Molecular mechanisms of activated protein C resistance

Properties of factor V isolated from an individual with homozygosity for the Arg⁵⁰⁶ to Gln mutation in the factor V gene

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Resistance to activated protein C (APC), which is the most prevalent pathogenetic risk factor of thrombosis, is linked to a single point-mutation in the factor V (FV) gene, which predicts replacement of Arg (R) at position 506 with a Gln (Q). This mutation modifies one of three APC-cleavage sites in the heavy chain of activated FV (FVa), suggesting that mutated FVa (FVa:Q⁵⁰⁶) is at least partially resistant to APC-mediated degradation. To elucidate the molecular mechanisms of APC-resistance and to investigate the functional properties of FV in APC resistance, FV:Q⁵⁰⁶ was purified from an individual with homozygosity for the Arg to Gln mutation. Intact and activated FV:Q⁵⁰⁶ were demonstrated to convey APC resistance to FV-deficient plasma. Thrombin- or factor Xa-activated FV:Q⁵⁰⁶ were found to be approx. 10-fold less sensitive to APC-mediated

degradation than normal FVa, at both high and low phospholipid concentrations. The degradation pattern observed on Western blotting suggested that FVa:Q⁵⁰⁶ was not cleaved at position 506. However, it was slowly cleaved at Arg³⁰⁶, which explains the partial APC sensitivity of FVa:Q⁵⁰⁶. FV is initially activated during clotting and then rapidly inactivated in a process which depends on the integrity of the protein C anticoagulant system. During clotting of APC-resistant plasma, FV:Q⁵⁰⁶ was activated in a normal fashion, but then only partially inactivated. In conclusion, the reduced sensitivity of FVa:Q⁵⁰⁶ to APC-mediated degradation is the molecular basis for the life-long hypercoagulable state which constitutes a risk factor for thrombosis in APC-resistant individuals.

INTRODUCTION

The activation of prothrombin by the phospholipid-bound complex between activated factor X (FXa) and activated factor V (FVa) is a central event of blood coagulation [1]. Factor V (FV) circulates in plasma as a multi-modular (from N-terminus, A1-A2-B-A3-C1-C2 modules) single-chain procofactor ($M_r = 330000$), and is activated during coagulation by thrombin and/or FXa [2]. FVa comprises the heavy chain ($M_r = 105000$; A1-A2 modules) linked by Ca²⁺-dependent bonds to the light chain ($M_r = 75000$; A3-C1-C2 modules). The activity of FVa is inhibited by the protein C anticoagulant system [3]. Activated protein C (APC) cleaves the heavy chain of FVa sequentially at Arg⁵⁰⁶, Arg³⁰⁶ and Arg⁶⁷⁹, which leads to complete loss of procoagulant activity [4]. The activity of APC is supported by two cofactors, the vitamin K-dependent protein S and the intact form of FV [5,6].

APC resistance has recently been identified as the most common genetic risk factor for venous thrombosis [reviewed in 7]. The observation that purified FV corrects APC resistance linked the phenotype to the FV molecule [8] and the APC-resistance phenotype was recently shown to be associated with a single G to A point mutation at nucleotide position 1691, predicting replacement of Arg⁵⁰⁶ by Gln [9–12]. As the APC-mediated cleavage at Arg⁵⁰⁶ is reported to be necessary for optimal exposure of the other cleavage sites in FVa [4], a likely molecular explanation for the APC-resistance phenotype is that the Arg⁵⁰⁶ to Gln mutation renders FVa partially or completely resistant to APC-mediated degradation. However, there is some controversy in the literature on this topic. Bertina et al. [9] reported FXa-activated FVa:Q⁵⁰⁶ [i.e. FVa with glutamine (Q) at position 506], present in aluminium hydroxide-adsorbed plasma from a homozygous individual, to be resistant to APC,

whereas the thrombin-activated form was found to be APC sensitive. On the other hand, Sun et al. [13] demonstrated that 50% of thrombin-activated FV from two heterozygous individuals was APC-resistant, suggesting that thrombin-activated FV:Q⁵⁰⁶ is resistant to APC-mediated degradation. To resolve this controversy, we have isolated FV:Q⁵⁰⁶ from the plasma of a homozygous individual and characterized some of its functional properties. Our results demonstrate that the APC-resistance phenotype is conveyed both by FV:Q⁵⁰⁶ and FVa:Q⁵⁰⁶ and that FVa:Q⁵⁰⁶ is resistant to cleavage by APC at position 506, which is the likely molecular mechanism for the APC-resistance phenotype. During the preparation of this article, a paper by Kalafatis et al. [14] appeared, which reports on the purification of FV:Q⁵⁰⁶ from two individuals with homozygosity for the Arg⁵⁰⁶ to Gln mutation. Kalafatis and colleagues demonstrated that mutated FVa (FVa:Q⁵⁰⁶) is not cleaved at position 506 by APC, but that APC slowly inactivates FVa:Q⁵⁰⁶ by cleavages both at Arg³⁰⁶ and Arg⁶⁷⁹. Our present study confirms and extends the results reported by Kalafatis et al. [14].

MATERIALS AND METHODS

Proteins

Human protein C was purified from fresh frozen plasma essentially as previously described [15], but with monoclonal antibody (mAb) (HpC-4) affinity chromatography as the last purification step. mAb HpC-4, which recognizes a calcium-stabilized epitope in the first epidermal growth factor-like (EGF) module of human protein C [16], was coupled to Affigel 10 (Biorad) according to the manufacturer's instructions. The column was equilibrated with 50 mM Tris/HCl/0.15 M NaCl/2 mM CaCl₂, pH 7.4, containing 10 mM benzamidine,

Abbreviations used: APC, activated protein C; FV, factor V; FVa, activated factor V; FV:Q⁵⁰⁶, factor V with glutamine (Q) at position 506; FV:R⁵⁰⁶, factor V with arginine (R) at position 506; FXa, activated factor X; mAb, monoclonal antibody; HPC4, mAb against protein C; HPS54, mAb against protein S; HFV30, mAb against factor V; EGF, epidermal growth factor; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; PEG, polyethylene glycol; APTT, activated partial thromboplastin time.

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and after sample application, the column was washed with the same buffer containing 1 M NaCl instead of 0.15 M NaCl. Protein C was eluted with 0.1 M acetic/acetate, pH 4.0, containing 10 mM benzamidine. Protein C was activated by thrombin as described [17]. Human protein S [18] and bovine α -thrombin [19] were purified as reported in the respective references. Bovine FXa, purified and activated as described earlier [20,21], was a gift from Dr. J. Stenflo (University Hospital, Malmö). mAb HPC4 (note, this is not the antibody which was used for purification), which recognizes the activation peptide of protein C [22], was a gift from Dr. C. T. Esmon (Oklahoma Medical Research Foundation, OK, U.S.A.). This antibody, which blocks the activation of protein C, does not inhibit the function of APC. mAb HFV30 was directed against human FV [8]. mAb HPS54, directed against the first EGF-module of human protein S, efficiently blocks the APC-cofactor activity of protein S [23].

Purification of factor V from plasma

APC-resistant plasma was obtained from an individual with homozygosity for the Arg⁵⁰⁶ to Gln mutation in the FV gene by plasmapheresis, after informed consent. Control plasma from subjects with normal APC ratios was obtained from the local blood bank. FV was purified from plasma as previously outlined [24], but with the following modifications. Freshly frozen citrated plasma (10 ml for each preparation) was thawed at 37 °C and the following protease inhibitors were added: 1 mM PMSF, 10 mM benzamidine, 1.5 μ g/ml aprotinin, 50 μ g/ml soybean trypsin inhibitor and 1 μ M D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (final concentrations). The plasma was subjected to barium citrate absorption and polyethylene glycol (PEG)-6000 precipitation as described. The 8–12% PEG pellet was resuspended in 20 mM Tris/0.1 M NaCl/2 mM CaCl₂, pH 7.5, containing 0.5 μ M PPACK, and applied to a Q-Sepharose fast-flow (Pharmacia) column (0.5 $\times$ 4 cm). The adsorbed proteins were eluted at 100 ml/h with a 20 ml linear gradient of NaCl (0.2–0.8 M). Fractions containing FV coagulant activity were pooled and subjected to (NH₄)₂SO₄ precipitation (75% saturation). The precipitate was resuspended in 50 mM Tris/0.1 M NH₄Cl/10 mM CaCl₂, pH 7.5, containing 0.5 μ M PPACK, and applied to a Sephacryl S-300 (Pharmacia) column (1 $\times$ 22 cm) equilibrated in the same buffer (flow rate 1 ml/min). The fractions were analysed by SDS/PAGE, Western blotting and with FV clotting assay. Fractions containing FV were pooled, dialysed against 50 mM Tris/0.15 M NaCl/2 mM CaCl₂, pH 7.5, and stored at –80 °C after the addition of 0.1% PEG-6000 (final concentration). The FV concentration in the pool was quantified by ELISA.

Electrophoretic and immunological methods

SDS/PAGE, using 5–15% gradient gels, was performed as described previously [25]. Samples were reduced with dithiothreitol and subsequently alkylated with iodoacetamide. The proteins were transferred to membranes [Immobilon (polyvinylidene difluoride); Millipore] and FV was detected using a rabbit polyclonal antiserum against human FV (Dako) as primary antibody, and an alkaline phosphatase-conjugated swine anti-rabbit antiserum as secondary antibody (Dako). The intensity of immunoreactive bands was quantified using a laser densitometer (Molecular Dynamics). FV was measured by ELISA using standard procedures. Microtitre plates (Linbro/Titertek) were coated with a polyclonal antibody against human FV (10 μ g/ml; Dako), samples were added and incubated for 2 h. Bound FV was detected with biotinylated HFV30 (2.25 μ g/ml). The wells were washed and incubated with a solution containing

streptavidin and biotinylated horseradish peroxidase (Dako A/S) for 30 min. After multiple washes, the amount of attached peroxidase was measured with 1,2-phenylenediamine (Dako A/S) and H₂O₂ diluted in 0.5 M citrate, pH 4. The reaction was stopped by addition of 0.5 M H₂SO₄ and the absorbance measured at 490 nm. Pooled human plasma was used to construct the standard curve.

Activation of factor V and inactivation of factor Va

FV (50 nM) in 50 mM Tris/0.15 M NaCl/2 mM CaCl₂, pH 7.5, containing 0.1% PEG-6000, was activated using bovine α -thrombin (3 units/ml final concentration) at 37 °C for 15 min. Thrombin was then neutralized by the addition of hirudin (final concentration 4.5 units/ml; Sigma). Alternatively, FV was activated by bovine FXa (final concentration of 5 nM) at 37 °C for 15 min in the presence of 50 μ M phospholipid (the same preparation as is used in the Coatest Factor VIII kit from Chromogenix; a gift from Dr. S. Rosén at Chromogenix). Conditions used for FV activation by thrombin and FXa were chosen to yield maximal FVa activity. FVa was incubated with human APC at 37 °C in the presence of 50 μ M or 50 nM phospholipid vesicles. Aliquots were removed at various times and assayed for FV procoagulant activity and analysed by SDS/PAGE and Western blotting.

Clotting assays

FV procoagulant activity was measured with a clotting assay, as described [24]. FV-deficient plasma (50 μ l; Stago) was incubated with sample (50 μ l) at 37 °C for 30 s. Simplastin Excel (100 μ l; Organon Teknica) was then added and the clotting time recorded. FV activity was calculated from standard curves based on human pooled plasma, assuming a plasma concentration of 1 unit/ml. In a modified APC-resistance test [26], purified FV or FVa (50 μ l) was added to FV-deficient plasma (50 μ l) and incubated at 37 °C with activated partial thromboplastin time (APTT) reagent (50 μ l; Coatest APC-resistance, Chromogenix) for 200 s. Clotting was initiated with 40 mM CaCl₂ (50 μ l) in the presence or absence of APC. The APC ratio is the clotting time measured in the presence of APC divided by the clotting time obtained in the absence of APC. The thrombin-generation potential of normal and APC-resistant plasma was measured essentially as described by Hemker et al. [27]. The reaction mixtures, consisting of 200 μ l of plasma and 50 μ l of 50 mM Tris/0.1 M NaCl, pH 7.35, were kept at 37 °C and magnetically stirred. After 5 min, coagulation was initiated by the addition of 50 μ l of thromboplastin (Simplastin Excel; final dilution 1/100) in CaCl₂ (10 mM final concentration). This dilution of thromboplastin yielded clotting times of around 35 s in both normal and APC-resistant plasma. Every 10 s, 2 μ l aliquots were drawn and added to microtitre wells containing 93 μ l of buffer [50 mM Tris/0.1 M NaCl/20 mM EDTA (pH 7.9)/1% PEG-6000] and 5 μ l of 4 mM S-2238, prewarmed at 30 °C. After 10 min, the reactions were stopped by addition of 60 μ l of concentrated acetic acid and the absorbance read at 405 nm.

Statistical analysis

The results are expressed as means $\pm$ S.E.M. of 3–5 different experiments.

RESULTS

Purification of FV:Q⁵⁰⁶

FV:Q⁵⁰⁶ was purified from plasma obtained from an APC-resistant individual with homozygosity for the G to A mutation

at nucleotide position 1691 in the FV gene, which predicts replacement of Arg⁵⁰⁶ with Gln. The purification procedure was devised to allow rapid isolation of FV from small amounts of plasma; approx. 15 µg of FV was obtained from 10 ml of normal or APC-resistant plasma. The purity of the isolated FV was between 50 and 70 %, as judged by its specific activity (approximately 65 units/mg) and by silver-stained SDS/PAGE.

APC resistance conveyed by FV:Q⁵⁰⁶ and FVa:Q⁵⁰⁶

FV-deficient plasma was reconstituted with FV:Q⁵⁰⁶ or FV:R⁵⁰⁶ [FV with arginine (R) at position 506] (either intact or thrombin-activated forms) and the anticoagulant response to APC tested in an APTT-based assay. Different concentrations of FV or FVa were tried (from 0 to 50 % of the normal plasma concentration) and the conditions giving the best discrimination between normal and mutated FV (or FVa) were selected for presentation (Figure 1). FV:Q⁵⁰⁶ conveyed APC resistance to the plasma, whereas the addition of FV:R⁵⁰⁶ resulted in plasmas with normal APC sensitivity (Figure 1A). Similar results were obtained when the thrombin-activated forms of FV:Q⁵⁰⁶ or FV:R⁵⁰⁶ were used and also when the two FV forms were activated by FXa instead of by thrombin (Figure 1B). This demonstrated that the APC-resistance phenotype was conveyed not only by intact FV:Q⁵⁰⁶ but also by its activated counterpart.

High concentrations of protein S do not correct APC resistance

Protein S is a cofactor of APC in the degradation of FVa but it is not known whether protein S is equally important for the three different APC-cleavage sites in FVa. To elucidate whether the APC resistance of FVa:Q⁵⁰⁶ could be overcome by high concentrations of protein S, increasing concentrations of protein S were added to plasma containing FVa:Q⁵⁰⁶ or FVa:R⁵⁰⁶ and the APC response was measured (Figure 2). A dose-dependent increase in the APC ratio was observed in plasma containing FVa:R⁵⁰⁶, whereas plasma containing FVa:Q⁵⁰⁶ remained APC resistant also at high concentrations of protein S. This demonstrates that the APC-resistance phenotype is not corrected by high concentrations of protein S, suggesting that the rate of APC-cleavage at Arg³⁰⁶ is unaffected by high concentrations of protein S. In the absence of APC, the addition of protein S did not affect the results of the APTT assay.

Figure 1 Intact and activated FV:Q⁵⁰⁶ convey APC-resistance

(A) Intact FV:Q⁵⁰⁶ (◆) or intact FV:R⁵⁰⁶ (◇), were added to FV-deficient plasma (10% of the normal FV concentration). The APC response of the reconstituted plasmas was measured using an APTT-based test. Final APC concentrations in the assay are given. (B) The same experiment using thrombin-activated FV (● and □) or FXa-activated FV (▲ and △). Filled symbols, FVa:Q⁵⁰⁶; open symbols, FVa:R⁵⁰⁶.

Figure 2 Increasing concentrations of protein S did not reverse APC resistance

FV-deficient plasma was supplemented with (●) thrombin-activated FVa:Q⁵⁰⁶ or (□) thrombin-activated FVa:R⁵⁰⁶ (to 20% of normal FV level). Increasing concentrations of human protein S (final concentrations in the assay given) were added to the plasma-APTT reagent mixture and the APTT test was performed in the absence or presence of 5.2 nM APC.

Figure 3 Resistance of thrombin-activated FVa:Q⁵⁰⁶ to degradation by APC

(A) (●) FVa:Q⁵⁰⁶ or (□) FVa:R⁵⁰⁶ (50 nM each) were incubated with 5.2 nM APC in the presence of 50 µM phospholipid vesicles. At the indicated time-points, samples were removed and the remaining FVa activity measured. (B) (●) FVa:Q⁵⁰⁶ or (□) FVa:R⁵⁰⁶ (50 nM each) were incubated with increasing concentrations of APC in the presence of 50 µM phospholipid vesicles. The remaining FVa activity was measured after 15 min of incubation. (C) (▲) FVa:Q⁵⁰⁶ or (◇) FVa:R⁵⁰⁶ (50 nM each) were incubated with 16 nM APC in the presence of 50 nM phospholipid vesicles. At the indicated time-points, the remaining FVa activity was measured. (D) (▲) FVa:Q⁵⁰⁶ or (◇) FVa:R⁵⁰⁶ (50 nM each) were incubated with increasing concentrations of APC in the presence of 50 nM phospholipid vesicles. The remaining FVa activity was measured after a 30 min incubation.

APC-mediated inhibition of procoagulant activities of FVa:Q⁵⁰⁶ and FVa:R⁵⁰⁶

The ability of APC to inhibit the procoagulant activities of thrombin-activated FVa:Q⁵⁰⁶ and FVa:R⁵⁰⁶ was investigated in a system containing purified components (Figure 3). To elucidate

Figure 4 APC-mediated degradation of FVa:Q⁵⁰⁶ and FVa:R⁵⁰⁶

(a) Aliquots from the same incubations as in Figures 3(A) and 3(B) were removed at different times (left panel) or at 15 min (right panel), and analysed by SDS/PAGE and Western blotting. Arrows denote fragments formed as a result of APC-cleavage. HC denotes the position of intact heavy chain. (b) Schematic representation of inactivation of FVa:R⁵⁰⁶ and FVa:Q⁵⁰⁶ heavy chains by APC. Fragments recognized by the polyclonal antibodies against human FV are shaded.

the importance of the phospholipid vesicles in these reactions, the experiment was performed at high and low phospholipid concentrations (50 μ M and 50 nM). At both phospholipid concentrations, FVa:R⁵⁰⁶ was more efficiently inactivated than FVa:Q⁵⁰⁶. However, at high phospholipid levels, the proteolytic reactions proceeded at higher rates than at low phospholipid concentrations and less APC was required to inhibit the activity of both FVa:R⁵⁰⁶ and FVa:Q⁵⁰⁶. This indicated that APC-mediated degradation of both normal and mutated FVa is a phospholipid-oriented process. At both phospholipid concentrations, the rates of inactivation of FVa:Q⁵⁰⁶ were approx. 10-fold lower than those of FVa:R⁵⁰⁶, and the dose-response curves suggested that FVa:Q⁵⁰⁶ was approx. 10-fold less sensitive to APC than was FVa:R⁵⁰⁶ (Figure 3).

APC-mediated degradation of FVa:Q⁵⁰⁶ and FVa:R⁵⁰⁶ as revealed by Western blotting

Thrombin-activated FVa:R⁵⁰⁶ and FVa:Q⁵⁰⁶ demonstrated different patterns of APC-mediated degradation, as judged by Western blotting (Figure 4). The polyclonal antiserum against human FV that was used, mainly recognized epitopes present in

the heavy chain ($M_r = 105000$) or in the B module ($M_r = 150000$), and it reacted only weakly with the light chain ($M_r = 75000$). For a proper interpretation of the degradation pattern on the Western blotting, it is important to note that the antiserum reacted with the C-terminal half of the heavy chain but not with the rest of the chain; the epitopes were located within residues 507–709 of the A2-module (illustrated in Figure 4b). In the presence of 50 μ M phospholipid, APC efficiently degraded FVa:R⁵⁰⁶. The loss of the heavy chain coincided with the loss of FV activity and with the appearance of a doublet band (M_r approx. 28000), which corresponded to the immunoreactive C-terminal half of the A2 domain of FV [4]. The rate of degradation of the heavy chain of FVa:Q⁵⁰⁶ was slower than that of FVa:R⁵⁰⁶, and proteolytic fragments were only observed at the highest concentrations of APC. The loss of the heavy chain of FVa:Q⁵⁰⁶ coincided with loss of FV activity and with the appearance of a heterogeneous band with M_r of approx. 56000 (Figure 4), which represented the A2 domain of FVa after cleavage at Arg³⁰⁶, and possibly at Arg⁶⁷⁹ [14]. The pattern of APC-mediated degradation on this Western blotting was consistent with the results of Kalafatis et al. [14], demonstrating that FVa:Q⁵⁰⁶ was not cleaved at position 506.

Figure 5 Importance of protein C and protein S in the activation of FV during clotting and subsequent FVa inactivation

APTT reagent (final dilution 1/30) was mixed with normal or APC-resistant plasma (300 μ l) and incubated during magnetic stirring at 37 °C for 3 min before recalcification. Clotting occurred between 2.5 and 3 min. Aliquots were removed at intervals, diluted in 50 mM Tris/0.15 M NaCl, pH 7.4, containing 0.1% PEG-6000, and assayed for FV activity. Normal (A) or APC-resistant (B) plasma was incubated for 3 min at 37 °C with 65 μ g/ml of polyclonal anti-(protein C) antibody (Dako) (◆), 100 μ g/ml of HPC4 mAb (against activation peptide) (△), or with 50 mM Tris/0.15 M NaCl, pH 7.4 (□, ●), before recalcification. Normal (C) or APC-resistant (D) plasma was incubated for 3 min at 37 °C with 50 μ g/ml of mAb against protein S (HPS54) (▽), or with 50 mM Tris/0.15 M NaCl, pH 7.4 (□, ●), before recalcification. The differences in maximum FV activity (at 4 min) observed in APC-resistant plasma in the presence or absence of antibodies (B and D) were not statistically significant (unpaired *t*-test).

Activation and inactivation of FV:Q⁵⁰⁶ and FV:R⁵⁰⁶ during coagulation

During blood coagulation *in vitro*, the initial activation of FV is followed by a loss of FVa activity, which is caused by FVa degradation mediated by APC which is formed during the clotting reaction [28]. To investigate whether the activation and inactivation processes were affected by the Arg⁵⁰⁶ to Gln mutation, clotting of normal and APC-resistant plasma was induced and the FV activity measured (Figure 5). The use of diluted APTT reagent, rather than platelet-rich plasma [28], resulted in relatively slow activation of the coagulation system, which allowed detailed monitoring of the process. The initial FV activation appeared to be similar in the two plasmas, but FVa formed in APC-resistant plasma was considerably more stable than that formed during clotting of normal plasma. That the FVa inactivation was in fact mediated by APC was evident from the reduced inactivation rate of FVa in normal plasma observed upon the addition of either polyclonal or monoclonal antibodies against human protein C (mAb HPC4, which inhibits protein C activation [22]). In contrast, the FVa activity in APC-resistant plasma remained unaffected by the protein C antibodies (Figures 5A and 5B). Indeed, in the presence of protein C antibodies, the rates of FVa inactivation of normal and APC-resistant samples were quite similar. To investigate the role of protein S in the FVa inactivation that followed upon clotting, a mAb against protein S (HPS54), which completely inhibited APC-cofactor activity of protein S [23], was added (Figures 5C and 5D). In normal plasma, HPS54 was equally efficient at inhibiting FVa inactivation as the anti-(protein C) antibodies, whereas the presence

Figure 6 Thrombin-generation potential of normal and APC-resistant plasma

Thrombin-generation curves of normal (□) and APC-resistant plasma (●) were measured using the chromogenic substrate S-2238 as described in the Materials and methods section.

of HPS54 in APC-resistant plasma did not significantly affect the course of FVa inactivation. These results suggest that the difference in FVa inactivation rates between normal and APC-resistant plasma is related to the different sensitivities of FVa:R⁵⁰⁶ and FVa:Q⁵⁰⁶ to degradation by APC.

Thrombin-generation potential of normal and APC-resistant plasma

During coagulation of plasma, a burst of thrombin generation occurs during and after the formation of the clot. The thrombin-generation potential of normal and APC-resistant plasma was found to be essentially identical (Figure 6), indicating that the FV:Q⁵⁰⁶ mutation did not influence the process to any major extent. In both plasmas, the thrombin concentration peaked approx. 2 min after initiation of the coagulation process and this was followed by a slow loss of thrombin activity, presumably as a result of inhibition of thrombin by antithrombin III. In this experiment, a diluted thromboplastin reagent was used to initiate the clotting process. As a result, the rate of thrombin generation was low and presumably very little APC was formed during the first minutes of the clotting reaction. This is probably why normal and APC-resistant plasma yielded identical thrombin-generation curves.

DISCUSSION

APC resistance, which is the most prevalent genetic risk factor of thrombosis, is linked to a single point-mutation in the FV gene, which predicts replacement of Arg⁵⁰⁶ in one of the APC-cleavage sites with Gln [9–12]. Partially purified FV from individuals with heterozygosity for FV:Q⁵⁰⁶ has been shown to transfer APC resistance to FV-deficient plasma [13]. We now demonstrate that purified FV:Q⁵⁰⁶, from an individual with homozygosity for the Arg⁵⁰⁶ to Gln mutation, conveys APC resistance to FV-deficient plasma, which is in agreement with the conclusion that the APC-resistance phenotype is caused by a molecular defect residing in the FV molecule. Thrombin- or FXa-activated FV:Q⁵⁰⁶ was also found to convey APC resistance, showing the molecular defect to be present in the parts of FV which constitute FVa.

During degradation of FVa by APC, three peptide bonds are sequentially cleaved in the heavy chain, at Arg⁵⁰⁶, Arg³⁰⁶ and

Arg⁶⁷⁹ [4]. FVa:Q⁵⁰⁶ is therefore expected to be less sensitive towards APC than FVa:R⁵⁰⁶. However, there is some disagreement in the literature as to the question of whether or not thrombin-activated FVa:Q⁵⁰⁶ is resistant to APC [9,14]. Our results, together with those recently reported by Kalafatis et al. [14], help resolve this problem. Kalafatis et al. [14] reported that the Arg⁵⁰⁶ to Gln mutation was associated with resistance to APC-mediated cleavage at position 506. However, FVa:Q⁵⁰⁶ was shown to be inactivated by APC-mediated cleavages both at Arg³⁰⁶ and Arg⁶⁷⁹, which led to the formation of two major heavy-chain fragments of 45 kDa (A1 domain) and 56/54 kDa (A2 domain). The conclusion from our study is the same, i.e. FVa:Q⁵⁰⁶ is not completely resistant to APC even though there is a major difference in APC sensitivity between FVa:Q⁵⁰⁶ and FVa:R⁵⁰⁶. APC was found to be approx. 10-fold less efficient at degrading thrombin-activated FVa:Q⁵⁰⁶ than thrombin-activated FVa:R⁵⁰⁶, at both high and low phospholipid concentrations. The same difference in APC sensitivity between normal and mutated FV was observed after activation of FV with FXa. The rate of degradation of both normal and mutated FV was dependent on the phospholipid concentration. At low phospholipid levels, the degradation of FVa:Q⁵⁰⁶ proceeded very slowly, and even after a 1 h incubation with a high concentration of APC only 30 % of FVa:Q⁵⁰⁶ was inactivated. It is possible that the reported differences in the APC sensitivity of mutated FVa are related to differences in experimental conditions, e.g. in the concentration of phospholipid that has been used. The influence of the phospholipid concentration on the rate of FVa degradation may be of physiological significance. Under normal conditions, the amount of negatively charged phospholipids in contact with blood is low. The decreased APC sensitivity of FVa:Q⁵⁰⁶, as compared to FVa:R⁵⁰⁶, at such low phospholipid concentrations probably leads to a hypercoagulable state which contributes to the increased thrombotic tendency.

During coagulation, FV is rapidly activated to FVa, which is then inactivated by APC which is formed during the clotting process [28]. In normal and APC-resistant plasma, no difference in the activation process of FV to FVa was observed and the thrombin generation potential was found to be similar in the two plasmas. However, after coagulation, normal FVa was rapidly inactivated while the FVa activity in the APC-resistant sample was only slowly and partially lost. The addition of antibodies to protein C reverted the inactivation of FVa in normal plasma to the degree observed in APC-resistant plasma. This agrees with the concept that APC plays an essential role in the inactivation of FVa after clotting *in vitro*. The effect of protein S on the stability of FVa during clotting of plasma was also examined with the help of a mAb against human protein S, which efficiently inhibited the APC-cofactor function of protein S. In normal plasma, FVa inactivation was counteracted by the protein S antibody, whereas the FVa activity of APC-resistant plasma was not significantly affected by the antibody. These results suggested that the impaired FVa inactivation observed after coagulation of APC-resistant plasma was caused by the poor sensitivity of FVa:Q⁵⁰⁶ to APC.

In conclusion, in addition to confirming the results of Kalafatis et al. [14] that FV:Q⁵⁰⁶ is not cleaved at position 506 by APC, our results provide new insights into the molecular mechanisms of APC resistance. Thus, we demonstrate that not only intact, but also thrombin- and FXa-activated FV:Q⁵⁰⁶, convey APC resistance on plasma and that APC resistance is not corrected by high concentrations of protein S. Moreover, the rate of APC-

mediated inactivation of FVa:Q⁵⁰⁶ was shown to depend on the phospholipid concentration, APC resistance being particularly pronounced at low levels of phospholipid. Furthermore, FV:Q⁵⁰⁶ was found to be activated normally during clotting, whereas the APC-mediated FV inhibition that followed upon clotting was delayed in APC-resistant plasma as compared to normal plasma. The increased stability of mutated FVa leads to increased thrombin generation, a hypercoagulable state and a life-long increased risk of thrombosis [12]. Heterozygosity for FV:Q⁵⁰⁶ is associated with a 5–10-fold increased risk of venous thromboembolism. The observation that even homozygosity is a relatively mild risk factor (50–100-fold increased risk) (reviewed in [7]), suggests that there are alternative mechanisms by which APC regulates the coagulation process. Normal degradation of FVIIIa and slow degradation of FVa:Q⁵⁰⁶ as a result of cleavages at Arg³⁰⁶ and Arg⁶⁷⁹ by APC help explain why APC resistance is such a mild risk factor for thrombosis.

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