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Autolysis loop restricts the specificity of activated protein C: Analysis by FRET and functional assays ☆

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Abstract

We previously demonstrated that the substitution of the autolysis loop (residues 143–154 in chymotrypsin numbering) of APC with the corresponding loop of trypsin (APC-Tryp$^{143-154}$) has no influence on the proteolytic activity of the protease toward fVa, however, this substitution increases the reactivity of APC with plasma inhibitors so that the mutant exhibits no anticoagulant activity in plasma. To further investigate the role of the autolysis loop in APC and determine whether this loop is a target for modulation by protein S, we evaluated the activity of APC-Tryp$^{143-154}$ toward fVa and several plasma inhibitors both in the absence and presence of protein S. Furthermore, we evaluated the active-site topography of APC-Tryp$^{143-154}$ by determining the average distance of the closest approach (L) between a fluorescein dye tethered to a tripeptide inhibitor, attached to the active-site of APC-Tryp$^{143-154}$, and octadecylrhodamine dyes incorporated into PCPS vesicles both in the absence and presence of protein S. The activity of APC-Tryp$^{143-154}$ toward fVa was identical to that of wild-type APC both in the presence and absence of protein S. However, the reactivity of APC-Tryp$^{143-154}$ with plasma inhibitors was preferentially improved independent of protein S. The FRET analysis revealed a dramatic change in the active-site topography of APC both in the absence and presence of protein S. Anisotropy measurements revealed that the fluorescein dye has a remarkable degree of rotational freedom in the active-site of APC-Tryp$^{143-154}$. These results suggest that the autolysis loop of APC may not be a target for modulation by protein S. This loop, however, plays a critical role in restricting both the specificity and spatial environment of the active-site groove of APC.

Keywords: Activated protein C; Protein S; Factor Xa; Trypsin; FRET

1. Introduction

Activated protein C (APC) is a vitamin K-dependent trypsin-like serine protease in plasma that down-regulates the clotting cascade by proteolytically degrading the procoagulant cofactors factor Va (fVa) and factor VIIIa (fVIIIa) which are essential cofactors for factors Xa and IXa in the extrinsic and intrinsic pathways of thrombin generation, respectively [1,2]. The anticoagulant activity of APC is markedly promoted by the cofactor function of protein S, another vitamin K-dependent protein in plasma [3,4]. Previous results have indicated that the N-terminus γ-carboxyglutamic (Gla) domain of protein S interacts with the same domain of APC to promote the anticoagulant function of APC on the surface of negatively charged phospholipid membranes [5,6]. The APC cleavage of two peptide bonds after Arg-506 and Arg-306 is required for the complete inactivation of fVa on the membrane surface [7–9]. It is known that the first cleavage by APC occurs with a higher catalytic efficiency independent of membrane [4,7]. On the other hand, the APC cleavage of the Arg-306 scissile bond is membrane-dependent and proceeds with a relatively slower catalytic efficiency in the absence of protein S [7]. The APC cleavage of the Arg-306 site is required for a complete elimination of the cofactor function of fVa in the clotting cascade [7,10]. Protein S markedly promotes the activity of APC toward both cleavage sites, though it has a greater

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cofactor effect toward the cleavage of the Arg-306 scissile bond [4,7]. It has been hypothesized that protein S functions by relocating the active-site topography of the membrane-bound APC, thereby optimizing the docking of the Arg-306 scissile bond into the catalytic pocket of the protease [11,12]. Thus, fluorescence resonance energy transfer (FRET) studies have shown that protein S lowers the average distance of the closest approach (L) between a fluorescein dye in the active-site of APC and octadecylrhodamine dyes incorporated into the PCPS vesicles by ~10 Å (95 Å in the absence and 85 Å in the presence of protein S), suggesting that the cofactor function of protein S may involve an alteration of the topographical orientation of the active-site of the membrane-bound APC [11].

Relative to trypsin and other vitamin K-dependent coagulation proteases, APC has a longer autolysis loop [13]. Previous results have indicated that shortening this loop improves the anticoagulant activity of APC [14]. In a recent study we also demonstrated that the substitution of the longer autolysis loop of APC from residues 143–154 (chymotrypsin numbering [15]) with the shorter loop of factor Xa (APC-FX$^{143-154}$) improved the anticoagulant activity of APC in a fVa degradation assay in the absence but not in the presence of protein S [16]. Nevertheless, the substitution of this loop with the corresponding loop of trypsin (APC-Tryp$^{143-154}$) did not alter the activity of the mutant toward fVa in the purified system, however, it completely abolished the anticoagulant activity of the protease in a plasma-based assay system [17]. Further studies revealed that the APC-Tryp$^{143-154}$ mutant has adopted a trypsin-like specificity, thus becoming susceptible to inhibition by plasma inhibitors, accounting for its lack of activity in plasma [17]. Interestingly, the amidolytic activity of the APC-Tryp$^{143-154}$ was also markedly improved, as evidenced by its 3–4-fold decreased K_m toward the cleavage of an APC-specific small synthetic chromogenic substrate [17]. The molecular basis for the overall improved catalytic activity of this mutant has not been investigated nor the effect of protein S on its proteolytic activity toward fVa in the purified system.

To determine whether protein S has any modulatory effect on the autolysis loop of APC, we employed FRET and activity assays to characterize the functional properties and active-site topographies of wild-type APC, APC-FX$^{143-154}$ and APC-Tryp$^{143-154}$ chimeras in the absence and presence of protein S. The results suggest that protein S has no effect on the amidolytic activity or reactivity of either wild-type or mutant proteases toward plasma inhibitors. FRET studies revealed that the active-site topography of APC-Tryp$^{143-154}$ has been dramatically altered both in the absence and presence of protein S, explaining its improved reactivity with plasma inhibitors.

2. Experimental procedures

2.1. Materials

Human plasma protein S was purchased from Enzyme Research Laboratories (South Bend, IN). Human factor Xa (fXa), fVa, antithrombin (AT), prothrombin and fluorescein-labeled Phe-Pro-Arg-ck (Fl-FPR) were purchased from Haematologic Technologies Inc. (Essex Junction, VT) and octadecylrhodamine (OR) was from Invitrogen (Carlsbad, CA). Human α_1-antitrypsin was purchased from Athens Research and Technology Inc. (Athens, GA). Dioleoylphosphatidylcholine (PC) and dioleoylphosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). EnzoLyte™ pNPP Alkaline Phosphatase Assay Kit was purchased from AnaSpec, Inc. (San Jose, CA). Transformed human umbilical vein endothelial cells were provided by Dr. C. Edgell (University of North Carolina at Chapel Hill, NC). The chromogenic substrate Spectrozyme PCa (SpPCa) was purchased from American Diagnostica (Greenwich, CT) and S2238 was from Kabi Pharmacia/Chromogenix (Franklin, OH). The active-site directed serine protease inhibitor *p*-aminobenzamidine (PAB), and the specific thrombin inhibitor hirudin were purchased from Sigma (St. Louis, MO).

2.2. Construction, expression and purification of recombinant APC mutants

Expression, purification and preparation of wild-type protein C and the chimeric mutants in which the autolysis loop of the protein from residues 143–154 has been replaced with the corresponding loop of either factor X (PC-FX$^{143-154}$) or trypsin (PC-Tryp$^{143-154}$) have been described [16,17]. The expressed proteins were purified by a combination of immunoaffinity and ion exchange chromatography using the HPC4 monoclonal antibody immobilized on Affi-gel 10 and FPLC Mono Q column, respectively, as described [16]. The protein C zymogens (~1 mg) were converted to APC by thrombin (50 μg) in 0.1 M NaCl, 0.02 M Tris–HCl, pH 7.4 (TBS) containing 5 mM EDTA for 2 h at 37 °C as described [16]. The APC derivatives were separated from thrombin and their concentrations were determined from the absorbance at 280 nm assuming a molecular mass of 56 kDa and an extinction coefficient ($E^{1\%}_{1\ cm}$) of 14.5 and by an active-site titration assay using recombinant protein C inhibitor (PCI) as described [18]. PCI was expressed and purified as described [18]. The purity of all APC derivatives was ensured by SDS-PAGE.

2.3. Anticoagulant activity

The APC concentration dependence of fVa inactivation was measured by a three-stage assay as described [16]. Briefly, in the first stage, fVa (2.5 nM) was incubated with different concentrations of each APC derivative (1–5 nM) for 5 min on 20 μM phospholipid vesicles composed of 80% PC and 20% PS in TBS containing 5 mM Ca^{2+}, 0.5 mg/mL BSA, and 0.1% polyethylene glycol 8000 (TBS/Ca^{2+}). In the second stage, the remaining fVa activity was determined in a prothrombinase assay measuring the fVa-catalyzed prothrombin activation by fXa as described [16]. The remaining activity of fVa was determined from the decrease in the rate of thrombin generation as monitored by an amidolytic activity assay in the third stage using 100 μM S2238. A similar assay was used to evaluate the catalytic activity of APC derivatives toward fVa in the presence of a saturating concentration of protein S (220 nM).

2.4. Phospholipid vesicle preparations

Phospholipid vesicles at a molar ratio of PC to PS of 4:1 containing or lacking different amounts of OR were prepared by the extrusion method as described [19]. Briefly, dried phospholipid mixtures were suspended at 1 mg/mL in 0.1 M NaCl, 0.05 M Hepes, pH 7.4, mixed for 10 min and passed twenty times through a 100 nm pore diameter polycarbonate membrane. The OR containing vesicles received various amounts of the dye in ethyl acetate prior to lyophilization and extrusion. The % yield of phospholipids recovery was determined by comparing the amount of choline from small samples before and after extrusion step by a colorimetric assay using Wako phospholipid B kit (Wako Chemicals USA, Inc., Richmond, VA). The % recovery for different preparations ranged from 85% to 90%. Phospholipid vesicles containing 100% PC vesicles were prepared by the same procedures for coating cuvettes. The concentration of OR in the acceptor-containing samples was determined from absorbance at 564 nm using a molar extinction coefficient of 95,400 M^{-1} cm^{-1} as described [11]. The acceptor density of OR (σ, in OR/Å^2) was calculated using molecular weights 786.1 and 810.0 Da for PC and PS, respectively, assuming that acceptors were distributed randomly at the phospholipid surface, and assuming that each phospholipid molecule occupies 70 Å^2 of the surface area as described [20].

2.5. Fluorescent labeling

All APC derivatives were incubated with 10-fold molar excess of Fl-FPR for 2 h at room temperature in the dark. The extent of active-site labeling was monitored by the loss of the enzymatic activity using SpPCa. Incubation was continued till more than 99.9% of the activity was inhibited. The free inhibitor or dye was separated from the labeled proteins by gel filtration on the PD-10 column followed by their extensive dialysis in 0.1 M NaCl and 0.05 M Hepes, pH 7.4 containing 5 mM Ca^{2+} at 4 °C in the dark. The extent of fluorescence labeling for all proteins was determined as described by Bock [21]. An extinction coefficient of 84,000 M^{-1} cm^{-1} at 498 nm was used to calculate the fluorescein concentration and the ratio $e_{280\ nm}/e_{498\ nm}=0.19$ was used to correct for the contribution of the dye to 280-nm absorbance of the proteins as described [21]. The average number of dyes per protein was determined to range from 0.6–0.8 for all APC derivatives. The maximum excitation and emission wavelengths for all fluorescein-labeled proteins were determined to be 493 nm and 521 nm, respectively.

2.6. Spectral measurements

All spectral measurements were performed using an Aminco-Bowman series 2 Spectrophotometer (Spectronic Unicam, Rochester, NY) as described [19]. The anisotropy of the fluorescein-labeled proteins (25–50 nM) was measured both in the absence and presence of saturating concentrations of PCPS vesicles (35 μM) and protein S (300 nM) using the excitation and emission wavelengths of 490 nm and 520 nm as described [19]. The anisotropy of OR labeled PCPS vesicles containing limiting concentration of acceptor (σ 0.7×10^{-4}) was measured as described [19]. Absorbance measurements were made using a Beckman Coulter DU 800 spectrophotometer. The values for the quantum yield (Q), spectral overlap between donor and acceptor dyes (J_{DA}), and the distance between donor and acceptor dyes at the 50% FRET efficiency (R_0) were calculated as described [11,19]. The values of Q and J_{DA} were separately measured for each fluorescein-labeled protein both in the absence and presence of PCPS vesicles and protein S.

2.7. Energy transfer measurements

Fluorescence energy transfer measurements were performed as described [19], except that the donor containing (cuvette D) and both donor (fluorescein) and acceptor (OR) containing cuvettes (cuvette DA) each received 50 nM fluorescein-labeled APC derivatives in 0.1 M NaCl, 0.05 M Hepes, pH 7.4, and 5 mM Ca^{2+} while blanks (cuvette B) and acceptor-containing cuvettes (cuvette A) received 50 nM of unlabeled APC derivatives in the same buffer. In case of measurements in the presence of protein S, 50 nM of fluorescein-labeled APC derivatives were first incubated with a saturating concentration of OR containing PCPS to obtain the maximum energy transfer in the DA sample followed by addition of protein S to a final concentration of 300 nM as described [11]. The net initial emission intensities were obtained by subtracting the initial intensities of A from DA $(F_{DA}-F_A)_o$ and B from D $(F_D-F_B)_o$. Samples D and B were then titrated with PCPS vesicles lacking the acceptor OR, while samples DA and A were titrated with PCPS vesicles containing the acceptor. Similarly, the intensities of A and B were subtracted from DA and D, respectively and the values were then corrected for dilutions (less than 4% at the end of titration). Following 5 min incubation emission intensities were measured. The ratio of the donor quantum yields (Q) in the D and DA samples based on their fluorescence emission intensities (F) is given by Eq. (1) as described [11]:

$$Q_D/Q_{DA} = \left[(F_D - F_B)/(F_D - F_B)_o\right]/\left[(F_{DA} - F_A)/(F_{DA} - F_A)_o\right] \quad (1)$$

at the end of each experiment, the fluorescent labeled proteins were released from the membrane surface by the addition of EDTA to 10 mM to ensure that energy transfer is reversible, however, in case of native APC the EDTA dissociation for APC from PCPS vesicles was too slow thus DTT was used to reduce the disulphide bond between the two APC chains, thereby releasing the fluorescein-labeled heavy chain from the vesicle surface and thus reverting the fluorescence intensity of the donor as described [11]. For calculating the distance of the closest approach by Eq. (2) below, the Q_D/Q_{DA} value was calculated by dividing the value before EDTA by value after the addition of EDTA. This normalization procedure corrects for the contribution of the acceptor inner filter effects and a potential membrane-binding-independent energy transfer as described [11,12].

2.8. Calculation of the distance of closest approach

Assuming that both donor and acceptor dyes are randomly and uniformly distributed ($\kappa^2=2/3$), the distance of closest approach (L) between the plane of the donor dye attached to the active-site of the protein and the plane of the acceptor dyes at the surface of PCPS vesicles can be determined using Eq. (2) as described [11]:

$$Q_D/Q_{DA} = 1 + \left(\pi\sigma R_o^2/2\right)(R_o/L)^4 \quad (2)$$

where π is 3.14, σ is the acceptor density at the membrane surface in OR/Å^2 and R_o is the distance at which the singlet–singlet energy transfer from the donor dye to the acceptor dye is 50% efficient. The net Q_D/Q_{DA} values were plotted as a function of product between R_o^2 and the acceptor density (σ) for at least 5–13 different energy transfer experiments to obtain the average value of L as described [11,19].

3. Results and discussion

3.1. Analysis of the anticoagulant activity of APC derivatives

The autolysis loop of APC is 5–6 residues longer than the corresponding loop of both trypsin and fXa (Fig. 1). We previously demonstrated that the substitution of this loop of APC with the same loop of fXa (APC-FX$^{143-154}$) improves the anticoagulant function of the mutant toward fVa, independent of protein S, in the purified system [16]. This is consistent with results of another study which demonstrated that when the autolysis loop of human APC was shortened in order to make it resemble the same loop of bovine APC, the anticoagulant function of APC was also improved [14]. Since the improvement in the anticoagulant function of APC-FX$^{143-154}$ was observed in the absence, but not in the presence of protein S [16] (Fig. 2), this observation raised the possibility that a cofactor function for protein S may involve the modulation of the structure of the autolysis loop of APC on the membrane surface. To further investigate this question, we evaluated the anticoagulant function of another APC mutant in which its autolysis loop was replaced with the same loop of trypsin (APC-Tryp$^{143-154}$). This APC chimera exhibited normal activity in the fVa degradation assay, and its activity was enhanced by protein S to a similar extent as the wild-type APC (Fig. 2). These results suggest that the protein S-independent enhancement in the activity of APC-FX$^{143-154}$ toward fVa is due to unique features of the fXa autolysis loop which can interact and cleave fVa with a higher degree of specificity, independent of protein S.

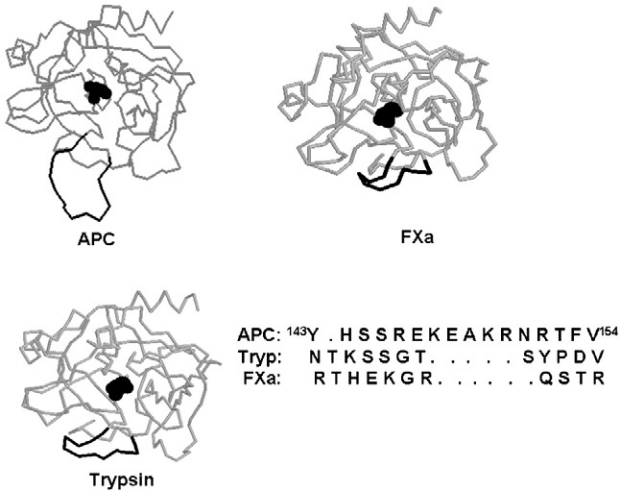

Fig. 1. Crystal structures of the catalytic domains of APC, trypsin and fXa and alignment of the sequence of the autolysis loops from residues 143–154. The backbone residues 143–154 of the autolysis loops are colored in black. The catalytic Ser-195 (black) is shown in the spacefill model in each protease.

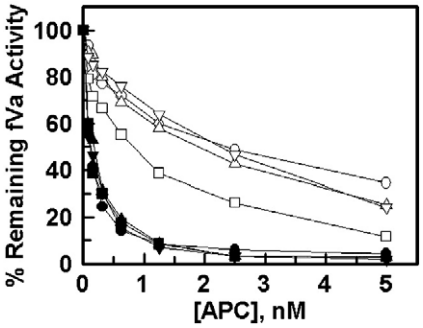

Fig. 2. Factor Va degradation by the APC derivatives in the absence and presence of protein S. The APC concentration dependence of fVa (2.5 nM) degradation by wild-type APC (○,●), APC-FX$^{143-154}$ (□,■), APC-FX$^{143-154(3A)}$ (△,▲) and APC-Tryp$^{143-154}$ (▽,▼) was carried out in the absence (open symbols) or presence of protein S (220 nM) (closed symbols) on PCPS vesicles (20 μM) in TBS/Ca^{2+}. After 5 min incubation at room temperature, small aliquots of the inactivation reaction was transferred to a 96-well assay plate and the remaining cofactor activity of fVa was determined by a prothrombinase assay as described under "Experimental procedures". The data for APC-FX$^{143-154}$ are derived from Ref. [16].

An important difference between the structure of the autolysis loop of fXa and trypsin is that this loop lacks a basic residue in trypsin, but it contains several positively charged residues in both APC and fXa (Fig. 1). Since the basic residues of the autolysis loop have been demonstrated to be critical for the specificity of fVa recognition by APC [22], we evaluated the activity of another APC-FX$^{143-154}$ mutant in which the three basic residues Arg-143, Lys147 and Arg-150 of the autolysis loop of the chimeric APC mutant were substituted with Ala (APC-FX$^{143-154(3A)}$). The results presented in Fig. 2 indicate that this mutant has an APC-like activity toward fVa both in the absence and presence of protein S. These results suggest that the basic residues of the autolysis loop in APC-FX$^{143-154}$ are responsible for the protein S-independent improvement in the activity of the mutant, possibly by the basic residues making a productive interaction with fVa in the absence of protein S. Analysis of the activity of the autolysis loop mutants in the plasma-based assay indicated that the anticoagulant activities of APC derivatives (in particular APC-Tryp$^{143-154}$) have been dramatically impaired (data not shown, see Ref. [17]). Analysis of the inhibition profiles of APC derivatives with three different plasma inhibitors, PCI, AT and α_1-antitrypsin have indicated that the reactivity of APC-Tryp$^{143-154}$ with all three inhibitors has been markedly improved, explaining a lack of anticoagulant activity for this mutant in plasma [17].

To determine whether protein S alters the specificity of APC derivatives in reaction with plasma inhibitors, their reactivity with the same plasma inhibitors mentioned above was evaluated in the presence of protein S on PCPS vesicles. The results suggested that protein S has no influence on the reactivity of APC derivatives with any one of these inhibitors (data not presented). Similarly, protein S did not influence the amidolytic activity of APC derivatives toward the hydrolysis of the chromogenic substrate SpPCa, nevertheless, the K_m for this substrate by APC-Tryp$^{143-154}$, was decreased ~4-fold independent of protein S (~200 μM and 50 μM, for wild-type and mutant APC, respectively). To determine the basis for the improvement in the K_m of APC-Tryp$^{143-154}$ for the chromogenic substrate, the ability of the mutant to bind the S1 site-specific probe of the trypsin-like serine proteases *p*-aminobenzamidine (PAB) was examined. Similar K_i values of ~40 μM PAB for wild-type APC and ~30 μM PAB for the mutant was observed in both the absence and presence of protein S, thus the mutant exhibiting insignificant improvement in its interaction with the S1 site-specific inhibitor. These results suggest that protein S does not modulate the autolysis loop of APC and that the basis for an improved K_m of the mutant toward the chromogenic substrate arises through interaction of the proteases with a residue(s) other than the P1 Arg residue of the substrate. Taken together these results suggest that grafting the autolysis loop of the less specific ancestral enzyme trypsin to APC endows a trypsin-like specificity for the mutant protease, thus improving its catalytic efficiency of the mutant with both the synthetic substrate and the plasma inhibitors. These results support our hypothesis that the autolysis loop of APC has evolved to restrict the specificity of APC, thereby allowing the protease to carry out its specialized function in the anticoagulant pathway.

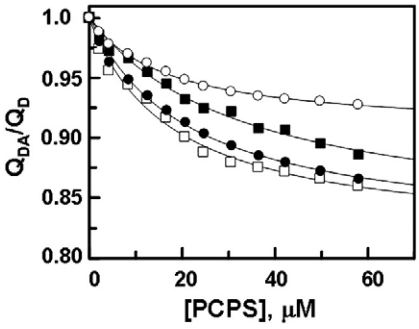

Fig. 3. Dependence of energy transfer on PCPS concentrations. The fluorescence labeled APC derivatives (50 nM each) in 0.1 M NaCl, 50 mM Hepes, 5 mM Ca^{2+}, pH 7.4 were titrated with PCPS vesicles containing or lacking OR at 25 °C as described under "Experimental procedures". The plots show the ratio of donor quantum yield (Q_{DA}/Q_D) in the presence or absence of OR for APC-WT (■), APC-FX$^{143-154}$ (●), APC-FX$^{143-154(3A)}$ (□) and APC-Tryp$^{143-154}$ (○) which were calculated using Eq. (1) as described under "Experimental procedures". An acceptor density of 5.0×10^{-4} was used in all three experiments. Nonlinear regression analyses of the titration data yielded K_D values of 33.2±4.2 μM for APC-WT, 21.0±1.5 μM for APC-FX$^{143-154}$, 17.7±2.0 μM for APC-FX$^{143-154(3A)}$, and 16.8±1.1 μM for APC-Tryp$^{143-154}$.

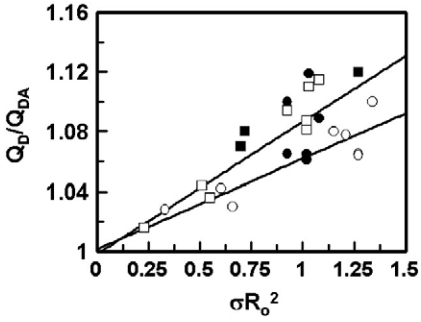

Fig. 4. Linear dependence of FRET upon acceptor density for APC derivatives in the absence of protein S. The Q_D/Q_{DA} ratios, obtained using different densities of acceptor in the range of $1.5–5.6\times10^{-4}$ OR/Å^2, are plotted as the function of σR_o^2 for APC-WT (■), APC-FX$^{143-154}$ (●), APC-FX$^{143-154(3A)}$ (□) and APC-Tryp$^{143-154}$ (○) as described under "Experimental procedures".

3.2. Active-site topography of autolysis loop mutants

Previous FRET studies have indicated that the average distance of the closest approach (L) between a fluorescein dye in the active-site of APC and octadecylrhodamine (OR) dyes, randomly incorporated into PCPS vesicles, is ~94 Å in the absence of protein S and ~84 Å in the presence of protein S [11,12]. Based on these results and the functional data showing a protein S-dependent improvement in the anticoagulant activity of APC, it has been hypothesized that protein S functions by altering the active-site topography of APC, thereby optimizing the recognition of the fVa scissile bonds by the membrane-bound protease [11,12]. To determine whether a similar mechanism accounts for the protein S-dependent improvement in the anticoagulant activity of the autolysis loop chimeras, the *L* values were determined for APC mutants by using essentially identical procedures described for wild-type APC in the past [11,12]. Thus, the change in the emission intensity of the donor fluorescein dye was monitored as a function of increasing concentrations of PCPS vesicles with or without the acceptor OR dye in both the absence and presence of a saturating concentration of protein S for all APC derivatives. Similar to previous results [11,12], insignificant changes in the fluorescence emission intensities of either wild-type APC or the mutants were observed if PCPS vesicles lacking OR were used for the titrations (data not presented). However, the fluorescence emission intensities of APC derivatives were significantly decreased if PCPS vesicles containing OR were used for the titrations. The EDTA or DTT reversible ratio of the donor quantum yields in the presence and absence of the acceptor (Q_{DA}/Q_D) was calculated according to Eq. (1). Plots of the data in the absence of protein S presented in Fig. 3 indicated that the changes in the quantum yields (Q_{DA}/Q_D) for APC-FX$^{143-154}$ and APC-FX$^{143-154(3A)}$ at all concentrations of OR containing PCPS are similar to those of wild-type APC (slightly lower for APC). However, the efficiency of energy transfer was significantly lower for the APC-Tryp$^{143-154}$ mutant, suggesting that either the position of the fluorescein dye in the active-site of this mutant is located at a higher distance from the membrane or that its orientation in the active-site pocket is different than that of the wild-type protease. Noting that the efficiency of energy

transfer depends on the density of OR (σ) on the membrane surface, the energy transfer was determined at several acceptor densities ranging from 1.5–5.6 × 10^{-4} and the average L values for each APC derivative both in the absence and presence of protein S were calculated using Eq. (2), assuming a random orientation of transition dipoles for donor and acceptor dyes (κ^2 = 2/3) as described previously [11,12]. Since the magnitude of the energy transfer depends upon the density of the acceptor OR on the membrane surface [11,12], it was also ensured that the plot of Q_D/Q_{DA} exhibited a linear dependence on the product between R_o^2 and the acceptor density (σR_o^2) as demonstrated in Fig. 4.

The L values obtained from these measurements (Table 1) for APC both in the absence (98 ± 5 Å) and presence of protein S (87 ± 2 Å) are in close agreement with the corresponding values obtained for APC in a previous study (94.3 ± 4.0 Å in the absence and 84 ± 1 Å in the presence of protein S) [11,12], supporting the reliability of measurements. Analysis of data presented inTable 1 suggests that protein S lowers the height of the active-site of both APC-$FX^{143-154}$ and APC-$FX^{143-154(3A)}$ to an extent similar to that of wild-type APC, however, the height of the active-site of APC-$Tryp^{143-154}$ appears to be significantly elevated and not sensitive to the absence or presence of protein S. To determine if protein S differentially changes the environment of the probe in the active sites of the APC derivatives, the anisotropy values for the labeled proteins were determined both in the absence and presence of a saturating concentration of protein S and PCPS vesicles. The results indicated that neither PCPS nor protein S influence the environment of the probe in the active-site pocket of APC derivatives, which is consistent with the literature [11,12]. The anisotropy values for wild-type (0.15) and APC-$FX^{143-154}$ (0.12) and APC-$FX^{143-154(3A)}$ (0.13) were similar, suggesting a nearly identical degree of rotational freedom for the fluorescein dye in the active-site pocket of these proteins. Interestingly,

Table 1
Distance of the fluorescein-labeled active sites of APC derivatives from OR labeled PCPS vesicles

APC	Ro (Å)	L (Å)	n	r (PCPS)
APC-WT	48 ± 1	98 ± 5	3	0.15
APC-WT + protein S	48 ± 1	87 ± 2	2	0.15
APC-$FX^{143-154}$	43 ± 1	94 ± 6	6	0.12
APC-$FX^{143-154}$ + protein S	43 ± 1	85 ± 3	2	0.12
APC-$FX^{143-154(3A)}$	43 ± 1	92 ± 6	9	0.13
APC-$FX^{143-154(3A)}$ + protein S	43 ± 1	84 ± 2	2	0.13
APC-$Tryp^{143-154}$	48 ± 1	111 ± 6	8	0.035
APC-$Tryp^{143-154}$ + protein S	48 ± 1	111 ± 2	2	0.035

The relative distances were determined from the efficiency of energy transfer (FRET) from the donor fluorescein dye in the active-site of each APC derivative to the acceptor OR anchored to PCPS vesicles with densities ranging from (1.5–5.6) × 10^{-4} OR/Å^2 in 0.1 M NaCl and 0.05 M Hepes (pH 7.4) containing 5 mM Ca^{2+} using Eq. (2) as described under "Experimental procedures". In all experiments κ^2 was assumed to be 2/3. The number of experiments used to calculate the average values are given as (n). The anisotropy (r) values for the fluorescein-labeled APC derivatives were measured at excitation and emission wavelengths of 493 nm and 521 nm, respectively. The anisotropy for the membrane-bound OR at the limiting concentration of the acceptor (σ = 0.7 × 10^{-4}) was determined to be 0.15.

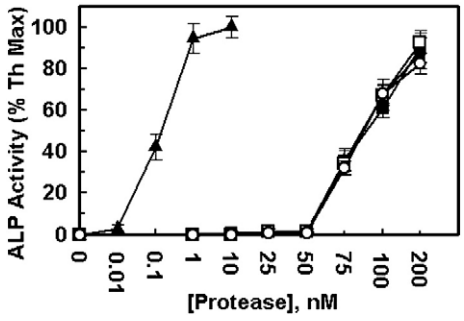

Fig. 5. PAR-1 exodomain cleavage by APC derivatives. Endothelial EA.hy926 cells were cultured in 24-well plates, transiently transfected with the ALP-PAR-1 cleavage reporter plasmid, and incubated with increasing concentrations of thrombin and APC derivatives for 1 h as described [24]. The activity of soluble alkaline phosphatase (ALP) in the cell culture supernatants was measured by using EnzoLyte™ pNPP Alkaline Phosphatase Assay Kit as described [24]. The activity of 10 nM thrombin is presented as 100%. All assays with the APC derivatives were carried out in the presence of 4 units/mL hirudin. The symbols are: APC-WT (■), APC-$FX^{143-154}$ (●), APC-$FX^{143-154(3A)}$ (□), APC-$Tryp^{143-154}$ (○), and thrombin (▲).

however, the anisotropy was significantly smaller for APC-$Tryp^{143-154}$ (0.035), suggesting a much higher degree of rotational freedom for the probe in the active-site pocket of this mutant. This increased free rotation for the fluorescence probe is the likely reason for a larger L value observed for this mutant. It implies that the trypsin autolysis loop swapping increases the available space in the active-site groove of the mutant protease, thereby allowing the free movement of the tethered fluorescent probe in the pocket. Noting that ~100 Å is the upper limit sensitivity for the FRET approach, the active-site distance measurements in the presence of protein S did not yield any further insight into the nature of the effect of protein S in the active-site topography of this APC mutant. These results, however, correlate well with the functional data, thus explaining the basis for the decreased K_m for the chromogenic substrate and improved reactivity with the plasma inhibitors for this APC mutant. Hence, it appears that, relative to trypsin, the autolysis loop of APC plays an important role in restricting the spatial environment of the active-site groove of APC and impeding the non-specific entry of plasma inhibitors into this pocket of the anticoagulant protease.

3.3. Cleavage of PAR-1 by APC derivatives

In addition to its anticoagulant effect, previous results have indicated that APC can also bind to endothelial protein C receptor to activate protease activated receptor 1 (PAR-1), thereby eliciting antiinflammatory and cytoprotective signaling responses in endothelial cells [23]. In light of the observed changes in the specificity of the autolysis loop swap mutants of APC toward plasma inhibitors, we wondered whether the autolysis loop of APC plays any role in the specificity of PAR-1 recognition by the protease. To answer this question, the catalytic activity of APC derivatives toward the receptor was evaluated by a sensitive colorimetric assay measuring the cleavage of P1 Arg-41 of PAR-1 in a reporter construct in which a soluble alkaline phosphatase (ALP) cDNA was coupled to the

exodomain of PAR-1 and the membrane spanning domain of tissue factor, thereby anchoring the cleavage reporter construct to the cell surface upon its transfection to endothelial cells [24]. As shown in Fig. 5, all APC derivatives cleaved the endothelial cell surface PAR-1 with similar efficiency. In agreement with previous results, APC cleaved the PAR-1 exodomain with ~750-fold lower catalytic efficiency than thrombin (Fig. 5). These results suggest that the autolysis loop of APC does not play a role in specific recognition of the Arg-41 scissile bond of PAR-1 expressed on the surface of endothelial cells. The PAR-1 cleavage efficiency of APC derivatives was not studied in the presence of protein S.

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