Protein C Thr315Ala variant results in gain of function but manifests as type II deficiency in diagnostic assays

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Key Points

• A novel PC mutation in a healthy subject results in type II PC deficiency as diagnosed by commercial kits.
• Recombinant expression and analysis reveals this is a gain-of-function mutant of PC that cannot be properly diagnosed by commercial kits.

Introduction

Protein C (PC) is a vitamin K–dependent plasma glycoprotein, which upon activation by thrombin in complex with thrombomodulin (TM), regulates the coagulation cascade through a feedback loop inhibition mechanism. PC deficiency is associated with an increased risk of venous thromboembolism (VTE). A recent cohort study aimed at establishing a normal PC range identified a healthy PC-deficient subject whose PC antigen level of 65% and activity levels of 50% (chromogenic assay) and 36% (clotting assay) were markedly low. The proband has a negative family history of VTE. Genetic analysis revealed the proband has a heterozygous missense mutation in which Thr-315 of the PC heavy chain has been substituted with Ala. We expressed this mutant in HEK-293 cells and purified it to homogeneity. A similar decrease in both anticoagulant and anti-inflammatory activities of the activated protein C mutant was observed in plasma- and cell-based assays. Interestingly, we discovered if functional assays were coupled to PC activation by the thrombin-TM complex, the variant exhibits improved activities in all assays. Sequence analysis revealed Thr-315 is a consensus M-linked glycosylation site for Asn-313 and that its elimination significantly (four- to fivefold) improves the maximum velocity of PC activation by the thrombin-TM complex, explaining the basis for the proband’s negative VTE pedigree. (Blood. 2015;125(15):2428-2434)

Protein C (PC) is a vitamin K–dependent plasma glycoprotein, which upon activation by thrombin in complex with thrombomodulin (TM), regulates the coagulation cascade through a feedback loop inhibition mechanism. PC deficiency is associated with an increased risk of venous thromboembolism (VTE). A recent cohort study aimed at establishing a normal PC range identified a healthy PC-deficient subject whose PC antigen level of 65% and activity levels of 50% (chromogenic assay) and 36% (clotting assay) were markedly low. The proband has a negative family history of VTE. Genetic analysis revealed that the proband has a heterozygous missense PROC (Thr-315Ala) was responsible for this apparent type II PC deficiency. In clinical laboratories, PC deficiency is initially diagnosed by the PC:Ag and PC:A levels, and type II deficiency is characterized by a lower activity level for APC. In both types of assays, PC in patient’s plasma is activated with an activator derived from the venom of a specific snake (Protac) followed by evaluation of APC activity employing either a plasma-based activated partial thromboplastin time (aPTT) assay or a synthetic peptide-based chromogenic assay. In a recent study aimed at establishing the normal range of PC in the healthy population, we identified a healthy subject whose PC:Ag and PC:A levels were 65%, 50% (chromogenic assay), and 36% (clotting assay) of the normal range, respectively. Noting a lack of family history of VTE for the proband, we decided to determine whether a mutation in the PC gene (PROC) was responsible for this apparent type II PC deficiency. Genetic analysis revealed that the proband has a heterozygous missense mutation in PROC leading to substitution of Thr-315 of the PC heavy chain with Ala. In this study, we expressed this PC mutant in HEK-293 cells and purified it to homogeneity. Following activation, we characterized the anticoagulant and anti-inflammatory properties of the


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APC mutant in purified, plasma-based, and cell-based assay systems. Consistent with results of the proband’s plasma assays, the APC mutant exhibited significantly decreased activity in all functional assays. Interestingly, further analysis revealed that if the functional anticoagulant and anti-inflammatory assays were coupled to activation of the zymogen by the physiological activator (thrombin-TM), the PC mutant exhibits improved activities in all assays. The complementary DNA sequence analysis indicates that Thr-315 is a consensus N-linked glycosylation site for Asn-313 and that its elimination significantly improves the maximum velocity (Vmax) of PC activation by thrombin-TM. Thus, this is a novel gain-of-function mutant of PC that cannot be diagnosed by current commercial assays.

Materials and methods

Analysis of TG in plasma

TG assay was performed using previously described standard methods.16 Each assay contained 80 μL citrated test plasma incubated with 20 μL PPP reagent containing 5 pmol tissue factor, 4 μM phospholipids, and 100 mM CaCl2. In some experiments, TG assay was performed in the presence of 2 to 10 nM soluble thrombomodulin (sTM). TG was determined by measuring the hydrolysis of a fluorogenic thrombin substrate as described.15,16 Three parameters—including lag time (minutes), peak height (peak, nM), and area under the curve, referred to the endogenous thrombin potential (ETP, nM min)—were used to assess TG dynamics and evaluate the anticoagulant activity of PC.

Expression and characterization of APC derivatives are described in the supplemental Materials, available on the Blood Web site.

Anticoagulant assays

The anticoagulant activity of recombinant APC derivatives was evaluated in both purified and plasma-based assay systems as described.17-19 Brieﬂy, FVIIIa (2.5 nM) was incubated with increasing concentrations of either wild-type APC (APC-WT) or APC-T315A (0-5 nM) on 25 μM phosphatidylcholine (PC, 80%)/phosphatidylserine (PS, 20%) vesicles (PC/PS) in Tris-buffered saline (TBS)/CaCl2. Following 10 minutes incubation at room temperature, the remaining FVIIIa activity was determined in a prothrombinase assay from the FVIIIa-catalyzed prothrombin activation by FXa as described.17,18 The prothrombinase assay was carried out for 30 seconds using 1 μM prothrombin and 1 nM FXa. The remaining FVIIIa activity was determined from the decrease of the FVIIIa-dependent rate of TG as determined by an amidolytic activity assay using S2238 (100 μM). The same assay was used to monitor the inactivation of FVIIIa by increasing concentrations of APC in the presence of protein S (100 nM) with the exception that incubation time was decreased to 1 minute. In an alternative assay, the activation of PC by thrombin-TM was coupled to the rate of FVIIIa degradation by the generated APC. In this case, FVIII (5 nM), thrombin (1 nM), protein S (100 nM), stTM (10 nM), PC derivatives (0-100 nM), and PC/PS vesicles (50 μM) were incubated in TBS/ CaCl2 for 8 minutes before adding FX (0.2 μM) and FDXa (2 nM) and measuring the rate of FVIII generation for 10 minutes.

The anticoagulant activities of APC-WT and APC-Th315Ala were also evaluated in plasma by a pPTT assay using a Start 4 fibrinometer (Diagnostic/ Stago, Asnières, France). Brieﬂy, 0.05 mL TBS containing 0 to 20 nM final concentrations of APC derivatives were incubated with a mixture of 0.05 mL of normal pooled plasma plus 0.05 mL of the aPTT reagent (Alexin) for 5 minutes before the initiation of clotting by the addition of 0.05 mL of 55 mM CaCl2 at 37°C as described previously.17-19

Endothelial cell permeability assay

The intracellular signaling activity of APC derivatives (5-100 nM, 3 hours) was evaluated in an endothelial cell permeability assay in response to lipopolysaccharide (LPS) (10 ng/mL, 4 hours) using transformed human umbilical vein endothelial (EA.hy926) cells as described elsewhere.20 The endothelial cell permeability in response to LPS 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 elsewhere.20

A variation of the cell permeability assay was used to evaluate the intracellular signaling function of APC derivatives, activated on endothelial cells by the thrombin-W215A/E217A mutant, which is known to activate PC normally, but has no PAR1-dependent signaling activity.21 In this case, cells were initially incubated with increasing concentrations of APC or PC (0-100 nM) plus the thrombin mutant (5 nM) for 3 hours before inducing cell permeability with LPS (10 ng/mL, 4 hours).

Expression of cell surface receptors

The effect of PC (0-80 nM) + thrombin-W215A/E217A on the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 on EA.hy926 cells in response to LPS was determined by a whole-cell enzyme-linked immunosorbent assay (ELISA) as described.20

Cell adhesion assay

The interaction of THP-1 cells with LPS-stimulated endothelial cells was evaluated by fluorescent labeling of THP-1 cells as described.22 THP-1 cells were labeled with the Vybrant DiD dye and then added to LPS (10 ng/mL)-stimulated EA.hy926 cells. Cells were allowed to adhere, the nonadherent THP-1 cells were washed off, and the fluorescence of the adherent cells was measured as described.22 The effect of PC (0-80 nM) + thrombin-W215A/E217A on THP-1-endothelial cell interaction was analyzed as described previously.22

NF-κB assay

The protective effect of PC zymogens on LPS-induced activation of nuclear factor κB (NF-κB) pathway in nuclear lysates of EA.hy926 cells was evaluated in a coupled assay using an ELISA-based nonradioactive transcription factor assay kit (Abcam, Cambridge, UK) according to the manufacturer’s protocol and as described elsewhere.20,22 Brieﬂy, confluent monolayers of endothelial cells were incubated with increasing concentrations of either PC-WT or PC-T315A (0-100 nM) plus the thrombin-W215A/E217A mutant (5 nM, 3 hours) before inducing NF-κB activation with LPS (10 ng/mL, 4 hours).

Results

Case presentation and PC assays

The healthy subject, a 33-year-old female with a negative personal and family history of VTE, voluntarily took part in a cohort study aimed at establishing normal ranges of anticoagulation proteins when she was...
diagnosed with PC deficiency. The total PC:Ag level in the proband’s plasma was 65% and the PC:A level, measured by both coagulation (aPTT) and chromogenic assays, were 36% and 50% of normal ranges, respectively. Genetic analysis identified a missense mutation in the PROC gene, resulting in substitution of Thr-315 of PC with Ala. In light of the proband’s negative VTE pedigree, we decided to express this mutant for further characterization. Thus, the PC mutant (PC-T315A) as well as its wild-type counterpart were expressed in HEK-293 cells and purified to near homogeneity, with the heavy chain of the PC mutant migrating slightly faster than wild-type (Figure 1A). The remaining cofactor activity of FV was determined by a prothrombinase (1 nM FXa and 1 μM prothrombin for 1 minute) assay as described in “Materials and methods.” (F-G) FVIIa (20 nM) degradation by APC-WT (○) and APC-T315A (△) in the absence (F, 30 minutes) and presence (G, 5 minutes) of protein S (110 nM) was analyzed by incubating increasing concentrations of each protease with the cofactor on PC/PS vesicles (50 μM) in TBS/Ca²⁺ in a 96-well assay plate. The remaining cofactor activity of FVIIa was determined by an intrinsic Tenase (1 nM FIXa and 200 nM FX for 2 minutes) assay as described in “Materials and methods.” Data in panels B-G are derived from 3 independent measurements (± standard deviation).

Figure 1. SDS-PAGE analysis of recombinant PC derivatives and their characterization in amidolytic, plasma-based clotting, and purified systems. (A) Recombinant PC-WT and PC-T315A (lanes 1 and 2, respectively) were fractionated on a 10% SDS-PAGE under nonreducing and reducing conditions (lanes 3 and 4, respectively). Lane 5 represents molecular mass standards in kDa. SC, single chain; HC-α, heavy chain α; HC-β, heavy chain β; LC, light chain. (B) Amidolytic activity of APC-WT (○) and APC-T315A (△) (5 nM each) toward the chromogenic substrate SpPCa. (C) Plasma clotting activity of APC-WT (○) and APC-T315A (△) were determined as a function of increasing concentrations of proteases (0-20 nM) at 37°C as described in “Materials and methods.” (D-E) FVas (2.5 nM) degradation by APC-WT (○) and APC-T315A (△) in the absence (D, 10 minutes) and presence (E, 1 minute) of protein S (110 nM) was analyzed by incubating increasing concentrations of each protease with the cofactor on PC/PS vesicles (25 μM) in TBS/Ca²⁺ in a 96-well assay plate. The remaining cofactor activity of FVa was determined by an intrinsic Tenase (1 nM FIXa and 200 nM FX for 2 minutes) assay as described in “Materials and methods.” Data in panels B-G are derived from 3 independent measurements (± standard deviation).
FVIII, protein S, PC, and sTM were all incubated on PC/PS vesicles and incubated with 1 nM thrombin on PC/PS vesicles for 8 minutes before initiating FVa generation by adding FIIa plus FX (Figure 2B). Similar to the inactivation of FVa, the anticoagulant function of the PC variant toward FVIIa was higher than that of wild-type PC in this assay (Figure 2B). Similar results were obtained in variations of these assays if FV, prothrombin, protein S, PC, and sTM were all incubated on PC/PS vesicles and TG was directly monitored by addition of a low concentration of FIIa (Figure 3C). This was also true if FV, prothrombin, FX, protein S, PC, and sTM were all incubated on PC/PS vesicles and TG was initiated by a low concentration of tissue factor–factor VIIa (10 pM) complex (Figure 3D). Taken together, these results suggest that the improved activation of the PC variant by the thrombin-TM complex more than compensates for its decreased catalytic function if these rather physiologically relevant coupled assay systems are employed to analyze the anticoagulant activity of the PC variant.

Analysis of TG in plasma

The anticoagulant activity of PC in the proband’s plasma was evaluated by TG with a commercial kit using a tissue factor concentration of 5 pM to initiate the clotting cascade. Analysis of TG in plasma was conducted in both the absence and presence of sTM (2-10 nM). The results in the presence of 10 nM sTM indicated a normal anticoagulant function for the proband’s plasma PC based on similar clotting parameters obtained in this test (Figure 4). In the absence of sTM, the values of lag time, peak, and ETP in plasma derived from the case were similar to those in normal control plasma (Figure 4). In the presence of 10 nM sTM, the proband’s plasma exhibited a similar reduction (or slightly lower) in lag time, peak, and ETP of TG. Interestingly, at a low concentration of sTM (2 nM), the T315A variant exhibited an anticoagulant advantage as evidenced by significantly lower values for these parameters (Figure 4). These results support the central hypothesis presented previously and may explain the molecular basis for the normal anticoagulant activity of the PC-Thr315Ala variant under physiological conditions.

Anti-inflammatory activity

APC can elicit potent cytoprotective signaling responses in endothelial cells in response to thrombin and proinflammatory cytokines. The modulation of LPS-induced endothelial cell permeability has been frequently used to probe the cytoprotective signaling function of APC variants. Thus, we evaluated the signaling activity of APC-WT and APC-T315A in an LPS-mediated permeability assay. Analysis of

Figure 2. Initial rate of PC activation by thrombin. (A) The time course of PC-WT (○) and PC-T315A (●) (1 μM each) activation by thrombin (1 nM) in complex with sTM (10 nM) was monitored in TBS/Ca²⁺. 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 “Materials and methods.” (B) The same as panel A except that the concentration dependence of PC activation by the thrombin-sTM complex was carried out in TBS/Ca²⁺ for 10 minutes and the rate of APC generation was determined as described previously. Data are derived from at least 3 independent measurements (± standard deviation). The solid lines in panel A are computer fits of data to a linear equation and those in panel B are fits of data to the Michaelis-Menten equation, yielding $K_m$ and $k_{cat}$ values of 2.7 ± 0.2 μM and 7.8 ± 0.3 minutes⁻¹ for APC-WT and 4.2 ± 0.4 μM and 34.5 ± 1.6 minutes⁻¹ for APC-T315A, respectively.

Analysis of PC activation

Analysis of the initial rate of PC activation indicated that the activation of the PC variant by the thrombin-TM complex has been improved four- to fivefold (Figure 2A). The PC concentration dependence of activation revealed that the improvement in the activation of the PC variant by the thrombin-TM complex is primarily due to a higher Vmax (Figure 2B). The activation of the PC variant by thrombin in the absence of TM was improved ~60% (data not shown). These results suggest that the glycosylation of Asn-313 plays an inhibitory role in the activation of PC by the thrombin-TM complex. This is consistent with previous mutagenesis studies demonstrating that the substitution of Asn-313 of PC with a Gln (N313Q) improves the activation of the zymogen mutant by the thrombin-TM complex. Nevertheless, the latter variant exhibited a reduced $K_m$ in activation by the thrombin-TM complex, whereas the T315A mutant has a higher Vmax. As demonstrated later in the coupled functional assays, this improved Vmax for the natural T315A variant of PC activation by the thrombin-TM complex is the most likely reason for the proband’s negative VTE history.

Coupling PC activation to its anticoagulant function

To investigate the hypothesis that the improvement in the Vmax of variant PC activation may be responsible for the proband’s negative VTE history, the activation of PC by the thrombin-TM complex was mechanistically linked to its anticoagulant function by coupling zymogen activation to protease function in both purified and plasma-based assay systems. In the purified system, FV, protein S, PC, and sTM were all incubated with 1 nM thrombin on PC/PS vesicles for 1 minute before initiating TG by the addition of FXa plus prothrombin (1.5 μM). Interestingly, it was discovered that, unlike the decreased anticoagulant effect of the APC variant, the PC variant exhibits improved anticoagulant function in this assay (Figure 3A). Thus, the APC variant inactivated FVa better than wild-type APC. The anticoagulant function of the PC variant was also evaluated in the intrinsic pathway using a similar coupled assay. Thus, FVIII, protein S, PC, and sTM were

Figure 1E-G) degradation in either the absence (Figure 1D,F) or presence (Figure 1E,G) of protein S supported results of other assays that the anticoagulant activity of APC-T315A has been negatively impacted by the mutation. The APC variant exhibited a similar half-life in plasma and an essentially identical reactivity profile toward specific plasma inhibitors (antithrombin, PC inhibitor, and α1-antitrypsin) in purified systems (data not shown).

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concentration dependence of APC activity in this assay indicated that the barrier protective activity of the APC variant is significantly impaired (Figure 5A). Thus, in contrast to effective barrier protective activity for 10 nM APC-WT, a markedly higher concentration (50-100 nM) was required to observe a similar barrier protective function for the APC variant (Figure 5A). To determine whether, similar to anticoagulant assays, coupling of PC activation to its cytoprotective function on cell surfaces can alter the outcome, we incubated endothelial cells with different concentrations of wild-type and variant PC zymogens followed by their activation with a thrombin derivative (W215A/E217A-thrombin or WE-thrombin), which is known to activate PC normally in the presence of TM, but is incapable of activating PAR1.20 This assay facilitated specific monitoring of the PAR1-dependent protective activity of the cell surface–generated APC, without interference from thrombin. Interestingly, when the activation of the PC zymogens was coupled to their activity by this strategy, the PC variant exhibited a markedly higher barrier protective activity than did wild-type PC (Figure 5B). Thus, in contrast to wild-type PC, which only exhibited a significant barrier protective activity at 80 nM zymogen, the same extent of barrier protective activity was observed with only a 20 nM variant zymogen (Figure 5B).

A similar improved cytoprotective activity for PC-T315A was observed in inhibiting NF-κB activation, cell adhesion molecule expression, and THP-1 cells binding to endothelial cells in response to LPS when the activity of the zymogen was evaluated in the same coupled assay using the thrombin-W215A/E217A mutant as the PC activator (Figure 5C-E). These results clearly suggest that the PC variant has significantly higher protective activity when its activation is mechanistically coupled to its cellular activity on cells expressing both EPCR and TM.

Discussion

In a cohort study aimed at establishing normal PC levels, we identified a healthy subject whose plasma tests of chromogenic and clotting-based assays indicated that the concentration dependence of APC activity in this assay indicated that the barrier protective activity of the APC variant is significantly impaired (Figure 5A). Thus, in contrast to effective barrier protective activity for 10 nM APC-WT, a markedly higher concentration (50-100 nM) was required to observe a similar barrier protective function for the APC variant (Figure 5A). To determine whether, similar to anticoagulant assays, coupling of PC activation to its cytoprotective function on cell surfaces can alter the outcome, we incubated endothelial cells with different concentrations of wild-type and variant PC zymogens followed by their activation with a thrombin derivative (W215A/E217A-thrombin or WE-thrombin), which is known to activate PC normally in the presence of TM, but is incapable of activating PAR1.20 This assay facilitated specific monitoring of the PAR1-dependent protective activity of the cell surface–generated APC, without interference from thrombin. Interestingly, when the activation of the PC zymogens was coupled to their activity by this strategy, the PC variant exhibited a markedly higher barrier protective activity than did wild-type PC (Figure 5B). Thus, in contrast to wild-type PC, which only exhibited a significant barrier protective activity at 80 nM zymogen, the same extent of barrier protective activity was observed with only a 20 nM variant zymogen (Figure 5B).

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assays followed by an ELISA, conducted by commercially available reagents, demonstrated that the subject may have type II PC deficiency. This prediction was based on results of these assays yielding significantly decreased PC activity levels of 36% (clotting) and 50% (chromogenic) and antigen level of 65% (ELISA) in the subject plasma. In light of a negative family history of VTE for the subject’s living parents and other family members, we decided to further study the underlying cause of this apparent type II PC deficiency and to understand whether it is environmentally acquired or has a genetic basis. Genetic analysis revealed a single-point mutation in the PROC gene of the subject, leading to substitution of Thr-315 of PC with an Ala. Following recombinant expression and zymogen activation, the catalytic activity of the variant APC is due to the loss of a secondary interaction of the protease with its natural substrates. The observation that the variant APC exhibited a normal amidolytic activity toward 2 substrates suggests that the lack of Asn-313 glycosylation caused by Thr-315 mutation reduces the stability of the variant PC in circulation. This modification is not surprising because TM together with the critical receptors EPCR and PAR1 are colocalized to lipid raft microenvironments, which is where the signaling molecules involved in the anti-inflammatory function of APC are also localized. Thus, when the EPCR-bound PC is activated by the thrombin-TM complex, the resulting APC is already primed to function in the anti-inflammatory pathways.

The basis for the lower PC level in the subject’s plasma is not known. However, given the established role of posttranslationally attached N-linked glycans to the stability of protein molecules, it is possible that the lack of Asn-313 glycosylation caused by Thr-315 mutation reduces the stability of the variant PC in circulation. This modification may also be responsible for the lower catalytic function of the APC variant in the functional assays, possibly because of the carbohydrate side chain of Asn-313 playing a productive role in the interaction of the protease with its natural substrates. The observation that the variant APC exhibited a normal amidolytic activity toward 2 chromogenic substrates and also bound to the active-site probe, PAB, with a normal affinity, is consistent with the hypothesis that the defect in the catalytic activity of the APC variant is due to the loss of a secondary
substrate binding site on the protease. There is a precedent for the carbohydrate side chains of APC contributing to its catalytic function; a recent study demonstrated that the mutation of another N-linked glycosylation site, Asn-329, improved the cytoprotective activity of the APC mutant.26 Thus, in addition to conferring stability, the carbohydrates of APC can modulate the catalytic function of the protease in anticoagulant and anti-inflammatory pathways. Nevertheless, the twofold instability and the same extent of loss in the catalytic activity of the APC-T315A variant is compensated by a four- to fivefold improvement in the activation of the PC zymogen variant by the thrombin-TM complex with the net effect being the APC variant in the subject’s plasma functioning normally in both anticoagulant and anti-inflammatory pathways. These findings suggest that the TM dependence of PC activation may need to be evaluated for diagnosing all PC deficiencies.

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References


Authorship

Contribution: Q.D. designed experiments, performed research, analyzed data, and contributed to writing of the manuscript; L.Y. prepared recombinant proteins and performed research; P.D. performed the inflammatory assays; X.W. designed experiments and supervised studies conducted in Ruijin Hospital with the proband’s plasma; and A.R.R. analyzed data, wrote the article, and supervised the project.

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Protein C Thr315Ala variant results in gain of function but manifests as type II deficiency in diagnostic assays

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