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Increased Tissue Factor-initiated Prothrombin Activation as a Result of the Arg^{506} → Gln Mutation in Factor V^{LEIDEN}*

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The effect of the Arg^{506} → Gln mutation in factor V^{LEIDEN} on thrombin generation was evaluated in a reconstituted system using the purified components of the tissue factor (TF) pathway to thrombin and the components of the protein C pathway. Recombinant full-length tissue factor pathway inhibitor (RTFPI) was included in the system because of a previously observed synergistic inhibitory effect of TFPI and the protein C pathway on TF-initiated thrombin generation. Thrombin generation initiated by 1.25 pM factor VIIa·TF in the absence of the protein C pathway components occurs following an initiation phase, after which prothrombin is quantitatively converted to 1.4 μM thrombin. The factor V^{LEIDEN} mutation did not influence thrombin generation in the reconstituted model in the absence of the protein C pathway. In the presence of 2.5 nM TFPI, 65 nM protein C, and 10 nM recombinant soluble thrombomodulin (Tm), thrombin generation catalyzed by normal factor V was abolished after the initial formation of 25 nM thrombin. In contrast, persistent thrombin generation was observed in the presence of factor V^{LEIDEN} in the same system, although the rate of thrombin generation was slower compared with the reaction without protein C and Tm. The rate of thrombin generation with factor V^{LEIDEN} increased with time and ultimately resulted in quantitative prothrombin activation. When the TFPI concentration was reduced to 1.25 nM, thrombin generation is still curtailed in the presence of normal factor V. In contrast, under similar conditions using factor V^{LEIDEN}, the protein C pathway totally failed to down-regulate thrombin generation. The dramatic effect of a 50% reduction in TFPI concentration on the inhibitory potential of the protein C pathway on thrombin generation catalyzed by factor V^{LEIDEN} suggests that the observed synergy between TFPI and the protein C pathway is directly governed by the TFPI concentration and by cleavage of the factor Va heavy chain at Arg^{506}. This cleavage appears to have a dramatic regulatory effect in the presence of low concentrations of TFPI. Markedly increased thrombin generation in the presence of both 1.25 nM TFPI and factor V^{LEIDEN} was also observed when antithrombin-III was added to the system to complete the natural set of coagulation inhibitors. Protein S (300 nM) had a minimal effect in the model on the inhibition of thrombin generation by protein C, Tm, and TFPI, with either normal factor V or factor V^{LEIDEN}. Protein S also failed to significantly potentiate the action of the protein C pathway in the presence of antithrombin-III in reactions employing normal factor V or factor V^{LEIDEN}. The absence of an effect of protein S in the model, which employs saturating concentrations of phospholipid, suggests that the reported interactions of protein S with coagulation factors are not decisive in the reaction. Altogether the data predict that TFPI levels in the lower range of normal values are a risk factor for thrombosis when combined with the Arg^{506} → Gln mutation in factor V^{LEIDEN}.

The procoagulant reactions leading to the generation of thrombin during the blood clotting process are governed by three vitamin K-dependent enzyme complexes, each of which is composed of a serine protease and a cofactor protein assembled on a membrane surface (1). The serine protease (factor VIIa, factor IXa, or factor Xa) in each case is derived from a plasma zymogen, while cofactors are regulated either by presentation (tissue factor) or proteolytic activation (factor Va or factor VIIIa). The procoagulant process is thought to be initiated when the small amounts of circulating two-chain factor VIIa bind to the tissue factor (TF)[1] (2) presented as a consequence of vascular disruption or an inflammatory process. The reaction initiated by the formation of this complex activates the serine protease components of both the intrinsic tenase (factor IXa·factor VIIIa) and prothrombinase (factor Xa·factor Va). The latter complex converts prothrombin to thrombin (for a review on blood coagulation, see Ref. 3). The formation of thrombin is tightly regulated by a system of the stoichiometric inhibitors tissue factor pathway inhibitor (TFPI) (4) and antithrombin-III (AT-III) (5, 6) and by the activated protein C (APC) pathway through which thrombin serves as its own down-regulator by complexing with constitutively expressed vascular thrombomodulin (Tm) (7). The resulting complex activates the plasma zymogen protein C to APC. APC down-regulates coagulation by proteolytic inactivation of the cofactor factor Va.

We have shown in earlier studies that the combination of the stoichiometric inhibitors TFPI, which regulates the factor VIIa·TF complex, and AT-III, an inhibitor of all the procoagulant serine proteases, and the protein C pathway behave synergistically in their attenuation of thrombin generation (8, 9).

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Portions of this work were presented at the Thirty-seventh Annual Meeting of the American Society of Hematology, December 1–5, 1995, Seattle, WA (42).

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[1] The abbreviations used are: TF, tissue factor; TFPI, tissue factor pathway inhibitor; AT-III, antithrombin-III; APC, activated protein C; Tm, thrombomodulin.

These studies demonstrated that the system produces threshold-limited bursts of thrombin when the procoagulant reactions proceed in the presence of the combined regulatory systems. The cooperative interactions between procoagulant and anticoagulant systems regulate thrombin generation such that it is limited by the extent of presentation of tissue factor and concentrations of pro- and anticoagulants.

Congenital and acquired alterations of either the procoagulant or anticoagulant pathways are associated with hemorrhagic disease or thrombosis. A factor V genetic mutation (factor V^{LEIDEN}) has recently been described that is present in approximately 5% of the Caucasian population and appears to be responsible for 20% of familial venous thrombosis (10, 11). The G → A mutation at nucleotide 1691 leads to the substitution of glutamine for arginine at position 506 in the factor V^{LEIDEN} molecule. The principle regulatory influence of the protein C pathway is associated with the inactivation of factor Va (9, 12), the cofactor of the prothrombinase complex. The normal proteolytic inactivation process involves cleavages of the factor Va molecule at Arg^{506}, Arg^{306}, and Arg^{679} in the heavy chain (13, 14). The substitution of glutamine for arginine at position 506 slows the inactivation process and therefore extends the kinetic lifetime of the expression of prothrombinase complex activity (14). The synergistic relationships between the stoichiometric inhibitors and the protein C system in the regulation of thrombin generation is a largely kinetic phenomenon (8, 9). We have therefore studied the influence of the slowed inactivation rate of factor V^{LEIDEN} on the expression of thrombin in a reconstituted system of procoagulant and anticoagulation reactions representative of the blood clotting process.

MATERIALS AND METHODS

Reagents—Phosphatidylserine from bovine brain, phosphatidylcholine from egg yolk, and Hepes were purchased from Sigma (St. Louis, MO). D-Phenylalanyl-L-arginine chloromethyl ketone was a gift from Dr. R. Jenny, Hematologic Technologies Inc. (Essex Junction, VT). Spectrozyme TH was purchased from American Diagnostica, Inc. (Greenwich, CT). S2366 was obtained from Chromogenix (Kabi Pharmacia Hepar, Inc.). Blue Sepharose was obtained from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade. The mouse monoclonal antibody αHFV-6 was provided by Dr. William Church, Department of Biochemistry, Antibody Core Facility, University of Vermont (Burlington, VT).

Proteins—Human coagulation factors X, IX, and prothrombin were isolated from freshly frozen plasma using the general methods of Bajaj *et al.* (15) and were depleted of trace contaminants and traces of active enzymes as described (8). Human protein C was purified by heparin-Sepharose and immunochromatography and treated with D-phenylalanyl-L-arginine chloromethyl ketone as described (9). Human protein S was a gift from Dr. R. Jenny or was purified from the protein S pool of the initial ion exchange chromatography step involved in the purification of the vitamin K-dependent clotting factors using blue Sepharose chromatography as described by Dahlbäck (16). The purified protein S prepared by the method described by Dahlbäck was depleted of trace amounts of contaminants and treated with D-phenylalanyl-L-arginine chloromethyl ketone as described (9). Human factor V was isolated from freshly frozen human plasma using the method of Nesheim *et al.* (17). Factor V^{LEIDEN} (Arg^{506} → Gln) was purified from plasma of patients who were homozygous for the factor V^{LEIDEN} mutation as described (14). AT-III was purified according to the method described by Griffith *et al.* (18). Recombinant factor VIII and recombinant tissue factor (residues 1–242 of the human sequence) were provided as gifts from Drs. Shu Len Liu and Roger Lundblad, Hyland division, Baxter Healthcare Corp. (Duarte, CA). Recombinant human coagulation factor VIIa was purchased from NOVO pharmaceuticals. Recombinant soluble thrombomodulin (Solulin) was provided as a gift by Dr. J. Morser, Berlex (Richmond, CA). Recombinant full-length TFPI produced in *E. coli* was provided as a gift from Dr. K. Johnson, Chiron Corp. (Emeryville, CA). Purified human factor Xa and purified human activated protein C were gifts from Dr. R. Jenny and Dr. P. Haley, Hematologic Technologies Inc. Tick anticoagulant peptide (TAP) was provided as a gift by Dr. S. Krishnaswamy, Hematology/Oncology Division, Emory University (Atlanta, GA). Hirudin was provided as a gift by Genentech (South San Francisco, CA).

Coagulation Factor Activation Experiments—Thrombin generation initiated by factor VIIa·TF in a reconstituted model using normal plasma protein concentrations was studied as described previously (19). TF was relipidated into 400 μM 75% phosphatidylcholine, 25% phosphatidylserine vesicles, for 30 min at 37 °C in 20 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4 (Hepes/Ca^{2+}). The relipidated TF was incubated with factor VIIa for 20 min at 37 °C to allow factor VIIa·TF complex formation. Factor V and factor VIII were added in microliter amounts of concentrated stock solutions to the equilibrated factor VIIa·TF mixture (total volume of the addition not exceeding 0.25% of the final reaction volume), and immediately thereafter the reaction was started by the addition of a solution containing factor X, factor IX, and prothrombin, which was prepared in Hepes/Ca^{2+}. The zymogen solution was preheated at 37 °C for 3 min before the addition to the factor VIIa·TF, factor V, and factor VIII mixture. When protein C, TFPI, AT-III, or protein S was included it was added to the factor X, factor IX, and prothrombin mixture. Thrombomodulin was added to the factor VIIa·TF mixture.

The final concentrations of the proteins in the reaction, chosen to represent mean plasma values, are 160 nM factor X, 90 nM factor IX, 0.7 nM factor VIII, 20 nM factor V, and 1.4 μM prothrombin (19). When added, protein C (65 nM), TFPI (2.5 nM), protein S (300 nM), and AT-III (3.4 μM) (20–23) were also present at their respective plasma concentrations in the reaction unless indicated otherwise. The final phosphatidylcholine/phosphatidylserine concentration in the reactions was 200 μM. Following initiation of the reaction, aliquots were withdrawn from the reaction mixture and quenched in either 20 mM EDTA, 20 mM Tris, 150 mM NaCl, pH 7.4, to assay for thrombin formation or in 2% SDS, 0.062 M Tris, 10% glycerol, 0.04% bromphenol Blue, pH 6.8, for SDS-polyacrylamide gel electrophoresis and immunoblotting. Assays for thrombin activity were performed using the chromogenic substrate Spectrozyme TH. The hydrolysis of the substrate was monitored by the change in absorbance at 405 nm using a Molecular Devices Vmax spectrophotometer. Thrombin measurements in samples withdrawn from experiments that included AT-III were performed by rapid quenching of samples in a solution containing 20 mM EDTA and 0.4 mM Spectrozyme TH, and thrombin generation was measured immediately as described (8). Thrombin generation was calculated from a standard curve prepared with serial dilutions of purified human α-thrombin. APC generation was measured using substrate S2366 in samples that were withdrawn from the reaction mixture and diluted 5-fold into excess hirudin (7 μM), tick anticoagulant peptide (2.31 μM), and 20 mM EDTA/Tris-buffered saline. APC generation was calculated from a standard curve prepared using serial dilutions of purified human APC. Some residual amidolytic activity was observed in the thrombin activity assay in the presence of hirudin in samples taken from reactions with Tm and protein C. This residual amidolytic activity, caused by generated APC, did not significantly affect the higher thrombin levels measured in the reactions and never exceeded 20% of the total amidolytic activity at the lowest thrombin levels measured. The SDS-quenched samples were analyzed using SDS-polyacrylamide gel electrophoresis under nonreducing conditions on 4–12% polyacrylamide gels essentially as described by Laemmli (24). Following SDS-polyacrylamide gel electrophoresis the proteins were transferred to nitrocellulose membranes for immunoblot analysis using the techniques described by Towbin *et al.* (25). Membranes were blocked for nonspecific binding with 5% nonfat dry milk in 0.05% Tween, Tris-buffered saline and incubated for 1.5 h with monoclonal antibody αHFV-6. This antibody recognizes an epitope on the heavy chain of factor V between residues 307 and 506 (14). This fragment is a final product of factor Va cleavage by APC. The products of factor V recognized by this antibody were visualized using peroxidase-conjugated horse anti-mouse IgG and the Renaissance chemiluminescent reagent obtained from DuPont. The membranes were stripped with 0.5 M NaCl, 0.1 M glycine, pH 2.7, to remove bound antibodies. The stripped membranes were blocked again with 5% nonfat milk and probed for prothrombin activation products using a polyclonal burro anti-prethrombin-1 antibody as described (19).

RESULTS

Effect of the Factor V^{LEIDEN} Mutation on Factor VIIa·TF-initiated Thrombin Generation in the Presence and Absence of the Protein C Pathway—The effect of the factor V^{LEIDEN} mutation was studied in reactions that were initiated with 1.25 pM factor VIIa·TF in the presence of 2.5 nM TFPI. TFPI was included in the reaction mixtures to provide the physiological

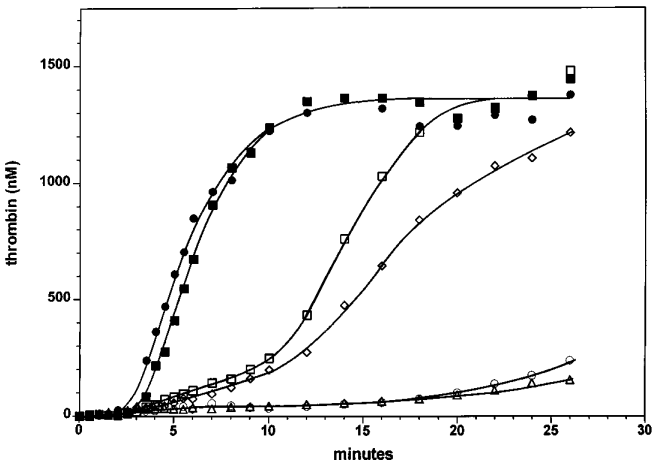

FIG. 1. **Thrombin generation by normal factor V and factor V^{LEIDEN} in the absence and presence of the protein C pathway.** Thrombin generation is initiated by 1.25 pM factor VIIa·TF (1.25 pM factor VIIa and 250 pM TF) in the presence of 2.5 nM TFPI. *Closed symbols* represent the control reactions for factor V (●) and factor V^{LEIDEN} (■) in the absence of the protein C pathway components. *Open symbols* show the reactions in the presence of the protein C pathway (65 nM protein C plus 10 nM thrombomodulin) with or without protein S (300 nM). ○, factor V and protein C pathway; □, factor V^{LEIDEN} and protein C pathway; △, factor V, protein C pathway, and protein S; ◇, factor V^{LEIDEN}, protein C pathway, and protein S.

kinetically transient factor VIIa·TF activity and because of a synergistic effect of TFPI and the protein C pathway observed in this model (9). Thrombin generation with normal factor V or factor V^{LEIDEN} in the absence of the protein C pathway occurred following an initial lag phase of 2.5 min, after which prothrombin was converted into thrombin at 180 nM·min^{-1} during the propagation phase. The profile was essentially the same for normal factor V and factor V^{LEIDEN} (Fig. 1, *filled symbols*).

In the presence of protein C and 10 nM Tm with normal factor V (Fig. 1, *open circles*), thrombin generation occurred at a reduced rate after a lag time of 2.5 min. At 5 min, thrombin generation ceased after ~25 nM thrombin was produced. Thus, at the factor VIIa·TF concentration used (1.25 pM) the protein C pathway eliminated the propagation phase of the reaction after a limited amount of thrombin was formed (9). In the case of factor V^{LEIDEN} (Fig. 1, *open squares*) the same lag time (2.5 min) was observed; however, subsequent thrombin generation, while slowed, continued at a rate of ~26 nM·min^{-1}. After 10 min, the rate of thrombin generation increased, ultimately rising at 12 min to the rate observed in the absence of protein C/Tm. Prothrombin was quantitatively converted to thrombin (1.4 μM) in the presence of factor V^{LEIDEN} after 20 min. The rate of APC generation (~3 nM·min^{-1}) was the same in reactions containing either normal factor V or factor Va^{LEIDEN} (data not shown). Thrombin generation with normal factor V in the presence of protein C, 10 nM Tm and 300 nM protein S (Fig. 1, *open triangles*) results in a nearly identical rate of thrombin generation as observed in the absence of protein S (Fig. 1, *open circles*). Thus, under the conditions used, protein S does not strongly potentiate the inhibitory effect of the protein C pathway. Protein S (300 nM) had only minimal influence on the initial rate of thrombin generation by factor V^{LEIDEN} (Fig. 1, *diamonds*) in the presence of the components of the protein C pathway. The rate of secondary thrombin generation in the presence of factor V^{LEIDEN} and the protein C pathway is decreased slightly by protein S (by approximately 30%) but still results in the quantitative activation of prothrombin. The lack of a major effect of protein S on thrombin generation under these conditions in the presence of factor Va^{LEIDEN} and the protein C pathway does not support a decisive role for protein S in accelerating the APC-dependent cleavage at Arg^{306} and subsequent inactivation of factor Va^{LEIDEN} as reported by Rosing *et al.* (26).

Proteolysis of Factor V and Factor V^{LEIDEN} in the Presence of the Protein C Pathway during TF-initiated Thrombin Generation—Analyses of the proteolytic activation and inactivation of normal factor V and factor V^{LEIDEN} by immunoblotting using an antibody reacting with an epitope on the heavy chain within residues 307–506 (αHFV-6) (14) showed that both cofactors were cleaved during the initial phase (0–1.5 min) of thrombin generation to generate the heavy chain (Fig. 2). Densitometric data of the factor Va heavy chain bands of the immunoblots is shown in Fig. 3. No factor V intermediate is observed at M_r = 280,000, indicating that there is no significant direct inactivation of intact factor V by cleavage of the procofactor at Arg^{306} (14). In the presence of 10 nM thrombomodulin and protein C (Fig. 2*A*), inactivation of normal factor Va occurs during the initiation phase of the reaction (1.5 min) due to initial cleavage at Arg^{506}. A subsequent cleavage at Arg^{306} gives rise to a M_r 30,000 product (14) (Fig. 2*A*). After 5 min, most of the heavy chain is degraded by APC, yielding the M_r 30,000 fragment, consistent with the elimination of prothrombinase activity at 5 min (Fig. 1, *open circles*).

Inactivation of factor Va^{LEIDEN} in the presence of protein C/thrombomodulin (Fig. 2*B*) is observed after 2.5 min by the appearance of the M_r 60,000 reactive band (residues 307–709) and a M_r 54,000 reactive band (residues 307–679) (14). These fragments are the consequence of cleavages at Arg^{306} and Arg^{679}. Compared with the data obtained with normal factor Va (Fig. 2*A*), the heavy chain of factor Va^{LEIDEN} is cleaved at a slower rate. Quantitation of the factor Va heavy chain by densitometry (Fig. 3, *squares*) shows that higher levels of the factor Va heavy chain are obtained in the presence of factor V^{LEIDEN} (*squares*) compared with normal factor V (*circles*). The complete proteolysis of the factor Va^{LEIDEN} heavy chain is accomplished only after 18 min. It is noteworthy that, during the initial 20 min of the reaction, the integrated concentration of the factor Va^{LEIDEN} heavy chain is approximately 3-fold greater than that of the normal factor Va heavy chain. The abundant amounts of thrombin and/or factor Xa formed during the course of the reaction cleave the 307–679 fragment resulting in M_r 43,000 and 40,000 fragments (Fig. 2*B*). Some remaining intact heavy chain is cleaved to generate a M_r 85,000 fragment.

The sustained activity of factor Va^{LEIDEN} in the presence of the protein C pathway is made obvious by following the analysis of prothrombin activation by immunoblotting. Prothrombin activation in reactions with factor V or factor V^{LEIDEN} is quantitative in the absence of the protein C pathway (Fig. 4, *A* and *B*) and evolves consistent with the lag and propagation phases observed for the generation of thrombin (Fig. 1, *filled symbols*). Consistent with the attenuation of thrombin generation catalyzed by normal factor V in the presence of the protein C pathway, quantitative prothrombin consumption was not observed under these conditions (Fig. 4*C*), indicating that the observed inhibition of thrombin generation is caused by the elimination of prothrombinase activity and is not the result of conversion of prothrombin to inactive products. In contrast, under similar conditions, quantitative prothrombin consumption ultimately occurred when the reaction was catalyzed by factor V^{LEIDEN} (Fig. 4*D*).

The Effect of TFPI Concentration on Thrombin Generation Catalyzed by Factor V^{LEIDEN} in the Presence of the Protein C Pathway and Protein S—The lower limit of normal TFPI concentration in plasma is thought to be ~1.25 nM (22). Experiments performed with a TFPI concentration of 1.25 nM, initi-

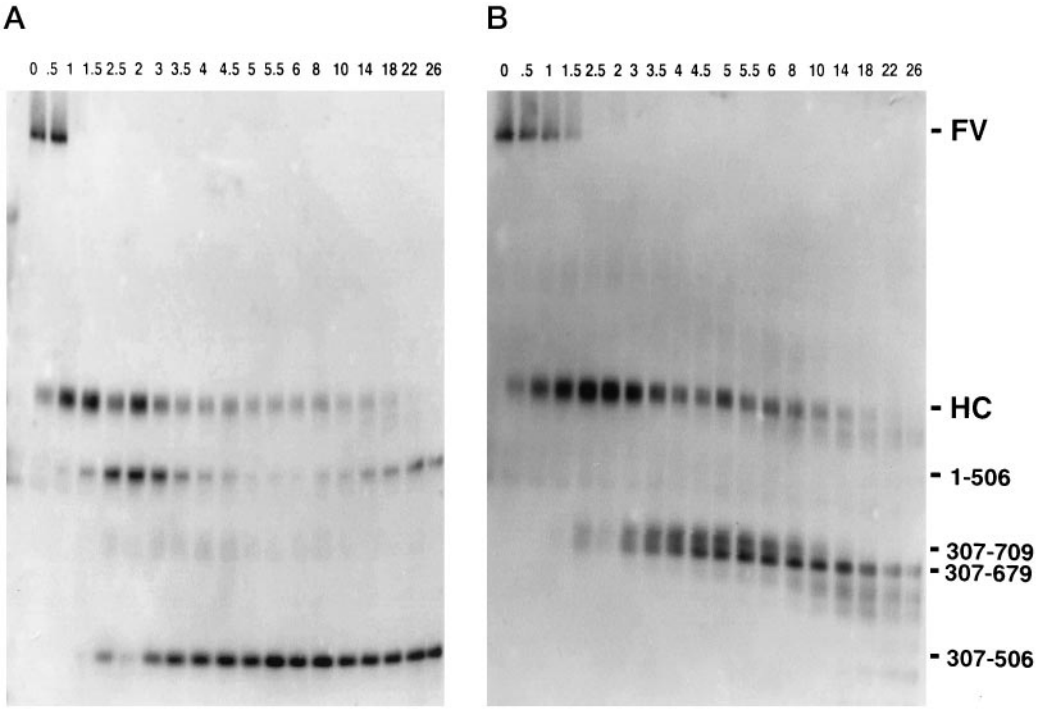

FIG. 2. **Proteolysis of factor V and factor V^LEIDEN in the presence of the protein C pathway during TF-initiated thrombin generation.** The activation and inactivation of factor V (*A*) and factor V^LEIDEN (*B*) in reactions shown in Fig. 1 was followed by immunoblotting with antibody α-HFV-6, which recognizes an epitope on the heavy chain between residues 307 and 506 (14). Immunoblots are shown for the reactions with protein C and 10 nM Tm. The time (min) of sample acquisition is listed *above* the *gel lanes*. Abbreviations and M_r are as follows: factor V (*FV*, M_r 330,000), factor V heavy chain (*HC*, M_r 105,000), and factor Va heavy chain fragments (*1–506*, M_r 75,000; *307–709*, M_r 60,000; *307–679*, M_r 54,000; *307–506*, M_r 30,000).

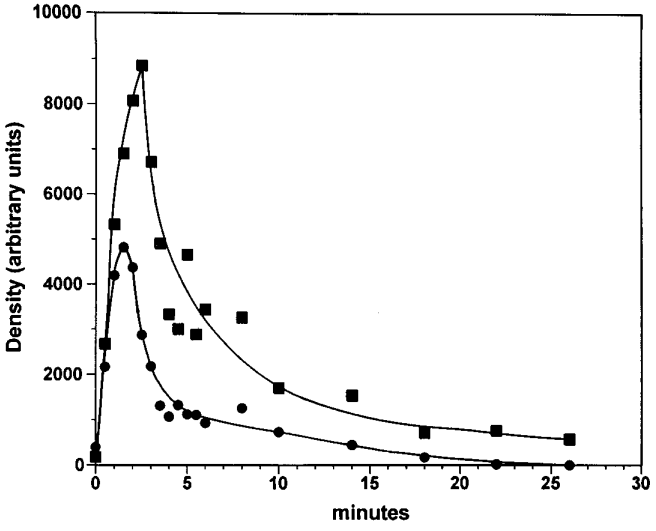

FIG. 3. **Quantitative analysis of the appearance and disappearance of the heavy chain of factor V and factor V^LEIDEN.** Densitometric quantitation of the intact heavy chain bands (M_r 105,000) of normal factor V (●) and factor V^LEIDEN (■) in the presence of the protein C pathway is shown. Data are derived from the immunoblots shown in Fig. 2.

ated by 1.25 pM factor VIIa·TF, are displayed in Fig. 5. Thrombin generation at 1.25 nM TFPI in the absence of the protein C pathway (*filled symbols*) occurs after a shorter lag period (1.5 min) and proceeds with a slightly higher rate in the propagation phase when compared with reactions with 2.5 nM TFPI (Fig. 1). No major difference is observed between reactions with normal factor V (*filled circles*) or factor V^LEIDEN (*filled squares*). In the presence of 1.25 nM TFPI (Fig. 5, *open circles*), thrombin generation by normal factor V was temporarily attenuated up to 10 min in the reaction by the protein C pathway (65 nM protein C, 10 nM thrombomodulin, 300 nM protein S).

In marked contrast, thrombin generation by factor V^LEIDEN in the presence of the protein C pathway components and 1.25 nM TFPI occurred after a lag time of 1.5 min and proceeded explosively (Fig. 5, *open squares*), resulting in a thrombin generation profile similar to the reaction without protein C and thrombomodulin (*filled squares*). Thus, in contrast to the effective down-regulation of thrombin generation observed with normal factor V by the protein C pathway at a 50% reduced TFPI concentration, the protein C pathway fails to inhibit thrombin generation by factor V^LEIDEN at this concentration of TFPI (Fig. 5, *open squares*). This is of particular significance, since thrombin generation with normal factor V (under these conditions) is effectively slowed down by the protein C pathway in the complete absence of TFPI (9). These data demonstrate the critical roles of both the cleavage site in factor Va at Arg^{506} and TFPI in the regulation of TF-initiated thrombin generation by the protein C pathway. The results also indicate that relatively small differences in TFPI concentrations within the range of "normal" plasma levels may have a significant influence on thrombin generation in individuals with the factor V^LEIDEN mutation.

Thrombomodulin Titration with Factor V^LEIDEN and Normal Factor V in the Presence of AT-III at Varying TFPI Concentrations—The dramatic effect of a 50% reduction in TFPI concentration on the inhibitory potential of the protein C pathway on thrombin generation catalyzed by factor V^LEIDEN suggests that the synergy observed between TFPI and the protein C pathway is directly governed by the TFPI concentration and the rate of factor Va inactivation via initial cleavage at Arg^{506}. This effect and/or the lack of a major effect of protein S could be a consequence of the absence of thrombin regulation by other physiological inhibitors such as AT-III. The effect of the factor V^LEIDEN mutation was therefore also evaluated in the presence

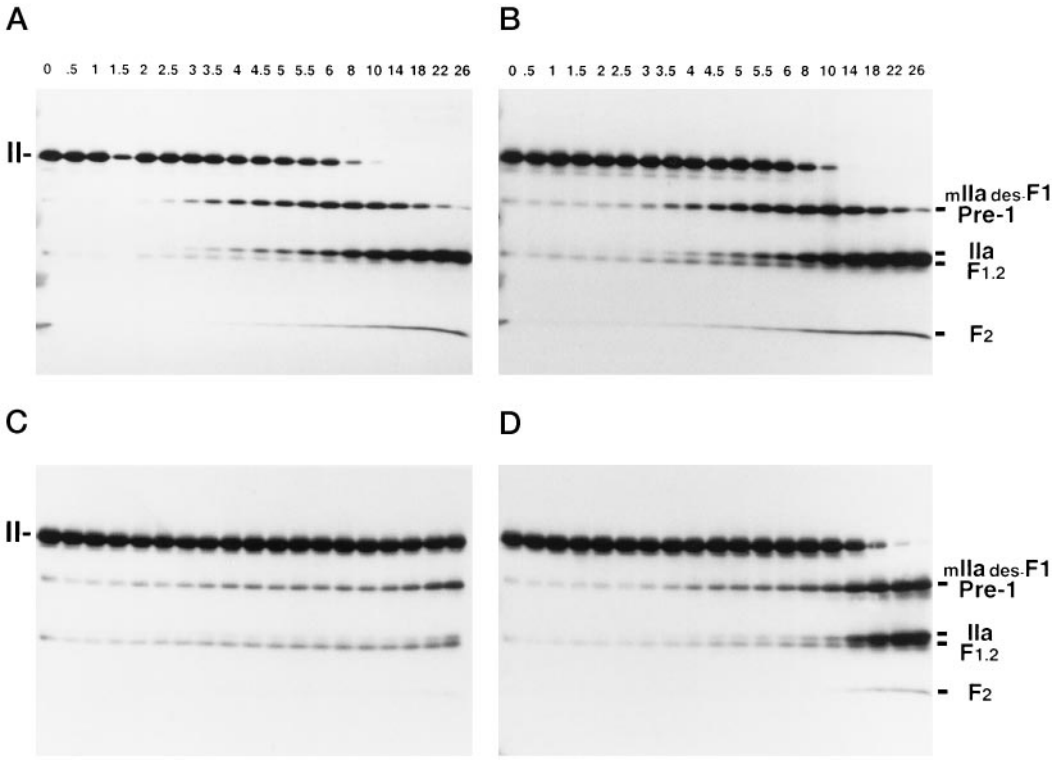

FIG. 4. **Effect of the factor V^LEIDEN mutation on prothrombin consumption as evaluated by immunoblotting for prothrombin and prothrombin products.** The activation of prothrombin in reactions shown in Fig. 1 was followed by immunoblotting with a burro polyclonal anti-human prethrombin-1 antibody. Immunoblots are shown for reactions with factor V (*A*), factor V^LEIDEN (*B*), factor V plus protein C and Tm (*C*), and factor V^LEIDEN plus protein C and Tm (*D*). The time (min) of sample acquisition is listed *above* the *gel lanes*. Abbreviations and M_r are as follows: prothrombin (*II*, M_r 72,000), prethrombin-1 (*Pre-1*, M_r 50,000), meizothrombin-des-fragment-1 (*mIIa des-F1*, M_r 50,000), α-thrombin (*IIa*, M_r 38,500), fragment 1·fragment 2 (*F1·2*, M_r 37,000), fragment 2 (*F2*, M_r 14,000).

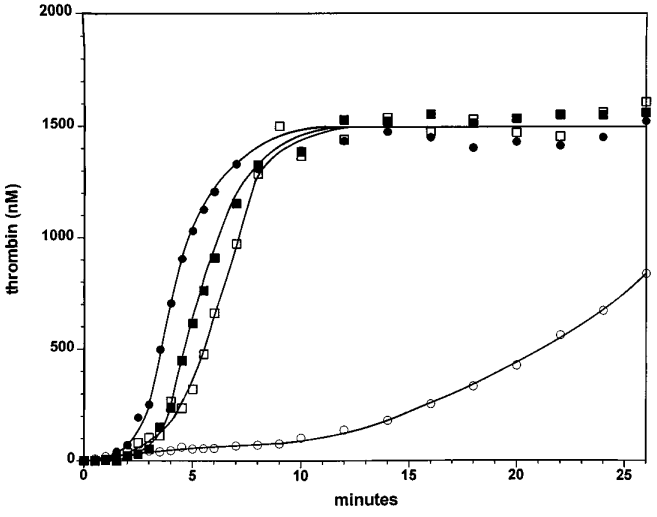

FIG. 5. **Effect of the factor V^LEIDEN mutation in the reconstituted model in the presence of 1.25 nM TFPI.** A representative experiment is shown with a 50% decreased TFPI concentration (1.25 nM). A concentration of 1.25 nM TFPI resembles the level of TFPI at the lower limit of the plasma TFPI values observed in normal individuals. The initiating factor VIIa·TF concentration is 1.25 pM (1.25 pM factor VIIa and 250 pM TF). Curves are shown for reactions in the absence of the protein C pathway for factor V (●) and factor V^LEIDEN (■) and in the presence of 65 nM protein C, 10 nM thrombomodulin, and 300 nM protein S with factor V (○) or factor V^LEIDEN (□).

of AT-III. Reactions performed with factor V or factor V^LEIDEN in the presence of AT-III (3.4 μM), protein C (65 nM), and TFPI at normal (2.5 nM) and 50% plasma concentrations (1.25) at various concentrations of thrombomodulin are presented in Fig. 6. *Panels A* and *C* show reactions with normal factor V; *panels B* and *D* are with factor V^LEIDEN. In all cases, thrombin generation was initiated with 5 pM factor VIIa·TF. This concentration of initiator was chosen because of an observed threshold between 1 and 5 pM factor VIIa·TF for explosive thrombin generation in the presence of 2.5 nM TFPI and AT-III.

Thrombin generation initiated with 5 pM factor VIIa·TF in the presence of protein C, 2.5 nM TFPI, and 3.4 μM AT-III in the absence of Tm reaches a peak concentration of thrombin of ~195 nM at 5 min (Fig. 6*A*, *filled circles*). Subsequently, the thrombin concentration declined to approximately 45 nM at 12 min and reached a plateau value of 10 nM at 20 min. In the absence of Tm, normal factor V and factor V^LEIDEN produce similar thrombin generation curves at 2.5 nM TFPI (Fig. 6, *A* and *B*, *filled circles*). Control reactions performed in the absence of protein C (data not shown) resulted in similar thrombin generation curves as observed in the presence of protein C. Tm decreases thrombin generation in the presence of protein C in a concentration-dependent manner without appreciably affecting the initiation phase of the reaction (Fig. 6, *A–D*). Higher Tm levels are needed to inhibit thrombin generation in the presence of factor V^LEIDEN (*panels B* and *D*) compared with normal factor V (*panels A* and *C*). For example, at 0.25 nM, Tm has no affect on the reaction with factor V^LEIDEN (compare Fig. 6*B*, *open* and *filled circles*), whereas for the reaction with normal factor V at this Tm concentration the peak level of thrombin generation is decreased by ~50% (compare Fig. 6*A*, *open* and *filled circles*). A 50% inhibition of thrombin generation in the presence of factor V^LEIDEN is observed with 1 nM Tm (Fig. 6*B*, *open squares*). These data demonstrate that a 4-fold higher Tm concentration is required to inhibit thrombin generation by 50% in the system comprising factor V^LEIDEN when compared with the system containing normal factor V. Similarly, in a reaction with normal factor V, 2.5 nM Tm (Fig. 6*A*, *open triangles*) causes a 90% reduction in the peak level of thrombin

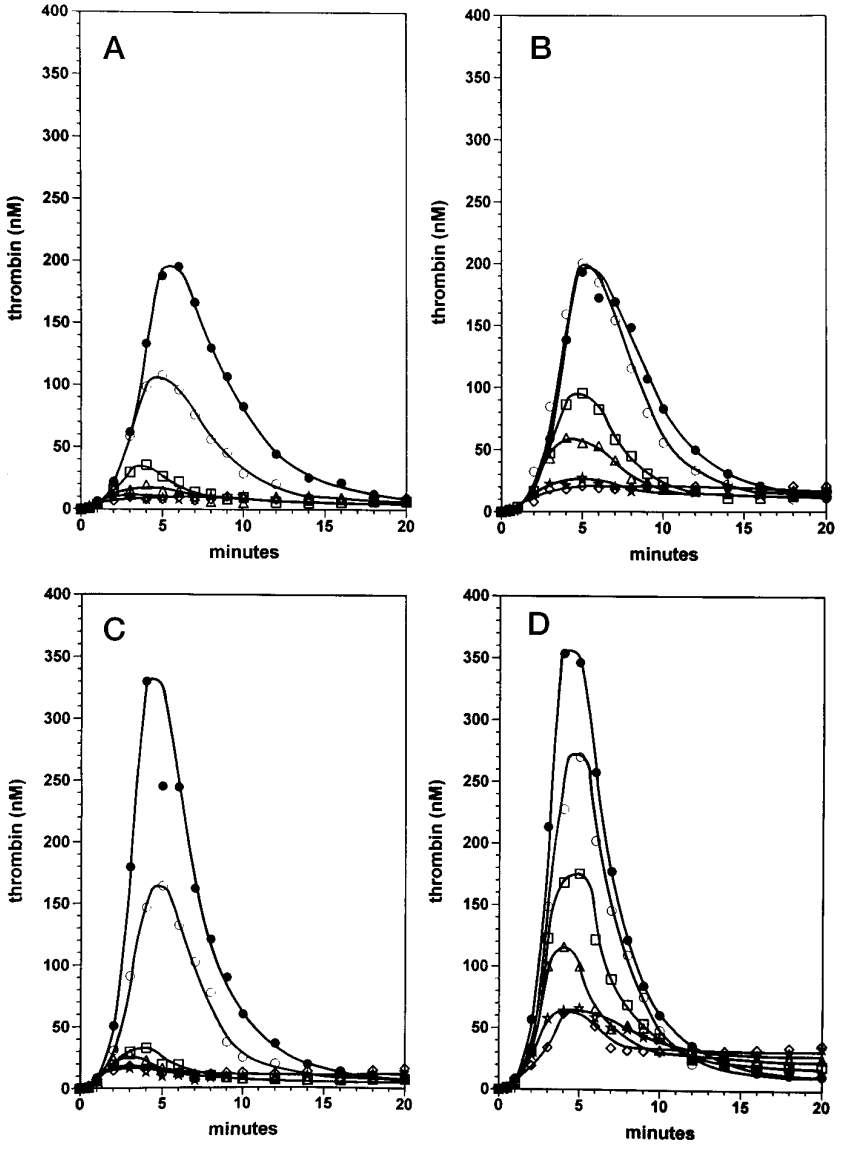

FIG. 6. **Effect of the factor V^LEIDEN mutation in reactions initiated in the presence of AT-III and a normal and 50% reduced TFPI concentration.** Reactions were initiated with 5 pM factor VIIa·TF (5 pM TF and 100 pM factor VIIa) in the presence of 3.4 μM AT-III, 65 nM protein C with factor V and 2.5 nM TFPI (*A*), factor V^LEIDEN and 2.5 nM TFPI (*B*), factor V and 1.25 nM TFPI (*C*), and factor V^LEIDEN and 1.25 nM TFPI (*D*). Thrombomodulin concentrations are 0 (●), 0.25 (○), 1 (□), 2.5 (△), 5 (★), or 10 nM (◇).

(20 nM), whereas a 4-fold higher Tm concentration (10 nM) is needed to reduce the peak level of thrombin generation by 90% in the case of factor V^LEIDEN (Fig. 6*B*, *open diamonds*). Analogous to this, 3-fold higher peak levels of thrombin are observed in the presence of 1–2.5 nM Tm (Fig. 6, *A* and *B*) in reactions containing factor V^LEIDEN compared with reactions with normal factor V. A similar but exacerbated set of results is seen when the TFPI concentration is reduced to 50% of normal (Fig. 6, compare panels *C* and *D*).

Fig. 7 displays the peak levels of thrombin observed in the reactions shown in Fig. 6 plotted *versus* the Tm concentration present in the reaction. The *open symbols* are for factor V^LEIDEN, while the *filled symbols* are for normal factor V. Note the dramatic effect of halving the TFPI concentration in the case of factor V^LEIDEN (*open squares*) observed for all Tm concentrations used. Tm not only decreases the peak levels of thrombin observed in the presence of AT-III but also shortens the time interval required before a steady state level of ~10–20 nM thrombin is reached. This thrombin level persists in all reactions over the 20-min time interval studied (Fig. 6). With 10 nM Tm, a 2-fold higher level of thrombin generation is observed with factor V^LEIDEN compared with normal factor V. The stable level of thrombin observed in the reactions at the later time points suggests that thrombin is being generated at a rate equal to its rate of inhibition, resulting in a steady-state thrombin concentration.

At 1.25 nM TFPI, the rate of thrombin generation with normal factor V (Fig. 6*C*) and factor V^LEIDEN (Fig. 6*D*) is increased when compared with reactions with 2.5 nM TFPI (Fig. 6, *A* and *B*). The reduced TFPI level results in ~1.7-fold higher peak levels of thrombin generation in the reactions without Tm (*filled circles*). In contrast, for normal factor V at Tm concentrations ≥1 nM, the difference observed in thrombin generation between the normal and 50% reduced TFPI concentration become almost negligible (Fig. 7, *filled symbols*). At 1.25 nM TFPI and in the presence of factor V^LEIDEN, up to a 6-fold increase in the peak level of thrombin is observed compared with reactions with normal factor V and 1.25 nM TFPI. This 6-fold increase in peak thrombin level is observed at 1 nM Tm (Fig. 6, compare *panels C* and *D*, *open squares*). The differences in peak levels of thrombin generation observed at low thrombomodulin concentrations caused by low TFPI or factor V^LEIDEN are virtually eliminated at 10 nM Tm (Fig. 7, *filled circles*, *filled squares*, and *open circles*).

The thrombin generation profiles observed in the presence of 10 nM Tm are compared in Fig. 8. Thrombin generation in the presence of 10 nM Tm evolves without the display of a peak level of thrombin in the reaction that contains normal levels of

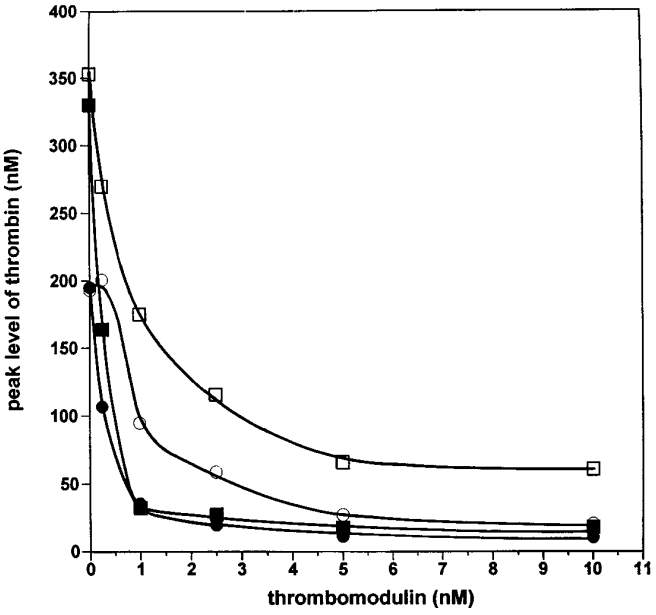

FIG. 7. **Maximum levels of thrombin observed at varying thrombomodulin concentrations in reactions with factor V and factor V^LEIDEN at a normal and 50% reduced TFPI concentration.** Reactions with factor V and 2.5 nM TFPI (●), factor V and 1.25 nM TFPI (■), factor V^LEIDEN and 2.5 nM TFPI (○), and factor V^LEIDEN and 1.25 nM TFPI (□) are shown. Data are derived from the curves presented in Fig. 6.

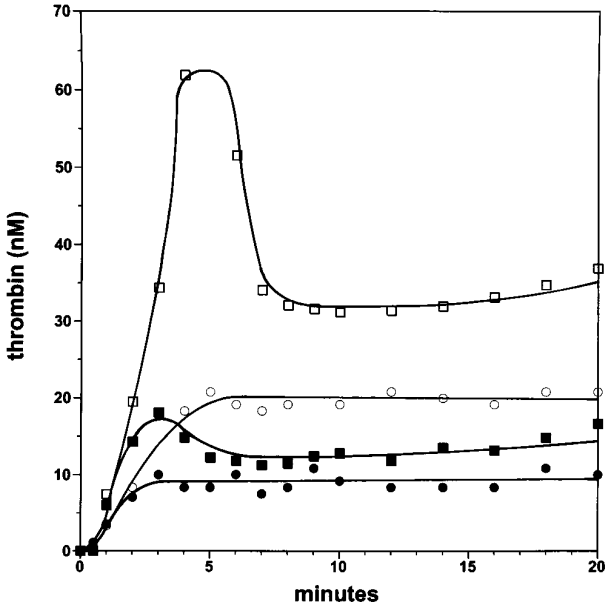

FIG. 8. **Thrombin generation curves in the presence of 10 nM thrombomodulin, protein C, and AT-III for reactions with normal and reduced TFPI concentration with normal factor V or factor V^LEIDEN.** Reactions with factor V and 2.5 nM TFPI (●), factor V and 1.25 nM TFPI (■), factor V^LEIDEN and 2.5 nM TFPI (○), and factor V^LEIDEN and 1.25 nM TFPI (□) are shown. Data are derived from Fig. 6.

TFPI and normal factor V (Fig. 8, *filled circles*). Similar curves are obtained at 10 nM Tm in the presence of either a reduced TFPI level and normal factor V (*filled squares*) or a normal TFPI concentration and factor V^LEIDEN (*open circles*). These reactions show only slight differences in thrombin generation (≤2-fold), and all result in persistent levels of thrombin between approximately 10 and 20 nM (Fig. 8). The isolated potential thrombotic states do not seem to result in highly increased thrombin generation when the reaction is strongly down-regulated by high Tm concentrations. In marked contrast, the combination of low TFPI and factor V^LEIDEN (Fig. 7, *open squares*) results in significantly increased levels of thrombin at 10 nM Tm. In contrast to the other reactions, thrombin generation occurs with a peak level of 60 nM with 1.25 nM TFPI and factor V^LEIDEN (Fig. 8, *open squares*), a level that is 6-fold higher compared with the level reached in the reaction with 2.5 nM TFPI and normal factor V.

The reaction with 1.25 nM TFPI and factor V^LEIDEN is inhibited by 85% with 10 nM Tm in the presence of AT-III (Fig. 6*D*, compare *open circles* and *diamonds*). In the experiment using the same conditions (1.25 nM TFPI, factor V^LEIDEN, 10 nM Tm) and in the absence of AT-III (Fig. 5, *open squares*), virtually no inhibition is observed. This demonstrates the change in the kinetics of thrombin appearance when the reaction is dampened by AT-III and TFPI (8).

Altogether these results strongly suggest that a 50% decrease in the TFPI concentration will significantly increase the prothrombotic state of individuals bearing the factor V^LEIDEN mutation.

Effect of Protein S as Cofactor of the Protein C Pathway on Thrombin Generation by Factor V^LEIDEN and Normal Factor V in the Presence of AT-III—The lack of a significant effect of protein S on the progress curves of thrombin generation in the presence of either normal factor V or factor V^LEIDEN (Fig. 1) could have been the result of the absence of AT-III. Based on the differences in progress curves of thrombin in the presence of the protein C pathway, 2.5 nM TFPI, and 3.4 μM AT-III in the experiment presented in Fig. 6, *A* and *B*, a similar experiment was performed in the presence of 2.5 nM thrombomodulin with normal factor V and factor V^LEIDEN in the absence or presence of 300 nM protein S. Thrombin generation with normal factor V was identical in the absence and presence of protein S (Fig. 9, *open symbols*). The progress curves obtained with factor V^LEIDEN (*filled symbols*) reached a 2.5-fold higher thrombin level compared with normal factor V. Protein S did not affect the thrombin generation profile by factor V^LEIDEN. These data demonstrate that under the conditions employed, in the presence of an excess of membrane surface, protein S has no potentiating effect on the attenuation of thrombin generation by the protein C pathway.

DISCUSSION

The effect of the Arg^{506} → Gln substitution in factor V^LEIDEN was evaluated in a reconstituted model which represents the tissue factor pathway to thrombin in the presence of the protein C system and TFPI. While thrombin generation by normal factor V is synergistically down-regulated and terminated by the combination of protein C, Tm, and TFPI (9), this was not observed for factor V^LEIDEN. TF-initiated thrombin generation in the presence of factor V^LEIDEN continued in the presence of physiological concentrations of TFPI, protein C, and high (10 nM) concentrations of soluble Tm. A reduction of 50% in the TFPI concentration essentially eliminated the ability of the protein C pathway to slow thrombin generation down in the presence of factor V^LEIDEN, while thrombin generation in the presence of normal factor V was still curtailed by the protein C pathway in the presence of 50% levels of TFPI. In the presence of AT-III and high (10 nM) Tm concentrations, a reduced TFPI level *or* the presence of factor V^LEIDEN results in regulated thrombin generation equivalent to control values. However, the combination of factor V^LEIDEN with a reduced TFPI level results in a 6-fold increase of the thrombin level observed in the control in the presence of the highest Tm concentrations evaluated.

The presented data show that the regulation of the tissue factor pathway by the protein C system is directly governed by the TFPI concentration and the inactivation of factor Va by

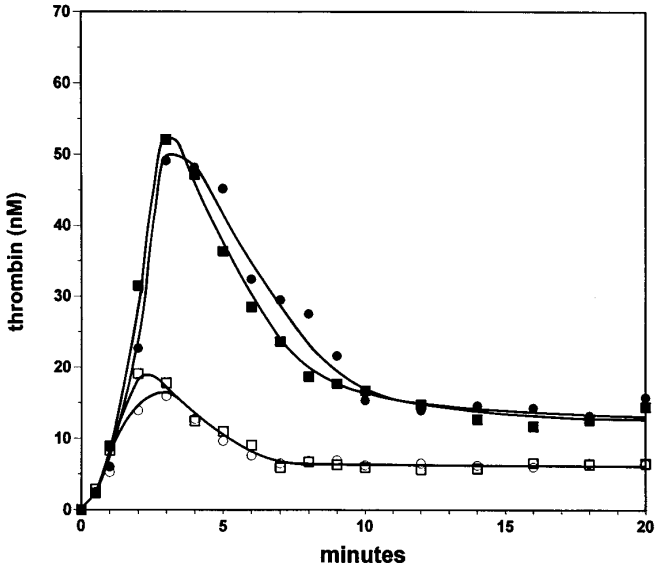

FIG. 9. **Effect of protein S on thrombin generation by factor V and factor V^LEIDEN in the presence of AT-III and TFPI.** Reactions are initiated by 5 pM factor VIIa·TF (5 pM TF and 100 pM factor VIIa) in the presence of 2.5 nM TFPI, 3.4 μM AT-III, 65 nM protein C, and 2.5 nM thrombomodulin in the presence of factor V (*open symbols*) or factor V^LEIDEN (*filled symbols*), in the absence (*circles*) or presence (*squares*) of 300 nM protein S.

initial cleavage at Arg^{506}. These results strongly suggest that the combination of homozygosity for factor V^{LEIDEN} and a mild TFPI deficiency would lead to a severe thrombotic state. Thus far no human deficiencies of TFPI have been reported (4). However, Huang *et al.* (27) have reported the lethal effect of homozygous TFPI deficiency in mice. Heterozygous TFPI-deficient mice appear without symptomatic disease. Our model is consistent with these results, since in the model, with the protein C pathway and AT-III, only marginal differences are observed when the TFPI concentration is at 50% of the mean normal concentration. Based on the profound effects of TFPI in combination with AT-III in our model, we proposed that a homozygous TFPI deficiency would result in a thrombotic status (8). In addition we propose, based on the present data, that the combination of a heterozygous TFPI deficiency with factor V^{LEIDEN} will result in a severe thrombotic tendency.

While the risk for thrombosis is increased 7-fold in individuals heterozygous for the factor V^{LEIDEN} mutation, an 80-fold increased risk of thrombosis is observed in cases of homozygosity for factor V^{LEIDEN} (28). The high prevalence of heterozygous cases of factor V^{LEIDEN} in the normal population results in a significant number of case reports in which this mutation is coinherited with other risk factors such as heterozygous protein C (29), protein S (30), or AT-III (31) deficiency. The greatly increased thrombotic risk with the combination of heterozygous inheritance of factor V^{LEIDEN} with protein C, protein S, or AT-III deficiency explains some of the variation in thrombotic risk associated with heterozygous factor V^{LEIDEN} (29–31). The data presented here suggest that reduced TFPI levels will be another factor that will contribute to variation in the risk of thrombosis when associated with factor V^{LEIDEN}.

The Arg^{506} → Gln substitution leads to partial resistance to inactivation of factor Va by APC (10, 11). Due to the absence of the APC cleavage site at Arg^{506}, the inactivation of factor Va^{LEIDEN} is dependent upon the relatively slower cleavage at Arg^{306} (13, 14).

In the reconstituted model, the generation of factor Xa and the factor Va light chain are the rate-limiting factors for the generation of thrombin (9). In a previous report we have demonstrated that in the presence of protein C and high concentrations of Tm, the heavy chain of factor V becomes inactivated by APC before the generation of the light chain is complete (9). Generation of the light chain of factor Va is reported to be essential when cofactor activity is tested in a clotting assay or in a prothrombinase assay using low factor Xa concentrations (32, 33). This means that the relative stability of the heavy chain of factor V^{LEIDEN} in the presence of the protein C pathway results in higher prothrombinase activity because of higher and persistent levels of intact heavy chain. The kinetic stability of the heavy chain associated with increasing concentrations of light chain can lead to high levels of fully active factor Va.

Cleavage of factor Va at Arg^{506} diminishes the rate of thrombin generation by decreasing the affinity of factor Xa for factor Va (34). This cleavage is accompanied by a relatively small loss in cofactor activity when assayed at saturating levels (5–10 nM) of factor Xa (14, 34). However, cleavage at Arg^{506} results in significant loss of cofactor activity when assayed at low concentrations of factor Xa (34). Thus, the observed increased and ongoing thrombin generation in the reconstituted model in the presence of factor V^{LEIDEN}, bearing the Arg^{506} → Gln substitution, indicates that the initial cleavage at Arg^{506} is necessary for efficient factor Va inactivation and the resulting arrest of thrombin generation.

Most provocative is the absence of a major effect of protein S in the model. Our data show only a small but significant potentiation of the protein C system by protein S. This observation is in contrast to studies that have reported 1) a cofactor function of protein S for APC in the inactivation of factor Va and factor VIII(a) (35, 36) and 2) a normalization of the APC inactivation of factor V^{LEIDEN} by acceleration of cleavage at Arg^{306} (26). The protein S used in this study supports increased inactivation of factor V^{LEIDEN} by APC in an isolated reaction performed under conditions identical to those described by Rosing *et al.* (26).[2] Hence, the lack of an effect of protein S in the reconstituted model is not caused by differences in the protein preparations. Based upon previous studies with isolated reactions, we had expected that protein S would become an important player by the elimination of the protection of factor Va by factor Xa from inactivation by APC (37, 38). Under our experimental conditions, however, protein S has no major inhibitory effect. The reported neutralization of the protecting effect of factor Xa by protein S on factor Va inactivation (38) was studied using nanomolar concentrations of factor Xa that were equimolar or higher than the factor Va used. Under the present experimental conditions and during the TF-mediated clotting of whole blood, only subnanomolar concentrations of factor Xa are formed (9, 39). However, nearly complete activation of factor V occurs during the initiation phase of the reaction, resulting in the generation of 20 nM of factor Va heavy chain. Thus, the protective effects of subnanomolar concentrations of factor Xa on the excess factor Va formed, although reproducible, may be insignificant at the concentration present in the biologically relevant reaction.

It should be mentioned that the function of protein S has been proposed to increase the membrane interaction of APC (35). The relatively high concentrations of phospholipids in the present model probably result in saturation of APC with respect to phospholipid binding, eliminating the requirement for optimal functioning of APC on protein S (35). The observations also suggest that the reported protein-protein interactions of protein S with Va or factor Xa (40, 41), which were hypothesized to play a role in the functioning of protein S as an

[2] Dr. J. Egan, unpublished results.

independent prothrombinase inhibitor, do not occur or are of no kinetic significance in this complex model.

The phospholipid for the procoagulant enzyme complexes is presumably principally provided *in vivo* by platelets. The exposure of the required membrane phospholipid is, however, dependent on stimulation of the platelets; thus, the concentration of phospholipids on which the reactions may occur varies with the accumulation and activation of platelets. The present model, with its relative excess of phospholipids, is more representative for a platelet aggregate with high concentrations of a procoagulant membrane surface. Under these conditions, protein S has no decisive effect on the thrombin generation reaction. The APC-dependent and -independent actions of protein S may be more prominent in reactions that are initiated with quiescent platelets.

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