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Cleavage at both Arg³⁰⁶ and Arg⁵⁰⁶ is required and sufficient for timely and efficient inactivation of factor Va by activated protein C*

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Abstract

Background—Activated protein C (APC) inactivates membrane-bound factor Va following cleavages of the heavy chain at Arg³⁰⁶, Arg⁵⁰⁶, and Arg⁶⁷⁹.

Objectives—To examine which cleavage is most important for inactivation, recombinant factor V molecules were constructed as follows: factor V^{306Q} (R³⁰⁶→Q), factor V^{506Q} (R⁵⁰⁶→Q), and factor V^{306Q/506Q} (R³⁰⁶→Q and R⁵⁰⁶→Q).

Methods—The recombinant molecules were expressed in mammalian cells, purified, and assayed prior and after incubation with APC and lipids for 30 min (factor V_{ai}) in clotting assays and in an assay using purified reagents and saturating concentrations of factor Va.

Results—Clotting assays demonstrated that factor V_{ai}^{WT}, factor V_{ai}^{306Q} and factor V_{ai}^{506Q} were devoid of activity, while factor V_{ai}^{306Q/506Q} maintained ~70% activity following a 30 minute incubation with APC. Prothrombinase assembled with all mutant cofactor molecules before and after treatment with APC had K_m values similar to values found with prothrombinase assembled with factor V_{ai}^{WT}. Prothrombinase assembled with factor V_{ai}^{WT} demonstrated 20-fold reduction in k_{cat}, while prothrombinase assembled with factor V_{ai}^{506Q} had a 2-fold reduction in k_{cat} as compared to prothrombinase assembled with factor V_{ai}^{WT}. In contrast, factor V_{ai}^{306Q} and factor V_{ai}^{306Q/506Q} didn't show any loss in k_{cat} under similar experimental conditions.

Conclusion—Our data demonstrate that: 1) the activity of an APC-treated factor Va molecule bearing a single mutation at Arg³⁰⁶ or Arg⁵⁰⁶ depends on the assay used; and 2) regardless of the assay employed, in the absence of the APC-cleavage sites at Arg³⁰⁶ and Arg⁵⁰⁶, the active cofactor is unable to be significantly inactivated by APC in the presence of a membrane surface.

INTRODUCTION

The coagulation cascade culminates in the activation of prothrombin by the prothrombinase complex (factor Xa, factor Va, Ca²⁺, on a membrane surface) [1]. It has been well

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established that factor Va increases the catalytic efficiency of prothrombin activation by the enzyme, factor Xa, by five orders of magnitude [2]. Thrombin promotes both clotting by activating platelets and therefore ensuring the formation of fibrin, and the arrest of coagulation by activating protein C (PC) [3,4].

Activated protein C (APC) and its cofactor, protein S (PS) in the presence of a membrane surface cleaves and inactivates the cofactors of the intrinsic and prothrombinase complexes, factor VIIIa and factor Va, respectively [3,5]. APC cleaves factor VIIIa at two peptide bonds, Arg³³⁶ and Arg⁵⁶². However, these two cleavages do not appear to be essential for factor VIIIa inactivation since cofactor inactivation occurs spontaneously following activation by thrombin and dissociation of the A2 domain [6–8]. APC also cleaves three peptide bonds in the heavy chain of human factor Va at Arg³⁰⁶, Arg⁵⁰⁶, and Arg⁶⁷⁹ [9]. The cofactor function of protein S is weak in purified systems, only enhancing the rate of APC inactivation of factor Va two-fold as compared to the inactivation of the cofactor by APC alone [10,11].

Extensive studies to determine the molecular mechanism of APC inactivation of factor Va have established that the first cleavage at Arg⁵⁰⁶ is kinetically favored and results in a molecule with slightly decreased cofactor activity [9,12]. It is also known that cleavage at Arg⁵⁰⁶ in factor Va is essential for efficient rate of cleavage at Arg³⁰⁶. Cleavage at Arg³⁰⁶ leads to full loss of cofactor function and the dissociation of the A2 domain from the rest of the molecule [9,11–13]. The role of cleavage at Arg⁶⁷⁹ has not yet been determined. It is noteworthy that APC can also cleave the procofactor, factor V, in the presence of a membrane surface at Arg³⁰⁶, Arg⁵⁰⁶, Arg⁶⁷⁹, and Lys⁹⁹⁴. It is the first cleavage at Arg³⁰⁶ that is the inactivating cleavage [9,14,15].

In individuals with factor V^{LEIDEN} cleavage and inactivation of the procofactor is delayed [16]. Two new mutations in the factor V gene have been reported: Arg³⁰⁶→Gly (factor V^{HONGKONG}) and Arg³⁰⁶→Thr (factor V^{CAMBRIDGE}) [17,18]. The factor V^{HONG KONG} mutation was found in two patients with venous thrombosis and in a non-thrombotic patient. Factor V^{CAMBRIDGE} was discovered in a patient that had thrombosis with resistance to APC. While earlier data clearly demonstrated that factor Va heavy chain only contains three cleavage sites for APC [9,12,14,19], two independent studies suggested the existence of additional secondary cleavage sites for APC on factor Va heavy chain [20,21]. The data showed that a recombinant molecule with Arg³⁰⁶, Arg⁵⁰⁶, and Arg⁶⁷⁹ mutated to glutamine is still able to be cleaved and inactivated very slowly following extended incubation with very high supra-physiological concentrations of APC because of cleavage of the heavy chain at a site around the Arg³⁰⁶ cleavage site [21,22]. These unexpected results stimulated our interest in further understanding the role of the Arg⁵⁰⁶ and Arg³⁰⁶ APC-cleavage sites and the assays used to measure cofactor activity. Through recombinant protein technology, factor V molecules were created with the two most significant inactivating cleavage sites of factor Va mutated to glutamine in order to determine which cleavage products maintain cofactor activity. Our data demonstrate that under physiological conditions cleavage of factor Va heavy chain at Arg⁵⁰⁶ and Arg³⁰⁶ is required and sufficient to efficiently inactivate the cofactor as initially established [9].

EXPERIMENTAL PROCEDURES

Materials and Reagents

Diisopropyl-fluorophosphate (DFP), O-phenylenediamine (OPD)-dihydrochloride, N-[2-Hydroxyethyl] piperazine-N'-2-ethanesulfonic acid (Hepes), Trizma (Tris base), and Coomassie Blue R-250, was purchased from Sigma (St. Louis, MO). Factor V-deficient plasma was obtained from Research Protein Inc. (Essex Junction, VT). Secondary anti-

mouse and anti-sheep IgG coupled to peroxidase were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). L- α -phosphatidylserine (PS) and L- α -phosphatidylcholine (PC) were obtained from Avanti Polar Lipids (Alabaster, AL). Chemiluminescent reagent ECL⁺ and Heparin-Sepharose were obtained from AmershamPharmacia Biotech Inc. (Piscataway, NJ). Normal reference plasma and chromogenic substrate Spectrozyme-TH were obtained from American Diagnostica Inc. (Greenwich, CT). Thromboplastin reagent was purchased from Organon Teknika Corp. (Durham, NC). Dansylarginine-N- (3-ethyl-1, 5-pentanediy) amide (DAPA), human thrombin, and human prothrombin were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Human APC and factor Xa were purchased from Enzyme Research Laboratories (South Bend, IN). Factor V cDNA was obtained from American Type Tissue Collection (ATCC# 40515 pMT2-V, Manassas, VA). All restriction enzymes were obtained from New England Biolabs (Beverly, MA). All molecular biology and tissue culture reagents, specific primers, and media were obtained from Gibco, Invitrogen Corp. (Grand Island, NY) or as indicated. Monoclonal antibodies to human factor V (α HFV_{HC}#17 and α HFV#1 coupled to Sepharose) were provided by Dr. Kenneth G. Mann (Department of Biochemistry, University of Vermont, Burlington, VT).

Construction of Recombinant Factor V Molecules

Recombinant factor V molecules, factor V^{306Q}, factor V^{506Q}, factor V^{306Q/506Q}, were constructed using Stratagene's QuikChange® XL Site-Directed Mutagenesis Kit. The factor V^{306Q} mutation was constructed with the following primers; 5' GC CCA AAG AAA ACC CAG AAT CTT AAG 3' (forward) and 5' CTT AAG ATT CTG GGT TTT CTT TGG GC 3' (reverse). The factor V^{506Q} mutation was constructed with the following primers; 5' GC AGA TCC CTG GAC CAG CGA GGA ATA CAG AGG GCA GC 3' (forward) and 5' CG TGC CCT CTG TAT TCC TCG CTG GTC CAG GGA TCT GC 3' (reverse). The bold, underlined bases indicated the mutated base pairs to insert the R → Q mutation. The double mutant Arg^{306Q/506Q} was constructed using the factor V Arg^{506Q} cDNA as template with the primers used to make the factor V^{306Q} mutant. The mutations were confirmed by DNA Sequencing (DNA Analysis Facility, Cleveland State University).

Transient Transfection of Recombinant Factor V Molecules

The purified wild type, factor V^{306Q}, factor V^{506Q}, and factor V^{306Q/506Q} plasmids were transfected into the COS-7L cells with fugene 6 (Roche Diagnostics) according to the manufacturer's instructions. Further treatment of the cells and collection of the media was performed as described [23].

Purification of Recombinant Factor V Molecules

Each recombinant protein was purified on a 2ml column of monoclonal antibody α HFV#1 coupled to Sepharose as described in detail [24]. The exact concentration of each preparation was verified using the ELISA previously described in our laboratory [25]. The activity and the integrity of the recombinant factor V molecules were confirmed by clotting assays using factor V deficient plasma and western blotting with monoclonal and polyclonal antibodies to factor V.

APC Inactivation of Recombinant Factor V/Va Molecules

The factor V species were first activated with thrombin to a final concentration of 1nM for 10 minutes at 37°C (designated factor Va). Immediately following this incubation, of 20 μ M PCPS were added and allowed to incubate at room temperature for 5 minutes. Then, APC was added at a 1:50 molar ratio and incubated at 37°C for an additional 30 minutes (designated factor Vai, for example, for a factor V molecule solution at 200 nM, 4 nM APC

was used). Although complete inactivation of factor Va^{WT} occurred following 10 minutes incubation with APC under the conditions described, the mixture was further incubated for an additional 20 minutes to detect possible additional APC cleavages the heavy chain of the cofactor as recently described [21].

Determination of Factor Va Clotting Activity

The clotting activity of the recombinant molecules was measured in a clotting assay using factor V-deficient plasma following activation of the cofactor by thrombin as described [23]. The clotting activities were also determined following inactivation of factor Va species with APC and PCPS. The specific activity of each recombinant factor Va molecule was calculated and reported in units/mg.

Gel Electrophoresis and Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were carried out using 5–15% gradient gels following reduction with 2% β -mercaptoethanol according to the method of Laemmli [26]. In some experiments the protein was transferred to polyvinylidene difluoride (PVDF) membranes following the method of Towbin et. al. [27]. Factor Va heavy chain and heavy chain inactivation fragments were probed with monoclonal antibody α HFV_{HC}#17 and visualized with chemiluminescence.

Factor Va Titrations

The ability of the recombinant factor V molecules to assemble in the prothrombinase complex and bind to the enzyme was measured in a discontinuous assay described in detail elsewhere [28,29]. The initial rate of thrombin generation was analyzed using Prizm (GraphPad) software by fitting the data to the model of one binding site. Dissociation constants reported in the text were derived directly from the fitted data.

Prothrombin Titrations

For the determination of the kinetic constants (K_m and k_{cat}) of prothrombinase, experiments were executed with a limiting amount of factor Xa. The amount of factor Va or factor Vai necessary to saturate factor Xa was calculated as described [30]. The initial rate of thrombin generation was analyzed with Prizm (GraphPad) software by fitting the data to the Michaelis-Menten equation. Kinetic constants reported in the text were derived directly from the fitted data.

RESULTS

Recombinant Factor V Molecules

Complete inactivation of factor Va by APC requires the presence of a membrane surface. However, factor Va can be cleaved by APC in the absence of a membrane surface at Arg⁵⁰⁶ and Arg⁶⁷⁹ retaining ~80% of its cofactor activity as assessed in a prothrombinase assay using purified reagents and saturating concentrations of factor Va with factor Xa. Nevertheless, when tested in a clotting assay, the same molecule has ~40% activity. It has been also shown that factor Va cleaved at Arg⁵⁰⁶ and Arg⁶⁷⁹ has reduced affinity for both factor Xa and prothrombin [31–33]. Conversely, when a membrane surface is present, cleavage at Arg³⁰⁶ occurs in addition to the cleavages at Arg⁵⁰⁶ and Arg⁶⁷⁹, and the resultant molecule has no activity no matter what assay is used.

To examine the consequences of the individual inactivating cleavages of factor Va by APC in the presence of a membrane surface, recombinant factor V molecules were constructed with R $\rightarrow$ Q mutation at the crucial APC cleavage sites; factor V^{306Q} (R³⁰⁶ $\rightarrow$ Q), factor

V^{506Q} (R⁵⁰⁶→Q), and factor V^{306Q/506Q} (R³⁰⁶→Q and R⁵⁰⁶→Q). Figure 1A is a schematic representation of the fragments resulting from complete digestion of membrane bound recombinant factor V/Va molecules by APC. Fragments from the recombinant molecules prior to and after activation with thrombin were identified following immunoblotting with a monoclonal antibody recognizing an epitope between amino acid residues 307–506 (figure 1B) [14, 15]. The fragments in black in figure 1A correspond to the fragments recognized by the antibody following cleavage by APC-membrane and shown in figure 1B. All recombinant procofactor molecules incubated with thrombin displayed complete activation as verified by the absence of single chain factor V and the appearance of the M_r 105,000 heavy chain of the cofactor (Fig. 1B, factor Va^{WT} in lane 1, factor Va^{306Q} in lane 4, factor Va^{506Q} in lane 7, and factor Va^{306Q/506Q} in lane 10). Membrane bound factor Vai^{WT} and factor Vi^{WT} displayed the final expected M_r 30,000 fragment (amino acid 307–506, Fig. 1B, lanes 2 and 3) following a 30 minutes incubation with APC and PCPS [9, 14]. Cleavage of factor Va^{306Q} and factor V^{306Q} by APC results in the appearance of a M_r 75,000 fragment indicating only cleavage at Arg⁵⁰⁶/Arg⁶⁷⁹ (Fig. 1B, lanes 5 and 6). Conversely, when the APC cleavage site at Arg⁵⁰⁶ is missing in factor Va^{506Q} and factor V^{506Q}, only the M_r 54/56,000 doublet is apparent indicating that these molecules were cleaved at Arg³⁰⁶/Arg⁶⁷⁹ (Fig. 1B, lanes 8 and 9). It is noteworthy that while following 15 minutes incubation with APC and PCPS factor Va^{506Q} still has 20% clotting activity (Fig. 2), the procofactor factor V^{506Q}, still has ~50% clotting activity under similar conditions (not shown), verifying the fact that factor V^{506Q} is more resistant to APC cleavage than factor V^{WT} as previously suggested using natural factor V^{R506Q} [16]. On the other hand, the factor Va^{306Q/506Q} and factor V^{306Q/506Q} molecules are unable to be cleaved by APC, and are only cleaved at Arg⁶⁷⁹ (Fig. 1B, lanes 11 and 12) resulting in a fragment of M_r 100,000 as previously demonstrated [15]. It is noteworthy that while factor Vai^{WT} (Fig. 1B, lane 2), factor Vai^{306Q} (Fig. 1B, lane 5) and factor Vai^{506Q} (Fig. 1B, lane 8), have no substantial clotting activity, there is still heavy chain remaining as assessed by immunoblotting with monoclonal antibody αHFV_{HC}#17 made against the heavy chain of the cofactor. In our experience, this is due to two major reasons: 1) the different affinity of the antibody for factor V, factor Va heavy chain, and the factor Va heavy chain derived fragments; and 2) the fact that there is some free factor Va heavy chain in solution (i.e not associated to the light chain). Since cleavage of human factor Va heavy chain by APC depends on its association with the light chain [34], free heavy chain can not be cleaved by APC. Thus, the minute amounts of heavy chain remaining following complete inactivation of the cofactor by APC are recognized by the monoclonal antibody that has higher affinity for the heavy chain than for factor V or factor Va heavy chain derived fragments. Thus, under the conditions described presence of heavy chain on the immunoblots doesn't necessary correlate with cofactor activity. Overall, the data demonstrate that the recombinant factor Va/V mutants display the expected fragments following incubation with a membrane surface and APC and can be used to further elucidate the molecular mechanism of APC-induced factor Va inactivation and the importance of each cleavage for cofactor inactivation.

Clotting Activity of Recombinant Factor V/Va Molecules Following APC

The importance of the first inactivating cleavage of factor Va at Arg⁵⁰⁶ is evident in patients with APC-resistance that have a molecular defect linked to a single point mutation (Arg⁵⁰⁶→Gln, designated factor V^{LEIDEN}) [14,35]. The factor V^{LEIDEN} mutation is present in 5% of the normal population in western countries [35] and has been shown to be a major risk factor for venous thrombosis [36]. Since factor Va^{LEIDEN} does not have the Arg⁵⁰⁶ cleavage site, APC inactivation of the molecule is delayed [14], due to slower rate of cleavage at Arg³⁰⁶. The published data clearly demonstrate that cleavage at Arg⁵⁰⁶ is necessary for timely and successful inactivation of the cofactor following cleavage at Arg³⁰⁶ [37].

We assayed all recombinant cofactor molecules for their clotting activity. Clotting times were recorded before and following 15 and 30 minutes of incubation with APC/PCPS vesicles. The results are shown in Figure 2. All factor Va species had similar clotting activities in the absence of APC. Following 15 minutes incubation with APC in the presence of a membrane surface, factor Va^{WT} is devoid of clotting activity. In contrast, factor Va^{306Q} and factor Va^{506Q} still possess 20% clotting activity whereas factor Va^{306Q/506Q} has approximately ~80% clotting activity. This data demonstrate that while under the experimental conditions used (1/50 enzyme substrate ratio) the wild type molecule is fully inactivated following a 15-minute incubation period with APC, factor Va^{306Q} and factor Va^{506Q} are partially resistant to APC. Following 30 minute incubation with APC and lipids, recombinant factor Va^{306Q/506Q} retains ~70% clotting activity while all other recombinant cofactor molecules are devoid of clotting activity. Since factor Va^{306Q/506Q} is not susceptible to APC-dependent cleavage and inactivation, the data demonstrate that cleavages at Arg³⁰⁶ and Arg⁵⁰⁶ are required and sufficient to inactivate factor Va under the conditions used.

Activity of prothrombinase assembled with factor Va and factor Va_i

In order to test the ability of the recombinant factor Va and factor Va_i molecules to assemble in prothrombinase and activate prothrombin, we used an assay employing purified reagents and a chromogenic substrate. We first evaluated the capability of recombinant factor Va molecules to bind plasma-derived factor Xa. The data demonstrate that factor Va^{WT}, factor Va^{PLASMA}, factor Va^{306Q}, factor Va^{506Q}, and factor Va^{306Q/506Q} have similar K_D values for plasma-derived factor Xa, indicating that the mutations do not affect the interactions governing prothrombinase assembly (Table 1). We also analyzed the effect of extended APC cleavage on the ability of the recombinant cofactors to bind factor Xa. Inactivation of the wild type cofactor increased the K_D for factor Xa by ten-fold (Table 1), while the mutated cofactors also have decreased affinity for factor Xa. Factor Va_i^{306Q} had a 5-fold increase in the K_D (Table 1) while factor Va_i^{506Q} had a 6-fold increase in the K_D (Table 1). The former value using recombinant proteins is remarkably similar to the K_D found when using APC inactivation of bovine factor Va in the absence of a membrane surface [13]. Under the experimental conditions used, factor Va_i^{306Q/506Q} had a ~3-fold increase in the K_D (Table 1). This data demonstrate that partial inactivation of factor Va results in a molecule that has decreased affinity for the enzyme within prothrombinase.

Next we tested the ability of the recombinant factor Va molecules to assemble within prothrombinase and activate prothrombin. The experiments were performed in the presence of limiting amounts of factor Xa (5pM) while the concentration of the cofactor was always saturating with respect to factor Xa. The results show that factor Va^{WT} (filled squares), factor Va^{306Q} (filled inverted triangles), factor Va^{506Q} (filled triangles), and factor Va^{306Q/506Q} (filled circles) have similar K_m (Table 1). All APC-treated cofactor molecules also have similar K_m values as the untreated cofactor molecules. These data demonstrate that cleavage of factor Va at either Arg³⁰⁶ or Arg⁵⁰⁶ is of no consequence for the interaction between prothrombinase and its substrate (prothrombin).

The catalytic efficiency of prothrombinase assembled with the APC-treated cofactor molecules under conditions where all factor Xa is saturated with factor Va, demonstrated that all the recombinant factor Va_i species with the exception of factor Va_i^{WT} and factor Va_i^{506Q} have similar k_{cat} values compared to the k_{cat} of prothrombinase assembled with factor Va^{WT} (Figure 3B). Prothrombinase assembled with saturating concentrations of factor Va_i^{WT} had a ~40-fold reduction in k_{cat}, while prothrombinase assembled with factor Va_i^{506Q} had only a 2-fold reduction in the catalytic efficiency for prothrombin activation (Fig. 3B). Overall these data demonstrate that 1) both cleavages are required for timely and efficient

inactivation of factor Va; and 2) cleavage at Arg³⁰⁶ appears to be more detrimental to cofactor activity than cleavage at Arg⁵⁰⁶ regardless of the assay used.

DISCUSSION

Proteolytic inactivation of factor Va by APC requires the presence of a membrane surface and cleavage at both Arg⁵⁰⁶ and Arg³⁰⁶. Cleavage at Arg⁵⁰⁶ is required for efficient rate of cleavage at Arg³⁰⁶ [9,14]. In the present study we show that the double mutant, factor Va^{306Q/506Q}, is vastly resistant to APC inactivation with 30% loss of activity following a 30 minutes incubation period. The double mutant was also fully active when used in an assay using purified reagents in the presence of saturating concentrations of factor Va and immunoblotting experiments revealed no additional heavy chain fragments that could be recognized by the monoclonal antibody used in this study when both factor Vi^{306Q/506Q} and factor Vai^{306Q/506Q} were incubated with APC in the presence of a membrane surface except of the fragment deriving from cleavage at Arg⁶⁷⁹ as previously shown [9,11,15]. These data confirm the fact that cleavage of factor Va heavy chain by APC at Arg⁵⁰⁶ and Arg³⁰⁶ is required and sufficient to inactivate factor Va under physiological conditions.

It has been long established that a single point mutation in the factor V gene, resulting in the Arg⁵⁰⁶→Gln substitution (factor V^{LEIDEN}), is a common risk factor for thrombosis because it results in APC-resistance. The fact that individuals with the factor V^{LEIDEN} mutation have a thrombotic tendency is a testimony of the importance of the cleavage site at Arg⁵⁰⁶ for the physiological function of the cofactor. However, cleavage at Arg⁵⁰⁶ alone does not result in complete inactivation of factor Va [9,11] and cleavage at Arg³⁰⁶ is required for complete inactivation of the cofactor by APC [9,11,12,14]. Table I shows that factor Vai^{306Q} (equivalent to factor Vai^{CAMBRIDGE}/factor Vai^{HONGKONG}) and factor Vai^{506Q} (equivalent to factor Vai^{LEIDEN}) which have been treated with APC and are cleaved at Arg⁵⁰⁶ and at Arg³⁰⁶ respectively, have similar K_D for factor Xa and similar K_m for prothrombin. However, figure 3 shows that factor Va^{506Q} has approximately half the k_{cat} of factor Va^{306Q} and should be therefore less procoagulant. Therefore, one would presume that mutation at Arg³⁰⁶ would cause a more severe APC-resistance as compared to a mutation at Arg⁵⁰⁶, leading to a severe thrombotic state. However, it has been well documented that while patients with the factor V^{LEIDEN} mutation have severe thrombotic episodes, individuals bearing the factor V^{CAMBRIDGE} mutation (Arg³⁰⁶→Thr) or the factor V^{HONGKONG} mutation (Arg³⁰⁶→Gly) do not appear to be at high risk for thrombosis [38]. Taken into account the data presented here and the importance of cleavage at Arg³⁰⁶ for the inactivation of the cofactor by APC, we can hypothesize that in individuals with factor V^{CAMBRIDGE} and factor V^{HONGKONG}, following cleavage at Arg⁵⁰⁶, the cofactor maybe cleaved by high concentrations of APC generated locally, at one of the sites surrounding the cleavage site at Arg³⁰⁶ as recently described [21,22]. Nevertheless, the physiological significance for cleavage at these recently identified sites when the Arg³⁰⁶ and Arg⁵⁰⁶ sites on the heavy chain of factor Va are available, remains to be established.

Two assays are used by many laboratories worldwide for the determination of factor Va cofactor activity: a clotting assay using factor V-deficient plasma and a prothrombinase assay using purified reagents. The clotting assay is performed at low (limiting) concentrations of factor Va, while the prothrombinase assay is normally conducted with high (saturating) concentrations of factor Va with respect to factor Xa. Nevertheless, regardless of the experimental conditions employed, and the final cofactor concentration used, both assays indirectly report on factor Va cofactor activity through the activation of prothrombin by prothrombinase. Our data show that in a clotting assay, any substitution of the two factor Va specific APC cleavage sites will result in a molecule that is still inactivated by APC compared to the wild type molecule, albeit with a slower rate. In

contrast, in the assay using saturating concentrations of factor Va, cofactor activity is sensitive only to cleavage at Arg³⁰⁶, verifying the importance for cleavage at this site for factor Va activity. Thus, according to the data presented herein, when focusing on the results obtained using the prothrombinase assay, one may suggest that cleavage at Arg⁵⁰⁶ alone is not important for cofactor inactivation since prothrombinase assembled with factor Vai^{306Q} behaves like prothrombinase assembled with the wild type molecule. However, it is well established worldwide that factor V^{LEIDEN} is an important risk for thrombosis and individuals with the Arg⁵⁰⁶→Gln mutation have severe thrombotic episodes. Thus, careful assessment of the detailed experimental conditions used in each study can reveal the significance of the findings relating to the physiological importance of each cleavage site.

Overall, our findings strongly suggest that conclusions pertaining to the physiological importance of APC cleavage sites in factor Va, that are usually inferred from in vitro studies studying natural or recombinant cofactor molecules, must be cautiously scrutinized with respect to the assay conditions, the nature of the recombinant molecules, and the concentration of APC used. This is especially important in view of the clinical relevance of the findings and their dissemination, with respect to the screening, identification, and treatment of patients with deep venous thrombosis. The data in the present study together with the bulk of findings provided in the literature the last 16 years, clearly demonstrate the importance and uniqueness of the APC cleavage sites at Arg³⁰⁶ and Arg⁵⁰⁶ for complete and efficient factor Va inactivation under physiological conditions.

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Abbreviations used are

PS	L- α -phosphatidylserine
PC	L- α -phosphatidylcholine
PCPS	small unilamellar phospholipids vesicles composed of 75% PC and 25% PS (w/w)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
factor Va^{WT}	recombinant human wild type factor Va
factor Va^{306Q}	recombinant human factor Va with the mutations R ³⁰⁶ →Q
factor Va^{506Q}	recombinant human factor Va with the mutations R ⁵⁰⁶ →Q
factor Va^{306Q/506Q}	recombinant human factor Va with the mutation R ³⁰⁶ →Q and R ⁵⁰⁶ →Q
factor Vai	membrane-bound factor Va treated with APC/PCPS for 30 minutes at 1/50 enzyme substrate ratio

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Figure 1.

Panel A. Schematic of factor Va heavy chain fragments. All fragments recognized by monoclonal antibody #17 are in black boxes while the rest of the heavy chain is depicted with white boxes. The heavy chain of factor Va is composed of two A domains. Cleavage of factor Va^{WT} at Arg⁵⁰⁶/Arg³⁰⁶/Arg⁶⁷⁹ results in a M_r 30,000 fragment that is recognized by a monoclonal antibody that recognizes an epitope between amino acids 307–506. In factor Va^{306Q} the antibody recognizes a fragment of M_r 75,000 following cleavage of the cofactor at Arg⁵⁰⁶/Arg⁶⁷⁹. In factor Va^{506Q} (equivalent to factor Va^{LEIDEN}) the antibody recognizes a doublet of M_r 54/56,000 following cleavages at Arg³⁰⁶/Arg⁶⁷⁹. Following cleavage of factor Va^{306Q/506Q} by APC the antibody recognizes a fragment of M_r 97,000 following cleavage at Arg⁶⁷⁹. **Panel B. Analysis of recombinant factor V molecules following cleavage by APC using an antibody specific to the heavy chain of factor Va.**

Recombinant Factor V molecules were subjected to activation by thrombin and inactivation by APC in the presence of a membrane surface as described in the “Experimental Procedures” section. Lane assignments are as follows; Lane 1 factor Va^{WT}, Lane 2 factor Va_i^{WT}, Lane 3 factor V_i^{WT}, Lane 4 factor Va^{306Q}, Lane 5 factor Va_i^{306Q}, Lane 6 factor V_i^{306Q}, Lane 7 factor Va^{506Q}, Lane 8 factor Va_i^{506Q}, Lane 9 factor V_i^{506Q}, Lane 10 factor Va^{306Q/506Q}, Lane 11 factor Va_i^{306Q/506Q}, and Lane 12 factor V_i^{306Q/506Q}. Positions of factor V/Va and factor V/Va fragments following cleavage by APC and stained with the monoclonal antibody αHFV_{HC}#17 that recognizes an epitope between amino acid residues 307–506 of factor V are shown on the right.

Figure 2. Clotting activity of recombinant factor Va molecules

Clotting times were measured as described in the “Experimental Procedures” section. The recombinant factor Va molecules were analyzed prior and following 15 and 30 minute incubation with APC in the presence of a membrane surface times. Bars are as follows: □ factor Va^{WT}, ▨ factor Va^{306Q}, ▩ factor Va^{506Q}, and ■ factor Va^{306Q/506Q}.

Fig. 3B

Figure 3.

Panel A. Prothrombin titrations. Thrombin generation experiments were carried out as described under “Experimental Procedures” by varying the substrate concentration (25 nM to 4 μ M) with 5 pM factor Xa saturated with the indicated factor Va species. Prothrombinase complex was assembled with recombinant purified factor Va^{WT} (*filled squares*), factor Va_i^{WT} (*open squares*), factor Va^{306Q} (*filled inverted triangles*), factor Va_i^{306Q} (*open inverted triangles*), factor Va^{506Q} (*filled triangles*), factor Va_i^{506Q} (*open triangles*), factor Va^{306Q/506Q} (*closed circles*), and factor Va_i^{306Q/506Q} (*open circles*). The data shown are the average of three different titrations performed in triplicate with at least three different preparations of purified proteins. **Panel B.** Comparison of k_{cat} values (expressed in min^{-1}) of recombinant factor Va species before and after APC inactivation.

Table 1

Functional Properties of recombinant factor Va molecules

Factor Va Species	K_{Dapp} (nM) ^a	K_m (μM) ^b
Factor Va ^{WT}	0.22 ± 0.03	0.13 ± 0.06
Factor Va _i ^{WT}	2.0 ± 0.4 ^c	0.5 ± 0.3 ^d
Factor Va ^{PLASMA}	0.2 ± 0.07	0.15 ± 0.01
Factor Va _i ^{PLASMA}	3.6 ± 1 ^c	ND ^e
Factor Va ^{306Q}	0.3 ± 0.04	0.13 ± 0.02
Factor Va _i ^{306Q}	1.35 ± 0.01	0.2 ± 0.01
Factor Va ^{506Q}	0.20 ± 0.01	0.3 ± 0.03
Factor Va _i ^{506Q}	1.15 ± 0.2	0.2 ± 0.02
Factor Va ^{306Q/506Q}	0.3 ± 0.03	0.15 ± 0.01
Factor Va _i ^{306Q/506Q}	0.7 ± 0.1	0.2 ± 0.05

^a Apparent dissociation constants of recombinant factor Va for plasma-derived factor Xa (K_{Dapp}) were determined as described in the “*Experimental Procedures*” section at limiting factor Xa concentrations (15 pM).

^b Apparent K_m , and k_{cat} (shown in Fig. 3B) were determined as described in the “*Experimental Procedures*” section at limiting factor Xa concentrations (5 pM) and saturating concentrations of factor Va. The constants reported were obtained directly from the fitted data. All graphs had an R^2 (goodness of fit) between 0.96–0.98.

^c Apparent dissociation constants of factor Va_i^{PLASMA} and factor Va_i^{WT} for plasma-derived factor Xa (K_{Dapp}) were determined directly from the fitted data as described in the “*Experimental Procedures*” section at limiting factor Xa concentrations (15 pM) using Prism and the equation for one binding site. Under the conditions employed the goodness of the fit (R^2) was 0.92 for factor Va_i^{PLASMA} and 0.93 for factor Va_i^{WT}.

^d The apparent K_m and k_{cat} of factor Va_i^{WT} was determined as described in the “*Experimental Procedure*” section at limiting factor Xa concentrations (5 pM) and saturating concentrations of factor Va. Following plotting of the data according to the Michaelis-Menten equation, the goodness of the fit (R^2) was 0.49.

^e ND, not done