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The effect of $Arg^{306} \rightarrow Ala$ and $Arg^{506} \rightarrow Gln$ substitutions in the inactivation of recombinant human factor Va by activated protein C and protein S

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Abstract

Factor Va (fVa) is inactivated by activated protein C (APC) by cleavage of the heavy chain at Arg^{306}, Arg^{506}, and Arg^{679}. Site-directed mutagenesis of human factor V cDNA was used to substitute $Arg^{306} \rightarrow Ala$ ($rfVa^{306A}$) and $Arg^{506} \rightarrow Gln$ ($rfVa^{506Q}$). Both the single and double mutants ($rfVa^{306A/506Q}$) were constructed. The activation of these procofactors by α-thrombin and their inactivation by APC were assessed in coagulation assays using factor V-deficient plasma. All recombinant and wild-type proteins had similar initial cofactor activity and identical activation products (a factor Va molecule composed of light and heavy chains). Inactivation of factor Va purified from human plasma (fVa^{PLASMA}) in HBS Ca^{2+} +0.5% BSA or in conditioned media by APC in the presence of phospholipid vesicles resulted in identical inactivation profiles and displayed identical cleavage patterns. Recombinant wild-type factor Va ($rfVa^{WT}$) was inactivated by APC in the presence of phospholipid vesicles at an overall rate slower than fVa^{PLASMA}. The $rfVa^{306A}$ and $rfVa^{506Q}$ mutants were each inactivated at rates slower than $rfVa^{WT}$ and fVa^{PLASMA}. Following a 90-min incubation with APC, $rfVa^{306A}$ and $rfVa^{506Q}$ retain approximately 30–40% of the initial cofactor activity. The double mutant, $rfVa^{306A/506Q}$, was completely resistant to cleavage and inactivation by APC retaining 100% of the initial cofactor activity following a 90-min incubation in the presence of APC. Recombinant fVa^{WT}, $rfVa^{306A}$, $rfVa^{506Q}$, and $rfVa^{306A/506Q}$ were also used to evaluate the effect of protein S on the individual cleavage sites of the cofactor by APC. The initial rates of $rfVa^{WT}$ and $rfVa^{306A}$ inactivation in the presence of protein S were unchanged, indicating cleavage at Arg^{506} is not affected by protein S. The initial rate of $rfVa^{506Q}$ inactivation was increased, suggesting protein S slightly accelerates the cleavage at Arg^{306}. Overall, the data demonstrate high specificity with respect to cleavage sites for APC on factor Va and demonstrate that cleavages of the cofactor at both Arg^{306} and Arg^{506} are required for efficient factor Va inactivation.

Keywords: activated protein C; factor Va inactivation; protein S; recombinant factor V

The generation of α-thrombin is a central event in the balance between hemostasis and thrombosis. *Prothrombinase*, the enzyme complex required to activate prothrombin, is composed of factor Xa and the cofactor, factor Va, associated in 1:1 stoichiometry on a phospholipid surface in the presence of Ca^{2+} (Nesheim et al., 1979b). The efficiency of prothrombin activation by the *prothrombinase* complex is five orders of magnitude greater than by factor Xa alone (Mann et al., 1990). Factor Va is formed by enzymatic cleavage of the single chain procofactor, factor V (M_r = 330,000), by α-thrombin and/or factor Xa (Fig. 1) (Mann et al., 1988). The cDNA for human factor V and the deduced amino acid sequence have been previously described (Kane & Davie, 1986; Jenny et al., 1987). The active cofactor, factor Va, is composed of a M_r = 105,000 heavy chain (A1–A2 domains, amino acid residues 1–709) derived from the NH_2-terminal portion of the procofactor and a M_r = 74,000 light chain derived from the COOH-terminal portion of factor V (A3–C1–C2 domains, amino acid residues 1546–2196). The two chains of the cofactor are non-covalently associated in the presence of Ca^{2+} ions (Esmon, 1979; Krishnaswamy et al., 1989).

The factor V molecule is post-translationally modified at multiple N-linked glycosylation sites in the B region as well as on the heavy and light chains (Bruin et al., 1987; Jenny et al., 1987). Factor V(a) is phosphorylated at Ser^{692} of the heavy chain by a casein kinase II-like kinase and on two residues of the light chain by an isoform of protein kinase C (Kalafatis et al., 1993b; Rand et al., 1994). The procofactor is also believed to be sulfated at Tyr^{664}, Tyr^{696}, Tyr^{698}, Tyr^{1494}, Tyr^{1510}, Tyr^{1515}, and Tyr^{1565} (Hortin, 1993) (Fig. 1). The effect of these posttranslational modifications on the function of the cofactor are not completely understood.

α-Thrombin is a potent procoagulant enzyme effecting the cleavage of soluble fibrinogen to form fibrin, which polymerizes

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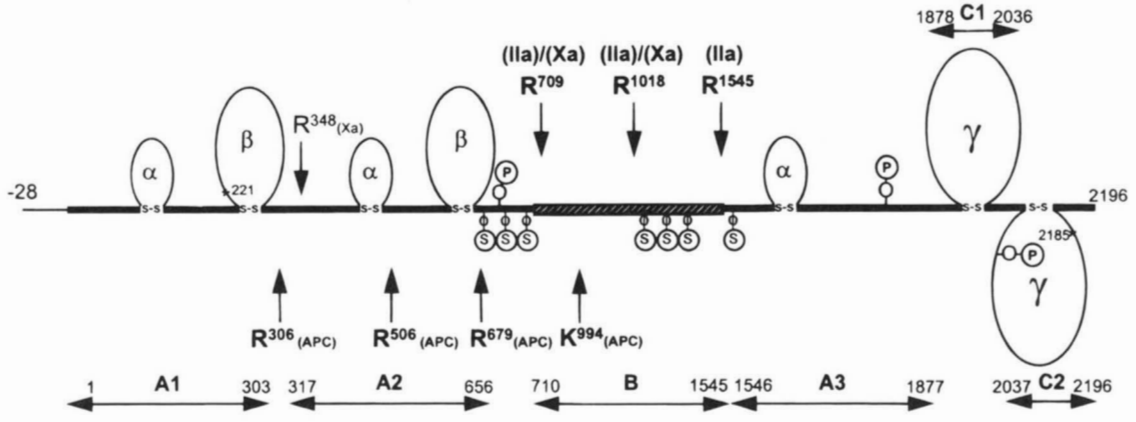

Fig. 1. Structural features of human factor V. Structural representation of the 2,196 amino acid factor V molecule. The molecule also contains a 28-amino acid leader peptide. The activating cleavage sites of the procofactor are depicted above the molecule. The inactivating cleavages are shown below the molecule. The positions of the free cysteines and disulfide bridges are shown. The sulfated tyrosine residues at amino acid positions 664, 696, 698, 1,494, 1,510, 1,515, and 1,565 are shown (Hortin, 1993). The sites of phosphorylation in the heavy and light chains are illustrated (Kalafatis et al., 1993b; Rand et al., 1994). The stars identify a point mutation and a polymorphism at amino acid positions 221 and 2,185, respectively (Murray et al., 1995).

to form the fibrin clot. Paradoxically, α-thrombin also acts as an anti-coagulant enzyme by binding to the endothelial cell receptor, thrombomodulin (Esmon et al., 1982). The thrombin–thrombomodulin complex alters the proteolytic specificity of α-thrombin and cleaves protein C to form the anticoagulant enzyme, activated protein C (APC). APC, in the presence of a membrane surface, inactivates factor Va by limited proteolysis of the heavy chain (Suzuki et al., 1983; Solymoss et al., 1988; Kalafatis & Mann, 1993; Kalafatis et al., 1994).

Biochemical studies using factor Va purified from human plasma demonstrate that inactivation of the cofactor is associated with APC cleavages of the heavy chain at Arg^{306}, Arg^{506}, and Arg^{679} (Fig. 1) (Kalafatis et al., 1994). On phospholipids, cleavage at Arg^{506} occurs first and results in a partially active molecule. The subsequent cleavage at Arg^{306} occurs efficiently only when the cofactor is present on a negatively charged phospholipid membrane surface and totally inactivates the molecule (Kalafatis & Mann, 1993; Kalafatis et al., 1994). The initial cleavage at Arg^{506} generates a fragment of M_r = 75,000 and a doublet of M_r = 28/26,000 (Fig. 2). Further cleavage of the M_r = 75,000 fragment at Arg^{306} generates fragments of M_r = 45,000 and 30,000, while cleavage of the M_r = 28/26,000 doublet at Arg^{679} generates a M_r = 22/20,000 doublet and a M_r = 6,000 peptide.

Factor V^{Leiden} is a mutant factor V molecule present in 5% of the normal population in western countries (Bertina et al., 1994). Factor V^{Leiden} contains an $Arg^{506} \rightarrow$ Gln substitution resulting from a point mutation at nucleotide 1691 in the factor V gene (Bertina et al., 1994). This amino acid substitution results in the elimination of the APC cleavage site at Arg^{506}. The factor V^{Leiden} molecule displays resistance to APC and has been shown to be a major risk factor for venous thrombosis (Rosendaal et al., 1995). Compared to purified plasma factor Va, APC inactivation of factor Va^{Leiden} is delayed, displaying a slower rate of cleavage by APC at Arg^{306} and Arg^{679} (Kalafatis et al., 1995a). The slowed loss of cofactor activity following APC cleavage of Factor Va^{Leiden} was shown to be a result of slowed cleavage at Arg^{306}, suggesting that cleavage at Arg^{506} is required for efficient inactivation of the cofactor (Kalafatis et al., 1995a).

Protein S has been reported to act as a cofactor for optimal expression of the anticoagulant activity of APC (Walker, 1981). The mechanism by which protein S facilitates APC inactivation of factor Va is not fully understood; however, the significance of protein S in the regulation of hemostasis has been demonstrated by the association of thrombosis with protein S deficiencies. Protein S has been reported to abolish the factor Xa-mediated protection of factor Va from cleavage by APC (Solymoss et al., 1988; Nesheim et al., 1982). Protein S has also been reported to accelerate the rate of APC cleavage of factor Va at Arg^{306} by 20-fold (Rosing et al., 1995).

The present study was undertaken to study the effect of cleavage at each site on cofactor inactivation. Although previous data have

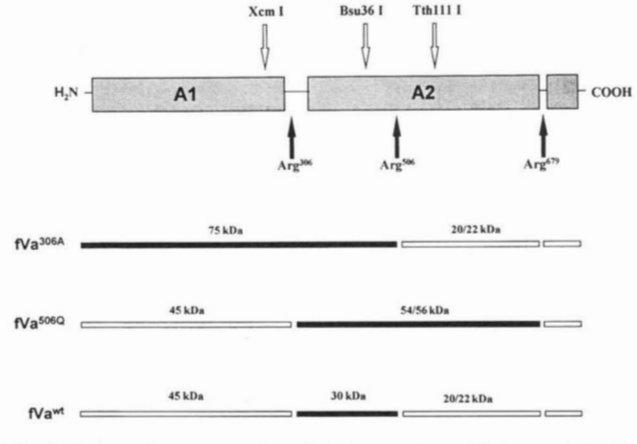

Fig. 2. Schematic representation of the human factor Va heavy chain during proteolytic inactivation by APC. Normal factor Va is inactivated following cleavage of the heavy chain at Arg^{506}, Arg^{306}, and Arg^{679} (Kalafatis et al., 1994). The relative positions of the cleavage sites for restriction endonucleases used during mutant construction (*Xcm* I, *Bsu*36 I, and *Tth*111 I) are shown. The APC cleavage products of the factor Va heavy chain that contain the $\alpha HFVa_{HC}$#6 epitope are represented by the black lines and were described elsewhere (Kalafatis et al., 1995a, 1995b, 1996).

characterized inactivation of factor Va^{Leiden} (fVa^{506Q}) (Kalafatis et al., 1995a), to date no data have been reported employing a mutant factor V molecule with an amino acid substitution at Arg^{306}. We have constructed $Arg^{306} \rightarrow Ala$ and $Arg^{506} \rightarrow Gln$ mutations of human factor V and expressed recombinant wild-type $Arg^{506} \rightarrow Gln$, $Arg^{306} \rightarrow Ala$, and $Arg^{306} \rightarrow Ala/Arg^{506} \rightarrow Gln$ factor V proteins in Chinese hamster ovary (CHO) cells. The recombinant human factor Va mutants were used to determine the contributions of the specific APC cleavage sites to the inactivation of factor Va. This study reports for the first time the kinetic parameters for mutant factor Va^{306A} inactivation by APC. Our study using the double mutant ($rfVa^{306A/506Q}$) also demonstrates the high specificity of APC cleavage sites on the factor Va molecule. The present study also examines the lipid dependence of the APC cleavages of factor Va at Arg^{306} and Arg^{506}. Finally, the influence of protein S on the specific APC cleavage sites of factor Va is evaluated.

Results

The pED-V, $pEDV^{306A}$, $pEDV^{506Q}$, and $pEDV^{306A/506Q}$ expression vectors were transfected into dihydrofolate-deficient CHO cells. Transfected cells were selected for dihydrofolate reductase and copy number was amplified using increasing concentrations of methotrexate. Production of recombinant factor V species was estimated using a radioimmunoassay for factor V (Tracy et al., 1982). The specific activity of the recombinant forms of factor Va were determined using concentrations obtained by radioimmunoassay of the media and activity measurements determined in clotting assays using factor V-deficient plasma. The factor V clones used in this study produced 0.5–3 μg/mL of recombinant factor V. These levels of recombinant human factor V expression are similar to those previously reported for stable (Pittman et al., 1994) and transient (Kane et al., 1990) transfectants of factor V. Specific activity of $rfVa^{WT}$ (730 U/mg) was similar to the activity of the fVa^{PLASMA} (750 U/mg). The $rfVa^{306A}$, $rfVa^{506Q}$, and $rfVa^{306A/506Q}$ mutants displayed similar specific activities at 1000, 890, and 570 U/mg, respectively. Following activation with α-thrombin, all forms of factor V used in this study produced an M_r = 105,000 heavy chain and an M_r = 74,000 light chain. The heavy chain appears as a doublet in all of our experiments. Following extended incubation of factor V with catalytic amounts of α-thrombin, or after a short incubation of factor V with high concentrations of α-thrombin, the heavy chain of factor Va appears as a doublet following reduction as a result of an additional proteolytic cleavage at the COOH terminus of the cofactor (Kalafatis et al., 1995b; Lu et al., 1995). The effect of this cleavage on factor Va cofactor activity has not been determined.

The potential influence of conditioned media on the APC cleavage sites and inactivation was evaluated using fVa^{PLASMA} in HBS Ca^{2+} +0.5% BSA or in conditioned media. Factor Va^{PLASMA} in the presence of PCPS vesicles displayed identical inactivation profiles during APC digestion in either HBS Ca^{2+} +0.5% BSA or in conditioned media (Fig. 3). Immunoblotting of aliquots removed

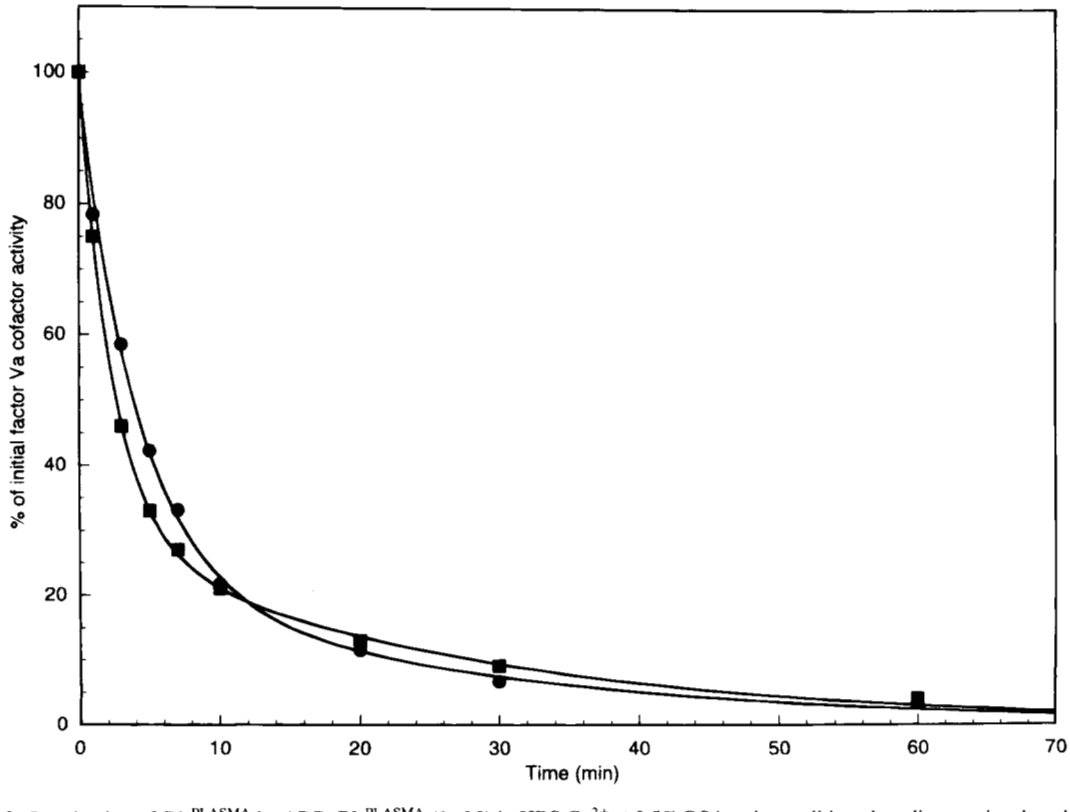

Fig. 3. Inactivation of fVa^{PLASMA} by APC. fVa^{PLASMA} (1 nM) in HBS Ca^{2+} +0.5% BSA or in conditioned media were incubated with PCPS vesicles (20 μM) for 5 min at 25 °C. The activity of factor Va was measured in a clotting time-based assay using factor V-deficient plasma. APC (0.1 nM) was then added. At selected time intervals aliquots were assayed for cofactor activity. Results are expressed as percent of initial cofactor activity as a function of time following APC addition. (filled circles), fVa^{PLASMA} control in HBS Ca^{2+}; (filled squares) fVa^{PLASMA} in conditioned media.

from the reaction mixtures at the corresponding time points displayed identical cleavage products (the appearance of an M_r = 75,000 fragment followed by an M_r = 30,000 fragment) (data not shown). These data suggest that there is no influence on activity that can be attributed to species present in the conditioned media and validate the use of recombinant factor Va mutants in conditioned media to evaluate the contribution of specific APC cleavages in the inactivation of the cofactor.

Following a 60-min incubation with PCPS vesicles in the absence of APC, fVa^{PLASMA}, $rfVa^{WT}$, $rfVa^{306A}$, $rfVa^{506Q}$, and $rfVa^{306A/506Q}$ retain 80–100% of the initial cofactor activity (data not shown) indicating that all forms of the cofactor used in the present study display approximately the same level of stability when prepared as described above. These data also indicate that there is no proteolysis of the cofactor associated with the conditioned media.

In the presence of PCPS vesicles and APC, fVa^{PLASMA} and $rfVa^{WT}$ were inactivated and displayed similar cleavage patterns. However, recombinant fVa^{WT} was inactivated at a slower rate than fVa^{PLASMA}. Factor Va^{PLASMA} and $rfVa^{WT}$, respectively, retain 40 and 70% of the initial cofactor activity after 5 min of incubation in the presence of PCPS and APC (Fig. 4, filled diamonds and open squares). In both cases loss of activity coincides with the appearance of the M_r = 75,000 fragment (residues 1–506) after 1–3 min of APC digestion and a decrease in the amount of heavy chain (M_r = 105,000) (Fig. 5A). The $rfVa^{WT}$ retained 60 and 40% of the initial cofactor activity after 10 and 20 min, respectively, whereas fVa^{PLASMA} retained 25 and 10% of the initial activity at equivalent time points (Fig. 4). This loss in activity coincides with the appearance of the M_r = 30,000 fragment (residues 307–506), which can be seen after 5 min of $rfVa^{WT}$ digestion with APC (Fig. 5A). The M_r = 75,000 fragment begins to disappear after 30 min as a result of cleavage at Arg^{306}. Following 90 min of APC digestion, fVa^{PLASMA} and $rfVa^{WT}$ retained 5–10% of the initial cofactor activity (Fig. 4). After 90 min of incubation in the presence of APC, cleavage of the $rfVa^{WT}$ heavy chain has not gone to completion (Fig. 5A). The residual heavy chain and/or partial activity of the M_r = 75,000 fragment may account for the remaining cofactor activity (Fig. 4). The nature of the discrepancy in the rate of inactivation seen using $rfVa^{WT}$ compared to fVa^{PLASMA} may be due to differences in post-translational modification of the recombinant cofactor produced in CHO cells (Fig. 1). Further discussion of the contributions of the specific cleavage sites using mutant species of the cofactor will therefore be compared to the inactivation of the $rfVa^{WT}$ species.

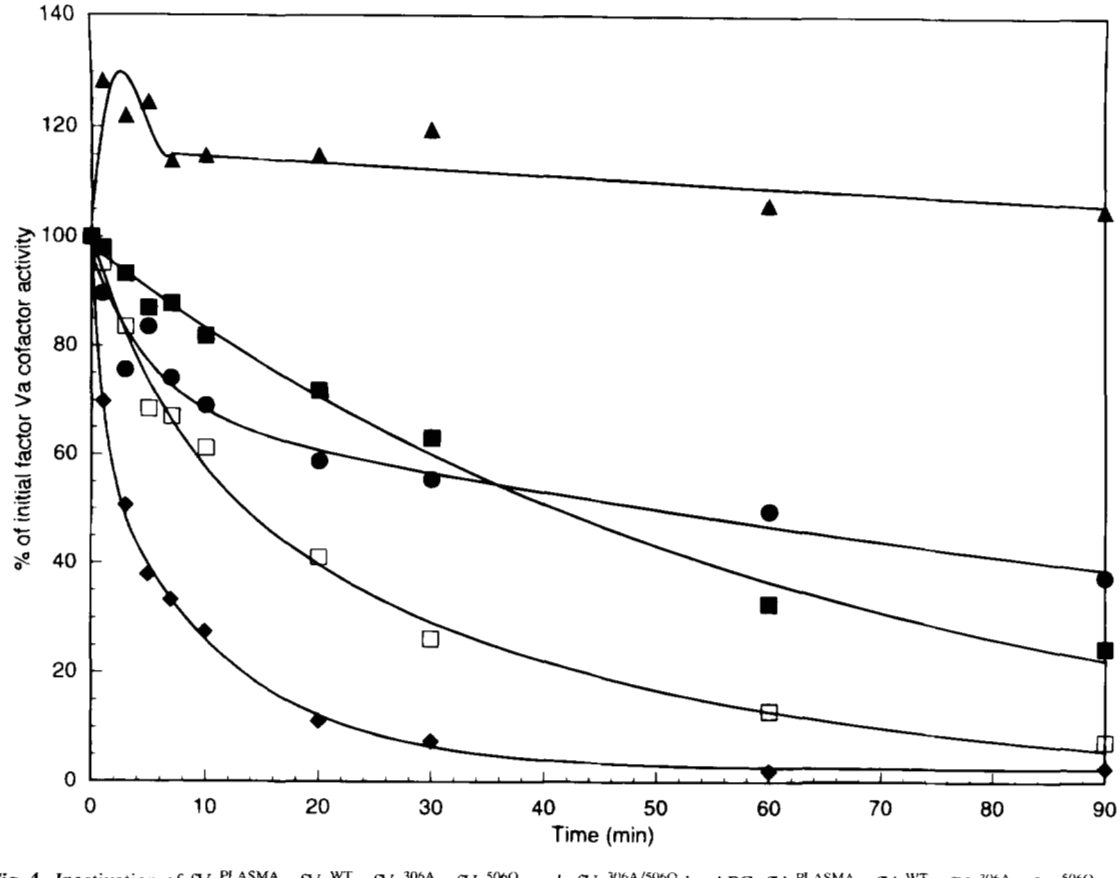

Fig. 4. Inactivation of fVa^{PLASMA}, $rfVa^{WT}$, $rfVa^{306A}$, $rfVa^{506Q}$, and $rfVa^{306A/506Q}$ by APC. fVa^{PLASMA}, $rfVa^{WT}$, $rfVa^{306A}$, $rfVa^{506Q}$, and $rfVa^{306A/506Q}$ (1 nM) were incubated with PCPS vesicles (20 μM) for 5 min at 25 °C. The activity of factor Va was measured in a clotting time-based assay using factor V-deficient plasma. APC (0.1 nM) was then added. At selected time intervals aliquots were assayed for cofactor activity. Results are expressed as percent of initial cofactor activity as a function of time following APC addition. At the same time intervals aliquots were withdrawn and analyzed by SDS-PAGE and shown in Figures 5, 6, and 7. (Open squares) $rfVa^{WT}$ with APC; (filled squares) $rfVa^{506Q}$ in the presence of APC; (filled circles) $rfVa^{306A}$ with APC; (filled triangles) $rfVa^{306A/506Q}$ with APC; (filled diamonds) fVa^{PLASMA} in the presence of APC.

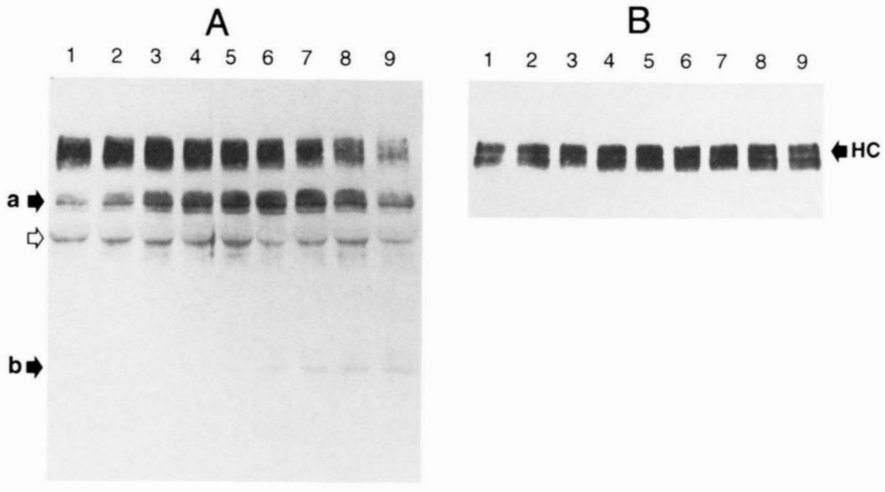

Fig. 5. Cleavage of $rfVa^{WT}$ and $rfVa^{306A/506Q}$. $rfVa^{WT}$ and $rfVa^{306A/506Q}$ were incubated with APC as described in the legend to Figure 4. At selected time intervals aliquots were analyzed on 5–15% linear gradient SDS-PAGE gels. Following transfer to nitrocellulose, fragments were visualized using monoclonal antibody $\alpha HFVa_{HC}$#6. **A:** Lane 1, $rfVa^{WT}$ with no APC; lanes 2–9, $rfVa^{WT}$ with APC at 1, 3, 5, 7, 10, 20, 30, and 60 min. **B:** Lane 1, $rfVa^{306A/506Q}$; no APC, control; lanes 2–9, same time points as in A. The position of the heavy chain of the cofactor (HC) is indicated at right of B. The position of the heavy chain fragments derived from factor Va following APC digestion are shown by the letters a and b to the left of A (a, M_r = 75,000 fragment containing amino acid residues 1–506; b, M_r = 30,000 fragment containing amino acid residues 307–506). The open arrowhead at the left of A indicates a component present in the conditioned media that migrates at the same level as albumin and reacts with the secondary antibody. This component is also visible in Figure 6.

In our studies using a clotting assay to test factor Va cofactor activity at low concentrations of factor Va (1 nM) in the presence of APC (0.1 nM), it is possible that some of the decrease in cofactor activity associated with added APC could be due to formation of a non-covalent APC–fVa complex (Nesheim et al., 1982; Krishnaswamy et al., 1986). Formation of a non-covalent APC–fVa complex would inhibit formation of the *prothrombinase* complex at low factor Xa concentrations (i.e., in the clotting assay), thereby potentially reporting the loss of factor Va cofactor activity that is not associated with cleavage by APC. Recombinant fVa^{WT} in the presence of PCPS vesicles and active site-blocked APC (EGRck–APC) retained 85% of the initial cofactor activity after one hour (data not shown). Gel electrophoresis and immunoblot analysis of $rfVa^{WT}$ incubated with EGRck–APC demonstrated no proteolysis of the cofactor (data not shown). These data indicate that the major loss in cofactor activity of factor Va, when incubated with PCPS vesicles and APC at the concentrations used in this study, can be directly attributed to the proteolytic activity of APC on factor Va and is not the result of impaired *prothrombinase* assembly attributable to the inhibition of factor Va-factor Xa interaction by non-covalent APC binding.

In the presence of APC and PCPS vesicles, $rfVa^{306A/506Q}$ consistently displayed an initial increase in cofactor activity. This increase in cofactor activity in the absence of cleavage sites at Arg^{306} and Arg^{506} suggests that cleavage of factor Va at Arg^{679} by APC may result in a cofactor with increased activity, as previously suggested (Kalafatis et al., 1996). APC cleavage at Arg^{679} may occur rapidly in the $rfVa^{306A/506Q}$ molecule generating an APC-resistant species due to the absence of the cleavage sites at Arg^{306} and Arg^{506}. The $rfVa^{306A/506Q}$ mutant retained 100% of the initial cofactor activity following a 90-min incubation (Fig. 4, filled triangles). Immunoblotting did not reveal any proteolytic degradation products of the cofactor, and no disappearance of the heavy chain(s) was evident (Fig. 5B). These data indicate that in the absence of the cleavage sites at Arg^{306} and Arg^{506} the cofactor is not inactivated by APC. The data also suggest that cleavage of the cofactor at Arg^{679} may generate a species of factor Va that displays a short-lived increase in cofactor activity. When factor Va is first cleaved at Arg^{679} by APC, the resulting species may display an increase in cofactor activity, whereas when cleavage at Arg^{679} is preceded by cleavage at Arg^{306} or at Arg^{506} the resulting product will display decreased cofactor activity (Kalafatis et al., 1996).

In the presence of PCPS vesicles and APC, inactivation of $rfVa^{506Q}$ occurs at a slower rate compared with $rfVa^{WT}$ (Fig. 4, filled squares and filled diamonds). Recombinant fVa^{506Q} retains 75% of the initial cofactor activity following a 20-min incubation in the presence of APC. This loss in activity coincides with disappearance of the heavy chain and the appearance of a M_r = 62/60,000 doublet (residues 307–709), which is generated as a result of cleavage at Arg^{306} (Fig. 6, fragment c). Prolonged incubation of $rfVa^{506Q}$ with APC resulted in continued loss of cofactor activity; however, the rate of inactivation is decreased when compared to $rfVa^{WT}$. Accumulation of the M_r = 62/60,000 doublet continued throughout the period of APC incubation (Fig. 6). The appearance of a smaller fragment (M_r = 54,000) following 60 min of incubation in the presence of APC suggests that the M_r = 62/60,000 product is also cleaved at Arg^{679} (Fig. 6, fragmented). Following a 90-min incubation in the presence of APC, $rfVa^{506Q}$ retains 25% of its initial cofactor activity (Fig. 4, filled squares). After a 90-min incubation in the presence of APC, the heavy chain has not been quantitatively cleaved to the M_r = 62/60,000 product. The residual heavy chain may account for the remaining cofactor activity. The data indicate that $rfVa^{506Q}$ is inactivated by cleavage of the heavy chain at Arg^{506}/Arg^{679}. These data support the conclusion that cleavage at Arg^{306} is required for complete inactivation of the cofactor but suggest that cleavage at Arg^{306} alone is insufficient for the efficient inactivation of the cofactor (Kalafatis et al., 1994).

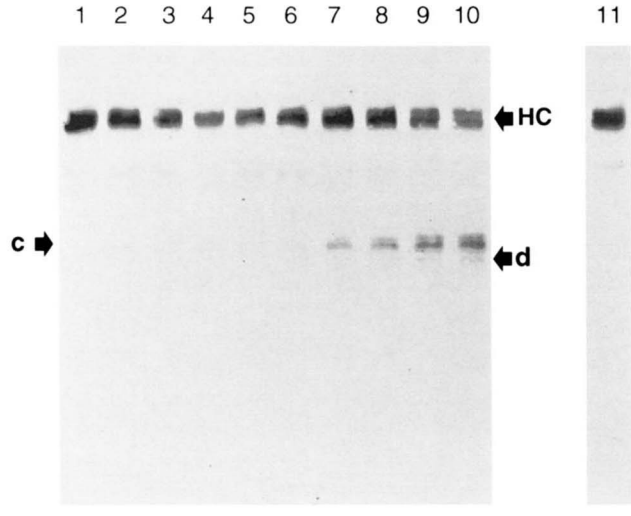

Fig. 6. Cleavage of $rfVa^{506Q}$. $rfVa^{506Q}$ was incubated with APC in the presence or absence of 20 μM PCPS as described in the legend to Figure 4. At selected time intervals aliquots were analyzed on 5–15% linear gradient SDS-PAGE gels. Following transfer to nitrocellulose, fragments were visualized using monoclonal antibody $\alpha HFVa_{HC}$#6. Lane 1, $rfVa^{506Q}$ with no APC, +PCPS; lanes 2–10, $rfVa^{506Q}$ with APC, +PCPS at 1, 3, 5, 7, 10, 20, 30, 60, and 90 min; lane 11, $rfVa^{506Q}$ with APC, −PCPS at 90 min. The position of the heavy chain of the cofactor (HC) is indicated. The position of the heavy chain fragments derived from factor Va following APC digestion are shown by the letters c and d (c, M_r = 62/60,000 doublet containing amino acid residues 307–709, d, M_r = 54,000 fragment containing amino acid residues 307–679).

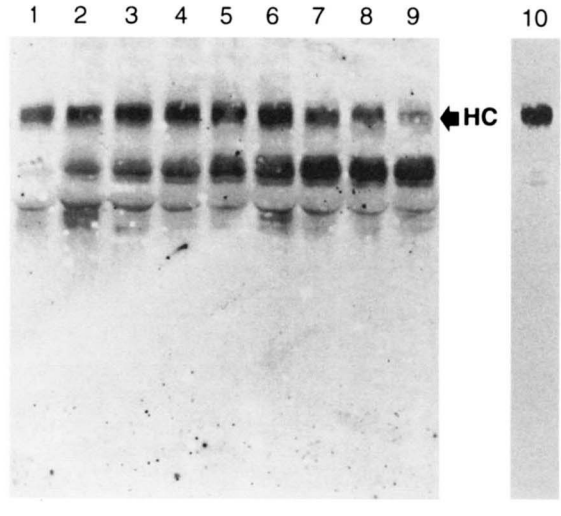

Fig. 7. Cleavage of $rfVa^{306A}$. $rfVa^{306A}$ was incubated with APC in the presence or absence of 20 μM PCPS as described in the legend to Figure 4. At selected time intervals aliquots were analyzed on 5–15% linear gradient SDS-PAGE gels. Following transfer to nitrocellulose, fragments were visualized using monoclonal antibody $\alpha HFVa_{HC}$#6. Lane 1, $rfVa^{306A}$ with no APC, +PCPS; lanes 2–9, $rfVa^{306A}$ with APC, +PCPS at 1, 3, 5, 7, 10, 20, 30, and 60 min. Lane 10, $rfVa^{306A}$ with APC, −PCPS at 60 min. The position of the heavy chain of the cofactor (HC) is indicated. The position of the heavy chain fragment derived from factor Va following APC digestion is shown by the letter a (as in Fig. 5).

In the presence of PCPS vesicles and APC, inactivation of $rfVa^{306A}$ occurs initially at a rate similar to $rfVa^{WT}$ (Fig. 4, filled circles and filled diamonds). The $rfVa^{306A}$, like $rfVa^{WT}$, retains 80% of the initial cofactor activity following a 3-min incubation in the presence of APC. This loss in activity coincides with a decrease in the amount of heavy chain present and an increase in the amount of an M_r = 75,000 fragment, which is generated as a result of cleavage at Arg^{506} (Fig. 7, fragment a). Prolonged incubation of $rfVa^{306A}$ with APC resulted in continued loss of cofactor activity; however, the rate of inactivation is decreased when compared to $rfVa^{WT}$. The slower rate of inactivation seen in $rfVa^{306A}$ after 3 min may be the result of cleavage at Arg^{679}. Accumulation of the M_r = 75,000 fragment continued throughout the period of APC incubation (Fig. 7). Following a 90-min incubation in the presence of APC, $rfVa^{306A}$ retains 40% of its initial cofactor activity (Fig. 4, filled circles). The heavy chain has not been completely cleaved following a 90-min incubation in the presence of APC (Fig. 7); however, the remaining cofactor activity (40% of initial cofactor activity) (Fig. 4) cannot be attributed to the remaining heavy chain because less than 10% of heavy chain remains at the end of the digestion (Fig. 7, lane 9). These data suggest, as previously described (Kalafatis et al., 1994), that the M_r = 75,000 fragment retains partial activity. The extent of cofactor activity of the M_r = 75,000 fragment is dependent on the assay used to measure cofactor activity (Kalafatis & Mann, 1997). Overall, the data indicate that $rfVa^{306A}$ is only partially inactivated by cleavage of the heavy chain at Arg^{506}/Arg^{679}. These data support the conclusion that cleavage at Arg^{306} is required for complete inactivation of the cofactor (Kalafatis et al., 1994).

The $rfVa^{306A}$ and $rfVa^{506Q}$ mutants were also incubated with APC (0.1 nM) in the absence of PCPS vesicles. Under these conditions there was no loss in activity over the 90-min incubation period (data not shown) and no cleavage of the cofactor at Arg^{506} or at Arg^{306} was evident (Figs. 6 lane 11 and 7 lane 10). These data indicate that at low concentrations of APC (0.1 nM) and factor Va (1 nM), cleavage of the cofactor at both Arg^{306} and at Arg^{506} requires the presence of a phospholipid surface.

Apparent second-order rate constants were calculated for the rates of inactivation and are shown in Table 1. Inactivation of fVa^{PLASMA} occurred at a faster rate compared to $rfVa^{WT}$. The initial rate of APC inactivation for $rfVa^{WT}$ and $rfVa^{306A}$ were nearly identical (2.3×10^7 M^{-1} s^{-1} versus 3.0×10^7 M^{-1} s^{-1}), consistent with previous work from our laboratory reporting that initial cleavage of factor Va by APC occurs at Arg^{506} (Kalafatis et al., 1994). Cleavage at Arg^{306} in $rfVa^{506Q}$ occurs at a 10-fold slower

Table 1. *Apparent second-order rate constants for the rates of factor Va inactivation by APC*

Factor Va species	Cleavage	M^{-1} s^{-1}
Va^{PLASMA}	Arg^{506}	1.5×10^8
	Arg^{306}	1.3×10^7
rVa^{WT}	Arg^{506}	2.3×10^7
	Arg^{306}	4.3×10^6
rVa^{306A}	Arg^{506}	3.0×10^7
rVa^{506Q}	Arg^{306}	2.5×10^6

rate compared to cleavage at Arg^{506} in $rfVa^{306A}$ or in $rfVa^{WT}$ (2.5×10^6 M^{-1} s^{-1} versus 2.3×10^7 M^{-1} s^{-1} and 3.0×10^7 M^{-1} s^{-1}). This finding is consistent with previous reports from our laboratory and others demonstrating that APC inactivation of factor Va^{Leiden} ($Arg^{506} \rightarrow Gln$) occurs at a slower rate than that observed for normal factor Va (Kalafatis et al., 1995a; Rosing et al., 1995). Cleavage at Arg^{306} in $rfVa^{WT}$ occurs at an approximately twofold faster rate compared to cleavage at Arg^{306} in $rfVa^{506Q}$ (4.3×10^6 M^{-1} s^{-1} versus 2.5×10^6 M^{-1} s^{-1}), suggesting that cleavage at Arg^{306} may occur, as suggested, at a faster rate when the cofactor has previously been cleaved at Arg^{506} (Kalafatis et al., 1995a). In the absence of the cleavage sites at Arg^{306} and Arg^{506} ($rfVa^{306A/506Q}$) inactivation of factor Va occurs at a rate 100-fold slower than that found for the wild type. Similarly, the inactivation of $rfVa^{WT}$ by active site blocked-APC (EGRck–APC) occurred at a rate similar to that seen for the double mutant.

For $rfVa^{WT}$, the lipid-dependent cleavage at Arg^{306} initially proceeds more slowly than cleavage at Arg^{506}, resulting in the sequential appearance of unique products at M_r = 75,000 and M_r = 30,000. The resulting products of intermediate cleavage of factor Va will bind factor Xa to form a functional *prothrombinase* complex; however, because each of the sequential cleavages results in lower affinity for this protease, the ultimate display of activity depends on the concentrations of factor Va and factor Xa used in the assay system. The most sensitive assays of factor Va function are clotting assays in which limited amounts of factor Va combine with factor Xa (≤10 pM), generated during the assay, to produce the thrombin necessary for clot formation. Because these assays involve the generation of factor Xa and *prothrombinase* complex formation, they will strongly reflect significant changes in the K_{diss} of factor Va for factor Xa (Rand et al., 1996).

Effect of protein S

The effect of protein S on factor Va inactivation by APC was evaluated using $rfVa^{WT}$, $rfVa^{306A}$, $rfVa^{506Q}$, and $rfVa^{306A/506Q}$. Recombinant fVa^{WT} and $rfVa^{306A}$ were inactivated by APC with similar rates in the presence or absence of protein S (490 nM) (Fig. 8A, D). The initial loss in cofactor activity for $rfVa^{WT}$ and $rfVa^{306A}$ is a result of cleavage of the factor Va heavy chain at Arg^{506}. The initial rate of inactivation of $rfVa^{506Q}$ by APC is increased when protein S is present in the reaction, suggesting

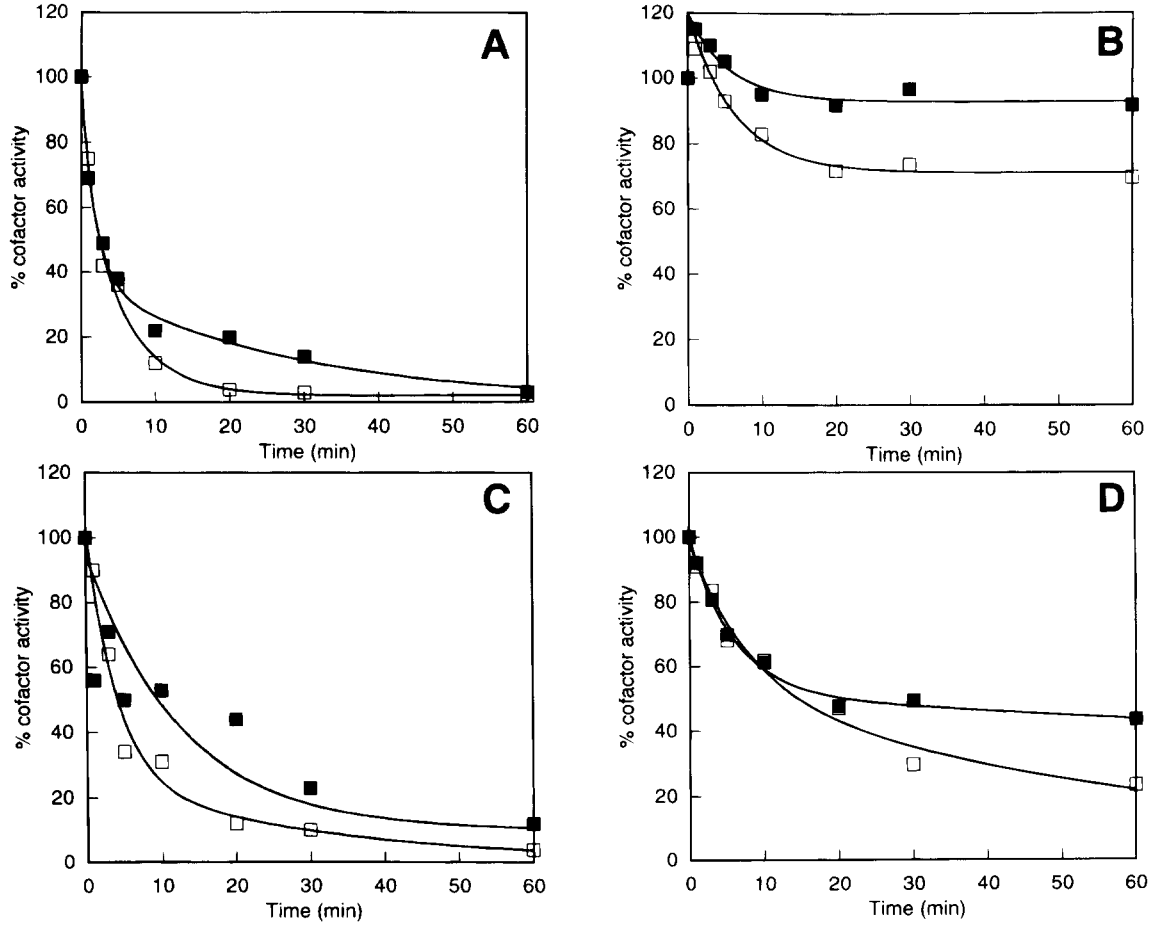

Fig. 8. Inactivation of $rfVa^{WT}$, $rfVa^{306A}$, $rfVa^{506Q}$, and $rfVa^{306A/506Q}$ by APC in the presence of protein S. $rfVa^{WT}$, $rfVa^{306A}$, $rfVa^{506Q}$, and $rfVa^{306A/506Q}$ were incubated with PCPS vesicles (20 μM) for 5 min at 25 °C in the presence or absence of protein S (490 nM). The activity of factor Va was measured in a clotting time-based assay using factor V-deficient plasma. APC (0.1 nM) was then added. At selected time intervals aliquots were assayed for cofactor activity. Results are expressed as percent of initial cofactor activity as a function of time following APC addition. (**A**) $rfVa^{WT}$; (**B**) $rfVa^{306A/506Q}$; (**C**) $rfVa^{506Q}$; (**D**) $rfVa^{306A}$. Filled squares, control, no protein S; open squares, Protein S (490 nM).

Table 2. *Apparent second-order rate constants for the initial rates of factor Va inactivation by APC in the presence or absence of Protein S*

Factor Va species	$M^{-1}\ s^{-1}$
rVa^{WT} − Protein S	8.6×10^7
rVa^{WT} + Protein S	1.4×10^8
$rVa^{306A\backslash 506Q}$ − Protein S[a]	3.1×10^7
$rVa^{306A\backslash 506Q}$ + Protein S[a]	2.7×10^7
rVa^{506Q} − Protein S	1.3×10^7
rVa^{506Q} + Protein S	4.2×10^7
rVa^{306A} − Protein S	2.8×10^7
rVa^{306A} + Protein S	2.1×10^7

[a]Represents ~20% loss in cofactor activity (see Fig. 8B, initial 5 min).

cleavage at Arg^{306} is accelerated (Fig. 8C). Apparent second-order rate constants calculated for the initial rates of APC inactivation in the presence and absence of protein S are presented in Table 2. Our data suggest that cleavage at Arg^{506} is not affected by the presence of protein S, whereas cleavage at Arg^{306} occurs at an approximately threefold faster rate in the presence of protein S. These data are consistent with our previous findings and qualitatively with work by Rosing et al. showing an increased rate of cleavage at Arg^{306} in the presence of protein S (Kalafatis & Mann, 1993; Rosing et al., 1995; Lu et al., 1996). However, our previous and present data show only a two-to threefold increase in the rate of cleavage at Arg^{306} in the presence of protein S when using fVa^{PLASMA} or $rfVa^{506Q}$. In contrast, Rosing et al. reported a 20-fold increase in the rate of factor Va inactivation by APC (normal factor Va or factor Va^{Leiden}) in the presence of protein S. A 20-fold increase in the rate of factor Va cleavage at Arg^{306} would essentially eliminate the effect of the factor V^{Leiden} mutation resulting in cofactor inactivation at a rate similar to that seen using normal factor Va. Recombinant $fVa^{306A/506Q}$ in the presence of APC displays an initial increase followed by a small decrease in cofactor activity. In the presence of protein S, the decrease in cofactor activity occurs at a faster rate (Fig. 8B). The absence of any other known APC cleavage sites in this factor Va molecule suggests that the APC cleavage at Arg^{679} may also be accelerated in the presence of protein S.

Discussion

From the data presented in this study and from previous work, we can conclude that inactivation of factor Va occurs in the following sequence:

$$Va \xrightarrow{k_{506}} Va_{506} \xrightarrow{k_{306}} Va_{306/506} \xrightarrow{k_{679}} Va_{306/506/679}$$

we can further conclude that

$$k_{506} > k_{306}$$

and under conditions where

$$k_{306} = k_{506} = 0$$

inactivation of factor Va by APC occurs at a rate equivalent to the rate of inactivation in the absence of APC.

The $rfVa^{WT}$ used in our experiments was inactivated at a slower rate compared to fVa^{PLASMA}. There was no significant difference in the specific activities of the two species of the cofactor (750 U/mg versus 724 U/mg, respectively) that could account for this discrepancy. The difference in the rate of inactivation may be attributable to alterations in the post-translational modification of the recombinant cofactor produced in CHO cells. Post-translational modifications of factor V purified from human plasma have been previously described and include phosphorylation (Mann & Fish, 1972; Kalafatis et al., 1993b), sulfation (Hortin, 1993), and glycosylation (Bruin et al., 1987; Jenny et al., 1987) of various amino acid residues of the procofactor (Fig. 1). Factor Va, which has been phosphorylated on the heavy chain by a platelet casein kinase II like kinase, is inactivated threefold faster by APC than the nonphosphorylated cofactor (Kalafatis, 1996). The phosphorylation states of the natural and recombinant forms of factor Va used in the present study have not been investigated.

Heeb et al. (1995), using similar conditions (200 pM fVa/0.1 nM APC), reported nearly identical levels of inactivation of fVa^{PLASMA} as obtained in our study using $rfVa^{WT}$. Recombinant fVa^{506Q} in the present experiments also displayed inactivation rates similar to those seen in studies using plasma purified fVa^{Leiden} (Heeb et al., 1995; Kalafatis et al., 1995a; Aparicio & Dahlbäck, 1996). Recombinant fVa^{506Q} and fVa^{Leiden} showed a similar degree of resistance to APC, retaining 30% of the initial cofactor activity following a 90-min incubation of the cofactor with APC, and PCPS vesicles.

Our data indicate that in the absence of PCPS vesicles, at low concentrations of APC (0.1 nM) and factor Va (1 nM), the cleavages at Arg^{506} and Arg^{306} both require the presence of a phospholipid surface. Previous data from our laboratory have shown that while cleavage of the cofactor at Arg^{506} is accelerated by PCPS (Kalafatis & Mann, 1993), it still occurs in the absence of a phospholipid surface, whereas cleavage at Arg^{306} requires the presence of a membrane surface (Kalafatis et al., 1994). The present data are consistent with these observations.

Data using EGRck–APC reveal essentially no inactivation by active site-blocked APC. Thus, under the experimental conditions used (0.1 nM APC), non-covalent APC binding does not influence *prothrombinase* assembly by competing with factor Xa for factor Va binding (Nesheim et al., 1982; Krishnaswamy et al., 1986). This result is predictable because the APC concentration used in this study was 100 times lower than the dissociation constant of APC for factor Va (K_d = 10 nM, Krishnaswamy et al., 1986). Hence, the major loss in cofactor activity in the presence of APC is solely attributable to proteolysis of the cofactor at specific cleavage sites.

It is noteworthy that previous data have demonstrated limited activity loss following cleavage of the cofactor by APC at Arg^{506} and Arg^{679} in an assay that measures thrombin formation and utilizes saturating concentrations of factor Xa (Odegaard & Mann, 1987; Kalafatis et al., 1994). A recent report by Nicolaes et al. (1995) demonstrates that the Arg^{506}/Arg^{679} cleaved cofactor has diminished binding capabilities for factor Xa. Therefore, at limiting factor Xa concentrations (i.e., as reported by clotting assay) cleavage at Arg^{506}/Arg^{679}, which appears to account for the loss in factor Va cofactor activity (Suzuki et al., 1983), represents a decrease in the affinity of factor Va for factor Xa within *prothrombinase* rather than a total loss in the catalytic efficiency of the *prothrombinase* complex.

Recently, Rosing et al. (1995), using a *prothrombinase* assay employing near saturating concentrations of factor Xa (5 nM),

reported a 20-fold increase in the rate of APC inactivation of normal factor Va and factor Va^{Leiden} in the presence of protein S (490 nM) due to acceleration of the cleavage at Arg^{306}. The physiological significance of this finding is somewhat questionable, given that acceleration of the Arg^{306} cleavage by 20-fold would essentially correct the defect in factor V^{Leiden}, resulting in cofactor inactivation at a rate similar to that seen using normal factor Va. It is noteworthy that the first case of factor V^{Leiden}, which was identified because of resistance to APC inactivation, was shown to have normal levels of protein S (Dahlback et al., 1993). Our present study, using a clotting assay, demonstrates that the rate of APC cleavage at Arg^{306} in factor Va is enhanced by approximately threefold in the presence of protein S. These data are in agreement with recent reports demonstrating a twofold increase in the inactivation rate of factor Va by APC in the presence of protein S (Kalafatis & Mann, 1993; Lu et al., 1996). Because the accelerating effect of protein S is only observed on the cleavage at Arg^{306}, the data demonstrate that in a clotting assay inactivation by cleavage at Arg^{306} may be underscored by the effect of cleavage at Arg^{506}, which occurs faster and results in a considerable decrease in the affinity of factor Va for factor Xa (Kalafatis et al., 1994; Rosing et al., 1995; Rand et al., 1996). As a conclusion, the effect of protein S on cleavage at Arg^{306} may not be as dramatic when cofactor activity is measured in a clotting assay compared to the effect of protein S on APC inactivation when cofactor activity is measured in a *prothrombinase* assay using saturating concentrations of factor Xa.

The present study demonstrates the effect of APC cleavage at each site on factor Va inactivation. We have constructed $Arg^{306} \rightarrow$ Ala and $Arg^{506} \rightarrow$ Gln mutations of human factor V and expressed recombinant wild-type, $Arg^{506} \rightarrow$ Gln, $Arg^{306} \rightarrow$ Ala, and $Arg^{306} \rightarrow$ Ala/$Arg^{506} \rightarrow$ Gln factor V proteins in Chinese hamster ovary (CHO) cells. This study also reports the kinetic parameters for mutant factor Va^{306A} inactivation by APC. Although previous data have characterized inactivation of natural factor fVa^{506Q} (Va^{Leiden}) (Kalafatis et al., 1995a), the present report investigates inactivation of a mutant factor V molecule with an amino acid substitution at Arg^{306}. The study using the double mutant ($rfVa^{306A/506Q}$) demonstrates the high specificity of APC cleavage sites on the factor Va molecule because no other APC cleavages in the double mutant were observed. The data suggest that, in the absence of cleavage at Arg^{306} or Arg^{506}, APC cleavage of factor Va at Arg^{679} does not contribute significantly to the inactivation of the cofactor. The present study also illustrates the lipid dependence of APC cleavage of factor Va at both Arg^{306} and Arg^{506}. Under the conditions employed, no cleavage of the cofactor occurs in the absence of a membrane surface. Cleavage at Arg^{506} has been reported in the absence of a membrane surface (Kalafatis & Mann, 1993), the present data demonstrate that at low APC the presence of a membrane surface is required for cleavage of the cofactor. This observation may be important in the in vivo situation in which limited membrane surfaces are available.

Overall our data demonstrate high specificity with respect to cleavage sites for APC on factor Va, and show that cleavage at both Arg^{306} and Arg^{506} are required for efficient factor Va inactivation. Wei et al. (1996) have reported that a minimum of two cleavages must occur on factor Va for inactivation. Therefore, the mechanism of inactivation of factor Va by APC may be similar to the mechanism by which factor VIIIa losses cofactor activity (Lollar & Parker, 1990; Fay et al., 1991).

Materials and methods

Materials and reagents

HEPES, methotrexate, 1-palmitoyl-2-oleoyl-phosphatidylserine (PS), 1-palmitoyl-2-oleoyl-phosphatidylcholine (PC), diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), and ϵ-amino-n-caproic acid (EACA) were purchased from Sigma (St. Louis, MO). Acrylamide, bis-acrylamide, TEMED, ammonium persulfate, and nitrocellulose were purchased from Bio Rad (Richmond, CA). Glutamylglycinylarginyl chloromethyl ketone (EGRck) and human APC were obtained as described previously (Kisiel, 1979; Nesheim et al., 1979a) and were gifts of Haematologic Technologies Inc. (Essex Junction, VT). α-Thrombin, factor V, and protein S were purified from human plasma as previously described (Bajaj & Mann, 1973; Lundblad et al., 1976; Katzmann et al., 1981; Dahlback, 1983; Kalafatis et al., 1993a). EGRck–APC was prepared as previously described (Krishnaswamy et al., 1986; Williams et al., 1989). The α-thrombin inhibitor, hirudin, was obtained from Genentech (South San Francisco, CA). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). α-MEM, Opti-MEM, and DME-F12 culture media, Lipofectin, agarose, T4 DNA Ligase, and calf intestinal alkaline phosphatase were purchased from Gibco BRL (Gaithersburg, MD). HyQ-CCM-5 and fetal bovine serum were purchased from Hyclone Laboratories Inc. (Logan, UT). Oligonucleotides were synthesized by Macromolecular Resources (Fort Collins, CO). Simplastin Excel was purchased from Organon Teknika Corp. (Durham, NC). Phospholipid vesicles composed of 75% PC and 25% PS were prepared as previously described (Barenholz et al., 1997) and the concentration was determined by phosphorous assay. Plasmid pBluescript KS(+) was purchased from Stratagene (La Jolla, CA). Plasmid pED-V was obtained from Debra Pittman (Genetics Institute, Cambridge, MA). The chemiluminescent substrate, Luminol, was purchased from DuPont NEN (Boston, MA). Restriction endonuclease digestions, ligation reactions, and dephosphorylation reactions were performed in the buffers provided using protocols recommended by the manufacturers. Goat anti-mouse IgG peroxidase was purchased from Southern Biotech (Birmingham, AL). CHO cells were grown at 37° in 10% CO_2. All plasmids were propagated in DH5α competent cells following transformation protocols recommended by the manufacturer (Gibco BRL). Plasmid DNA was isolated using Qiagen plasmid purification columns (Chatsworth, CA).

Plasmid construction

The factor V cDNA sequence was cleaved from pED-V by the restriction endonuclease *Sal* I and ligated to *Sal* I-digested dephosphorylated pBluescript KS(+) to make plasmid pBSV. The $Arg^{306} \rightarrow$ Ala and $Arg^{506} \rightarrow$ Gln mutant DNA fragments were constructed using the PCR based method previously described by Nelson and Long (1989) with the oligonucleotide primers listed in Table 3. The $Arg^{306} \rightarrow$ Ala mutant fragment was cleaved with *Xcm* I and *Bsu*36 I and ligated to *Xcm* I/*Bsu*36 I cleaved pBSV to make plasmid $pBSV^{306A}$. The $Arg^{506} \rightarrow$ Gln mutant fragment was cleaved with *Bsu*36 I and *Tth*111 I and ligated to *Bsu*36 I/*Tth*111 I cleaved pBSV to make plasmid $pBSV^{506Q}$. The $Arg^{306} \rightarrow$ Ala/ $Arg^{506} \rightarrow$ Gln double mutant was constructed by ligating the *Xcm* I/*Bsu*36 I cleaved $Arg^{306} \rightarrow$ Ala mutant DNA fragment to *Xcm* I/*Bsu*36 I cleaved pBSV506Q to make plasmid $pBSV^{306A/506Q}$. The mutant factor V cDNA sequences were then cleaved from plasmids

Table 3. *Oligonucleotide primers used for mutagenesis*

Primer	Sense[a]	cDNA position[b]	Sequence[c]
A^{306}	Forward	1,083–1,101	5′ GAAAACC**GC**GAATCTTAAG 3′
B^{306}	Inverse	1,497–1,517	5′ *GGGGTACTAGTAACCCGGGC*TGAACTGCTCTGATCATGGT 3′
C^{306}	Forward	941–960	5′ CAGCCATCACCCTTGTCAGT 3′
A^{506}	Forward	1,682–1,691	5′ TGGACAGG**CA**AGGAATACAG 3′
B^{506}	Inverse	1,954–1,972	5′ *GGGGTACTAGTAACCCGGGC*CAGTGAAGTGGATGGTCAA 3′
C^{506}	Forward	1,417–1,435	5′ TATAGCATTTACCCTCATG 3′
D	Inverse	None	5′ *GGGGTACTAGTAACCCGGGC* 3′

[a]Primer sense in relation to human factor V cDNA coding strand.
[b]Numbering of Jenny et al. (1987).
[c]Boldface type indicates substituted codon. Underlined bases indicate the position of directed mutations. Italics indicate xeno sequence.

$pBSV^{306A}$, $pBSV^{506Q}$, and $pBSV^{306A/506Q}$ with the restriction endonuclease *Sal* I and ligated to *Sal* I cleaved dephosphorylated pED (Kaufman et al., 1991) to create plasmids $pEDV^{306A}$, $pEDV^{506Q}$, and $pEDV^{306A/506Q}$. All mutations were confirmed by sequencing using a Taq DyeDeoxy Terminator Cycle Sequencing kit (ABI) and an ABI 373A DNA sequencer.

Cell culture and transfection

CHO DUKX (Urlaub & Chasin, 1980) cells were maintained in DME-F12 with 10% FBS. Cells were seeded at a density of 5×10^5 cells per 10 cm culture dish 24 h prior to transfection. Immediately prior to transfection 6 mg of plasmid DNA was suspended in 300 mL of Opti-MEM media. In a separate tube 20 mL of Lipofectin was suspended in 300 mL of Opti-MEM. The DNA/Lipofectin solutions were combined and incubated at room temperature for 15 min. The cells were washed with 10 mL of Opti-MEM immediately preceding transfection. Opti-MEM (5.4 mL) was added to the DNA/Lipofectin mix and the solution was added to the cells. Cells were incubated for 16–20 h then washed with 10 mL DME-F12 10% FBS. After 48 h the cells were split 1:4 and grown in α-MEM selection media with 10% FBS. The cells were grown until colonies were present, at which time colonies were isolated and propagated in 24-well culture plates. When the cells reached confluence they were transferred to six-well plates, at which time the supernatants were assayed for factor V production by immunoblot using a polyclonal antibody for factor V (Katzmann et al., 1981). Clones producing factor V were subjected to further selection by growth in the presence of 0.02 or 0.1 mM methotrexate. Following selection, cells were maintained in α-MEM with 10% FBS. The specific activity of recombinant forms of factor Va were estimated using concentrations obtained using a radioimmunoassay for factor V and activity measurements determined in clotting assays using factor V-deficient plasma. All factor V clones used in this study produced 0.5–3 μg/mL of recombinant factor V. These levels of expression are similar to those previously reported for stable and transient transfectants of human factor V. Specific activity of $rfVa^{WT}$ (724 U/mg) was similar to the activity of the fVa^{PLASMA} (750 U/mg). The $rfVa^{306A}$, $rfVa^{506Q}$, and $rfVa^{306A/506Q}$ mutants displayed similar specific activities at 1,000, 886, and 568 U/mg, respectively.

Preparation of recombinant factor V

CHO cells producing wild-type or mutant factor V were grown to confluence in 10 cm culture dishes. The cells were washed twice with 5 mL of HyQ-CCM-5 serum-free media then grown in 3 mL of HyQ-CCM-5 for 24 h. The media was collected and centrifuged at 10,000 $\times$ *g* for 20 min. The supernatant was removed and incubated with 1 mM DFP, 1 mM PMSF, 1 mM EACA, and 10 μM FPR-ck for 30 min at 25° to inhibit potential proteolysis and inactivation by proteases that may be present in the culture media. The media was then dialyzed twice against 4 L of HBS Ca^{2+} (20 mM HEPES, 0.15 M NaCl, 5 mM $CaCl_2$, pH = 7.4). Conditioned media were collected in the same manner from confluent dishes of CHO DUKX cells.

Factor V/Va proteolysis by thrombin and APC

Recombinant factor V was activated to factor Va by α-thrombin (0.44 U/ml, ~4.5 nM). Following a 5-min incubation at 25°, activity of the α-thrombin was inhibited by the addition of 10 nM hirudin and factor Va cofactor activity was measured in a clotting assay using factor V-deficient plasma as described (Nesheim et al., 1981). Briefly, a standard curve was constructed using serial dilutions of normal pooled plasma, assuming that normal plasma contains 1 U/ml of factor V (20 nM). All conditioned media supernatants were tested for cofactor activity following α-thrombin activation. All recombinant species of factor Va were adjusted to the level of cofactor activity of the cell line producing the lowest amount of factor V ($rfVa^{306A}$) by the addition of conditioned media. Under these conditions the starting concentration of all factor V species was 1 nM. Plasma factor Va was added to conditioned media at an equivalent concentration of 1 nM. PCPS vesicles were added to a final concentration of 20 μM followed by addition of 0.1 nM APC. At selected time intervals aliquots of the mixture were assayed for clotting activity (Nesheim et al., 1981). The factor Va samples were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at the same time intervals. All SDS-PAGE gels were produced according to the method of Laemmli (1970). The proteins were transferred to nitrocellulose using a Trans-Blot SD semidry transfer apparatus (Towbin et al., 1979) and displayed using the monoclonal antibody $aHFVa_{HC}$#6, which is directed against the heavy chain of factor Va and recognizes an

epitope located between amino acid residues 307 and 506 of the cofactor (Fig. 2) (Kalafatis et al., 1995a, 1995b).

The effect of protein S on APC inactivation of factor Va was also studied. PCPS vesicles (20 μM) and protein S (490 nM) were added to recombinant species of factor Va for 5 min at room temperature. The reaction was started by the addition of APC (0.1 nM). Aliquots of the mixture were removed and assayed for factor Va cofactor activity in a prothrombin time assay using factor V-deficient plasma as previously described (Nesheim et al., 1981).

Apparent second-order rate constants for the rates of APC inactivation of factor Va were obtained from exponential curve fits of the data presented in Figures 4 and 8.

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