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# An Antifibrinolytic Mechanism Describing the Prothrombotic Effect Associated with Factor $V^{Leiden}$*

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**Factor Va is the essential cofactor in prothrombinase-dependent activation of prothrombin. Resistance of Factor $Va^{Leiden}$ to inactivation by activated protein C (APC) contributes to thrombotic tendencies in subjects with the variant due, in part, to the inability to terminate thrombin production which increases both fibrin accretion and the frequency of thrombus formation. A reduced ability to inhibit thrombin generation, however, may lead to the stabilization of a clot through the activation of thrombin activatable fibrinolysis inhibitor (TAFI). This hypothesis was tested by determining the profibrinolytic effect of APC on lysis time using clots formed with plasma from either homozygous normal ($n$ = 4) or homozygous factor $V^{Leiden}$ ($n$ = 4) subjects. Clots were formed in the presence of tissue-type plasminogen activator, thrombin, phosphatidylcholine/phosphatidylserine vesicles, $Ca^{2+}$, and various concentrations of APC. Approximately 10-fold more APC was required to reduce lysis time from 140 to 50 min in clots containing factor $V^{Leiden}$ compared to normal factor V. This effect was specific to the form of factor V present in plasma since identical results were obtained in an appropriately reconstituted purified system, which included both TAFI and either form of factor V purified from pooled plasma. In the absence of TAFI, APC did not affect clot lysis in experiments with either normal factor V or factor $V^{Leiden}$. During the various lysis assays performed with purified components, clots were solubilized and the proteolytic alterations in factor V/Va were assessed by Western blotting using a specific factor Va heavy chain monoclonal antibody. The heavy chain of factor $Va^{Leiden}$ persisted for as long as 60 min, in the presence of 6.3 nM APC indicating sustained activity of factor $Va^{Leiden}$ during the lysis assay. In contrast, no factor Va heavy chain was present after the first 5.0 min in clots formed in the presence of normal factor V and 6.3 nM APC. These combined data indicate that factor $Va^{Leiden}$ specifically attenuates the profibrinolytic effect of APC. Thus, an impaired TAFI-dependent profibrinolytic response to APC in APC-resistant individuals appears to be an additional factor contributing to the prothrombotic tendencies in subjects with factor $V^{Leiden}$.**

Prothrombinase is the enzymatic complex responsible for prothrombin activation and is composed of the cofactor, factor Va, and the protease, factor Xa, associated on a membrane surface in the presence of $Ca^{2+}$ (1). Single-chain plasma factor V is activated by thrombin to produce factor Va. The presence of factor Va in the prothrombinase complex increases the catalytic efficiency of prothrombin activation by 5 orders of magnitude as compared with that of factor Xa alone. Activation and inactivation of factor Va, therefore, constitute key regulatory events in the generation of thrombin (2).

Prothrombinase is regulated, in part, by activated protein C (APC)[1] which is formed by limited proteolysis of protein C by thrombin in complex with the cofactor thrombomodulin (3). APC inhibits prothrombinase through proteolytic inactivation of the cofactor, factor Va. Proteolytic inactivation of factor Va by APC occurs through three sequential cleavages of the heavy chain at residues $Arg^{506}$, $Arg^{306}$, and $Arg^{679}$ (4). Cleavage at $Arg^{506}$ is necessary for efficient exposure of the inactivating cleavage sites at $Arg^{306}$ and $Arg^{679}$. Cleavage at $Arg^{306}$ only occurs efficiently when the cofactor is membrane-bound and reduces factor Va cofactor activity by 80%. Remaining cofactor activity is lost following a membrane-independent cleavage at $Arg^{679}$ (4). APC can also prevent activation of the procofactor through proteolysis at 4 sites: $Arg^{306}$, $Arg^{506}$, $Arg^{679}$, and $Lys^{994}$. Cleavage at $Arg^{306}$ occurs first and appears to preclude activation of factor V (4).

A G → A substitution at nucleotide 1691 in the factor V gene results in an $Arg^{506}$ → Gln mutation in the factor V molecule (factor $V^{Leiden}$, factor $V^{R506Q}$) that, upon activation, is resistant to inactivation by APC (5). Although factor $V^{Leiden}$ is inactivated by APC in the presence of a membrane surface with a rate similar to that observed for normal factor V, factor $Va^{Leiden}$ is inactivated by APC with a rate 5–10 times slower than similarly derived normal factor Va (6, 7). Cleavage of both factor $V^{Leiden}$ and factor $Va^{Leiden}$ by APC correlates primarily with cleavage at both $Arg^{306}$ and $Arg^{679}$ producing a $M_r$ = 54,000 fragment (6), whereas, cleavage of both normal factor V and factor Va correlates with cleavage at both $Arg^{306}$ and $Arg^{506}$ resulting in the appearance of $M_r$ = 30,000 fragment.

Recent data have demonstrated that APC is also profibrinolytic. The profibrinolytic mechanism of APC in clots formed from plasma is attributed to its ability to attenuate thrombin generation since high concentrations of thrombin are required to activate thrombin activatable fibrinolysis inhibitor (TAFI, which is also known as plasma procarboxypeptidase B) (8–10). Activated TAFI (TAFIa) exhibits carboxypeptidase B-like activity (8, 9) and is responsible for delayed fibrinolysis (8). Resistance of factor $Va^{Leiden}$ to inactivation by APC may result in more frequent formation of thrombi resulting from inadequate regulation of thrombin production. A previously unappreciated mechanism by which resistance of factor Va to inactivation by

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[1] The abbreviations used are: APC, activated protein C; TAFI, thrombin activatable fibrinolysis inhibitor; tPA, tissue plasminogen activator; PAGE, polyacrylamide gel electrophoresis; PCPS, phosphatidylcholine/phosphatidylserine.

APC could potentiate thrombosis is through the sustained activation of TAFI, thereby increasing the resistance of a thrombus to tissue plasminogen activator (tPA)-induced lysis. This hypothesis was tested by comparing the profibrinolytic potential of APC in clots produced from plasma or purified components containing either factor V or factor V$^{Leiden}$.

## EXPERIMENTAL PROCEDURES

*Materials*—Recombinant tPA (activase) was provided by Dr. G. Vehar of Genentech (South San Francisco, CA). The lyophilized powder was dissolved in water to give a final concentration of 1.0 mg/ml, from which working stock solutions were prepared as described previously (11). Fibrinogen, plasminogen, recombinant $\alpha_2$-antiplasmin, prothrombin, thrombin, PCPS vesicles, antithrombin III, and factor Xa were isolated, prepared, and concentrations were determined as described previously (8). Purified human APC was provided by Dr. Paul Haley (Haematologic Technologies Inc., Essex Junction, VT). Factor V was isolated from pooled normal plasma and from pooled plasma of 4 patients genotyped and determined to be homozygous for factor V$^{Leiden}$, as described previously (6). For experiments using whole plasma, citrated fresh frozen plasma, containing either normal factor V or factor V$^{Leiden}$, was thawed at 37 °C and dialyzed extensively against 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS).

*Lysis Assay*—Plasma was initially diluted with HBS such that the $A_{280}$ was 16 (typically a 1/3 dilution) and reconstituted with 10 $\mu$M PCPS vesicles. Clots (100 $\mu$l) were formed from the plasma by the addition of plasma to the wells of a microtiter plate containing 2 $\mu$l of separated aliquots of thrombin/$Ca^{2+}$ (final concentrations of 6.0 nM and 5.0 mM, respectively) and tPA (final concentration of 294 pM) and APC (final concentration 0–50 nM). The plate was transferred to a Thermomax microtiter plate reader (Molecular Devices, Sunnyvale, CA) that was maintained at 37 °C, and the turbidity at $\lambda$ = 405 nm was monitored at 2.5-min intervals over the duration of the experiment. Lysis profiles, generated by plotting relative absorbance ($A_{405}$/max $A_{405}$) as a function of time, were used to determine lysis times. Lysis time is operationally defined as the time required to attain the transition midpoint in the reduction from the maximum turbidity to baseline values. Relative lysis time is calculated by dividing the lysis time determined at a given concentration of APC by the lysis time determined in the absence of APC. In experiments using purified components, plasma was replaced with a solution comprising fibrinogen (3.0 $\mu$M), plasminogen (0.9 $\mu$M), recombinant $\alpha_2$-antiplasmin (0.5 $\mu$M), prothrombin (0.7 $\mu$M), antithrombin III (0.3 $\mu$M), TAFI (50 nM), and factor V (6.7 nM) reconstituted in HBS and supplemented with PCPS vesicles (10.0 $\mu$M). The lysis assay was carried out as previously indicated except a 2-$\mu$l aliquot of factor Xa (final concentration of 100 pM) was also included in the well of the microtiter plate.

*Identification of Factor V Fragments*—A series of identical clots were produced from plasma containing either factor V or factor V$^{Leiden}$ in the presence of various concentrations of APC. At various times (0, 5, 10, and 60 min), the clots were solubilized and the reactions were quenched by the addition of 100 $\mu$l of 0.2 M sodium acetate, pH 4.5. The samples were then diluted in sample preparation buffer (1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, and 10% glycerol with bromphenol blue in 0.2 M Tris, pH 8.0) and subjected to SDS-PAGE using a 5–15% gradient gel (12). Following transfer to nitrocellulose (13), cleavage products of factor V were detected with MoAb FVa$_{HC}$#6 (6, 14), which recognizes an epitope on factor V between amino acid residues 307 and 506, and visualized using chemiluminescence (Chemiluminescence Detection Kit; DuPont NEN).

## RESULTS AND DISCUSSION

*Factor V$^{Leiden}$ Confers Resistance to the Profibrinolytic Effect of APC*—The profibrinolytic effect of APC was determined for clots produced from dialyzed and diluted plasma samples obtained from 4 normal subjects and 4 individuals homozygous for factor V$^{Leiden}$. Fig. 1, *a* and *b*, represents the lysis profiles of clots formed from a representative plasma sample containing normal factor V and factor V$^{Leiden}$, respectively, in the presence of various concentrations of APC (0–50 nM). Substantially more APC was required to achieve a similar reduction in clot lysis when plasma containing factor V$^{Leiden}$ was used in the assay (compare Fig. 1*a* (◇) and Fig. 1*b* (●)). The magnitude of the difference is better represented in Fig. 1*c* which is a plot of the average lysis times of the samples as a function of the APC

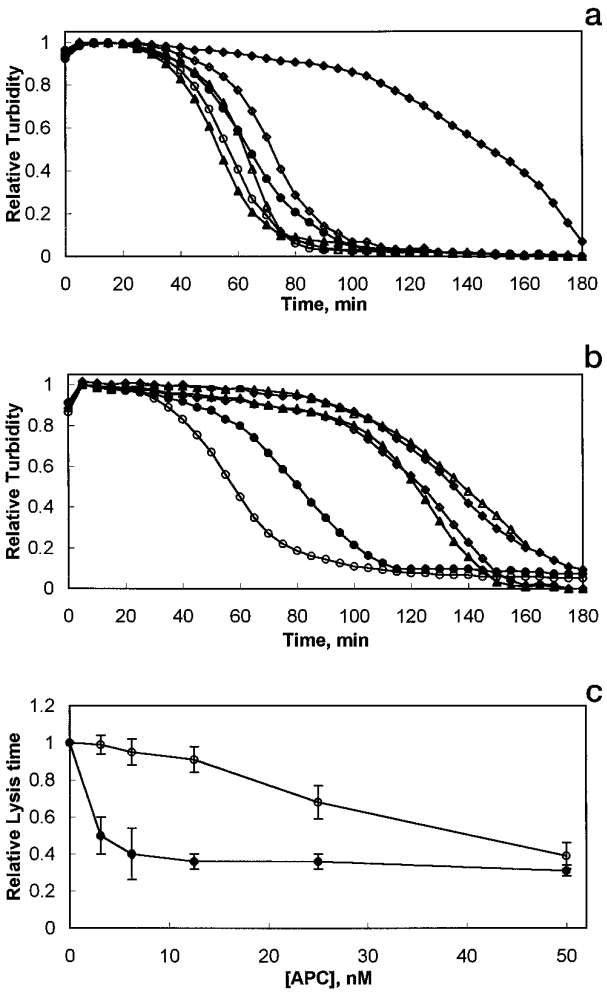


FIG. 1. **The effect of APC on fibrinolysis of clots formed from plasma.** Relative turbidity is plotted as a function of time for clots formed from plasma of an individual with normal factor V (*a*) and plasma from an individual with factor Va$^{Leiden}$ (*b*) in the absence of APC (♦) and the presence of 3.1 (◇), 6.3 (▲), 12.5 (△), 25 (●), and 50 (○) nM APC. *c* shows lysis times that were determined, in duplicate, from similar profiles representing either 4 individuals with normal factor V (●) or 4 individuals with factor Va$^{Leiden}$ (○), expressed in values relative to the lysis time observed in the absence of APC and plotted as a function of the APC concentation.

concentration. The $EC_{50}$ for APC in clots formed from plasma containing normal factor V was approximately 3 nM, whereas, for plasma containing factor V$^{Leiden}$, it was 27 nM. Similarly, the effect of APC on the reduction of lysis time was maximum at 5 nM in clots formed from normal plasma, whereas concentrations of APC ≥ 50 nM were necessary to achieve the same lysis time in clots formed using plasma containing factor V$^{Leiden}$.

The near identity of the maximum absorbance due to clot formation in all cases indicates that the amount of fibrin formed was similar. Further, since APC, at concentrations as high as 50 nM, did not alter the maximum absorbance change, it is concluded that APC did not appreciably affect clot structure either directly or indirectly by inhibiting the activation of prothrombin *in situ*. Clots formed from plasma containing normal factor V respond to lower concentrations of APC when compared to clots formed from plasma containing factor V$^{Leiden}$. These data indicate that the profibrinolytic effect of APC is attenuated in clots formed from plasma containing the APC-resistant factor V$^{Leiden}$ molecule.

An alternative explanation for the reduced potency of APC in clots formed from plasma containing factor V$^{Leiden}$ is that the

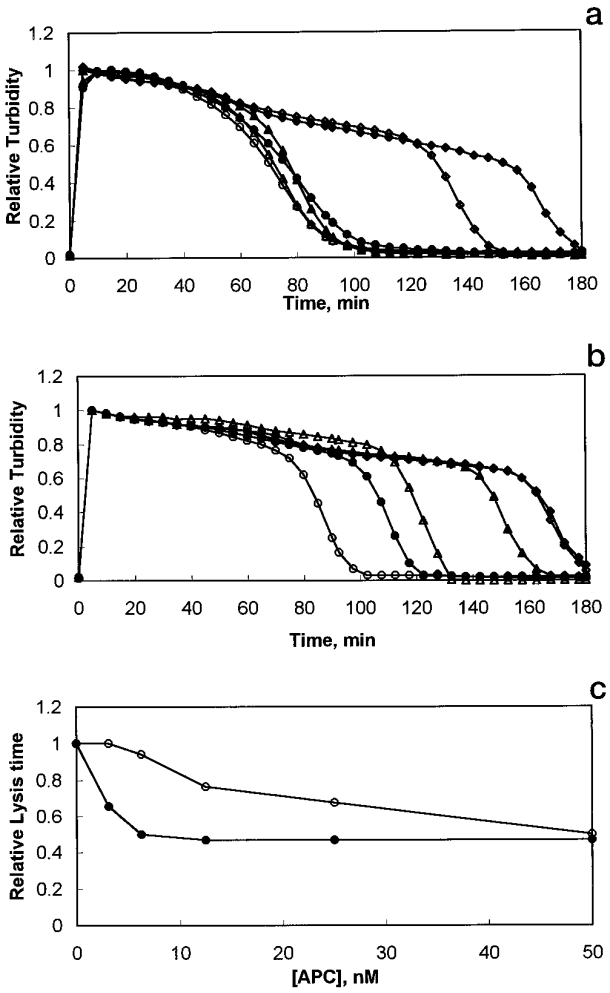


FIG. 2. **The effect of APC on fibrinolysis of clots formed from purified reagents.** Relative turbidity is plotted as a function of time for clots formed from a solution comprising fibrinogen (3.0 $\mu$M), plasminogen (0.9 $\mu$M), recombinant $\alpha_2$-antiplasmin (0.5 $\mu$M), prothrombin (0.7 $\mu$M), antithrombin III (0.3 $\mu$M), and TAFI (50 nM) reconstituted in HBS and supplemented with PCPS vesicles (10.0 $\mu$M) in the presence of 6.7 nM normal factor V (*a*) or 6.7 nM factor V$^{Leiden}$ (*b*) and in the absence of APC (♦) and the presence of 3.1 (◇), 6.3 (▲), 12.5 (△), 25 (●), and 50 (○) nM APC. *c* depicts the lysis times that were determined from the lysis profiles representing clots produced in the presence of normal factor V (●) or in the presence of factor Va$^{Leiden}$ (○), expressed in values relative to the lysis time observed in the absence of APC, and plotted as a function of the APC concentration (*c*).

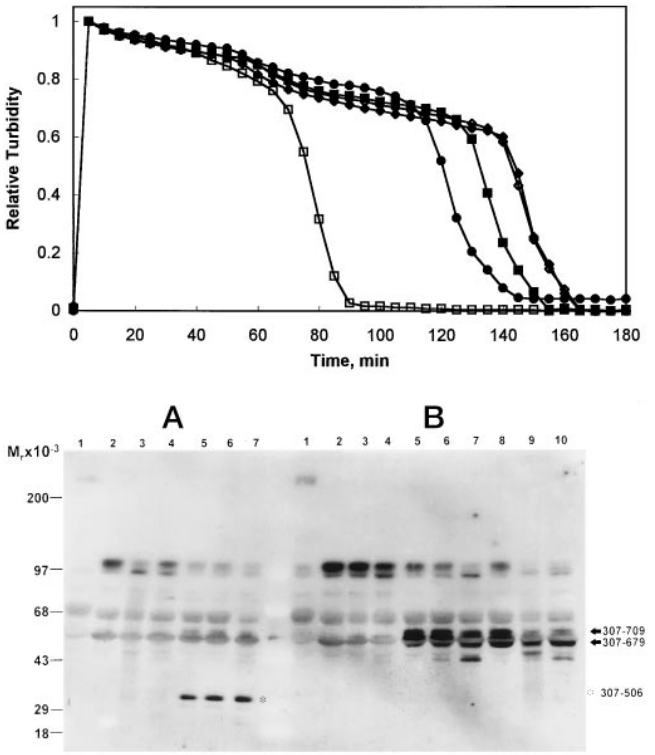


FIG. 3. **Analysis of factor Va generation and inactivation during fibrinolysis.** The *upper panel* represents the lysis profiles of clots formed from purified components with normal factor V in the absence of APC (◇) and the presence of 6.3 nM APC (□) or with factor V$^{Leiden}$ in the absence of APC (♦) and the presence of 6.3 nM APC (■) and 50 nM APC (●). At various time intervals, samples were prepared for SDS-PAGE as described under "Experimental Procedures." Following transfer to nitrocellulose, fragments of factor V were visualized using MoAb$_{HC}$#6 and shown in the *lower panel*. *A*, normal factor V; *B*, factor V$^{Leiden}$. *Lane 1*, starting material before clot formation; three time points were prepared (5, 10, and 60 min) for each APC concentration; *lanes 2*, *3*, and *4*, no APC; *lanes 5*, *6*, and *7*, 6.3 nM APC; *lanes 8*, *9*, and *10*, *panel B* only, 50 nM APC. The time points in *A* (*lanes 3* and *4*) are reversed. Molecular weight markers are shown to the *left* of *A*, and identification of fragments of factor V are shown to the *right* of *B*.

effect is modified by another, as yet, unidentified component which is found only in the plasma of subjects with factor V$^{Leiden}$. To circumvent this possibility, normal factor V and factor V$^{Leiden}$ were purified, and the effect of APC on lysis time was assessed in a purified system reconstituted with either normal factor V or factor V$^{Leiden}$ in the presence and absence of TAFI. Utilization of this system, therefore, allows differences in the effect of APC on lysis time to be attributed specifically to the form of factor V present. Fig. 2, *a* and *b*, represents the effect of APC on lysis profiles for clots formed from purified components with normal factor V and factor V$^{Leiden}$, respectively.

Lysis times were enhanced by APC when clots were formed in the presence of TAFI and either form of factor V. As was observed in the plasma-based system, APC was less effective in enhancing lysis of clots formed in the presence of factor V$^{Leiden}$. The plot of lysis time as a function of APC concentration, shown in Fig. 2*c*, quantitatively demonstrates the relative profibrinolytic potential of APC in clots formed from either normal factor V or factor V$^{Leiden}$. Under these conditions, the $EC_{50}$ values for normal factor V and factor V$^{Leiden}$ were 3 and 22 nM, respectively. Lysis times of clots produced in the absence of TAFI were invariant at 75 min regardless of either the concentration of APC or form of factor V present. These data are in excellent agreement with those determined using the plasma-based system and demonstrate that the difference in the profibrinolytic effect of APC is exclusively dependent upon both the factor V species present in the assay as well as the presence of TAFI.

*Cleavage of Factor V(a) by Activated Protein C during the Lysis Assays*—Previous experiments have demonstrated that near-quantitative conversion of prothrombin to thrombin during the fibrinolytic assay is necessary to activate TAFI (8, 11). Functional factor Va, therefore, is required during the assay to form the prothrombinase complex and ensure adequate prothrombin activation. Five identical lysis reactions were initiated for each assay condition and at times 0, 5, 10, and 60 min a fibrin clot, representing each condition, was solubilized and the reactions were concomitantly quenched. The fifth reaction was allowed to proceed until completion and was used to determine lysis time.

Fig. 3, *upper panel*, shows the lysis profiles of clots formed from purified components with normal factor V or factor V$^{Leiden}$ in the absence and presence of APC. The lysis times were essentially identical for clots formed with either form of factor V (150 min) in the absence of APC; however, 6.3 nM APC significantly shortened lysis time only in clots formed in the

presence of normal factor V (75 min) but not in the presence of factor V$^{Leiden}$ (135 min). In this particular assay, 50 nM APC was only marginally able to shorten the lysis time of clots formed in the presence of factor V$^{Leiden}$ (118 min).

The Western blot (Fig. 3, *lower panel*), shows the fragments produced by activation/inactivation of both factor V species during the lysis assays. In the absence of APC, the heavy chain of normal factor Va was evident at the initial time point but was consumed by 60 min during the lysis assay. This is most likely due to the action of plasmin which is formed during the late stages of the assay (15). For this reason, comparison of the presence of active forms of factor Va was limited to the initial (<10 min) time points of the lysis assay. In the presence of 6.3 nM APC, the heavy chain of normal factor Va has been completely degraded within the first 5 min giving rise to a fragment of $M_r$ = 30,000 indicating complete inactivation of either factor V and factor Va by APC (Fig. 3, *lower panel*, *lane 6A*). In contrast, even in the presence of 50 nM APC, some heavy chain of Va$^{Leiden}$ persisted after 5 min (Fig. 3, *lower panel*, *lane 8B*). A fragment of factor Va$^{Leiden}$ ($M_r$ = 60,000) was also apparent and represents a portion of the heavy chain comprising amino acid residues 307–709. Based on the presence of this fragment and its relative cofactor activity, in addition to the presence of the factor Va heavy chain, it was estimated that as much as 25% of the initial activity of Va$^{Leiden}$ remains at 5 min (6). Similar analyses indicated that in the presence of 6.3 nM APC ≥ 50% of the initial activity remained after 5 min (Fig. 3, *lower panel*, *lane 5B*). These data indicate persistence of cofactor activity in the presence of high concentrations of APC and factor Va$^{Leiden}$, which in turn is responsible for thrombin generation and subsequent TAFI activation.

Although interpretation of the data shown in Fig. 3 (*lower panel*) demonstrates the presence of functional factor Va during the lysis experiments it is not possible, with these data alone, to reject the possibility that factor V and not factor Va is the substrate for APC, since cleavage of either substrate would prevent thrombin generation and subsequent TAFI activation. The rate of inactivation of both normal factor V and factor V$^{Leiden}$ is similar. However, a difference in the profibrinolytic effect of APC was observed in these studies. We must conclude, therefore, that activation of factor V must have occurred prior to inactivation by APC since only the rate of inactivation of the two cofactors is significantly different (6). Since APC is present at time zero, there is the potential to preclude activation of the pro-cofactor in which case the magnitude of the difference between the $EC_{50}$ values reported is underestimated. However, the 7–10-fold difference in the $EC_{50}$ for the profibrinolytic effect of APC is in accordance with the reported difference in the rate of inactivation of the two cofactors as assessed using a prothrombinase activity assay (6, 7).

## CONCLUSIONS

The balance between coagulation and anticoagulation can affect the resistance of a clot to fibrinolysis at a given concentration of tPA. In this report, we demonstrate that resistance of factor Va$^{Leiden}$ to inactivation by APC produces a clot that is also resistant to the profibrinolytic effect of APC. Therefore, regulation of the prothrombinase complex, specifically through modulation of factor Va cofactor activity, plays a pivotal role in determining the relative stability of a clot to fibrinolysis. Since this effect is not observed in the absence of TAFI, these results highlight a potential physiological role for TAFI in disease states involving deregulation of coagulation. Further, we propose that the thrombotic tendencies in subjects with factor V$^{Leiden}$ is likely due to the inability of APC to effectively inactivate factor Va$^{Leiden}$ which would contribute to thrombosis through the production of thrombi more resistant to fibrinolysis due to sustained activation of TAFI and subsequent inhibition of fibrinolysis in addition to other effects caused by impaired regulation of thrombin generation.

It is conceivable that the magnitude of the initial thrombotic response predicts the stability of the newly formed thrombus. Other factors in addition to the form of factor V which affect thrombin formation would also affect clot stability by modulating TAFI activation. If a thrombus, initially formed, is able to withstand an initial fibrinolytic response, then a stable thrombus ensues. In contrast, a thrombus will be dissolved if the initial fibrinolytic response is greater than its antifibrinolytic potential. In this manner, factors such as factor XI, which was reported to positively feedback into the coagulation cascade (16), may be essential in the areas of high fibrinolytic potential and not a prerequisite in areas where the initial fibrinolytic response is less effective. Therefore, the formation and maintenance of a thrombus may be governed not only by the ability to generate thrombin at a site of injury but also upon the magnitude, temporal, and spatial arrangement of the components comprising the thrombus.

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