Secondary Substrate-binding Exosite in the Serine Protease Domain of Activated Protein C Important for Cleavage at Arg-506 but Not at Arg-306 in Factor Va*

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Proteolytic inactivation of activated factor V (FVa) by activated protein C (APC) is a key reaction in the regulation of hemostasis. We now demonstrate the importance of a positive cluster in loop 37 of the serine protease (SP) domain of APC for the degradation of FVa. Lysine residues in APC at positions 37, 38, and 39 form a secondary binding site for FVa, which is important for cleavage of FVa at Arg-506 while having no effect on Arg-306 cleavage. In contrast, topological neighbors Lys-62, Lys-63, and Arg-74 in APC appear of minor importance in FVa degradation. This demonstrates that secondary binding exosites of APC specifically guide the proteolytic action of APC, resulting in a more favorable degradation of the 506–507 peptide bond as compared with the 306–307 bond.

The protein C pathway provides a major anticoagulant mechanism, which is important for regulation of hemostasis (1–3). The key component in this pathway is the vitamin K-dependent protein C, which circulates in plasma as an inactive zymogen to a serine protease (4). Protein C is composed of a light chain (containing a γ-carboxyglutamatic acid (Gla) domain and two epidermal growth factor (EGF)-like domains), which is linked by a disulfide bridge to the heavy chain that comprises the activation peptide and the serine protease (SP) domain. After activation by the thrombin-thrombomodulin complex on endothelial cell surfaces, activated protein C (APC) regulates blood coagulation by cleaving and inhibiting the activated forms of factor V (FVa) and factor VIII (FVIIIa). FVa and FVIIIa are membrane-bound cofactors to factor Xa (FXa) and factor IXa (FIXa) in the prothrombinase (PTase) and tenase complex, respectively. FV/FVa and FVIIIa are the only known physiological substrates for APC. Until now, the structural background for this narrow substrate specificity is not clearly understood (5). Peptide inhibition studies showed the involvement of the region 149–154 (chymotrypsinogen numbering)² within the SP domain of APC in FVa binding (6). Furthermore, recently published loop grafting experiments and site-directed mutagenesis data confirm that residues in loop 148 play an important role in the kinetic properties of APC and as such could contact directly FVa during the APC-catalyzed inactivation (7, 8).

Computer analysis of the x-ray structure of Gla-domainless APC (9), theoretical screening of its molecular surface (10) and some experimental data (11–13) have indicated the importance of an electropositive region within the SP domain of APC for protein-protein interaction. Thus, a cluster of basic residues located on loops 37, 60, and 70 was shown to be involved in direct interactions with thrombomodulin during the activation process of protein C and with heparin, the protein C inhibitor (PCI) and α1-antitrypsin (7, 13, 14).

In the present study, we have examined the role of positively charged amino acids in loops 37, 60, and 70 of APC in the degradation of FVa. The basic residues in loop 37 were found to form a substrate-binding exosite for FVa that is important during cleavage of Arg-506, whereas they have virtually no effect on the FVa Arg-306 cleavage rate.

EXPERIMENTAL PROCEDURES

Construction, Expression, and Purification of Recombinant Human Protein C Variants—Recombinant protein C variants were created by polymerase chain reaction-based site-directed mutagenesis, expressed in HEK293 cells (ATCC CRL-1573), purified and activated following procedures previously described (13, 15). Similar expression levels (2.7–6.7 mg/liter) and amidolytic activities (kcat and km) of wt and APC variants demonstrated the functional integrity of the proteins (13). Prothrombinase Assay—Time courses of FVa inactivation by APC were determined by following the loss of FVa cofactor activity as a function of time (16). Therefore, 0.8 nM plasma-purified human FVa was incubated with 0.17–0.8 nM wt or APC variants in the absence or presence of 500 nM protein S and 25 μM phospholipid vesicles (1090 dioleoylphosphatidylserine (DOPS)/dioleoylphosphatidylcholine (DOPC) M/M). The remaining cofactor activity of FVa was determined by quantification of the rate of FXa-catalyzed prothrombin activation, as described previously (16).

Kinetic Data Analysis—kcat and Vmax values were obtained from Lineweaver-Burk plots and kcat was calculated from the Michaelis-Menten equation (17, 18). Rate constants for APC-catalyzed Arg-506 and Arg-306 cleavage were calculated using a method described previously (16). Briefly, data from time courses of FVa inactivation were fitted to an equation describing the APC-catalyzed inactivation of FVa using non-linear least squares regression analysis (16). This equation describes a model for inactivation where FVs is initially randomly cleaved at either Arg-306 or Arg-506. Cleavage at Arg-306 leads to a complete loss of FVa cofactor activity, whereas cleavage at Arg-506

* This work was supported by grants from the Swedish Medical Research Council, a Senior Investigator’s grant from the Foundation for Strategic Research, and a grant from La Fondation pour La Recherche Médicale. The costs of publication of this article were defrayed in part " in accordance with 18 U.S.C. Section 1734. Solely to indicate this fact.

² The numbering of amino acid positions in protein C corresponds to the chymotrypsinogen nomenclature.

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This paper is available on line at http://www.jbc.org


RESULTS AND DISCUSSION

Insights into Structure-Function Relationships Involved in the Cleavage of FVa by APC—In a recently created model of the three A-domains of FV, we observed that several positively charged residues are located around Arg-506 and Arg-306 (19). These positive charges could guide and orient FVa into the relatively electronegative active site of APC during the initial approach while repulsing the positively charged loops of the enzyme. We also noted that Arg-506 is located on a well defined loop-like structure, not protruding significantly outside the molecular surface, which could insert into the catalytic cleft of APC after some structural changes. In addition, we predicted the Arg-306 segment to adopt either an α-helical conformation or a long, potentially not well structured loop, which protrudes relatively far outside the molecular surface. Whereas the orientation of the Arg-506 and Arg-306 regions in the active site of APC are not easily envisioned, the Arg-306 site is certainly the most difficult to predict. A preliminary model of the FVa-APC complex has recently been reported (20) thereby helping to analyze this interaction. Despite this progress, the exact definition of residues in contact is still not clear because several parameters such as the relative orientation of the proteins with regard to the membrane plane, the organization of the FV domains and the conformation of the FVa loops 306 and 506 are ill-defined. As a consequence, we observe different sets of atomic interactions between FVa and APC depending on which structure of loop 506 or 306 is used.3 For instance, docking Arg-506 into the active site of APC suggests several possible contact regions between the enzyme and the substrate around APC loop 37. In contrast, the same experiments performed for the Arg-306 site do not clearly highlight important contacts between FVa and the loops lining the catalytic cleft of APC. In this study, we have performed site-directed mutagenesis of APC to define which residues in the loop 37 area of the enzyme interact with FVa. This information will help determine and

3 B. Villoutreix, unpublished data.

refine the orientation of the Arg-506 and Arg-306 sites in the catalytic cleft of APC. Such data are indeed essential for both functional characterization of this important macromolecular complex and structural understanding of the enzymatic reactions. To define rationally the location and type of substitution to introduce in protein C, we analyzed the structures of several serine proteases, including PC sequences from different species (data not shown). We decided to produce five PC variants with amino acid exchanges at positions 37, 38, 60a, 61, 62, 63, and 74 to probe the region of interest.

Anticoagulant Response of APC Variants in Normal Human Plasma—The effect of different APC variants on the APTT of normal human plasma was tested (Fig. 1). Because of the anticoagulant property of wt APC, a concentration-dependent prolongation of the clotting time was detected. The two APC variants K37S/K38Q/K39Q and K37S/K38Q/K39Q/K62N/K63D showed reduced anticoagulant response, whereas APC variants R74Q and K62N/K63D expressed only slight reduction of anticoagulant response toward normal human plasma compared with that of wt APC. These results suggested the cluster of three lysines in loop 37 to be important for the interaction between APC and FV/FVas and/or FVIIIa.

Loop 37 Variants and the Selective Influence on the Arg-506 Cleavage in FVa—To elucidate the effect of the APC variants on the degradation of FVa, a system with purified components was used. In this experiment, FVa was incubated with APC in the presence of negatively charged phospholipid vesicles and the remaining FVa activity determined in a PTase assay. A typical inactivation time of FVa by wt APC appears biphasic (Fig. 2), in agreement with the results on record (5). Under these conditions, this biphasic shape is due to the APC-catalyzed cleavage of FVa plasma-derived human FVa by 0.8 nM wt or variant APCs were performed, and rate constants for Arg-506 and Arg-306 cleavage were calculated as described previously (16). Because of the different velocities of FVa Arg-506 and Arg-306 cleavage, wt APC induces a biphasic FVa inactivation time course. The figure represents one of three independent experiments. The calculated kinetic constants are given in Table I.
acid Arg-74 in the so-called calcium-binding loop (loop 70) play 506 in FVa but not for the cleavage at position Arg-306. In APC, are indispensable for the APC-mediated cleavage at Arg- amino acids Lys-37, Lys-38, and Lys-39, located on loop 37 of APC have no influence on the cofactor activity of protein S in This demonstrates that these mutations in the SP domain of stimulated to the same degree by protein S (data not shown).

In this case, a transient 60-kDa fragment was observed and in contrast, the 30-kDa fragment comprising residues 307–506 is readily observed on the blots already early during the reaction. It should be noticed that for practical reason, the FVa concentration used in this experiment is higher than of the experiment illustrated in Fig. 2, which explains the faster cleavage at 306 (16). The APC variant K37S/K38Q/K39Q/K62N/K63D yielded a different degradation pattern (Fig. 3B). In this case, a transient 60-kDa fragment was observed and in addition, the 30-kDa fragment was generated more slowly than in the case of wt APC. The obtained pattern is compatible with a reduction in the rate of cleavage at Arg-506 and with an unaffected cleavage at Arg-306, thus supporting the conclusions from the functional analysis of Fig. 2.

**FVa Degradation Catalyzed by APC in the Presence of Protein S**—The activity of APC toward FVa is stimulated by the non-enzymatic cofactor, protein S. The exact mechanism of this stimulation is still not defined. It is known that protein S specifically accelerates the APC-induced FVa cleavage at position Arg-306 (20-fold) and relocates the active site of APC closer to the membrane surface (21–23). Protein S interacts directly with residues 493–506 in FVa (24) and therefore could stimulate the APC-induced FVa Arg-306 cleavage and/or protect the FVa Arg-506 region from APC degradation. To evaluate the effect of protein S on the interaction of the APC variants with FVa, time courses for APC-catalyzed FVa inactivation were performed in the presence of protein S. The inactivation rates for Arg-506 were unaffected by the presence of protein S, whereas with respect to the Arg-306 cleavage, all variants were stimulated to the same degree by protein S (data not shown). This demonstrates that mutations in the SP domain of APC have no influence on the cofactor activity of protein S in the APC-catalyzed cleavage of Arg-306 in FVa.

In conclusion, our results indicate that positively charged amino acids Lys-37, Lys-38, and Lys-39, located on loop 37 of APC, are indispensable for the APC-mediated cleavage at Arg-506 in FVa but not for the cleavage at position Arg-306. In contrast, amino acids Lys-62, Lys-63 in loop 60, and/or amino acid Arg-74 in the so-called calcium-binding loop (loop 70) play no or only a minor role in the Arg-506 and Arg-306 cleavages. Because loops 37 and 60 form one side of the wall lining the catalytic cleft, we can conclude that only loop 37 constitutes a secondary binding exosite for FVa critical for appropriate orientation of APC toward FVa and that this applies only for the Arg-506 site. At a structural level, loop 37 could indeed interact with FVa residues Asp-577 and Asp-578 as suggested by Pellequer (20) or with other surrounding negatively charged residues, yet to be defined experimentally. Interestingly, the autolysis loop of APC (loop 148) is suggested to play a similar role in the degradation of FVa as the now reported loop 37, discriminating between the 306 and 506 cleavages (8). This suggests that the 506 segment is in sandwich between loop 37 and loop 148 of APC and that these two regions, but not residues 62, 63, and 74 of APC contact FVa directly. In contrast, all these residues play minor roles for the cleavage at Arg-306. These results also support preliminary docking experiments of the Arg-306 site into APC, in which it is observed that the FV Arg-306 loop segment protrudes far outside the molecular surface of FVa as compared with the 506 loop. As such, when Arg-306 enters the S1 pocket of APC, at least the loops 37, 60, and 148 of Arg-306 make no clear contact with FVa. The absence of favorable electrostatic guidance for cleavage at Arg-306 could partly explain the much slower inactivation rate at this site when compared with cleavage at Arg-506. The results

![Table 1](image)

**Table 1.** Apparent second order rate constants for APC-mediated cleavage of Arg-306 and Arg-506 in FVa

Data from time courses of FVa inactivation such as those illustrated in Fig. 2 were fitted to an equation describing the APC-catalyzed inactivation of FVa using nonlinear least squares regression analysis (16). For APC variants giving poor FVa inhibition, APC concentrations at both 0.5 nM and 8 nM were used to derive the kinetic constants. Three independent experiments were used to derive the kinetic constants.

| APC | $k_{506}$ \(M^{-1}s^{-1}\) | $k_{306}$ \(M^{-1}s^{-1}\) |
|-----|----------------|----------------|
| Wild type | 4.97 \(\pm 0.18 \times 10^7\) | 1.18 \(\pm 0.11 \times 10^6\) |
| K62N/K63D | 5.80 \(\pm 0.21 \times 10^7\) | 1.42 \(\pm 0.12 \times 10^5\) |
| K37S/K38Q/K39Q | 3.41 \(\pm 0.65 \times 10^7\) | 7.92 \(\pm 0.09 \times 10^6\) |
| K37S/K38Q/K39Q/K62N/K63D | 4.02 \(\pm 0.32 \times 10^7\) | 3.91 \(\pm 0.60 \times 10^6\) |
| R74Q | 2.82 \(\pm 0.38 \times 10^7\) | 5.19 \(\pm 0.98 \times 10^5\) |

![Figure 3](image)
now presented provide important information about the orientation of FVa in the active site of APC. Further definition of the interface will require the production of several FV variants able to complement the mutations in APC. A very accurate three-dimensional model of this important macromolecular complex confirmed by experimental data should soon emerge from such investigation and shed new light onto key antithrombotic and anti-inflammatory reactions in the human body.

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