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Importance of individual activated protein C cleavage site regions in coagulation Factor V for Factor Va inactivation and for Factor Xa activation

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Activated protein C (APC) cleavage of Factor Va (FVa) at residues R^{506} and R^{306} correlates with its inactivation. APC resistance and increased thrombotic risk are due to the mutation R506Q in Factor V (FV). To study the effects of individual cleavages in FVa by APC and the importance of regions near the cleavage sites, the following recombinant (r) human FVs were prepared and purified: wild-type, Q^{306}-rFV, Q^{506}-rFV, and $Q^{306}Q^{506}$-rFV. All had similar time courses for thrombin activation. Q^{506}-rFVa was cleaved by APC at R^{306} and was moderately resistant to APC in plasma-clotting assays and in prothrombinase assays measuring FVa residual activity, in agreement with studies of purified plasma-derived Q^{506}-FVa. Q^{306}-rFVa was cleaved by APC at R^{506} and gave a low APC-resistance ratio similar to Q^{506}-rFVa in clotting assays, whereas unactivated Q^{306}-rFV gave a near-normal APC-resistance ratio. When FVa residual activity was measured after long exposure to APC, Q^{306}-rFVa was inactivated by only $\leq 40\%$ under conditions where Q^{506}-rFVa was inactivated $> 90\%$, supporting the hypothesis that efficient inactivation of normal FVa by APC requires cleavage at R^{306}. In addition, the heavy chain of Q^{306}-rFVa was cleaved at R^{506} much more rapidly than activity was lost, suggesting that FVa cleaved at only R^{506} is partially active. Under the same conditions, $Q^{306}Q^{506}$-rFVa lost no activity and was not cleaved by APC. Therefore, cleavage at either R^{506} or R^{306} appears essential for significant inactivation of FVa by APC. Modest loss of activity, probably due to cleavage at R^{679}, was observed for the single site rFVa mutants, as evidenced by a second phase of inactivation. $Q^{306}Q^{506}$-rFVa had a low activity-to-antigen ratio of 0.50–0.77, possibly due to abnormal Factor Xa (FXa) binding. Furthermore, $Q^{306}Q^{506}$-rFV was very resistant to cleavage and activation by FXa. $Q^{306}Q^{506}$-rFV appeared to bind FXa and inhibit FXa's ability to activate normal FV. Thus, APC may downregulate FV/Va partly by impairing FXa-binding sites upon cleavage at R^{306} and R^{506}. This study shows that R^{306} is the most important cleavage site for normal efficient inactivation of FVa by APC and supports other studies suggesting that regions near R^{306} and R^{506} provide FXa-binding sites and that FVa cleaved at only R^{506} retains partial activity.

Keywords: activated protein C; activated protein C resistance; blood coagulation; Factor Va; Factor Xa.

Coagulation Factor Va (FVa) is a cofactor for Factor Xa (FXa) that increases by ≈500-fold the k_{cat} for conversion of prothrombin to thrombin in the prothrombinase complex [1–4]. FVa is down-regulated by proteolytic inactivation by activated protein C (APC) [5–7], and the importance of APC as a negative regulator of blood coagulation is illustrated by the association of an increased risk for venous thrombosis with heterozygous deficiency of protein C, and of potentially fatal purpura fulminans at birth with homozygous deficiency [8–11].

APC resistance of patient plasma (i.e. the failure of plasma to show normal prolongation of clotting times with addition of APC) was recently found to be associated with 20–50% of cases of venous thrombosis [12–15]. The defect was shown to reside in the FV gene, and in over 90% of cases was associated with a G-to-A mutation at nucleotide 1691, resulting in Arg506Gln at an APC cleavage site in Factor V (FV) [16–19]. This mutation is found in ≈5% of the Caucasian population. The phenotype is variable and often asymptomatic, probably depending on the presence of additional genetic and/or acquired thrombotic risk factors [20–23]. Plasma samples from homozygous subjects exhibit a very pronounced resistance to prolongation of clotting time by APC and these individuals are at higher risk of venous thrombosis than heterozygous subjects.

Past studies [24–26] have demonstrated the presence of two important APC cleavage sites in the heavy chain of FV, one at arginine 306 and another at arginine 506. Recent studies [27–29] using plasma-derived normal and Arg506Gln-FVa (Q^{506}-FVa) demonstrated that Q^{506}-FVa was inactivated $> 90\%$ by APC, although at a rate ≈8–10-fold more slowly than normal FVa. The inactivation of Q^{506}-FVa was accompanied by cleavage at R^{306}. Thus, cleavage at R^{506} may not result in complete inactivation of FVa, and functional inactivation of FVa may require cleavage at R^{306}. The significance of a third APC-cleavage site at R^{679} is less clear [25].

In order to clarify the importance of the three heavy-chain

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Abbreviations: APC, activated protein C; APTT, activated partial thromboplastin time; FV and FVa, Factors V and Va; FXa, Factor Xa; HBS, Hepes-buffered saline (0.05 M Hepes, 0.1 M NaCl, 0.02% azide, pH 7.4); NHP, pooled normal human plasma; pAPMSF, p-amidino-phenylmethylsulfonyl fluoride; PPACK, Phe-Pro-Arg chloromethylketone.

Enzymes: Activated protein C (EC 3.4.21.69); Factor Xa (EC 3.4.21.6); thrombin (EC 3.4.21.5).

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cleavage sites for APC inactivation of FVa, wild-type recombinant human FV molecules were prepared, as well as the mutants Arg306Gln (Q^{306}-rFV), Arg506Gln-rFV (Q^{506}-rFV) and a mutant containing both substitutions ($Q^{306}Q^{506}$-rFV). Although previous studies with wild-type human and murine rFV were performed with conditioned media [30–35], the rFVs were purified for the present studies. The rFVas were compared with each other and with purified plasma-derived normal FVa and Q^{506}-FVa as to activity, time course of activation by thrombin and FXa, proteolytic cleavages by APC, and resistance to APC in clotting assays and in purified component prothrombinase assays measuring FVa residual activity. Insights were gained concerning the relative importance of the three APC cleavages in inactivating FVa and possible functions of FVa regions near APC cleavage sites. Preliminary reports of this work were presented at the American Society of Hematology Meeting, December 1996 [35a] and at the XVIth Congress of the International Society on Thrombosis and Haemostasis [36].

MATERIALS AND METHODS

Plasma-derived proteins

Unless otherwise specified, protein C [37], plasma-derived FV [38] and prothrombin [39] were purified and activated as described previously. Heavy chain of FVa was prepared from FVa on a Sepharose (Pharmacia, Parsipanny, NJ, USA) column coupled with 3 mg·mL^{-1} FV anti-(heavy chain) mAb 3B1 (kindly provided by Drs Tilman Hackeng and Bonno Bouma). FVa light chain and activation peptides were eluted with 5 mM EDTA, and heavy chain was then eluted with 2 M NaCl and dialyzed against Hepes-buffered saline (HBS). Antibodies to FVa heavy chain were raised in rabbits and IgG fraction was prepared. Rabbit anti-(FV) was obtained from Accurate Chemical and Scientific (Westbury, NY, USA). Part of the IgG fraction was biotinylated [40]. Thrombin and FXa were obtained from Enzyme Research Laboratories (South Bend, IN, USA) and FXa activity was determined by active-site titration. The mAb to FVa light chain was obtained from Hematologic Technologies (Burlington, VT, USA).

Construction of FV mutant-expression vectors

Wild-type rFV contained in pBSII has been described previously [31]. The mutations R506Q and R306Q were introduced using overlap-extension PCR mutagenesis. For the Q^{506} mutant, FV cDNA was used as template and two PCR products were generated, one that contained sequences spanning residues 1–510 and another that spanned residues 499–536. The 3′ primer for the N-terminus-coding PCR product and the 5′ primer for the C-terminus-coding PCR product were complementary to one another and contained the appropriate mutation at residue 506 (5′-CTGGACAGG*CAA*GGAATACAG-3′). Purification of the N-terminal and C-terminal fragments followed by a joining PCR reaction using the 5′ primer as the N-terminal product and the 3′ primer as the N-terminal PCR product yielded a *Cla*I–*Kpn*I fragment that was reintroduced into the FV cDNA in the vector pBSII. The Q^{306} mutation was constructed in a similar manner using the mutagenic primer (5′-AAA-GAAAACC*CAG*AATCTTAAG-3′). To construct a FV cDNA that had both the Q^{506} and Q^{306} mutations, the strategy described for the construction of Q^{506} was used except that the template used had a mutation coding for Q^{306}. Mutant cDNAs were subsequently subcloned from pBSII into the expression vector pED [41] and used for DNA sequence analysis as well as functional analysis.

Expression and preliminary analysis of rFV molecules

Wild-type and mutant rFV expression plasmids were transfected into COS-1 cells [30]. At 48-h post-transfection the cells were metabolically labeled using ^{35}S-methionine/cysteine (Promix, Amersham, UK) and immunoprecipitated with FV-specific antiserum (Dako Corp., Carpinteria, CA, USA) [31]. rFVs were subjected to SDS/PAGE before and after activation with 10 nM thrombin [31] to confirm that the characteristic heavy chains, light chains and activation polypeptides were released.

Functional analysis of rFV in culture supernatant

Conditioned media were collected for 24 h using serum-free OPTI-MEM (Gibco, MD, USA) at 48-h post-transfection of COS-1 cells. rFV activity in the media was measured in a FV clotting assay using FV-deficient plasma [31,42]. One hundred microliters of diluted media were incubated with 100 μL FV-deficient plasma (George King Biomedical Inc., Overland Park, KS, USA) at 37 °C for 1 min. Thromboplastin (100 μL, Sigma Corp., St Louis, MO, USA) was added and incubated for 3 min. After the addition of 100 μL of 25 mM $CaCl_2$ clotting time was measured using an Electra 750 coagulometer. rFV activity was calculated using a standard curve consisting of dilutions of pooled normal human plasma (NHP) (George King Biomedical Inc., 1 U·mL^{-1}).

Purification of rFV

The culture supernatant of each rFV (250–400 mL) was thawed at 37 °C, placed on ice and treated with 1 mM $PhCH_2SO_2F$ (Sigma), 10 mM benzamidine (Sigma) and 10 nM Phe-Pro-Arg chloromethylketone (PPACK) (Chemica Alta, Edmonton, Alberta, Canada). The mixture was centrifuged for 15 min at 10 000 ***g*** and 4 °C, and the supernatant was loaded at 4 °C onto a 2-mL column of CNBr–Sepharose (Pharmacia) coupled with 2 mg of anti-(FV heavy chain) mAb AHV 5101 (Hematologic Technologies) at a flow rate of 0.4 mL·min^{-1}. The column was then washed with HBS containing 5 mM $CaCl_2$, 2 mM benzamidine and 5 nM PPACK, and then with the same buffer containing 0.3 M NaCl until less than 0.5 μg·mL^{-1} rFV activity was detected in the eluate. The column was taken to room temperature, washed at 0.1 mL·min^{-1} with 50 mL of the same buffer containing a final concentration of 0.5 M NaCl and then with the same buffer containing a final concentration of 1.8 M NaCl until all detectable rFV activity was eluted. Fractions were chilled to 5 °C and analyzed for SDS/PAGE profile, FV activity and FV antigen. Pooled fractions were treated with 1 mM diisopropyl fluorophosphate (Sigma), concentrated, stabilized with 0.2% BSA, dialyzed against HBS-5 mM $CaCl_2$ and frozen in aliquots at −80 °C.

Activation of rFVs

rFVas were prepared by activation of each rFV with 10 nM thrombin in 20% glycerol for 20 min at 37 °C. Thrombin was neutralized with a 1.2-fold molar excess of hirudin (Sigma) and FVa aliquots were frozen. In some experiments, time courses of activation of each rFV were performed, using 1 nM thrombin or 1 nM FXa with 10 μM phospholipid vesicles containing 20% bovine brain phosphatidyl serine and 80% bovine liver phosphatidyl choline (Sigma). Aliquots were tested over time for rFVa activity in prothrombinase assays.

Prothrombinase assays

rFV/Va activity was measured by prothrombinase assays [40,43] with 20 pM FV, FVa, rFV or rFVa, 1 nM FXa, 25 μM phospholipid vesicles, 0.3 μM prothrombin, HBS, 0.5% BSA and 5 mM $CaCl_2$, unless otherwise specified. In this assay, FV became fully activated in < 20 s following the addition of prothrombin to start the reaction. Aliquots were removed from the reaction mixture over time and quenched in buffer containing 10 mM EDTA. The rate of thrombin formation was assessed with thrombin substrate CBS 34.47 (American Bioproducts, Parsippany, NJ, USA) using the Kineticalc program on a Biotek microtiter plate reader (Winooski, VT, USA). To monitor the time course of activation of rFVs by thrombin or FXa, only 40 pM FXa and 10 μM phospholipid vesicles (final concentrations) were employed in the assays to prevent further FV activation during measurement [32]. A standard of stable, purified plasma-derived FV was prepared, frozen in aliquots and checked periodically for activity against FV in NHP. The concentration of FV in the standard was determined in the ELISA described below and it had the same activity as plasma FV when activated with thrombin.

For some experiments, rFVa was preincubated for various times with APC in the presence of 25 μM phospholipids prior to assay of rFVa residual activity in prothrombinase assays. Controls showed that the same results were obtained whether or not APC was inactivated with 100 μM *p*-amidino-phenylmethanesulfonyl fluoride (pAPMSF) (Chemicon, Carpenteria, CA, USA) for 20 min at the end of the preincubation time prior to assay for rFVa activity. Therefore, inactivation of rFVa during the time required for measurement of residual rFVa activity (2 min) in the presence of protective FXa was negligible. Controls showed that pAPMSF did inactivate APC and did not affect the prothrombinase assays after 20 min of decay.

APC-resistance assays

Activated partial thromboplastin time (APTT) was determined for each rFV or rFVa in FV-depleted plasma in the presence or absence of 1 $\mu g \cdot mL^{-1}$ APC [28]. The ratio of the clotting time in the presence of APC to that in the absence of APC (APC-resistance ratio) was determined and compared with that of normal and homozygous Q^{506}-FV plasma and purified plasma-derived FV, Q^{506}-FV, FVa and Q^{506}-FVa.

SDS/PAGE and immunoblot analysis

SDS/PAGE was performed on 4–15% minigels. Gels were silver stained or transferred to Immobilon membranes (Millipore, Bedford, MA, USA) and subjected to immunoblotting [28]. Incubation mixtures contained 2.4 nM FVa or rFVa, 25 μM phospholipids and 400 pM APC unless otherwise stated. Aliquots of the incubation mixtures containing 32 ng FVa or rFVa were taken over time and boiled for 2 min with SDS prior to electrophoresis and immunoblotting. At the same time, aliquots were taken for measurement of FVa residual activity. Immunoblots were developed with rabbit anti-(FV heavy chain IgG) (10 $\mu g \cdot mL^{-1}$) combined with anti-(FV heavy chain) mAb VE (Enzyme Research Laboratories) at 2 $\mu g \cdot mL^{-1}$, followed by biotin-donkey anti-(rabbit IgG) and biotin-goat anti-(mouse IgG) (Pierce, Rockford, IL, USA) each diluted 1 : 1000. Further development employed streptavidin–alkaline phosphatase (Pierce) 1 : 500 and color development with BCIP/NBT substrate (Bio-Rad, Hercules, CA, USA). In some experiments, ^{125}I-secondary antibodies were used instead of the biotin–streptavidin system. These immunoblots were exposed to a phosphoimaging screen for 24 h. The screen was read on a Phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA) and bands were quantitated.

Ligand-binding analysis

Binding of FXa to immobilized FVs was detected using the biotin–streptavidin system described above. The detecting antibodies were mAb anti-(FX) (Biodesign, Kennebunk, ME, USA) followed by biotin-goat anti-(mouse IgG) (Pierce).

FV antigen detection

A variation of an ELISA method for the determination of FV antigen was used [28]. Microtiter plates were coated with 5 $\mu g \cdot mL^{-1}$ rabbit anti-(FV IgG). Wells were blocked and samples or standards consisting of dilutions of NHP (beginning at 1 : 100) were prepared in 0.5% BSA/HBS and incubated in the wells for 1 h at 23 °C and 16 h at 4 °C. Bound FV was detected with biotin-rabbit anti-(FV IgG) followed by streptavidin–alkaline phosphatase and p-nitrophenyl phosphatase substrate. Absorbance at 405 nm was taken, standard curves were constructed and unknowns were calculated. Standard curves for dilutions of NHP were parallel to those for dilutions of purified FV.

RESULTS

Construction and expression of APC-cleavage-site mutants of FV

To directly assess the significance of each of the APC-cleavage sites in FV, mutant rFVs were generated containing an Arg-to-Gln substitution at residues 306, 506 or both. FV procoagulant activity in the conditioned media ranged from 2.0 to 2.4 $\mu g \cdot mL^{-1}$ from cells transfected with the wild-type and mutant FV expression plasmids but not from transfected cells that did not receive DNA.

rFVs were metabolically labeled with [^{35}S]-methionine/cysteine and, after a 4-h chase, the media were harvested for immunoprecipitation of rFV. A 330-kDa band observed by SDS/PAGE was immunoprecipitated from cells expressing rFV, Q^{506}-rFV, Q^{306}-rFV and $Q^{306}Q^{506}$-rFV but not from mock-transfected cells (Fig. 1). Similar quantities of secreted proteins were detected for the wild-type and each mutant, indicating that the mutations did not significantly interfere with their folding to impair their secretion. The apparent molecular mass of the rFV agrees with previous reports for rFV [44] and for plasma-derived FV. When immunoprecipitated rFVs were activated with thrombin and then resolved by SDS/PAGE, the wild-type and mutant molecules yielded the characteristic 150-kDa activation peptide, 102-kDa heavy chain and the 74-kDa light chain (Fig. 1) expected for FV.

Purification of wild-type and mutant rFV

rFV activity and antigen eluted from the mAb anti-(FV) Sepharose column with similar patterns for each rFV. Recovery was 100–200 μg rFV from 200 to 400 mL of culture supernatants that contained 400–800 μg rFV. Approximately half of each rFV was subsequently lost during concentration and dialysis. Each rFV was pooled in two parts that contained the ascending and descending limbs (pools I and II) of the rFV peaks.

SDS/PAGE analysis showed that the ascending limb contained visible contaminants, while the descending limb appeared

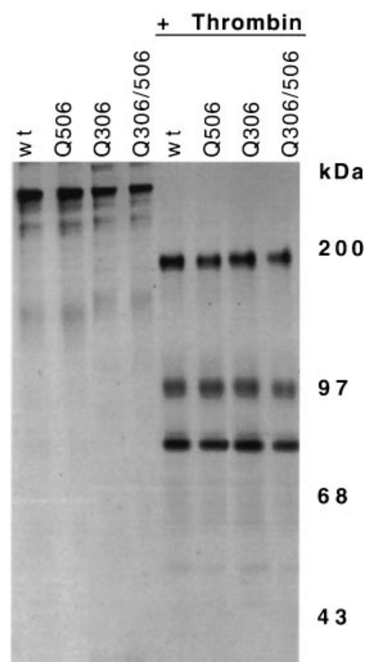

Fig. 1. SDS/PAGE analysis of ^{35}S-labeled recombinant FV molecules in culture supernatant. Metabolically labeled rFV was immunoprecipitated from conditioned medium as described above. Lanes 1–4 contained untreated rFVs, while rFVs in lanes 5–8 were activated with thrombin. Lanes 1 and 5, wild-type rFV; lanes 2 and 6, Q^{506}-rFV; lanes 3 and 7, Q^{306}-rFV; lanes 4 and 8, $Q^{306}Q^{506}$-rFV. The positions of molecular mass markers are given in the right margin.

$>$ 90% pure, yet no functional differences were detected in the rFV in the respective ascending and descending limbs of the rFV peaks. Figure 2A shows a silver-stained gel of the nonreduced descending limb pools of each rFV and Fig. 2B shows a wild-type rFV preparation, reduced and nonreduced. The majority of

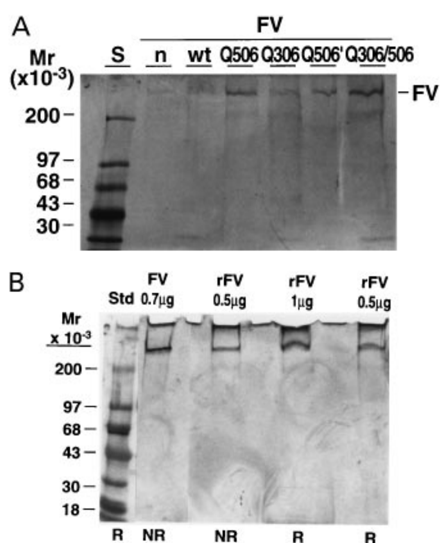

Fig. 2. SDS/PAGE of purified rFactor V molecules. (A) SDS/PAGE and silver stain of a nonreduced preparation of each rFV. (B) SDS/PAGE and silver stain of plasma-derived FV and wild-type rFV, reduced with dithiothreitol (R) and nonreduced (NR). The positions of molecular weight standards (S) are indicated on the left margins. n indicates normal plasma-derived FV. Q506′ indicates plasma-derived Q506-FV.

Table 1. Specific activity of recombinant Factor V molecules.

rFV type	Activity/ Antigen[a]	Activity/ Antigen[b]
Wild-type		
Pool I	1.2	0.9
Pool II	1.0	1.0
Q^{506}		
Pool I	3.4	2.9
Pool II	1.9	2.6
Q^{306}		
Pool I	0.9	1.0
Pool II	1.0	1.0
$Q^{306}Q^{506}$		
Pool I	0.4	0.5
Pool II	0.6	0.5
Peak tube	0.5	0.4

[a] In the first Activity/Antigen column, these parameters were determined immediately after purification and concentration of FVs. A standard prothrombinase assay was used with 1 nM FXa and an ELISA as described above. The peak tube during purification of $Q^{306}Q^{506}$-rFV by immunoaffinity chromatography was retained in its original tube without pooling, dialysis, or concentration. [b] In the second Activity/Antigen column, values were determined in aliquots of rFV frozen for 1–5 months. Activity was taken as the maximal activity obtained over time during activation with 2 nM thrombin as determined in a prothrombinase assay using 40 pM FXa as described above. Values were the average of at least three different determinations on different dates.

rFV was single chain migrating at 330 kDa. Part of the rFV was of lower apparent molecular mass, ≈190 kDa, possibly due to proteolytic cleavage near the middle of the B domain between the heavy and light chain regions, yielding fragments of ≈150 kDa and 190 kDa, possibly representing residues 1–1018 and 1019–2196 as reported previously [32, 45].

Ratio of activity to antigen for rFV molecules

The activity and antigen values for the ascending and descending limb pools (I and II) of each rFV were determined immediately after purification, and then from aliquots frozen and assayed at later times. For the immediate activity measurements, saturating FXa (1 nM) and phospholipids (25 μM) were used in a standard prothrombinase assay. For the later activity measurements, a time course for activation by thrombin was performed and the maximum activity obtained was recorded, as determined in a prothrombinase assay with 40 pM FXa and 10 μM phospholipids. In either case (Table 1), the wild-type and Q^{306}-rFVa expressed activity-to-antigen ratios near 1 and near that of plasma-derived FVa. However, Q^{506}-rFVa expressed ratios of activity to antigen of $\geq$ 1.9, while two preparations of $Q^{306}Q^{506}$-rFVa expressed ratios of 0.50 (Table 1) and 0.77 (not shown). The latter finding suggests that mutations at both 306 and 506 may synergistically affect the ability of FVa to bind FXa and/or prothrombin, and thus may impair FVa activity. Immunoblotting analysis for heavy-chain epitopes of $Q^{306}Q^{506}$-rFVa showed that all single chain had been converted to heavy chain by thrombin (Fig. 6), even though its activity-to-antigen ratio was somewhat low. Similar results, as shown in Table 1, were obtained for a second preparation of each rFV.

We questioned whether the finding of a high activity-to-antigen ratio for Q^{506}-rFV might be observed for human individuals with plasma Q^{506}-FV. We examined the activity-to-antigen ratio of Q^{506}-FV in the plasma of seven homozygous individuals, compared with FV in NHP. Q^{506}-FV activity in homozygous plasma was evaluated in prothrombinase assays as

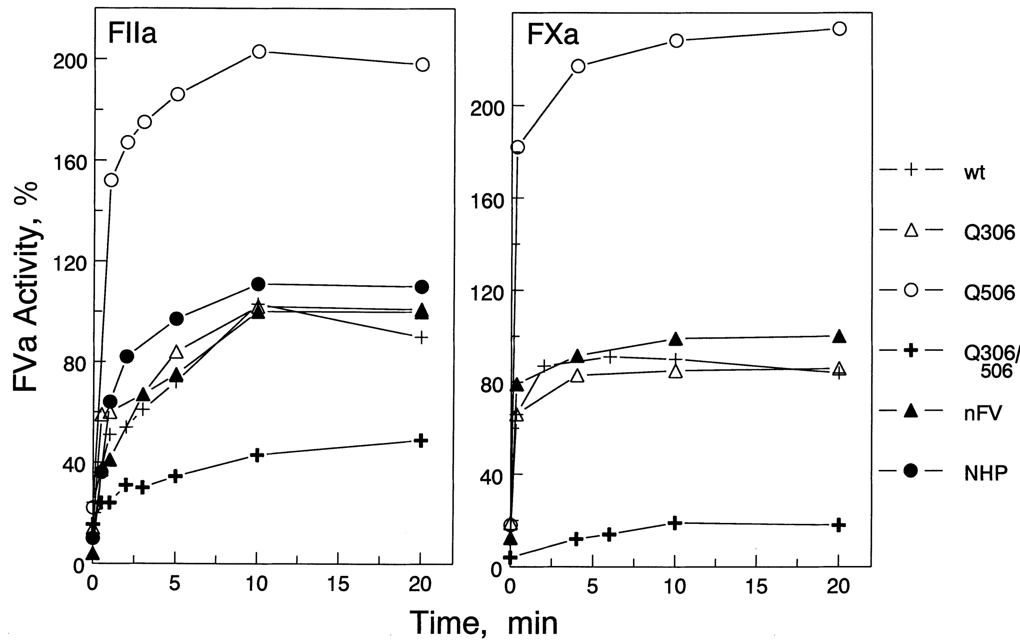

Fig. 3. Time course for activation of FV molecules. FVs (1 nM) were incubated with 1 nM thrombin (FIIa) (left panel) or 1 nM FXa and 10 μM phospholipids (right panel). At various times, aliquots were diluted for assay of FVa activity in prothrombinase assays using 40 pM final FXa. 100% activity was defined as the maximum activity exhibited by normal plasma-derived FV (nFV). No further increase in activity was observed up to 40 min for any of the activated FVs.

described above for NHP and FVs. Q^{506}-FV plasma from one individual prepared on several different dates had near-normal FVa activity, but lower than normal antigen, so that the activity-to-antigen ratios were 2.1, 1.6 and 1.3, respectively. However, six unrelated subjects homozygous for Q^{506}-rFV did not have a FV activity-to-antigen ratio greater than 1.1, and one normal sibling of the first individual had an activity-to-antigen ratio of 1.4. Therefore, the high activity-to-antigen ratio for Q^{506}-rFVa and one homozygous individual with Q^{506}-FV was probably not directly related to this mutation and likely represents variability in human subjects or cultured cells that synthesize FV, possibly due to post-translational modifications.

FXa activation, and not thrombin activation, is defective in the double mutant $Q^{306}Q^{506}$, but not in either single mutant

Time courses were examined for activation of plasma-derived FV and each preparation of rFV by 1 nM thrombin, using 400 pM of each rFV as determined by antigen level. When subsequently assayed in prothrombinase assays, FV in NHP, purified plasma-derived FV, wild-type and various mutant rFVs had reasonably similar time courses for activation by thrombin (Fig. 3, left panel). A rapid phase of activation was observed in the first 2 min, followed by modest increases in activity up to 10 min. However, as indicated in Table 1 and in data not shown, three independent preparations of Q^{506}-rFVa had approximately twofold the maximal activity of normal plasma-derived and wild-type rFVa, while two preparations of $Q^{306}Q^{506}$-rFV had 50–77% the maximal activity of normal plasma-derived and wild-type rFVa. This was true for both the ascending and descending limb pools of each type of rFV.

Time courses for FXa activation were also examined for each pool of each rFV, using 1 nM FV, 1 nM FXa and 10 μM phospholipids (Fig. 3, right panel). For plasma-derived FV, wild-type rFV, Q^{306}-rFV and Q^{506}-rFV, the time courses of activation by FXa were slightly more rapid but the maximal FVa activities achieved were reasonably similar to those seen for thrombin activation. However, $Q^{306}Q^{506}$-rFV was very resistant to activation by FXa and activity values did not increase above those shown in Fig. 3 during 40 min of activation.

Immunoblots such as those shown in Fig. 4 revealed that very little heavy chain was generated from $Q^{306}Q^{506}$-rFV during 40 min of FXa activation, although all $Q^{306}Q^{506}$-rFV single chain was fully converted to heavy chains (and light chains not shown) during 5 min of thrombin activation. Other FVs were mostly converted to heavy and light chains by FXa activation within 5–15 min (Fig. 4). A second preparation of $Q^{306}Q^{506}$-rFV and unpurified $Q^{306}Q^{506}$-rFV from conditioned medium were also resistant to FXa activation (not shown). Figure 4 also illustrates that the antibodies used for immunoblotting recognize heavy chain > B domain cleaved FV (150 kDa) > single chain FV. This was not due to a difference in transfer efficiencies, since ^{125}I-labeled FV and FVa had similar transfer efficiencies under the conditions used. The 150-kDa fragment was only

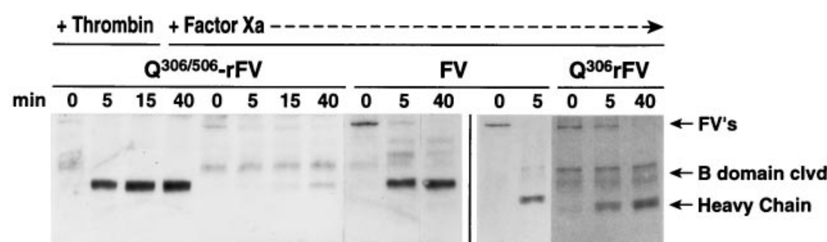

Fig. 4. Immunoblot of FV molecules activated with FXa. Aliquots from incubation mixtures as in Fig. 3 were withdrawn at various times as indicated. Aliquots were boiled with SDS and subjected to immunoblotting for FVa heavy-chain epitopes. The positions of single-chain FV, heavy chain and FV cleaved at or near residue 1018 in the B domain are noted.

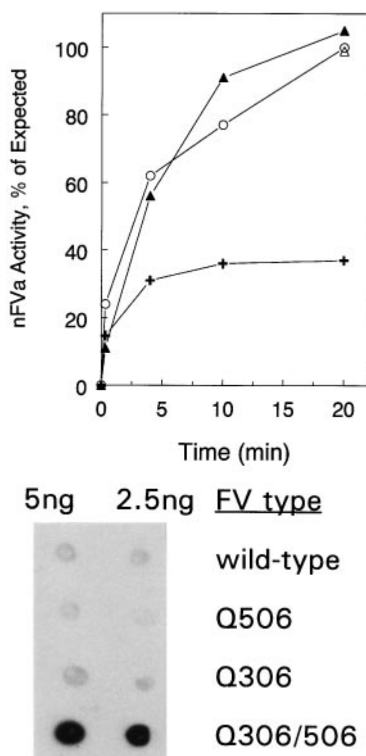

Fig. 5. Inhibition of FXas ability to activate normal FV in the presence of $Q^{306}Q^{506}$-rFV. (top) FXa (730 pM final) and 10 μM phospholipid vesicles were preincubated for 20 min in duplicate with 545 pM of normal plasma-derived (n) FV (▲), $Q^{306}Q^{506}$-rFV (+), wild-type rFV (△) or buffer alone (○). After maximal activation of FVs was achieved (as established by assay of FVa activity), fresh nFV (432 pM) was added to one aliquot of each incubation mixture (*t* = 0 on the *x*-axis). Subaliquots were then taken of all mixtures at times indicated and diluted into assay mixtures for FVa activity as described, at 40 pM final FXa. FVa activity in the samples without fresh nFV added was subtracted from the corresponding sample with fresh nFV added to determine nFVa activity generated from the freshly added nFV. nFVa activity was expressed as a percentage of the expected activity generated. Generation of nFVa activity from the freshly added nFV by FXa was inhibited by the presence of $Q^{306}Q^{506}$-rFV/Va, but not by nFV/Va or wild-type rFV/Va. (bottom) Ligand binding of FXa with various FVs. FVs as indicated were spotted on nitrocellulose paper and blocked. The paper was incubated with 40 nM FXa, 2 mM $CaCl_2$ and 5 μM phospholipid vesicles. After washing, bound FXa was detected as described. Significantly more FXa remained bound to $Q^{306}Q^{506}$-rFV than to other FVs. Controls with no FXa had negligible signals (not shown).

faintly visible in silver-stained gels (Fig. 2) and by analysis of immunoprecipitated ^{35}S-labeled protein (Fig. 1). However, on immunoblots it appeared intense compared with single chain FV and disproportional to its true mass.

$Q^{306}Q^{506}$-rFV inhibits FXa's ability to activate normal FV

The mechanism of poor activation of $Q^{306}Q^{506}$-rFV by FXa was explored further. The slow generation of the heavy chain of $Q^{306}Q^{506}$-rFV by FXa (Fig. 4) could be due to poor binding of the unactivated double mutant to FXa, or alternately, to binding in a nonproductive manner. In the latter case, FXa would not cleave $Q^{306}Q^{506}$-rFV and therefore might remain bound and inhibited in its ability to cleave and activate other FVs. To test these possibilities, FXa was preincubated alone or with various FVs. After maximal FVa activity was generated, the FXa mixtures were supplemented with fresh plasma-derived normal (n) FV. In Figure 5, left panel, we show that FXa that had been preincubated with $Q^{306}Q^{506}$-rFV was inhibited in its ability to generate activity from freshly added nFV, while FXa that was preincubated alone or with nFV or wild-type FV was not inhibited. This suggests that some of the $Q^{306}Q^{506}$-rFV remained bound to FXa in a nonproductive manner. Indeed, in the ligand blot in Fig. 5, top panel, we show that FXa incubated with FVs spotted on nitrocellulose paper remained bound to $Q^{306}Q^{506}$-rFV to a far greater extent than to other FVs.

Q^{306} and Q^{506} FV and FVa are both partially resistant to APC as measured in APTT assays

Clinically, APC resistance is defined in terms of APC-resistance ratios in APTT-clotting assays, where the APC-resistance ratio is the ratio of the clotting time in the presence of APC divided by the clotting time in the absence of APC. Therefore, the APC-resistance ratio was determined for each rFV and rFVa and compared with the ratios for purified plasma-derived FV and Va and for purified Q^{506}-FV and Va from an individual homozygous for the Arg506Gln mutation. Each test sample was diluted into FV-deficient plasma for testing. As seen in Fig. 6, FV in NHP, purified FV and Va, and wild-type rFV and Va all had mean APC-resistance ratios ≥ 2.5. Q^{506}-rFV and Va and Q^{306}-rFVa had mean APC-resistance ratios of ≈1.3–1.6, similar to the APC ratio for plasma-derived Q^{506}-rFV. Q^{306}-rFV had an intermediate mean APC-resistance ratio of 2.1. $Q^{506}Q^{306}$-rFV and its activated form were completely APC resistant with ratios close to 1.0.

In prothrombinase assays, the order of increasing resistance to APC inactivation is wild-type-, Q^{506}-, Q^{306}-, and $Q^{306}Q^{506}$-rFVa

Figure 7 illustrates the time courses for inactivation of 80 pM (panel A) or 2.4 nM (panel B) plasma-derived and rFVas by 400 pM APC, where residual FVa activity was measured in prothrombinase assays with limiting FVa. For each type of FVa, the symbols represent the averaged data from two to five experiments. FVa activity was compared with controls with no APC, where activity varied no more than 10% during a 110-min incubation.

The averaged data for plasma-derived FVa and wild-type rFVa were best fit by a biphasic least-squares analysis of FVa activity vs. time, resulting from cleavages at R^{506} and R^{306}, as seen below using immunoblotting analysis of samples taken simultaneously from experiments in Fig. 7B. Plasma-derived nFVa and wild-type rFVa (80 pM–3 nM) were inactivated at rates (in percentage inactivation/time) dependent on the dose of APC from 33 pM to 2 nM. Wild-type rFVa was more slowly inactivated than plasma-derived FVa at low dose APC (33 pM, data not shown).

Q^{506}-rFVa was inactivated by 400 pM APC more slowly than plasma-derived or wild-type FVa (Fig. 7), with an initial rate of inactivation at least three times slower than that of nFVa, similar to results for purified plasma-derived Q^{506}-FVa [28]. Q^{506}-rFVa was inactivated at essentially the same rate as plasma-derived Q^{506}-FVa, and the initial rate of inactivation of each was seven times slower than that of plasma-derived FVa at 33 pM APC (data not shown). The time course data for Q^{506}-rFVa were best fit by a two-phase calculation, attributable to cleavage at R^{306}, possibly followed by R^{679}. In panel B, the second phase might

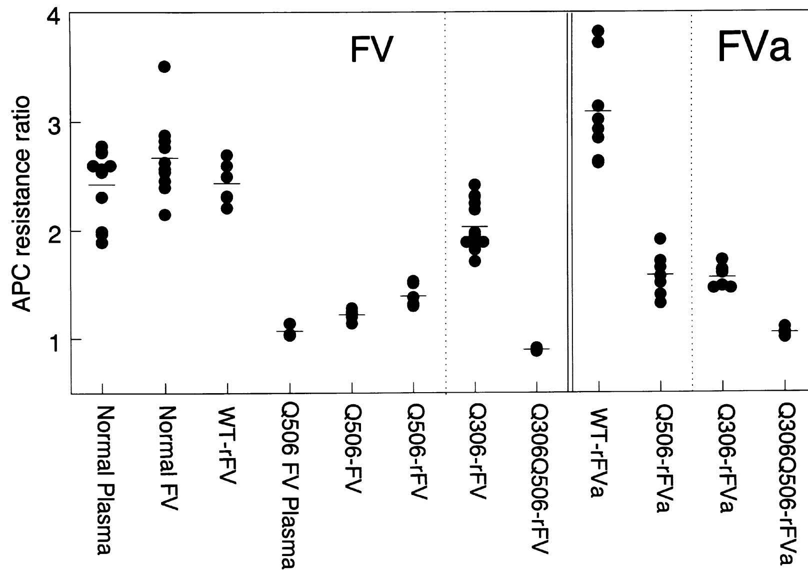

Fig. 6. APC-resistance ratio of FV and FVa molecules based on APTT assays. Each type of FV or its thrombin-activated form was added to FV-deficient plasma and analyzed ± APC in an APTT assay. The ratio of clotting time with APC to the clotting time without APC was calculated in each case. Purified FV or FV in normal plasma was used at a final concentration of 1 nM, while FVas were used at 40 pM final. Data are from several different days.

possibly be distorted by a type of product inhibition that has been reported due to FVa light-chain interaction with APC at high concentrations of FVa up to 200 nM [46]. This effect should be small at 80 pM FVa as used in Fig. 7, panel A, since the K_d for APC interaction with FVa light chain was reported as 7 nM.

Q^{306}-rFVa was very resistant to inactivation (Fig. 7), and Q^{306}-rFVa activity almost reached a plateau at ≥ 60% during the 1-h time course. This suggests that cleavage by APC at R^{306} in nFVa is necessary for efficient inactivation, consistent with previous reports [27–29]. Cleavage at R^{506}, and perhaps also at R^{679}, would appear to be responsible for a maximum of 40% loss of activity in 1 h in nFVa. Thus, the concept of a partially active form of FVa, cleaved at R^{506}, appears tenable.

The double mutant $Q^{306}Q^{506}$-rFVa was almost totally resistant (± 10%) to inactivation by APC under all conditions tested (Fig. 7). This suggests that cleavage at other sites, such as R^{679}, either does not occur in this mutant or contributes little to its loss of activity. Thus, cleavage at either R^{306} or at R^{506} is necessary for any significant loss of FVa activity. However, a second

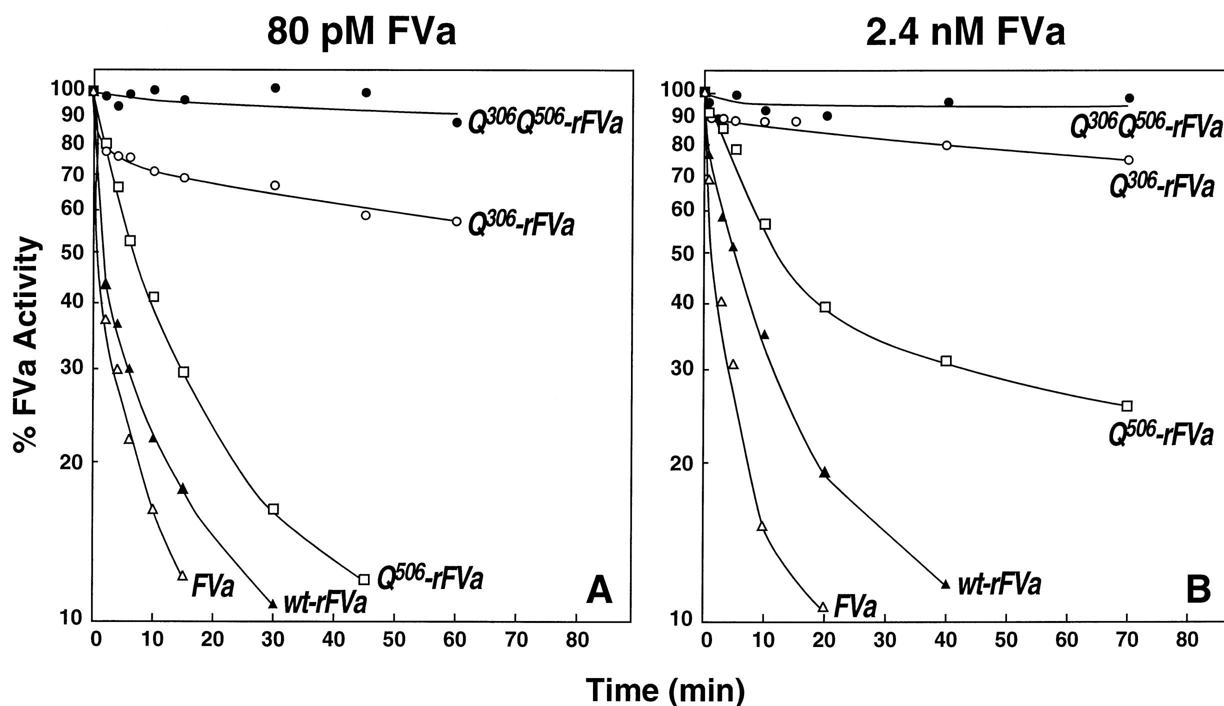

Fig. 7. APC inactivation of FVa molecules at two concentrations of FVa. (A) Thrombin activated FVas (80 pM) were incubated in separate wells of a polypropylene v-well plate with 20 µM phospholipids. At various times from 0 to 58 min, APC was added to selected wells to 400 pM. At the end of 60 min, the mixtures were assayed for residual FVa activity (compared with controls without APC) in prothrombinase assays as described above. (B) Incubation mixtures contained 2.4 nM FVa, 400 pM APC and 20 µM phospholipids. Aliquots were removed over time and assayed for residual FVa activity in prothrombinase assays at the same time that samples were taken for immunoblot analysis (shown in Fig. 8). Controls without APC remained constant in activity (± 10%) over the time course of the experiment.

phase of inactivation was detected in the case of Q[306]-rFVa and Q[506]-rFVa, suggesting that cleavage at R[679] may occur in these single-site mutants and make a small contribution to their loss of activity.

In comparing results for 80 pM vs. 2.4 nM FVas in Fig. 7A and Fig. 7B, data were qualitatively similar, with somewhat slower rates of inactivation at 2.4 nM FVas than at 80 pM FVas. Thus, similar inactivation data as described above were obtained at very different FVa concentrations, and also under conditions where APC or FVa was in excess. Data for inactivation of FVa and rFVas at a low concentration of 33 pM APC were subjected to least-squares analysis using a biphasic fit according to Rosing *et al*. [26, 29]. The Rosing laboratory concluded that inactivation of FVa is first order in both APC and FVa up to a concentration of 5 nM FVa, allowing the calculation of first-order rate constants [29]. In our experiments, the respective mean rate constants for k506 and k306 were determined to be 55 and 9.4 ($\times 10^6$ $M^{-1} \cdot s^{-1}$). The k506 was 138 for normal FVa and 41 for wild-type rFVa and Q[306]-rFVa, accounting for the slower rate of inactivation of the wild-type rFVa as compared with plasma-derived FVa at low APC concentration. If the second phase of inactivation in the single-site mutants was due to R[679] cleavage, the k679 was 0.6×10^6 $M^{-1} \cdot s^{-1}$. The k306 values for inactivation of plasma-derived FVa, wild-type and Q[506]-rFVa were similar in our experiments, although cleavage at R[506] was reported to provide a 1.4 to 1.7-fold enhancement of cleavage at R[306] [29, 33].

Immunoblot analysis correlates APC cleavages of mutant rFVa molecules with their resistance to inactivation

Time-dependent APC-cleavage patterns of the heavy chains of plasma-derived FVa and rFVas were evaluated by immunoblotting (Fig. 8A–D) simultaneously as samples were taken for the activity measurements in Fig. 7B. The identity of heavy-chain fragments as indicated was established previously by N-terminal amino acid sequencing [25, 28], except the fragment tentatively labeled 680–709. This fragment was recognized by a mAb whose epitope maps to the C-terminus of the heavy chain, residues 683–709. We were unable to obtain enough material for sequencing and confirmation of the identity of this fragment.

For plasma-derived FVa and wild-type rFVa (Fig. 8A, B), the earliest heavy-chain fragments observed were those at 78 kDa (residues 1–506) and 28 kDa (residues 507–709), corresponding to cleavage at R[506] (Fig. 8). The intensity of the 1–506 fragment was at a maximum within 1 min, and disappeared after 5 min. A band at 44 kDa (residues 1–306) increased in intensity up to about 10 min, corresponding to cleavage at R[306]. This cleavage would be predicted to form an additional fragment of approximately 28 kDa, corresponding to residues 307–506, but that band was not efficiently recognized by our antibodies and a doublet near 28 kDa was barely visible in some lanes of some blots. The initial 28 kDa fragment corresponding to residues 507–709 diminished after 20 min in association with the appearance of an additional faint band of ~ 25 kDa (not seen on these blots), most likely corresponding to cleavage at R[679]. The latter fragment of 25 kDa corresponding to residues 507–679 was not recognized by the anti-(heavy chain) mAb used in the detection mixture and was only weakly recognized by the polyclonal antibodies in the detection mixture. In some cases a low molecular mass fragment (< 14 kDa) was also observed at ≈ 20 min (Fig. 8A, C, right panels). This fragment probably represents residues 680–709 due to cleavage at R[679], based on its recognition by a particular mAb as discussed above.

The time-dependent APC-cleavage patterns of Q[306]-rFVa

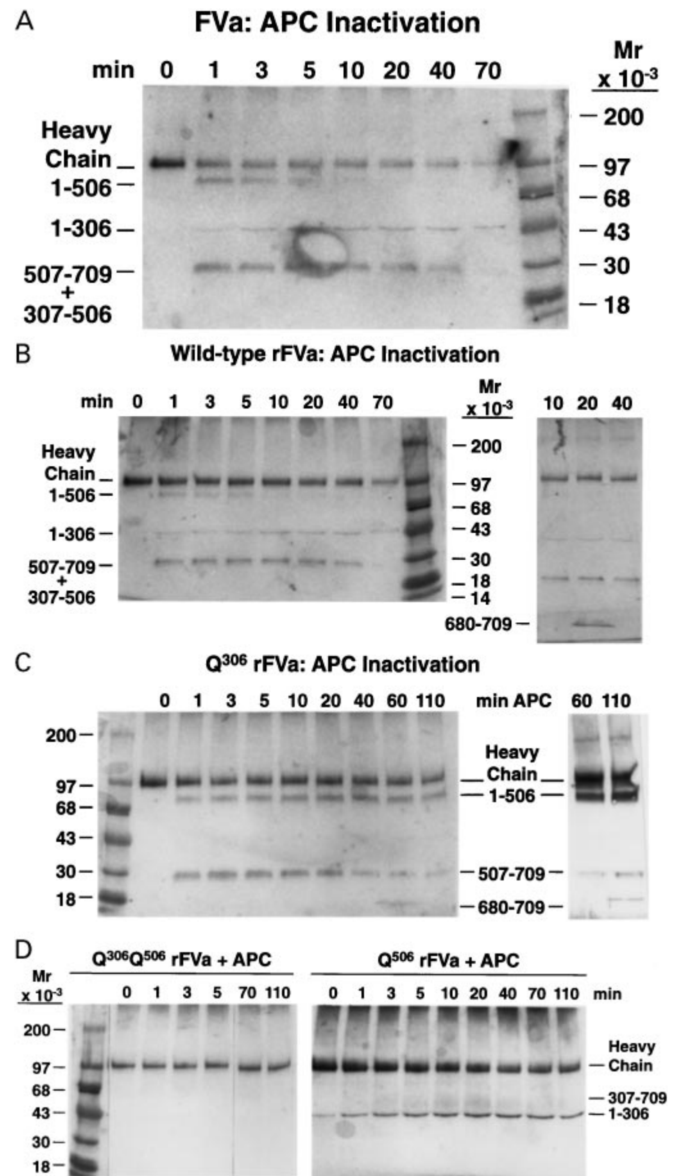

Fig. 8. (A–**D) Immunoblots for heavy chains of FVa molecules incubated with APC.** Aliquots of incubation mixtures in Fig. 7(B) were boiled with SDS and subjected to immunoblotting for heavy-chain epitopes. The positions of molecular weight standards (S) are shown in the margins, as well as the location of various FVa heavy chain fragments. At the right side of B and C, a transient fragment of < 14 kDa is noted that could be due to cleavage at R[679] to produce the fragment 680–709, but this remains to be verified by sequencing.

heavy chain revealed only fragments of 78 kDa, 28 kDa (Fig. 8C) and 25 kDa (not visible in this figure), corresponding to cleavages at R[506] followed by R[679]. On many blots a transient fragment was observed of low molecular mass (< 14 kDa) that probably represents residues 680–709, due to cleavage at R[679] (Fig. 8C, right and left panels). No fragments were detected that would result from cleavage at or near residue 306.

The APC-cleavage pattern of Q[506]-rFVa revealed only fragments of 44 kDa (residues 1–306) and 58 kDa (residues 307–709), corresponding to cleavage at R[306] (Fig. 8D) A diminution of the 58-kDa band (307–709) after 20 min suggested that cleavage at R[679] may have occurred in concert with the second phase of inactivation in Fig. 7. The appearance of a 44-kDa

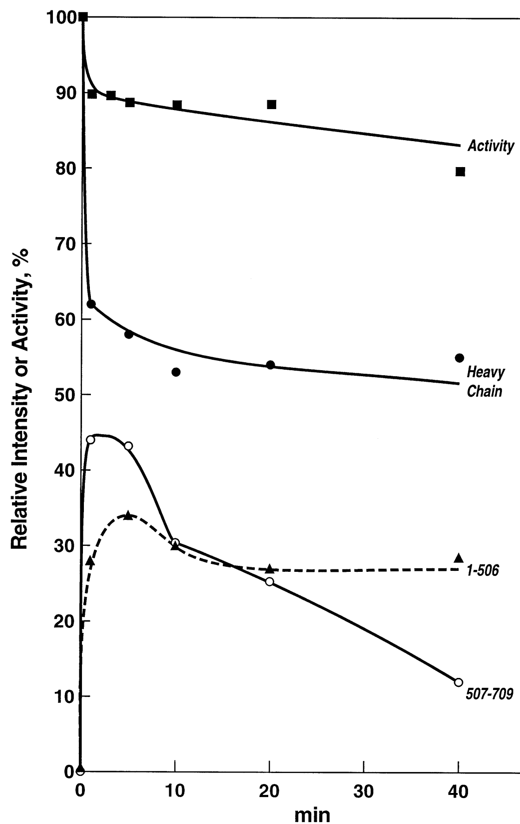

Fig. 9. Quantitation of heavy chain and heavy-chain fragments of Q^{306}-rFVa incubated with APC. An incubation mixture of Q^{306}-rFVa with APC as in Fig. 8(C) was subjected to immunoblotting for heavy-chain epitopes using ^{125}I-secondary antibody and phosphoimaging. Bands were quantitated relative to the intensity of the heavy chain band without addition of APC (100%). The relative intensity of a given band can be compared over time even though the intensity of a band of one mobility can not be compared directly with the intensity of a band of a different mobility.

fragment in the first lane, without APC, is likely due to spillover from the next lane, as this fragment was not observed on several other blots of Q^{506}-rFVa, even after 110 min of incubation in the absence of APC.

The Q^{306}Q^{506}-rFVa heavy chain appeared completely resistant to APC because no change in the immunoblot pattern of its heavy chain was seen with or without APC incubation for 60 min (Fig. 8C), consistent with functional data. Either the double mutant was not cleaved by APC at R^{679}, or the cleavage was not detected and did not measurably affect the molecule's activity (Fig. 7).

Quantitation of heavy-chain fragments during inactivation of Q^{306}-rFVa

Selected immunoblots of Q^{306}-rFVa inactivation time courses were subjected to phosphoimaging. Under the conditions used, the signal for heavy chain was proportional to the mass of heavy chain. In Fig. 9, bands from an immunoblot of a time course for inactivation of Q^{306}-rFVa were quantitated relative to the intensity of the heavy chain in the absence of APC (assigned 100%). There was a rapid cleavage at R^{506} in the first minute of incubation, producing the fragments 1–506 and 507–709 (Figs 8C and 9). The heavy-chain band decreased to 62% of its original intensity in 1 min, whereas activity only decreased to 90% of its original value in 1 min. The great disparity between activity and amount of heavy chain lost during 40 min of incubation supports the concept that FVa cleaved at only R^{506} retains partial activity, as suggested by others [24, 29]. It was reported that APC cleavage at the three equivalent Arg residues in bovine FVa heavy chain results in dissociation of the A2 domain, whereas cleavage at only the last two sites (R505 and R662) results in molecules that are mostly noncovalently associated [46].

While fragment 1–506 remained relatively constant in intensity during 1–40 min of incubation of Q^{306}-rFVa with APC, fragment 507–709 diminished significantly by 10–40 min (Fig. 9). This can also be seen visually in Fig. 8A–C. Thus, fragment 507–709 is cleaved, possibly at R^{679}. This suggests that the second, slow phase of inactivation of Q^{306}-rFVa and Q^{506}-rFVa (Fig. 7) may be due to cleavage at R^{679}, leading to modest additional loss of activity.

DISCUSSION

This is the first report of studies using purified rFVs. Wild-type rFVa had similar activity and behaved in a manner very similar to plasma-derived FVa with respect to activation by thrombin or FXa and inactivation by APC. In agreement with previous studies of plasma-derived FVa by Kalafatis *et al.* [25], there was preference in both wild-type rFVa and plasma-derived FVa for an initial APC cleavage at R^{506}, followed by cleavage at R^{306}. Visual inspection of the wild-type and normal FVa inactivation time course patterns and polypeptide patterns suggested a third time-dependent step of inactivation which could involve other cleavages such as cleavage at R^{679}. The immunoblot fragments were consistent with a slow cleavage at or near R^{679}.

Wild-type rFVa was inactivated somewhat more slowly at low doses of APC, as also observed recently by Egan *et al.* [33]. The reason for the slower cleavage at R^{506} in the rFVas compared with plasma-derived FVa could not be determined. A higher degree of glycosylation of heavy chain and light chains of FVa can reduce sensitivity to APC [47–49]. However, by immunoblot analysis for light-chain and heavy-chain epitopes it did not appear that either the light chains or the heavy chains were more heavily glycosylated than those of plasma-derived FVa. When wild-type and plasma-derived FVs were treated with N-glycanase and sialidase [47], they each became more sensitive to APC, but treated wild-type rFV was still somewhat less sensitive to APC than treated plasma-derived FVa. rFVs did not react significantly differently with anti-(phosphotyrosine) or anti-(phosphoserine) than did plasma-derived FV, so a difference in phosphorylation was not demonstrated. It is possible that the degree of sulfation or some other post-translational modification varied. Plasma-derived FV can be heterogeneous with respect to phosphorylation, sulfation and glycosylation [47,50,51].

The susceptibility of the mutant Q^{506}-rFVa to APC inactivation was essentially the same as that of Q^{506}-FVa purified from the plasma of homozygous APC-resistant patients. Q^{506}-rFVa was modestly resistant to APC in clotting assays, and in purified component reaction mixtures, the initial rate of its inactivation by 33 pM APC was approximately seven times slower than that of normal FVa. These results for the recombinant variant, Q^{506}-rFVa, indicate that APC resistance in patients can be explained by the presence of Q^{506}-FV alone. Q^{506}-rFVa could be $>$ 90% inactivated, although more slowly than normal FVa. This observation strengthens the hypothesis [25, 27–29] that efficient inactivation of FVa requires cleavage at R^{306}, and accordingly, proteolytic fragments due to cleavage at R^{306} in Q^{506}-rFVa were observed. Mutation at 506 does not appear to substantially affect cleavage at R^{306}, as judged by comparing the rates of formation

of the 1–306 fragment in Fig. 8 A, B and D, and by comparing the similar k306s for plasma-derived FVa, wild-type rFVa and Q^{506}-rFVa. The mutant Q^{306}-rFVa could not be fully inactivated by APC under conditions where Q^{506}-rFVa was > 90% inactivated, indicating that R^{306} is the most important APC-cleavage site for normal FVa inactivation. Proteolytic fragments were observed due to APC cleavage in Q^{306}-rFVa at R^{506} and at later time points, due to probable cleavage at R^{679}. If mutation occurred at R^{306} of FV in humans, it might be predicted to cause more severe thrombophilia than the Arg506Gln mutation. Since this does not involve a CpG sequence, it may not be a frequent event. However, reports of the FV mutation Arg306Gly in two thrombotic and one nonthrombotic Hong Kong Chinese subjects and the mutation Arg306Thr in one thrombotic patient in England were published recently [52, 53].

Wei *et al.* [54] reported that APC cleavage at R^{306} is not necessary for inactivation of rFV or rFVa. In contrast, under the conditions of our studies, our data suggest that cleavage of R^{306} is necessary for efficient APC inactivation of FVa. However, in clotting assays, the APC-resistance ratio for unactivated Q^{306}-rFV in plasma is intermediate between that of normal plasma-derived or wild-type rFV and FVa, and that of Q^{306}-rFVa, Q^{506}-rFV or Q^{506}-rFVa (Fig. 6), indicating that the mechanism of inactivation of Q^{306}-rFV is distinct from that of Q^{306}-rFVa. Further studies will be needed to elucidate differences in mechanisms of inactivation of FV vs. FVa [25,36].

The double mutant $Q^{306}Q^{506}$-rFVa was extremely resistant to APC, with no significant loss of activity (± 10%) under all conditions tested and an APC-resistance ratio of 1.0. No heavy-chain fragments were observed on immunoblots of $Q^{306}Q^{506}$-rFVa incubated with APC. This suggests that either R^{679} cleavage did not occur in this mutant or that it contributed little to inactivation. Thus, cleavage either at R^{306} or at R^{506} is needed for any significant inactivation of FVa. However, the biphasic inactivation curves for Q^{506}-rFVa and Q^{306}-rFVa suggest that R^{679} cleavage probably contributes in a minor way to APC inactivation of these molecules, and fragments observed on immunoblots suggest that cleavage occurred at R^{679} in Q^{306}-rFVa and wild-type rFVa. It is possible that cleavage at 306 or 506 facilitates cleavage at 679. Nonenzymatic inhibition is not likely to explain the final phase of inactivation, as an active site mutant of APC (S360 A) that inhibits FVa without cleaving it [55] has a maximum effect in seconds, not in the ≥ 20 min seen here.

$Q^{306}Q^{506}$-rFV was very resistant to cleavage and activation by FXa, but not by thrombin. Thus APC cleavage or mutation at residues 306 and 506 may alter FXa-binding sites in FVa and in FV, and this supports our other studies demonstrating FXa-binding sites near these residues [56, 57]. In a three-dimensional model of the A domains of FVIIIa [58], the residues homologous to R^{306} and R^{506} in FVa are close spatially, and thus these regions in FVa may form an extended binding site for FXa. However, the mechanism of poor FXa activation of $Q^{306}Q^{506}$-rFV did not appear to involve inability of the double mutant to bind FXa. Rather, $Q^{306}Q^{506}$-rFV bound to FXa in a nonproductive manner in which the ability of FXa to generate activity from normal FV was inhibited (Fig. 5). The combined studies of the behavior of $Q^{306}Q^{506}$-rFV and $Q^{306}Q^{506}$-rFVa with FXa raise the interesting question of how the mode of binding of FXa to FV differs from that of FXa to FVa. Regions near FV residues 306 and 506 appear to be involved in either case.

A FXa-binding site and/or a prothrombin-binding site is possibly altered in $Q^{306}Q^{506}$-rFVa, because it a has a low activity-to-antigen ratio when thrombin activated. Since there is evidence from several of our studies using FVa or APC peptides [56,57,59–61] that a FXa-binding site(s) overlaps with an APC-binding site(s), altered binding of APC to the double mutant could also explain its resistance to R^{679} cleavage. The protection of FVa from APC by FXa [62] was shown to be exerted at the R^{506} site [26]. If binding sites for FXa and APC exist near both residues 306 and 506 in FVa, as suggested in our other studies [56,57,59,61], a double mutation may have a synergistic effect with respect to the altered binding of FXa and APC. This notion is further supported by the observation that Q^{506}-FV is strongly resistant to nonenzymatic inhibition by the active-site mutant of APC (S360 A) [55], suggesting that binding to APC is altered in Q^{506}-FV.

It is interesting to note that the specific activity of thrombin-activated $Q^{306}Q^{506}$-rFVa was ~ 50–77% based on comparison of activity with antigen and was approximately the same as seen in Q^{306}-rFVa that had been inactivated by APC, i.e. ~ 60% of maximum activity after 1 h. Thus, mutation at both sites appears to produce a FVa molecule about equivalent in activity to a molecule mutated at the 306 site and cleaved at the 506 site. It is possible to speculate that FXa binding may be impaired in either case. Upon titration with FXa, we found an apparent functional K_d of 35 pM for wild-type rFVa and Q^{306}-rFVa, in agreement with Ye and Esmon [63]. After the heavy chain of Q^{306}-rFVa was > 70% cleaved by APC, the apparent functional K_d increased to 80 pM, suggesting impairment of FXa-binding sites.

Use of normal FVa, wild-type rFVa and the several R^{306} and R^{506} APC cleavage site rFV mutants allows estimation of average rate constants for APC cleavages at R^{506}, R^{306} and R^{679} in FVa of 55, 9.4 and $0.6 \times 10^{6} M^{-1} \cdot s^{-1}$, respectively, which is in reasonable agreement with other reports [29, 33, 54]. Cleavage at R^{679} appears to contribute in a minor way to loss of FVa activity, although this was not proven. Since the APC-resistance ratio of Q^{306}-rFV differs from that of Q^{306}-rFVa, these studies also show that the mechanisms for inactivation of Q^{306}-rFV differ notably from those for Q^{306}-rFVa. FVa cleaved at R^{506} and R^{679} retains ~ 60% activity while FVa cleaved at R^{306} exhibits < 10% activity under the same conditions. Thus, the most important APC-cleavage site for efficient APC inactivation of FVa is R^{306}. The Q^{506} mutation in FV may be a relatively mild risk factor for thrombosis partly because it creates a deficiency in a rapid but mild anticoagulant mechanism, namely, the conversion of fully active FVa to partially active FVa cleaved by APC at R^{506}.

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REFERENCES

1. Rosing, J., Tans, G., Govers-Riemslag, J.W.P., Zwaal, R.F.A. & Hemker, H.C. (1980) The role of phospholipids and factor Va in the prothrombinase complex. *J. Biol. Chem.* **255**, 274–283.
2. Mann, K.G., Nesheim, M.E., Hibbard, L.S. & Tracy, P.B. (1981) The role of factor V in the assembly of the prothrombinase complex. In *Annals of the New York Academy of Sciences* (Walz, D.A. & McCoy, L.E., eds.), pp. 378–388. New York Academy of Sciences, New York.
3. Tracy, P.B. & Mann, K.G. (1983) Prothrombinase complex assembly on

the platelet surface is mediated through the 74,000-dalton component of factor Va. *Proc. Natl Acad. Sci. USA* **80**, 2380–2384.
4. Krishnaswamy, S., Church, W.R., Nesheim, M.E. & Mann, K.G. (1988) Activation of human prothrombin by human prothrombinase: influence of factor Va on the reaction mechanism. *J. Biol. Chem.* **262**, 3291–3299.
5. Kisiel, W., Canfield, W.M., Ericsson, L.H. & Davie, E.W. (1977) Anticoagulant properties of bovine plasma protein C following activation by thrombin. *Biochemistry* **16**, 5824–5831.
6. Odegaard, B. & Mann, K. (1987) Proteolysis of factor Va by factor Xa and activated protein C. *J. Biol. Chem.* **262**, 11233–11238.
7. Suzuki, K., Stenflo, J.A., Dahlbäck, B. & Teodorsson, B. (1983) Inactivation of human coagulation factor V by activated protein C. *J. Biol. Chem.* **258**, 1914–1920.
8. Griffin, J.H., Evatt, B., Zimmerman, T.S., Kleiss, A.J. & Wideman, C. (1981) Deficiency of protein C in congenital thrombotic disease. *J. Clin. Invest.* **68**, 1370–1373.
9. Branson, H.E., Katz, J., Marble, R. & Griffin, J.H. (1983) Inherited protein C deficiency and a coumarin-responsive chronic relapsing purpura fulminans syndrome in a neonate. *Lancet* **2**, 1165–1168.
10. Mannucci, P.M. & Vigano, S. (1982) Deficiencies of protein C, an inhibitor of blood coagulation. *Lancet* **2**, 463–466.
11. Bertina, R.M., Broekmans, A.W., van der Linden, I.K. & Mertens, K. (1982) Protein C deficiency in a Dutch family with thrombotic disease. *Thromb. Haemost.* **48**, 1–5.
12. Dahlbäck, B., Carlsson, M. & Svensson, P.J. (1993) Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc. Natl Acad. Sci. USA* **90**, 1004–1008.
13. Svensson, P.J. & Dahlbäck, B. (1994) Resistance to activated protein C as a basis for venous thrombosis. *N. Engl. J. Med.* **330**, 517–522.
14. Griffin, J.H., Evatt, B., Wideman, C. & Fernández, J.A. (1993) Anticoagulant protein C pathway defective in majority of thrombophilic patients. *Blood* **82**, 1989–1993.
15. Koster, T., Rosendaal, F.R., de Ronde, H., Briet, E., Vandenbroucke, J.P. & Bertina, R. (1993) Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden thrombophilia study. *Lancet* **342**, 1503–1506.
16. Sun, X., Evatt, B. & Griffin, J.H. (1994) Blood coagulation factor Va abnormality associated with resistance to activated protein C in venous thrombophilia. *Blood* **83**, 3120–3125.
17. Bertina, R.M., Koeleman, B.P.C., Koster, T., Rosendaal, F.R., Dirven, R.J., de Ronde, H., van der Velden, P.A. & Reitsma, P.H. (1994) Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* **369**, 64–67.
18. Greengard, J.S., Sun, X., Xu, X., Fernández, J.A., Griffin, J.H. & Evatt, B. (1994) Activated protein C resistance caused by Arg506Gln mutation in factor Va. *Lancet* **343**, 1361–1362.
19. Voorberg, J., Roelse, J., Koopman, R., Büller, H., Berends, F., ten Cate, J.W., Mertens, K. & van Mourik, J.A. (1994) Association of idiopathic venous thromboembolism with single point-mutation at Arg^{506} of factor V. *Lancet* **343**, 1535–1536.
20. Koeleman, B.P.C., Reitsma, P.H., Allaart, C.F. & Bertina, R.M. (1994) Activated protein C resistance as an additional risk factor for thrombosis in protein C-deficient families. *Blood* **84**, 1031–1035.
21. Zöller, B., He, X. & Dahlbäck, B. (1995) Homozygous APC-resistance combined with inherited type I protein S deficiency in a young boy with severe thrombotic disease. *Thromb. Haemost.* **73**, 743–745.
22. Gandrille, S., Greengard, J.S., Alhenc-Gelas, M., Juhan-Vague, I., Abgrall, J.F., Jude, B., Griffin, J.H., Aiach, M. & French Network on the behalf of INSERM (1995) Incidence of activated protein C resistance caused by the ARG 506 GLN mutation in factor V in 113 unrelated symptomatic protein C-deficient patients. *Blood* **86**, 219–224.
23. Brenner, B., Zivelin, A., Lanir, N., Greengard, J.S., Griffin, J.H. & Seligsohn, U. (1996) Venous thromboembolism associated with double heterozygosity for R506Q: mutation of factor V and for T298M: mutation of protein C in a large family of a previously described homozygous protein C-deficient newborn with massive thrombosis. *Blood* **88**, 877–880.
24. Kalafatis, M. & Mann, K.G. (1993) Role of the membrane in the inactivation of factor Va by activated protein C. *J. Biol. Chem.* **268**, 27246–27257.
25. Kalafatis, M., Rand, M.D. & Mann, K.G. (1994) The mechanism of inactivation of human factor V and human factor Va by activated protein C. *J. Biol. Chem.* **269**, 31869–31880.
26. Rosing, J., Hoekema, L., Nicolaes, G.A.F., Thomassen, M.C.L.G.D., Hemker, H.C., Varadi, K., Schwarz, H.P. & Tans, G. (1995) Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *J. Biol. Chem.* **270**, 27852–27858.
27. Kalafatis, M., Bertina, R.M., Rand, M.D. & Mann, K.G. (1995) Characterization of the molecular defect in factor V^{R506Q}. *J. Biol. Chem.* **270**, 4053–4057.
28. Heeb, M.J., Kojima, Y., Greengard, J. & Griffin, J.H. (1995) Activated protein C resistance: molecular mechanisms based on studies using purified Gln^{506}-factor V. *Blood* **85**, 3405–3411.
29. Nicolaes, G.A.F., Tans, G., Thomassen, M.C.L.G.D., Hemker, H.C., Pabringer, I., Varadi, K., Schwarz, H.P. & Rosing, J. (1995) Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *J. Biol. Chem.* **270**, 21158–21166.
30. Pittman, D.D. & Kaufman, R.J. (1993) Site-directed mutagenesis and expression in mammalian cells to evaluate structure–function relationships in coagulation factors V and VIII. *Methods Enzymol.* **222**, 236–260.
31. Marquette, K.A., Pittman, D.D. & Kaufman, R.J. (1995) The factor V B-domain provides two functions to facilitate thrombin cleavage and release of the light chain. *Blood* **86**, 3026–3034.
32. Keller, F.G., Ortel, T.L., Quinn-Allen, M.A. & Kane, W.H. (1995) Thrombin-catalyzed activation of recombinant human factor V. *Biochemistry* **34**, 4118–4124.
33. Egan, J.O., Kalafatis, M. & Mann, K.G. (1997) 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. *Protein Sci.* **6**, 2016–2027.
34. Yan, T.L., Cui, J., Rehemtulla, A., Yang, A., Moussalli, M., Kaufman, R.J. & Ginsburg, D. (1998) The structure and function of murine factor V and its inactivation by protein C. *Blood* **91**, 4593–4599.
35. Thorelli, E., Kaufman, R.J. & Dahlback, B. (1997) Cleavage requirements for activation of factor V by factor Xa. *Eur. J. Biochem.* **247**, 12–20.
35a. Heeb, M.J., Rehemtulla, A., Moussalli, M., Kojima, Y., Griffin, J.H. & Kaufman, R.J. (1996) Importance of individual activated protein C cleavage sites in the heavy chain of factor V and Va. *Blood* **88**, 440a.
36. Heeb, M.J., Rehemtulla, A., Moussalli, M., Kojima, Y. & Griffin, J.H. (1997). Arginine-306 is the most important activated protein C cleavage site for efficient inactivaiton of factor Va, but not of factor V. *Thromb Haemostas* (Suppl.) **598** (Abstract).
37. Gruber, A., Griffin, J.H., Harker, L. & Hanson, S.R. (1989) Inhibition of platelet-dependent thrombus formation by human activated protein C in a primate model. *Blood* **73**, 639–642.
38. Tans, G., Rosing, J., Thomassen, M.C., Heeb, M.J., Zwaal, R.F.A. & Griffin, J.H. (1991) Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. *Blood* **77**, 2641–2648.
39. DiScipio, R.G., Hermodson, M.A., Yates, S.G. & Davie, E.W. (1977) A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. *Biochemistry* **16**, 698–706.
40. Heeb, M.J., Mesters, R.M., Tans, G., Rosing, J. & Griffin, J.H. (1993) Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. *J. Biol. Chem.* **268**, 2872–2877.
41. Kaufman, R.J., Davies, M.V., Wasley, L.C. & Michnick, D. (1991) Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus. *Nucleic Acids Res.* **19**, 4485–4490.
42. Nesheim, M.E., Eid, S. & Mann, K.G. (1981) Assembly of the

prothrombinase complex in the absence of prothrombin. *J. Biol. Chem.* **256**, 9874–9882.

43. Mesters, R.M., Houghten, R.A. & Griffin, J.H. (1991) Identification of a sequence of human activated protein C (residues 390–404) essential for its anticoagulant activity. *J. Biol. Chem.* **266**, 24514–24519.
44. Pittman, D.D., Marquette, K.A. & Kaufman, R.J. (1994) Role of the B-domain for factor VIII and factor V expression and function. *Blood* **84**, 4214–4225.
45. Monkovic, D.D. & Tracy, P.B. (1990) Activation of human factor V by factor Xa and thrombin. *Biochemistry* **29**, 1118–1128.
46. Mann, K.G., Hockin, M.F., Begin, K.J. & Kalafatis, M. (1997) Activated protein C cleavage of factor Va leads to dissociation of the A2 domain. *J. Biol. Chem.* **272**, 20678–20683.
47. Fernández, J.A., Hackeng, T.M., Kojima, K. & Griffin, J.H. (1997) The carbohydrate moiety of factor V modulates inactivation by activated protein C. *Blood* **89**, 4348–4354.
48. Rosing, J., Bakker, H., Thomassen, M.C.L.G.D., Thomassen, L., Hemker, H. & Tans, G. (1993) Characterization of two forms of human factor Va with different cofactor activities. *J. Biol. Chem.* **268**, 21130–21136.
49. Ortel, T.L., Quinn-Allen, M.A., Keller, F.G., Peterson, J.A., Larocca, D. & Kane, W.H. (1994) Localization of functionally important epitopes within the second C-type domain of coagulation factor V using recombinant chimeras. *J. Biol. Chem.* **269**, 15898–15905.
50. Kalafatis, M., Rand, M.D., Jenny, R.J., Ehrlich, Y.H. & Mann, K.G. (1993) Phosphorylation of factor Va and factor VIIIa by activated platelets. *Blood* **81**, 704–719.
51. Pittman, D.D., Tomkinson, K.N., Michnick, D., Selighsohn, U. & Kaufman, R.J. (1994) Posttranslational sulfation of factor V is required for efficient thrombin cleavage and activation and for full procoagulant activity. *Biochemistry* **33**, 6952–6959.
52. Chan, W.P., Lee, C.K., Kwong, Y.L., Lam, C.K. & Liang, R. (1998) A novel mutation of Arg306 of factor V gene in Hong Kong Chinese. *Blood* **91**, 1135–1139.
53. Williamson, D., Brown, K., Luddington, R., Baglin, C. & Baglin, T. (1998) Factor V Cambridge: a new mutation ($Arg^{306} \rightarrow Thr$) associated with resistance to activated protein C. *Blood* **91**, 1140–1144.
54. Wei, C.-J., Quinn-Allen, M.-A. & Kane, W.H. (1995) Cleavage at arginine 306 is not required for inactivation of factor V by activated protein C (APC). *Blood* **86**, 615a. (Abstract).
55. Gale, A.J., Sun, X., Heeb, M.J. & Griffin, J.H. (1997) Nonenzymatic anticoagulant activity of the mutant serine protease Ser360Ala-activated protein C mediated by factor Va. *Protein Sci.* **6**, 132–140.
56. Heeb, M.J., Kojima, Y., Hackeng, T.M. & Griffin, J.H. (1996) Binding sites for blood coagulation factor Xa and protein S involving residues 493–506 in factor Va. *Protein Sci.* **5**, 1883–1889.
57. Kojima, Y., Heeb, M.J., Gale, A., Hackeng, T.M. & Griffin, J.H. (1998) Binding sites for coagulation factor Xa involving residues 311–335 in factor Va. *J. Biol. Chem.* **273**, 14900–14905.
58. Pemberton, S., Lindley, P., Zaitsev, V., Card, G., Tuddenham, E.G.D. & Kemball-Cook, G. (1997) A molecular model for the triplicated A domains of human factor VIII based on the crystal structure of human ceruloplasmin. *Blood* **89**, 2413–2421.
59. Heeb, M.J., Kojima, Y., Hackeng, T.M. & Griffin, J.H. (1994) Functional studies of factor Va residues 493–506 related to activated protein C resistance. *Blood* **84**, 391a. (Abstract).
60. Mesters, R.M., Heeb, M.J. & Griffin, J.H. (1993) Interactions and inhibition of blood coagulation factor Va involving residues 311–325 of activated protein C. *Protein Sci.* **2**, 1482–1489.
61. Kojima, Y., Heeb, M.J., Hackeng, T.M., Gale, A. & Griffin, J.H. (1997) Binding sites for coagulation factor Xa and activated protein C involving residues 311–335 in factor Va. *Thromb. Haemost.* (Suppl.) **June**, 423–424(Abstract).
62. Nesheim, M.E., Canfield, W.M., Kisiel, W. & Mann, K.G. (1982) Studies of the capacity of factor Xa to protect factor Va from inactivation by activated protein C. *J. Biol. Chem.* **257**, 1443–1447.
63. Ye, J. & Esmon, C.T. (1995) Factor Xa-factor Va complex assembles in two dimensions with unexpectedly high affinity: an experimental and theoretical approach. *Biochemistry* **34**, 6448–6453.