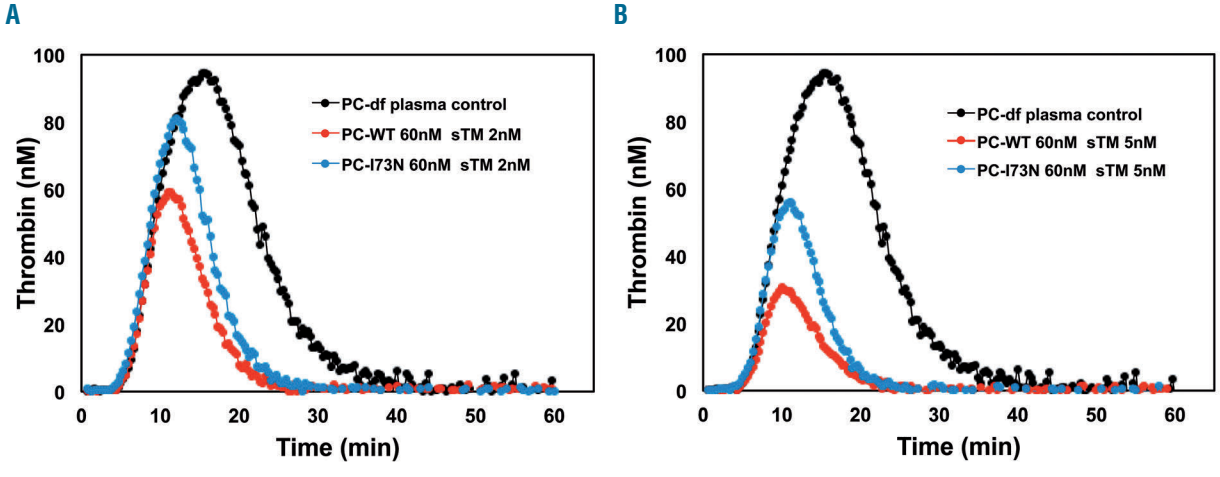
Activated protein C sequentially cleaves two peptide



C

Group name	Control	APC-WT 5nM	APC-WT 10nM	APC-I73N 5nM	APC-I73N 10nM	APC-R352Q 5nM	APC-R352Q 10nM
Lag time (min)	6.25±0	6.92±0	6.25±0.33	6.08±0.17	7.25±0	5.75±0.5	6.08±0.5
ETP (nM*min)	1011.12±34.84	236.66±77.06	134.86±1.37	785.11±111.17	409.22±20.59	232.49±42.68	117.89±18.83
Peak (nM)	69.69±1.78	19.6±9.88	7.03±0.39	76.24±12.57	36.1±2.23	18.25±5.32	5.77±1.35
ttPeak (min)	13.94±0	15.45±2.51	19.63±0.67	11.43±0.17	13.44±0.17	13.61±0.67	18.45±1.17

Figure 6. Assessment of the activated protein C (APC)-mediated inhibition of thrombin generation. Citrated normal plasma (80 μL) was incubated with 20 μL PPP-Reagent Low (1 pM TF) and APC-WT (red circles), APC-I73N (blue circles) or APC-R352Q (green circles): 5 nM APC in (A) and 10 nM APC in (B) or buffer control (black circles). The kinetics of thrombin generation was monitored by measuring the hydrolysis of a fluorogenic thrombin substrate. (C) The lag time (LT, min), peak height (Peak, nM), time to peak (ttPeak, min) and endogenous thrombin potential (ETP, nM*min) were deduced from thrombin generation curves as described in "Methods". min: minutes.



C

Group name	PC-df plasma control	PC-WT-60nM sTM-2nM	PC-WT-60nM sTM-5nM	PC-I73N-60nM sTM-2nM	PC-I73N-60nM sTM- 5nM
Lag time (min)	6.5±0.17	6±0	5.67±0	6.17±0.17	6±0
ETP (nM*min)	1400.23±72.79	523.95±9.59	257.18±3.87	764±3.56	462.28±13.87
Peak (nM)	93.98±4.49	58.79±0.21	30.39±0.54	80.3±1.18	55.95±1.65
ttPeak (min)	15.17±0.17	11.33±0	10.33±0	12.33±0	11±0

Figure 7. Assessment of the activated protein C (APC)-mediated inhibition of thrombin generation in protein C-deficient plasma. Citrated protein C-deficient plasma (80 μL) was supplemented with buffer control (black circles), protein C-wild type (WT) (red circles), or protein C-I73N (blue circles) (60 nM each) and either 2 nM sTM (A) or 5 nM sTM (B) and incubated with 20 μL PPP-Reagent Low (1 pM TF). The kinetics of thrombin generation were monitored by measuring the hydrolysis of a fluorogenic thrombin substrate. (C) The lag time [LT, minutes (min)], peak height (Peak, nM), time to peak (ttPeak, min) and endogenous thrombin potential (ETP, nM*min) were deduced from thrombin generation curves as described in "Methods". min: minutes.

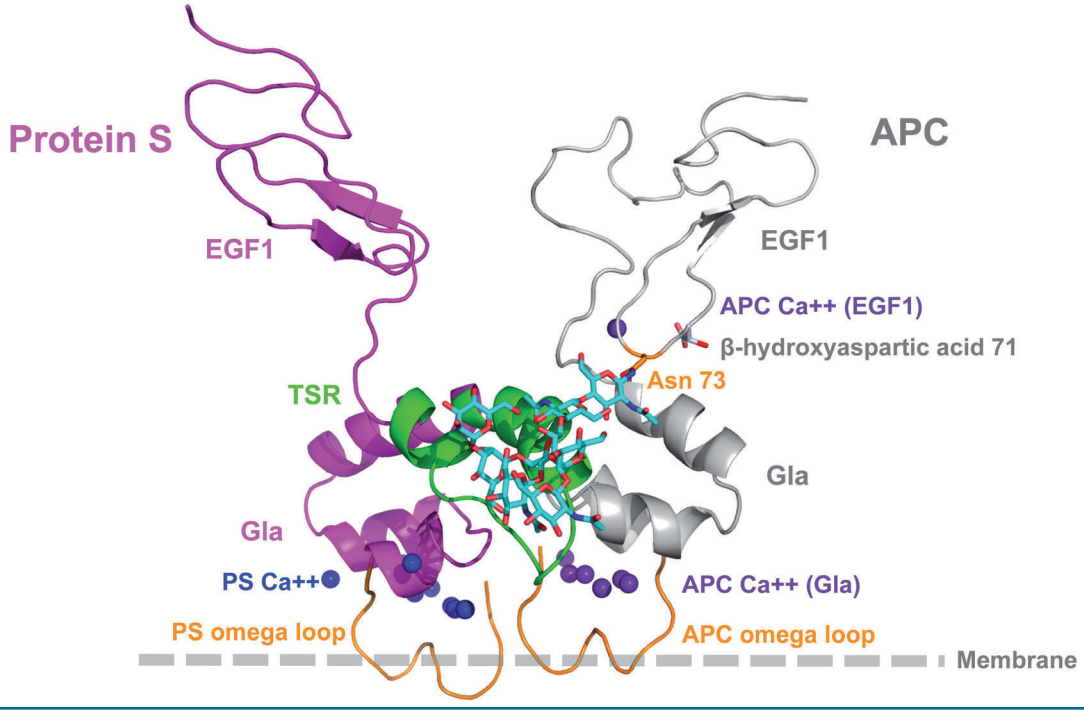


Figure 8. Computational model of the activated protein C (APC)-protein S complex. Molecular models of APC Gla-EGF1 (gray) and PS Gla-TSR-EGF1 (magenta and green) domains were docked as previously described.[25] The overall orientation agrees with several previous mutations that were modeled in the APC and protein S complex while positioning the two membrane binding omega loops side by side anchored in the phospholipid membrane. Following replacement of Ile73 with Asn, several rotamers were tested interactively and most of them could fit in the structure without creating steric clashes. An orientation of Asn representing most of the low energy rotamers was selected and a glycan was grafted and energy minimized: cyan with heteroatoms in blue (N) and red (O). The carbohydrate chain points toward the TSR and Gla domains of protein S. A key APC residue for calcium binding at position 71 (β-hydroxyaspartic acid) and the Ca^{++} ion are also shown (see main text for more details). The figure was generated using PyMol molecular graphic program (Schrodinger, Cambridge, MA, USA).

bonds after Arg506 and Arg306 to inactivate FVa.[36-38] Unlike cleavage of the Arg506 site, which is faster and membrane-independent, the APC cleavage of Arg306 is slower and membrane-dependent.[36-38] Thus, it has been hypothesized that the co-factor function of protein S preferentially improves cleavage of Arg306 to a greater extent than the cleavage of Arg506.[37,38] To determine whether the defective protein S-dependent activity of APC-I73N is due to a slower cleavage of the Arg306 site, the anticoagulant activity of the mutant toward FVa-Leiden was compared to APC-WT. In FVa-Leiden, Arg506 is mutated to Gln so the anticoagulant activity of APC is only monitoring cleavage of Arg306. The findings that both APC-WT and APC-I73N inactivated FVa-Leiden with similar rates in the absence of protein S but the activity of APC-I73N in the presence of protein S was markedly impaired, suggest that the co-factor function of protein S in promoting the catalytic efficiency of APC-I73N toward the Arg306 site of FVa has been adversely affected. Nevertheless, these results do not exclude the possibility that the protein S-dependent activity of APC-I73N toward cleavage of Arg506 has also been impaired.

Previous results have indicated that APC may interact with specific sites within three regions of protein S including Gla-domain, thrombin-sensitive region (TSR), and EGF1-domain.[8-12] In a recent study, we identified a DVT patient who had a mutation on residue 74 (Gly74 to Ser substitution) of APC EGF1-domain, and similar to the patient in the current study, a protein S-dependent anticoagulant defect in the APC mutant was determined to be responsible for thrombosis in the patient.[25] A computational docking model of the APC Gla-EGF1 and protein S Gla-TSR-EGF1 in this previous study indicated that the substitution of Gly74 with Ser may impose steric constraints for interaction between APC and protein S.[25] In the current study, we used the same computational approach and introduced a standard N-glycan chain at Asn73, which is solvent exposed in the mutant APC. Several rotamers were tested and all low energy conformations positioned the Asn73 side-chain toward the solvent in the direction of the docked protein S (Figure 8). Based on this computational model, we hypothesize that the carbohydrate side chain of the mutant Asn would be oriented toward TSR and Gla domains of protein S (Figure 8), thereby impeding a proper interaction between the two proteins. It should be noted that due to a low resolution of this molecular model and the flexibility of the loop carrying the mutation, the exact orientation of the glycan chain in EGF1 of the mutant APC cannot be predicted with any great certainty. However, results of coagulation assays strongly suggest that this glycan interferes with the interaction between EGF1-domain of APC and one of the three regions of protein S (Gla-TSR-EGF1) which have been identified as interactive-sites on the co-factor by molecular modeling and mutagenesis studies. Another potential mechanism through which glycosylation can impede the interaction of APC-I73N with protein S is by altering the Ca^{2+} affinity of EGF1-domain. The high affinity EGF1 Ca^{2+} binding site of APC (Kd approx. 100 μM) is centered around β-hydroxyaspartic acid at position 71.[39] Ca^{2+} bind-

ing to this site makes a key contribution to APC interaction with protein S.[39] Due to close proximity of residue 73 to this functionally important metal ion binding-site, it is possible a glycosylated Asn73 impairs the affinity of APC-I73N for protein S by altering the affinity of EGF1 for Ca^{2+}. Since this site has a much higher affinity for Ca^{2+} than the Gla-domain of APC (Kd low mM range), evaluating the effect of mutagenesis on the affinity of EGF1-domain for Ca^{2+} was not feasible by functional assays.

In summary, our results strongly suggest that substitution of Ile73 with Asn introduces a new N-linked glycosylation site on EGF1-domain of APC. This modification in the compound heterozygote patient leads to a weaker affinity of APC-I73N for protein S, thereby causing anticoagulant defect and recurrent DVT. Noting the anticoagulant defect is observed only in the presence of protein S, results further suggest the I73N mutation would be most harmful under conditions where the co-factor level is low (i.e. pregnancy, oral contraceptive use, etc.).[40,41] This hypothesis is consistent with the observation that recurrent DVT in the affected patient was associated with pregnancy (28th week, left lower limb DVT), during the postpartum period (3rd and 4th weeks, lower limb DVT and mesenteric venous thrombosis, respectively) and when taking oral contraceptives (mesenteric venous thrombosis after finishing one year of anticoagulant therapy). The I73N mutation does not adversely affect the anti-inflammatory signaling function of APC. Molecular modeling predicts the newly attached N-linked glycan on Asn73 can impede with the proper interaction of APC EGF1-domain with Gla-TSR-EGF1 domains of protein S on the membrane surface. This modification appears to not only weaken the affinity of APC for protein S but also adversely affect functionally important protein S-dependent topographical changes in the active-site of APC, thereby impairing its anticoagulant function on the membrane surface.

Acknowledgments

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References

1. Esmon CT. Molecular events that control the protein C anticoagulant pathway. Thromb Haemost. 1993;70(1):29-35.
2. Walker FJ, Fay PJ. Regulation of blood coagulation by the protein C system. FASEB J. 1992;6(8):2561-2567.
3. Foster D, Davie EW. Characterization of a cDNA coding for human protein C. Proc Natl Acad Sci. (USA) 1984;81(15):4766-4770.
4. Stenflo J. Structure-function relationships of epidermal growth factor modules in vitamin K-dependent clotting factors. Blood. 1991;78(7):1637-1651.
5. Mather T, Oganessyan V, Hof P, et al. The 2.8 Å crystal structure of Gla-domainless activated protein C. EMBO J. 1996;15(24):6822-6831.
6. He X, Rezaie AR. Identification and characterization of the sodium-binding site of activated protein C. J Biol Chem. 1999;274(8):4970-4976.
7. Yang L, Prasad S, Di Cera E, Rezaie AR. The conformation of the activation peptide of protein C is influenced by Ca2+ and Na+ binding. J Biol Chem. 2004;279(37):38519-38524.
8. Preston RJ, Ajzner E, Razzari C, et al. Multifunctional specificity of the protein C/activated protein C Gla domain. J Biol Chem. 2006;281(39):28850-28857.
9. Norstrom EA, Steen M, Tran S, Dahlbäck B. Importance of protein S and phospholipid for activated protein C-mediated cleavage in factor Va. J Biol Chem. 2003;278(27):24904-24911.
10. Ahnström J, Andersson HM, Canis K, et al. Activated protein C cofactor function of protein S: a novel role for a γ-carboxyglutamic acid residue. Blood. 2011;117(24):6685-6693.
11. Villoutreix BO, Teleman O, Dahlbäck B. A theoretical model for the Gla-TSR-EGF-1 region of the anticoagulant cofactor protein S: from biostructural pathology to species-specific cofactor activity. J. Comput. Aided Mol Des. 1997;11(3):293-304.
12. Andersson HM, Arantes MJ, Crawley JT, et al. Activated protein C cofactor function of protein S: a critical role for Asp95 in the EGF1-like domain. Blood. 2010;115(23):4878-4885.
13. Yegneswaran S, Wood GM, Esmon CT, Johnson AE. Protein S alters the active site location of activated protein C above the membrane surface. A fluorescence resonance energy transfer study of topography. J Biol Chem. 1997;272(40):25013-25021.
14. Yegneswaran S, Smirnov MD, Safa O, Esmon NL, Esmon CT, Johnson AE. Relocating the active site of activated protein C eliminates the need for its protein S cofactor. A fluorescence resonance energy transfer study. J Biol Chem. 1999;274(9):5462-5468.
15. Hackeng TM, Yegneswaran S, Johnson AE, Griffin JH. Conformational changes in activated protein C caused by binding of the first epidermal growth factor-like module of protein S. Biochem J. 2000;349 Pt 3:757-764.
16. Dahlbäck B. The protein C anticoagulant system: inherited defects as basis for venous thrombosis. Thromb. Res. 1995;77(1):1-43.
17. Griffin JH, Evatt B, Zimmerman TS, et al. Deficiency of protein C in congenital thrombotic disease. J Clin Invest. 1981;68(5):1370-1373.
18. Jalbert LR, Rosen ED, Moons L, et al. Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest. 1998;102(8):1481-1488.
19. Reitsma PH, Poort S, Bernardi F, et al. Protein C deficiency: a database of mutations. Thromb Haemost. 1993;69(1):77-84.
20. Mackie I, Cooper P, Lawrie A, Kitchen S, Gray E, Laffan M. Guidelines on the laboratory aspects of assays used in haemostasis and thrombosis. Int J Lab Hematol. 2013;35(1):1-13.
21. Bode W, Mayr I, Baumann U, Huber R, Stone SR, Hofsteenge J. The refined 1.9 Å crystal structure of human α-thrombin: interaction with D-Phe-Pro-Arg chloromethеylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. EMBO J. 1989;8(11):3467-3475.
22. Yang L, Manithody C, Rezaie AR. Contribution of basic residues of the 70-80-loop to heparin binding and anticoagulant function of activated protein C. Biochemistry. 2002;41(19):6149-6157.
23. Yang L, Rezaie AR. Calcium-binding sites of the thrombin-thrombomodulin-protein C complex: possible implications for the effect of platelet factor 4 on the activation of vitamin K-dependent coagulation factors. Thromb Haemost. 2007;97(6):899-906.
24. Ding Q, Yang L, Dinarvand P, Wang X, Rezaie AR. Protein C Thr315Ala variant results in gain of function but manifests as type II deficiency in diagnostic assays. Blood. 2015;125(15):2428-2434.
25. Chen C, Yang L, Villoutreix BO, Wang X, Ding Q, Rezaie AR. Gly74Ser mutation in protein C causes thrombosis due to a defect in protein S-dependent anticoagulant function. Thromb Haemost. 2017;117(7):1358-1369.
26. Grinnell BW, Walls JD, Gerlitz B. Glycosylation of human protein C affects its secretion, processing, functional activities, and activation by thrombin. J Biol Chem. 1991;266(15):9778-9785.
27. Rezaie AR, Esmon CT. Tryptophans 231 and 234 in protein C report the Ca(2+)-dependent conformational change required for activation by the thrombin-thrombomodulin complex. Biochemistry. 1995;34(38):12221-12226.
28. Walker FJ. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. J Biol Chem. 1981;256(21):11128-11131.
29. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci U S A.

1993;90(3):1004-1008.
30. Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature. 1994;369(6475):64-67.
31. Pineda AO, Zhang E, Guinto ER, Savvides SN, Tulinsky A, Di Cera E. Crystal structure of the thrombin mutant D221A/D222K: the Asp222:Arg187 ion-pair stabilizes the fast form. Biophys Chem. 2004;112(2-3):253-256.
32. Qureshi SH, Yang L, Manithody C, Iakhiaev AV, Rezaie AR. Mutagenesis studies toward understanding allostery in thrombin. Biochemistry. 2009;48(34):8261-8270.
33. Rezaie AR, Kittur FS. The critical role of the 185-189-loop in the factor Xa interaction with Na+ and factor Va in the prothrombinase complex. J Biol Chem. 2004;279(46):48262-48269.
34. Dang QD, Di Cera E. Residue 225 determines the Na(+)-induced allosteric regulation of catalytic activity in serine proteases. Proc Natl Acad Sci U S A. 1996;93(20):10653-10656.
35. Greengard JS, Fisher CL, Villoutreix B, Griffin JH. Structural basis for type I and type II deficiencies of antithrombotic plasma protein C: patterns revealed by three-dimensional molecular modelling of mutations of the protease domain. Proteins. 1994;18(4):367-380.
36. Kalafatis M, Mann KG. Role of the membrane in the inactivation of factor Va by activated protein C. J Biol Chem. 1993;268(36):27246-27257.
37. Nicolaes GA, Tans G, Thomassen MC, et al. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and Factor VaR506Q by activated protein C. J Biol Chem. 1995;270(36):21158-21166.
38. Rosing J, Hoekema L, Nicolaes GA, et al. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem. 1995;270(46):27852-27858.
39. Ohlin AK, Landes G, Bourdon P, Oppenheimer C, Wydro R, Stenflo J. Beta-hydroxyaspartic acid in the first epidermal growth factor-like domain of protein C. Its role in Ca2+ binding and biological activity. J Biol Chem. 1988;263(35):19240-19248.
40. Boerger LM, Morris PC, Thurnau GR, Esmon CT, Comp PC. Oral contraceptives and gender affect protein S status. Blood. 1987;69(2):692-694.
41. Comp PC, Thurnau GR, Welsh J, Esmon CT. Functional and immunologic protein S levels are decreased during pregnancy. Blood. 1986;68(4):881-885.