Factor VIIa/Tissue Factor Generates a Form of Factor V with Unchanged Specific Activity, Resistance to Activation by Thrombin, and Increased Sensitivity to Activated Protein C†

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ABSTRACT: Factor VIIa, in complex with tissue factor (TF), is the serine protease responsible for initiating the clotting cascade. This enzyme complex (TF/VIIa) has extremely restricted substrate specificity, recognizing only three previously known macromolecular substrates (serine protease zymogens, factors VII, IX, and X). In this study, we found that TF/VIIa was able to cleave multiple peptide bonds in the coagulation cofactor, factor V. SDS−PAGE analysis and sequencing indicated the factor V was cleaved at Arg^{679}, Arg^{709}, Arg^{1018}, and Arg^{1192}, resulting in a molecule with a truncated heavy chain and an extended light chain. This product ($FV_{TF/VIIa}$) had essentially unchanged activity in clotting assays when compared to the starting material. TF reconstituted into phosphatidylcholine vesicles was ineffective as a cofactor for the factor VIIa cleavage of factor V. However, incorporation of phosphatidylethanolamine in the vesicles had little effect over the presence of 20% phosphatidylserine. $FV_{TF/VIIa}$ was as sensitive to inactivation by activated protein C (APC) as thrombin activated factor V as measured in clotting assays or by the appearance of the expected heavy chain cleavage products. The $FV_{TF/VIIa}$ could be further cleaved by thrombin to release the normal light chain, albeit at a significantly slower rate than native factor V, to yield a fully functional product. These studies thus reveal an additional substrate for the TF/VIIa complex. They also indicate a new potential regulatory pathway of the coagulation cascade, i.e., the production of a form of factor V that can be destroyed by APC without the requirement for full activation of the cofactor precursor.

Factor V is a nonenzymatic protein cofactor (MW 330 000) whose proteolytic activation is required for efficient thrombin production during hemostasis [reviewed in (*1*) and (*2*)]. Like the homologous factor VIII molecule, factor V is composed of multiple A and C domains and a single B region. Native, single-chain factor V has little procoagulant activity, and it is only after limited proteolysis that it expresses full cofactor activity, playing an important role as a phospholipid-bound cofactor for factor Xa in the activation of prothrombin. Thrombin is considered the physiological activator of factor V and is the most potent activator, catalyzing the cleavage of three peptide bonds at Arg^{709}, Arg^{1018}, and Arg^{1545}. Factor Va, the resultant activated form, is composed of the 105 kDa heavy chain (A1 and A2 domains) and the 71/74 kDa light chain (A3, C1, and C2 domains),which are derived from the N-terminal and C-terminal domains, respectively, of factor V. The two chains are held together by a tightly bound calcium ion. The B region is released as two activation fragments of 71 and 150 kDa. The light chain of factor Va binds the phospholipid membrane, whereas the heavy chain interacts with prothrombin. Once factor Va is bound to the membrane, both the heavy and light chains contribute to the binding of factor Xa. Factor Va lacking the heavy chain region Asp^{683}−Arg^{709} is reported to have reduced factor Xa cofactor activity and demonstrates impaired binding of factor Xa and prothrombin, suggesting that the C-terminal part of the heavy chain is required for optimal interaction with factor Xa and prothrombin (*3*).

Factor V is sensitive to several other proteases which lead to molecules with different levels of cofactor activity. These include factor Xa (*4*), calpain (*5*), and cathepsin G (*6*). A calcium-dependent proteinase from vascular endothelial cells has also been described with factor V activating activity (*7*). Elastase (*6*, *8*) and plasmin (*9*) have been shown to activate and subsequently inactivate the cofactor. In addition to enzymes of potential physiological significance, several snake venom proteases have proven useful as reagents either for the activation of factor V (*10*) or in structure/function studies of the factor V molecule (*3*, *11*).

Factor Va can also be inactivated by limited proteolysis catalyzed by activated protein C (APC)[1] (*12*). Loss of cofactor activity is correlated with cleavage of the factor Va heavy chain at Arg^{506} and Arg^{306}. Factor V can be inactivated

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by cleavage at Arg^{306}, albeit at a significantly slower rate than that of the activated cofactor (*12*). The physiological importance of this reaction is illustrated by the severe thromboembolic complications that result from deficiencies of protein C or its cofactor, protein S (*13*). A polymorphism in the factor V molecule in which the Arg^{506} is substituted with a glutamine results in a molecule that is resistant to cleavage by APC and is also a major risk factor for thrombosis (*14*).

Factor VIIa is the plasma serine protease responsible for initiating the blood clotting cascade in both normal hemostasis and a variety of thrombotic disorders. The integral transmembrane glycoprotein tissue factor (TF) is an essential cofactor for its activity (*15*). Although free factor VIIa has limited enzymatic activity, binding to TF causes a large, reversible increase in its catalytic activity that can be detected with tripeptidyl-amide substrates as well as macromolecular substrates. The only documented macromolecular substrates for the TF/VIIa complex are the serine protease zymogens, factor IX (*16*), factor X (*17*), and factor VII itself in a process known as autoactivation (*18*, *19*).

In this study, we identify factor V as an additional substrate for the TF/VIIa complex. Although limited proteolysis of factor V by TF/VIIa does not appreciably increase the cofactor activity of the factor V molecule, it results in a form of factor V that is rendered resistant to full activation by thrombin and highly sensitive to complete inactivation by activated protein C (APC). Thus, proteolysis of factor V by TF/VIIa may represent an additional regulatory pathway of the coagulation cascade.

EXPERIMENTAL PROCEDURES

Materials. Human factor VIIa (*20*), human factor Xa (*21*), human activated protein C (*22*), and bovine α-thrombin (*23*) were purified as described. Recombinant human TF was expressed in *E. coli* and purified as described (*24*). TF was relipidated by the octyl β-D-glucopyranoside dialysis method (*20*). Human factor V was isolated from fresh frozen plasma by a modification of the method of Kane and Majerus (*25*, *26*). Additional inhibitors added to the original plasma included ε-aminocaproic acid (Sigma) and soybean trypsin inhibitor (Sigma). Human prothrombin was purified from plasma (*27*) and further purified by immunoaffinity chromatography as described (*28*).

Goat polyclonal antibody to bovine factor Va was affinity-purified over a 2-mL Affigel 15 bovine factor Va heavy or light chain column (3 mg of heavy or light chain/mL of resin). Antibody was eluted from the column with 0.1 M glycine, pH 2.1, and fractions were immediately neutralized with 1 M Tris-HCl, pH 8.0.

Phospholipids for TF relipidation were from Avanti. Phospholipids for other uses were from bovine brain and purchased from Sigma. Bovine serum albumin (BSA), gelatin, MOPS, Tris-HCl, and salts were also from Sigma. Factor V deficient human plasma was prepared by the method of Bloom et al. (*29*).

Preparation of Phospholipid Vesicles. Extruded vesicles (100 nm) were prepared (*26*) in 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.02% sodium azide at 5 mg/mL phospholipid using a Nucleopore membrane (Avestin) as described (*26*). Phosphatidylserine (PS), phosphatidylcholine (PC) vesicles contained 20% PS, 80% PC, and phosphatidylethanolamine (PE) vesicles contained 40% PE, 20% PS, and 40% PC.

Electrophoretic Analysis. SDS−PAGE was performed with either 6% or 6−20% gradient acrylamide gels using the Laemmli buffer system (*30*). Gels were silver-stained by the method of Morrissey (*31*). For Western blots, gels were transferred to PVDF membranes (Millipore) using a semi-dry transfer apparatus (Bio-Rad) at 10 V for 30 min. After blocking the membranes with 2% BSA in TBS, 0.05% Tween 20, the membranes were incubated with an affinity-purified goat anti-bovine factor Va subunit (heavy or light chain) for 30 min at room temperature with gentle agitation. Blots were washed 3 times (5 min each) with 0.1 M NaCl, 0.02 M Tris-HCl, 0.05% Tween 20, pH 7.5. Alkaline phosphatase conjugated rabbit anti-goat IgG (0.1 μg/mL) (Pierce) was then added for 30 min, followed by washing as above. The blots were developed with AttoPhos reagent (Vistra fluorescence Western kit; Amersham) and imaged on a Storm Phosphorimager (Molecular Devices).

Amino-Terminal Analysis of Factor V Cleavage Products. Factor V (400 nM) was digested with relipidated TF/VIIa in PC/PS vesicles (1:20 enzyme to substrate molar ratio) for 3 h at 37 °C to ensure total cleavage of factor V. This product is referred to as $FV_{TF/VIIa}$. After reduction, samples were electrophoresed on 6% SDS−PAGE and transferred to an Immobilon-P membrane (Millipore Corp.). Following transfer, the membrane was stained for 5 min with 0.1% Coomassie Blue R-250 in 10% methanol, 5% acetic acid and destained with 50% methanol, 5% acetic acid (3 × 5 min). Finally, the membrane was rinsed with water (3 × 5 min), air-dried overnight, and stored at −20 °C. Amino-terminal amino acid sequencing of cut bands was performed in Dr. Kenneth Jackson's laboratory (Molecular Biology Research Facility, W. K. Warren Medical Research Institute, Oklahoma City).

Factor V Activity Assay. Factor V coagulant activity was assayed in a one-stage clotting assay using factor V-deficient human plasma. Clot formation was detected using a coagulometer (Diagnostica Stago Model ST4). Assays were performed at 37 °C. Factor V activity was determined based on reference to a standard curve constructed with normal human plasma, which was assigned a value of 100%.

Cleavage of Human Factor V by TF/VIIa, Factor Xa, and Thrombin. Factor V (100 nM) was incubated at 37 °C with relipidated human TF/VIIa in PS/PC vesicles (5 nM TF, 50 μg/mL phospholipid), human factor Xa (20 nM) in the presence of 50 μg/mL PS/PC vesicles, or bovine α-thrombin (0.1 nM) in a reaction mixture containing 100 mM NaCl, 20 mM HEPES, pH 7.5, 5 mM $CaCl_2$, and 0.2 mg/mL BSA. In some experiments, TF/VIIa relipidated in PE/PS/PC vesicles was also used. At the indicated times, samples were removed and diluted appropriately for assay into 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, containing 0.1% gelatin. Factor V coagulant activity was immediately determined. A separate sample was also subjected to SDS−PAGE. BSA

[1] Abbreviations: TF, tissue factor; TF/VIIa, TF/factor VIIa complex; APC, activated protein C; B, activation fragment of factor V; HC, factor Va heavy chain; LC, factor Va light chain; $FV_{TF/VIIa}$, factor V derivative resulting from cleavage by the TF/VIIa complex; FV_{Xa}, factor V derivative resulting from cleavage by factor Xa; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; BSA, bovine serum albumin; S2238, D-Phe-(pipecolyl)-Arg-pNA; PAGE, polyacrylamide gel electrophoresis.

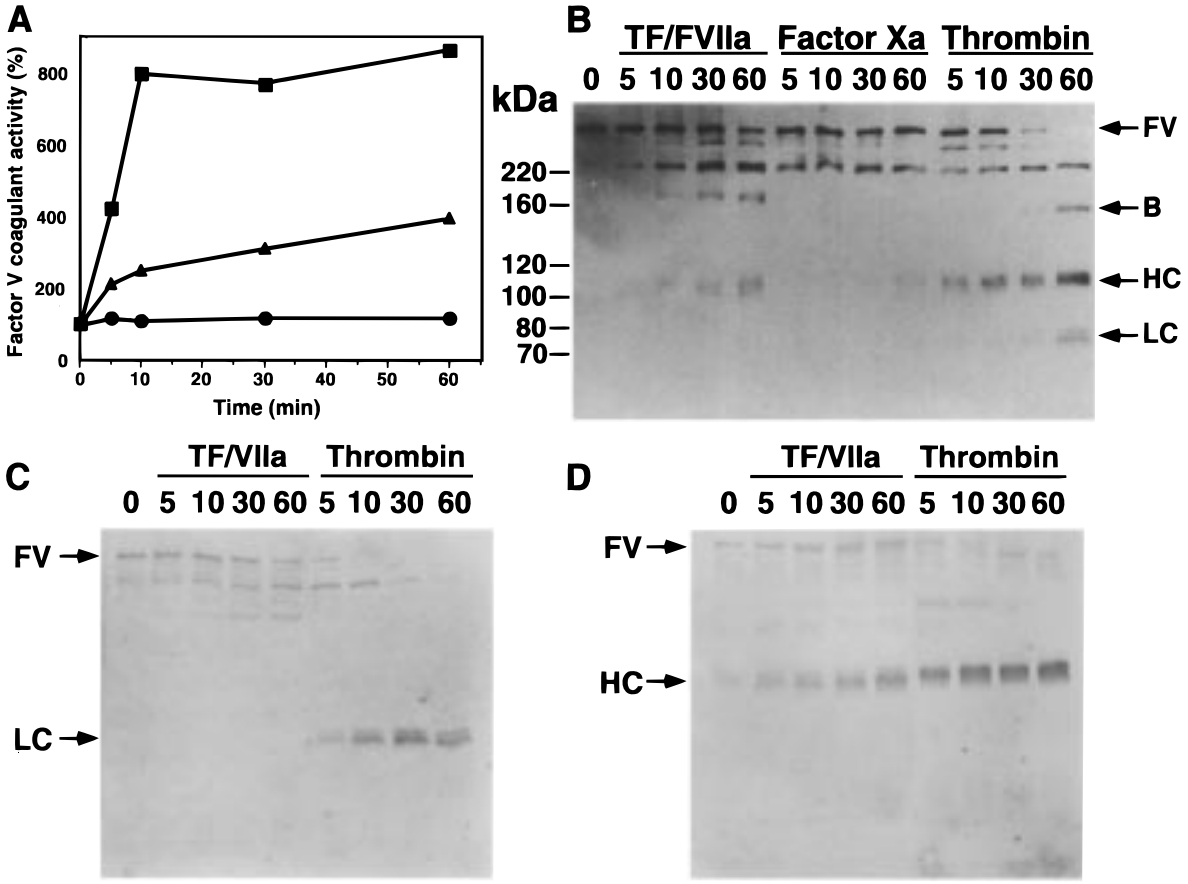

FIGURE 1: Cleavage of factor V by TF/VIIa, factor Xa, and thrombin. Purified factor V (100 nM) was incubated with TF/VIIa (5 nM) (●), factor Xa (5 nM) in the presence of 50 μg/mL phospholipid (▲), or α-thrombin (0.1 nM) (■) as described under Experimental Procedures. At the times indicated in minutes, samples were assayed for factor V activity (panel A) or subjected to SDS−PAGE on 6% polyacrylamide gels. Gels were either stained with silver as described (panel B) or further subjected to Western blot analysis with anti-light chain (panel C) or anti-heavy chain (panel D) antibody as described under Experimental Procedures. The position of the factor V (FV), activation fragment (B), factor Va heavy chain (HC), and the light chain doublet (LC) are indicated, as are the positions of molecular mass markers. Data are representative of five replicate experiments.

was omitted from the reaction buffer in experiments in which silver staining of gels was required.

Inactivation of Factor V and Factor V Derivatives by Human APC. Factor V (100 nM) was incubated for 60 min with TF/VIIa, factor Xa, or thrombin as described above, after which 0.1 nM APC was added to the mixtures. PS/PC vesicles (50 μg/mL) were also added to the thrombin-treated samples. At the times indicated on the figures, samples were removed, and factor V coagulant activity was measured by the one-stage clotting assay. Additional samples were subjected to SDS−PAGE analysis.

RESULTS

Cleavage of Factor V by the TF/VIIa Complex. The effect of TF/VIIa on the structure and function of factor V was monitored by functional assay (Figure 1A), SDS−PAGE (Figure 1B), and Western blot analysis (Figure 1C,D) and compared to the products of factor V incubated with factor Xa or thrombin. Although there was no increase in factor V activity, gel analysis indicated a time-dependent cleavage of factor V to four products of molecular weight 220 000, 170 000, 106 000 and 80 000 by TF/VIIa. The same pattern was observed if the reaction was run in the presence of the factor Xa inhibitor, tick anticoagulant protein or hirudin, indicating none of the cleavages are due to factor Xa or thrombin contamination in the factor V preparation. Additional experiments in which factor VIIa was omitted from the reaction showed no cleavage of the factor V, indicating that the observed activity was not due to protease contamination of the recombinant tissue factor. At low factor Xa concentration, the cleavage products observed by Monkovic et al. were observed (*4*), although higher concentrations of

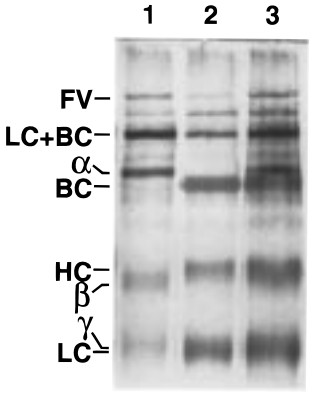

FIGURE 2: SDS−PAGE comparison of factor Va and $FV_{TF/VIIa}$. Purified factor V (100 nM) was incubated with TF/VIIa (5 nM) or thrombin (0.1 nM) for 3 h at 37 °C as described under Experimental Procedures. Samples were subjected to 6% SDS−PAGE and stained with silver. Lane 1, $FV_{TF/VIIa}$; lane 2, factor Va; lane 3, a mixture of $FV_{TF/VIIa}$ and factor Va.

Table 1: Amino-Terminal Sequence of Factor V Fragments after TF/VIIa Digestion[a]

cycle no.	LC + BC, 220 kDa	α, 170 kDa	β, 105 kDa	γ, ≈ 80 kDa
1	T	N	A	S/K
2	F	L	Q	F/M
3	H	S	L	R/H
4	?P	P	R	?N/D
5	L	A	Q	S/R
fragment identification	1019−2196	1193−2196	1−679	679−1018(1192?) 709−1018(1192?)

[a] Human factor V (400 nM) was incubated with 20 nM TF/VIIa at 37 °C for 3 h and was subjected to SDS−PAGE and transferred to a membrane as described under Experimental Procedures. The bands indicated in Figure 2 were excised and subjected to amino-terminal sequencing. LC, light chain; BC, B chain.

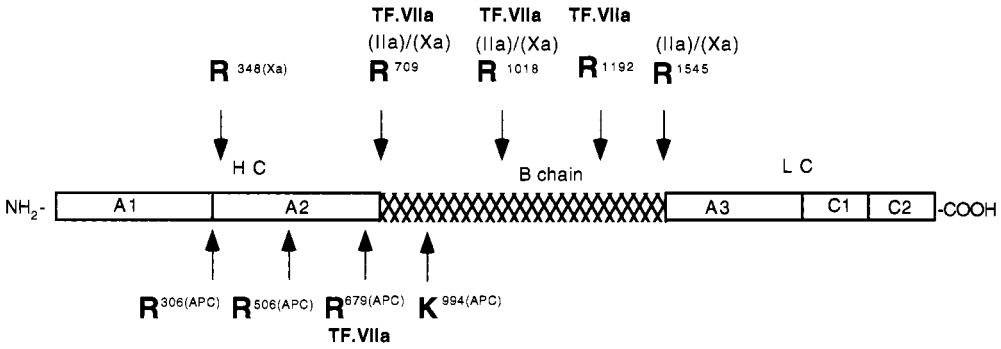

FIGURE 3: Schematic representation of the proteolytic cleavages of human factor V by TF/VIIa, factor Xa, and thrombin.

factor Xa or longer reaction time resulted in light chain release (*32*) (data not shown). The specific activity of the factor Xa activated factor V (FV_{Xa}) was approximately one-third that of thrombin-activated factor V under the conditions of cleavage and assay used here.

To identify the TF/VIIa cleavage sites in factor V, the bands corresponding to LC + BC, α, β, and γ in Figure 2 were subjected to N-terminal sequence analysis. The results are shown in Table 1. The fragment LC + BC is identical to that obtained as an intermediate in thrombin digestions (*33*). The N-terminal sequence and small size of the β band are compatible with it being a truncated form of the heavy chain. It is apparent in lane 3 that the α band is not the normal B chain produced by thrombin. Amino-terminal sequence, size, and blotting properties indicate it contains part of the B chain attached to the light chain. The γ band resulted in two sequences, compatible with cleavage at 679 and 709 in the factor V. Based on size, these fragments presumably result from these cleavages in addition to cleavage at positions 1018 or 1092. No bands corresponding to the normal light chain resulting from cleavage at 1545 could be identified. Figure 3 illustrates the location of the sites sensitive to cleavage by thrombin, factor Xa, and TF/VIIa.

The ability of TF/VIIa to activate factor X is sensitive to the phospholipid composition of the vesicles in which the TF is reconstituted (*24*, *34*). It was therefore of interest to determine if the phospholipid composition affected factor V cleavage. TF was relipidated into vesicles composed of PC, 20% PS/80% PC, or 40% PE/20% PS/40% PC, complexed with factor VIIa, and incubated with factor V. On occasion, a slight enhancement of the cleavage rate was observed when PE was present, as determined by the production of heavy chain (Figure 4). However, this enhancement was not reproducible. TF/VIIa in PC vesicles was

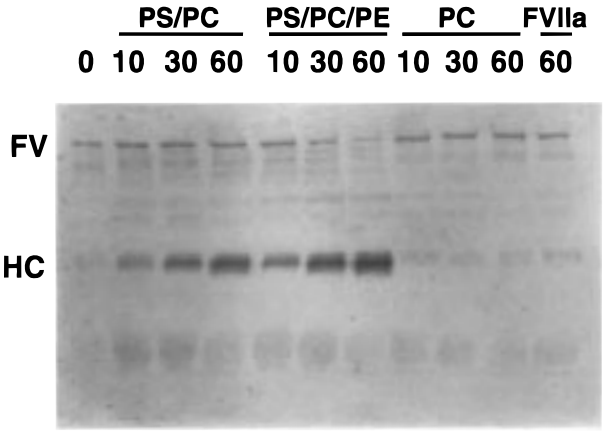

FIGURE 4: Effect of phospholipid composition on TF/VIIa cleavage of factor V. TF/VIIa complex was formed with TF incorporated in phospholipid vesicles composed of PC, PC/PS, or PC/PS/PE. Factor V (100 nM) was incubated with 5 nM TF/VIIa complex as described under Experimental Procedures. At the time points indicated in minutes, samples were subjected to 6% SDS−PAGE and transferred to PVDF membranes. Fragments were visualized with immunopurified polyclonal anti-factor V heavy chain. Controls incubated with factor VIIa in the absence of TF but the presence of PC/PS vesicles were also included.

inactive in this reaction, most likely because factor V does not bind efficiently to these vesicles (*35*). Factor VIIa, without TF but in the presence of PS/PC vesicles, was also inactive.

Effect of Thrombin on $FV_{TF/VIIa}$. To determine whether $FV_{TF/VIIa}$ could be further activated to fully functional factor Va, factor V was incubated with TF/VIIa for 3 h at 37 °C to ensure total cleavage of factor V to the final products indicated in Figure 2 and Table 1. Thrombin at 10 nM (Figure 5A) or 1 nM (Figure 5B−D) was then added. As control, factor V was incubated for 3 h in buffer before the addition of thrombin. $FV_{TF/VIIa}$ could be activated by thrombin

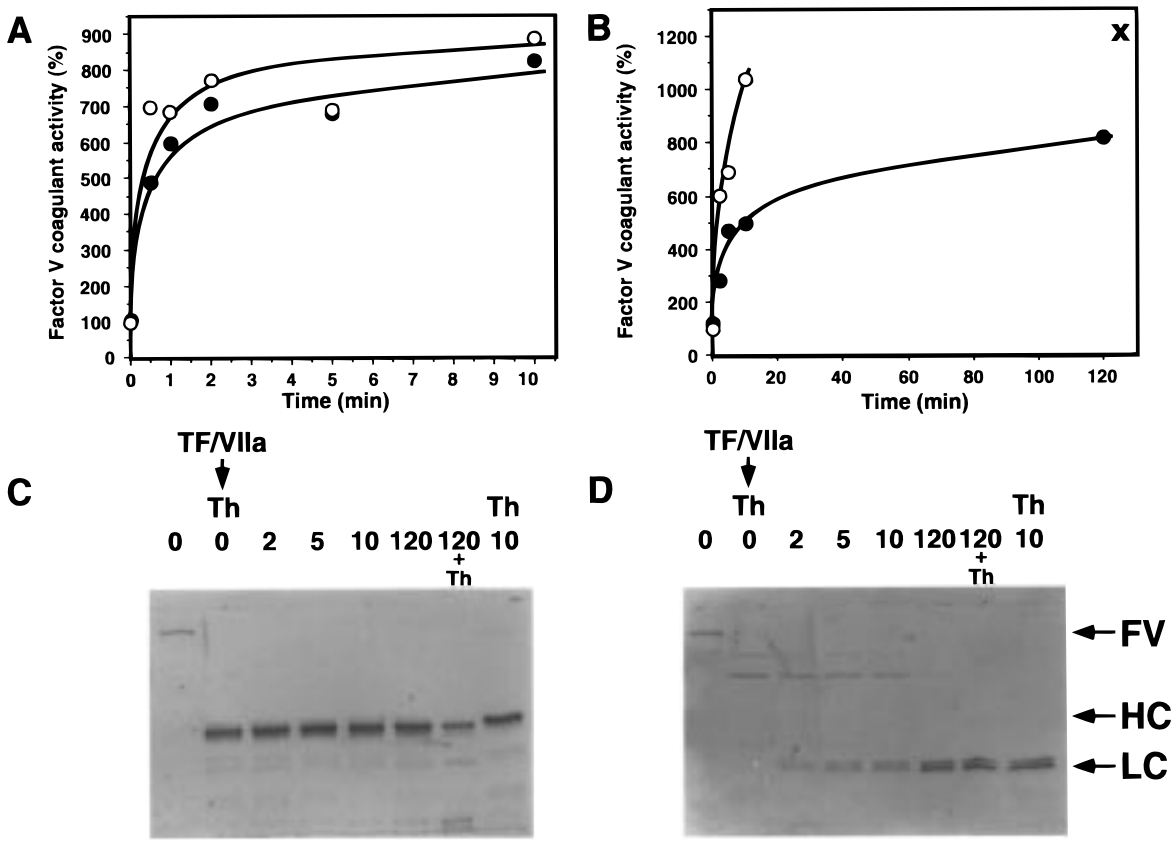

FIGURE 5: Comparison of the ability of thrombin to activate factor V and FVTF/VIIa. Panel A: Purified human factor V (100 nM) was incubated with (●) or without (○) TF/VIIa (10 nM) on PS/PC vesicles at 37 °C. After 3 h, thrombin (10 nM) was added. At the indicated times after thrombin addition, factor Va activity was determined in factor V deficient plasma as described under Experimental Procedures. Panels B−D: Purified human factor V (100 nM) was incubated with (●) or without (○) TF/VIIa (10 nM) on PS/PC vesicles at 37 °C. After 3 h, thrombin (1 nM) was added. After 2 h of incubation, excess thrombin (12.5 units/mL) was added to fully activate any remaining $FV_{TF/VIIa}$ (×). At the times after thrombin addition indicated, aliquots of the reaction mixture were assayed for factor Va activity (panel B) or subjected to 6% SDS−PAGE. Following transfer to PVDF membranes, the proteolytic fragments were visualized using the polyclonal specific anti-heavy chain (panel C) or anti-light chain antibodies (panel D). Lane 1 contains the factor V starting material. Lane 8 is a sample of the thrombin (1 nM)-activated factor V after 10 min of activation. Lane 7 corresponds to the $FV_{TF/VIIa}$ fully activated by thrombin denoted by the × in panel B. The positions of factor V (FV), factor Va heavy chain (HC), and factor Va light chain (LC) are indicated.

to a product whose activity was equivalent to factor Va (Figure 5A). This demonstrated the light chain could be released from $FV_{TF/VIIa}$, which was confirmed by immunoblotting experiments (data not shown). The truncated heavy chain did not affect activity in the clotting assay. Examination of the early part of the curve, however, indicated that the rate of activation of $FV_{TF/VIIa}$ might be impaired. To investigate this possibility further, $FV_{TF/VIIa}$ was incubated with 1 nM thrombin. An additional control included incubation with TF/VIIa and thrombin simultaneously to be sure the presence of TF/VIIa did not itself inhibit thrombin activation of $FV_{TF/VIIa}$. At the lower thrombin concentration, the difference in activation rate was much more pronounced (Figure 5B). This was reflected in a much slower release of the light chain from $FV_{TF/VIIa}$ than from native factor V (Figure 5C,D). As can be seen in the figures, thrombin released significantly less light chain from $FV_{TF/VIIa}$ in 120 min (center lanes) than from untreated factor V in 10 min (far right lane). A burst of activation was noted before a continued slow rise. Other experiments are consistent with this being due to the activation of the common 220 000 kDa degradation product present in the original factor V preparation. This intermediate is more readily cleaved by thrombin than the 170 000 kDa intermediate formed by TF/VIIa. All of the $FV_{TF/VIIa}$ was activatable as indicated by full activation upon the addition of excess thrombin at the end of the experiment. Controls in which TF/VIIa and thrombin were added simultaneously showed activation rates equivalent to those observed when thrombin alone was used, indicating the presence of the TF/VIIa complex itself was not responsible for the slower cleavage rate of $FV_{TF/VIIa}$ (data not shown).

$FV_{TF/VIIa}$ Prothrombinase Activity in a Purified System. To further characterize the activity of $FV_{TF/VIIa}$, the activity of $FV_{TF/VIIa}$ either before or after activation with thrombin was compared to that of factor V and factor Va in purified systems employing factor Xa, prothrombin, and phospholipid. The factor V activation profiles based on plasma factor V clotting assays (see Figure 5) suggested that factor V and $FV_{TF/VIIa}$ might exhibit a lag in the prothrombin activation time course. Indeed, the time course of prothrombin activation with $FV_{TF/VIIa}$ did exhibit a lag that was at least as great as that observed with factor V (Figure 6A). This same behavior, only slightly more exaggerated, was seen when thrombin generation at a set time was measured as a function of increasing factor Xa concentration (Figure 6B). When the low factor Xa concentrations are examined (Figure 6C), the differences are even larger between factor V and $FV_{TF/VIIa}$ and their corresponding thrombin-activated forms.

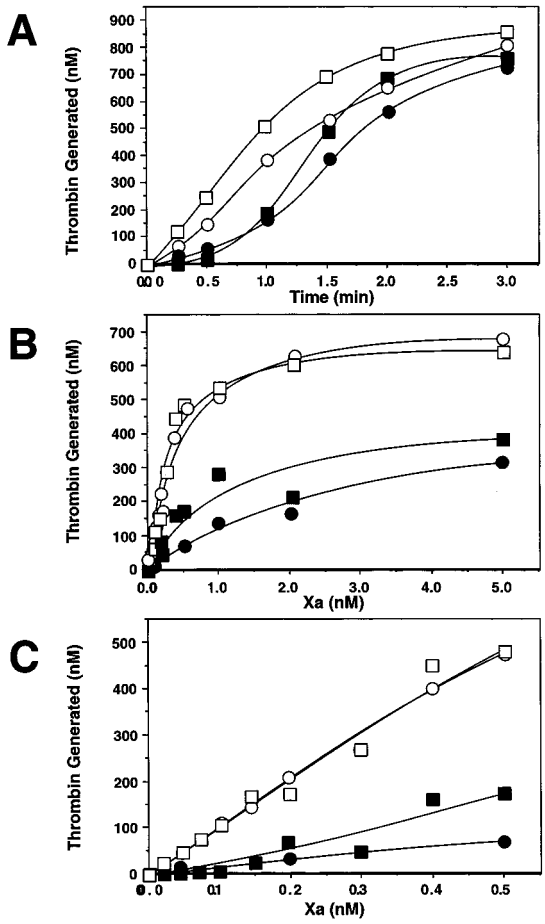

FIGURE 6: Comparison of the prothrombinase activities of factor V and factor V derivatives in a purified system. Factor V (■), $FV_{TF/VIIa}$ (●), $FV_{TF/VIIa}$ activated with thrombin (○) and factor Va (□) were used as the source of factor V activity in prothrombinase assays. Panel A: Prothrombin (1 μM) was mixed with the factor V derivative (0.5 nM) and phospholipid (50 μg/mL, 20% PS, 80% PC) in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.5% BSA, 2 mM $CaCl_2$ at room temperature and the reaction started by the addition of 0.5 nM factor Xa. At the times indicated, 20 μL samples were removed to 80 μL of buffer containing 10 mM EDTA, pH 8, instead of the $CaCl_2$. S2238 (20 μL of 2 mM) was then added and the rate of hydrolysis measured in a V_{max} microplate reader (Molecular Devices). Thrombin concentration was determined by comparison to a standard curve of known thrombin concentrations. Panels B and C: Prothrombinase reactions were performed as in panel A except that the concentration of factor Xa was varied as indicated on the *x*-axis. Reactions were carried out for 1 min before quenching with EDTA.

Sensitivity of $FV_{TF/VIIa}$ to Cleavage by Activated Protein C. Factor V (100 nM) was incubated with TF/VIIa (5 nM), factor Xa (20 nM), thrombin (0.1 nM), or buffer for 1 h. At that time, APC (0.1 nM) was added, and the time courses of inactivation were determined (Figure 7A). The activity of the $FV_{TF/VIIa}$ was reduced by incubation with APC. As expected, the factor Xa and thrombin-treated factor V were also readily inactivated while factor V was resistant. Interestingly, when the factor V activity of each incubation was normalized to 100% after the preincubation stage, it became apparent that the $FV_{TF/VIIa}$ and factor Va were equivalently sensitive to inactivation by APC (Figure 7B). With higher concentrations of APC, factor V was inactivated nearly completely in a similar time frame (Figure 7B, insert). Immunoblot analysis indicated that the cleavage products of APC proteolysis of $FV_{TF/VIIa}$ were the same as those obtained

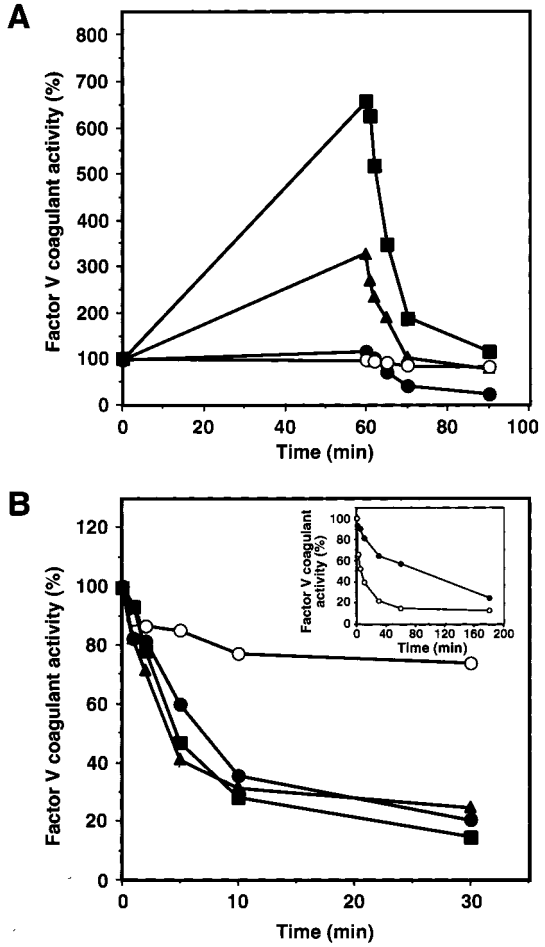

FIGURE 7: Comparison of the inactivation of human factor V and factor V derivatives by activated protein C. Purified human factor V (100 nM) was incubated with TF/VIIa (5 nM) (●), factor Xa (20 nM, 50 μg/mL phospholipid) (▲), thrombin (0.1 nM) (■), or buffer (○) at 37 °C. After 1 h, APC (0.1 nM) was added. Phospholipid (50 μg/mL) was also added to the thrombin- or buffer-incubated samples. Panel A: At the times indicated, factor V coagulant activity was determined as described under Experimental Procedures. Panel B: The data of panel A, normalizing the activity present in each reaction at 60 min incubation to 100%. Time of incubation with APC is indicated on the *x*-axis. Panel B, insert: Factor V (100 nM) was incubated with 0.1 nM (●) or 1.0 nM (○) APC for the times indicated.

when factor Va or FV_{Xa} was the starting material (Figure 8). $FV_{TF/VIIa}$ which was subsequently activated with thrombin was inactivated by APC at a rate similar to the other factor V derivatives (data not shown). Thus, precleavage at Arg^{679} does not facilitate further cleavage by APC.

DISCUSSION

Previously, the TF/VIIa complex was known to recognize only three macromolecular substrates, all of which are serine protease zymogens (factors VII, IX, and X). However, factor V, the protein cofactor for factor X, also circulates as an inert precursor, and it also requires limited proteolysis for activation. Accordingly, in this study we investigated if the TF/VIIa complex was able to recognize factor V as a substrate, possibly converting it to a form with factor Xa cofactor activity. We found that factor V was cleaved by the TF/VIIa complex at multiple sites, resulting in a form of factor V whose susceptibilty to inactivation by APC was equal to that of thrombin-activated factor V. Interestingly,

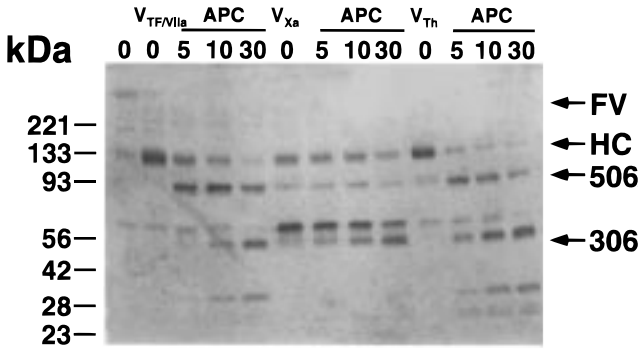

FIGURE 8: Immunoblot analysis of the APC cleavage patterns of $FV_{TF/VIIa}$, FV_{Xa}, and factor Va. Samples corresponding to the reaction time points of Figure 7 were analyzed on 6−20% SDS−PAGE gradient gels (approximately 120 ng of factor V per lane). Fragments were visualized following transfer to PVDF and staining with the polyclonal anti-heavy chain specific antibody. Lane 1, factor V control; lane 2, factor V after incubation with TF/VIIa for 60 min; lanes 3−5, $FV_{TF/VIIa}$ after incubation with APC for 5, 10, or 30 min, respectively; lane 6, factor V after incubation with factor Xa plus phospholipid for 60 min; lanes 7−9, FV_{Xa} after incubation with APC for 5, 10, or 30 min, respectively; lane 10, factor V incubation with thrombin plus phospholipid for 60 min; lanes 11−13, factor Va after incubation with APC for 5, 10, or 30 min, respectively. The positions of the molecular mass markers are indicated on the left. The positions of factor V (FV), heavy chain (HC), and the APC heavy chain cleavage products at Arg^{506} (506) or Arg^{306} (306) are indicated on the right.

however, factor V cleaved by TF/VIIa did not exhibit increased procoagulant activity. This is in marked contrast to the cleavage of factor V by a variety of other proteases (both physiological and nonphysiological), which either directly convert factor V to a form with significant cofactor activity or convert it to a form that can be rapidly activated by thrombin (*36*).

Considering the restricted specificity of TF/VIIa, it is interesting to note the cleavages in factor V that the complex performs. Of the four sites observed, only one is unique to TF/VIIa, Arg^{1192}. Two (Arg^{709} and Arg^{1018}) are shared with thrombin and factor Xa, and the third (Arg^{679}) is shared with APC. The sequences surrounding the cleavage sites in factor V catalyzed by TF/VIIa are given in Table 2, along with the cleavage sites of the three previously known macromolecular substrates of TF/FVIIa (factors VII, IX, and X). The amino acids at the P_2 position of the cleavages sites in factors VII, IX, and X tend to have relatively small side chains (Gly, Thr, and Ser), although Val, which occurs in bovine factor X, is somewhat larger (Table 2). For the cleavage sites in FV, some of the amino acid side chains at the P_2 position fit this pattern (i.e., Thr, and to a lesser extent, Ile), while others are somewhat bulkier (Gln and Pro). Some insights into the substrate specificity of this enzyme have been obtained by comparing the cleavage rates of small peptidyl-amide substrates (although only a limited number of substitutions at the P_2 and P_3 positions have been investigated to date). Notably, Pro, which occurs at the P_2 position in one of the factor V cleavage sites identified in this study, is relatively common at the P_2 position in peptidyl substrates that are preferred by TF/FVIIa (*37*, *38*). Amino acid preferences at the P_3 to P_5 positions in TF/FVIIa substrates are less clear, as there is little obvious similarity in sequence of the naturally occurring substrates at these positions. In addition, these positions are usually either absent (P_4 and P_5 residues) or occupied by D-amino acids (P_3 residues) in synthetic peptidyl substrates. Notably, however, all four of the factor V cleavage sites identified in this study have hydrophobic residues at the P_4 position, and most have amino acids with relatively small side chains at the P_3 position.

Little is known regarding the preference of TF/FVIIa for amino acids in the P′ positions of its substrates. This is because the high degree of sequence conservation of P′ residues in the natural substrates of TF/FVIIa likely results from the fact that these sequences fold back into the respective protease domains to stabilize the oxyanion hole, and so may be highly conserved solely for this reason. Furthermore, P′ amino acids are absent from peptidyl-amide or peptidyl-ester substrates. Indeed, there is little obvious similarity between the P′ residues in factors VII, IX, and X and the factor V cleavage sequences reported here. However, we note that all of the factor V cleavage sites have polar amino acids at the P'_1 and P'_3 positions, and all have hydrophobic amino acids at the P'_2 position.

Direct comparison of the product of TF/VIIa cleavage with those reported by other investigators using diverse enzymes is difficult. As has been discussed previously by others (*32*, *39*), the apparent activity observed is grossly affected by the assay conditions employed (factor Xa concentration, phospholipid composition and concentration, etc). This is perhaps most strikingly illustrated by the recent report from Dahlbäck's group in which factor V mutated to be totally thrombin-resistant gave a clotting activity similar to that of native factor V (*40*). When multiple cleavage sites are available, the order and rate of cleavage can vary depending upon the exact conditions used for the proteolysis reactions. If an assay is used in which active thrombin can be generated, this also can lead to variation in results, depending on the rate of the thrombin generation and the sensitivity of the

Table 2: Cleavage Sites in Factor V and Other Known Macromolecular Substrates of TF/FVIIa

protein[a]	sequence of cleavage site										ref
	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	
1. Hum, Bov, Rab factor VII	K/N	P	Q	G	R	I	V	G	G	K/H	*48*−*50*
2. Hum, Bov factor X	N/S	N/Q	L/V	T/V	R	I	V	G	G	Q/R	*51*, *52*
3. Hum, Bov, Rab factor IX[b]	S/K	K	L/I	T	R	A	E/T	A/T	V/I	F	*53*−*55*
4. Hum, Bov, Rab factor IX[b]	N/D	D/E	F	T/S	R	V/I	V	G	G	E	53−55
5. Hum factor V (R^{679})	V	M	A	T	R	K	M	H	D	R	*c*, 1
6. Hum factor V (R^{709})	A	L	G	I	R	S	F	R	N	S	*c*, 1
7. Hum factor V (R^{1018})	P	L	S	P	R	T	F	H	P	L	*c*, 1
8. Hum factor V (R^{1192})	E	L	I	Q	R	N	L	S	P	A	c

[a] Hum, human; Bov, bovine; Rab, rabbit. [b] Line 3, cleavage at (or homologous to) Arg^{145}−Ala^{146} in human factor IX; line 4, cleavage at (or homologous to) Arg^{180}−Val^{181} in human factor IX. [c] Cleavage site as determined in this study; factor V protein sequence from ref (*1*).

factor V(a) species to further cleavage. In the case of $FV_{TF/VIIa}$, the situation is even more complex since the factor Xa required for any functional assay can itself cleave the $FV_{TF/VIIa}$ to release active light chain (*32*). Once the prothrombinase complex is formed, this results in essentially a 1:1 enzyme (FXa) to substrate ($FV_{TF/VIIa}$) ratio, overcoming most kinetic obstacles. Thus, attempts to determine the basis for the low activity gave inconclusive results in purified systems (data not shown).

The observation that after cleavage with the TF/VIIa complex factor V is converted to factor Va more slowly by thrombin, could be the result of several mechanisms. One or more sites cleaved by TF/VIIa could be directly involved in binding to thrombin, perhaps at one of its exosites. Alternatively, TF/VIIa cleavage could result in conformational changes that alter the conformation around Arg1545 (or other scissile bonds) or the accessibility of these bonds. These same possibilities could account for why the TF/VIIa complex does not appear to cleave at Arg1545. In this case, however, there is an additional possibility that the sequence or conformation surrounding Arg1545 is not favorable for cleavage by the TF/VIIa complex. Since the structure of factor V is unknown, and there is limited information about the structure−function relationships involved in rapid factor V activation by thrombin, it is not possible to distinguish among the above possibilities.

Recently, Thorelli, et al. (*41*) have reported that thrombin cleavage at Arg^{709} and/or Arg^{1018} yielded factor V molecules that were still able to function as APC cofactors. The product of TF/VIIa cleavage described in this study may be a natural counterpart to the factor V molecule engineered by those authors and would be expected to retain similar cofactor activity. The rate of cleavage of factor V by TF/VIIa is probably too slow to play a major role in regulation of the coagulation system in blood. However, the process could have a function in the control of thrombin generation in the extravascular space. In recent years, it has become apparent that control of thrombin formation in the extravascular space is important (*42*), but as yet the control mechanisms are not completely understood. Extravascular cells express thrombin receptors, and prothrombin synthesis has been demonstrated in some of these cells (*43*). Factor VII has been reported to be synthesized by monocytes (*44*) and to be present in lymph (*45*), allowing factor VII access to extravascular cells. Alternatively, factor VII/VIIa bound to monocyte tissue factor could potentially enter the extravascular space during monocyte extravasation. Recently, a potential mechanism for protein C deposition in the extravascular space has been elucidated. The endothelial cell protein C receptor capable of binding both protein C and APC has been shown to localize in caveolae (*46*), organelles that have been reported to be involved in endothelial cell transcytosis (*47*). Thus, a potential scenario is in place for the TF/VIIa-mediated cleavage of any factor V that should gain access to the extravascular space either through vascular leakage by or association with inflammatory cells during extravasation (*48*). This modified factor V would then be resistant to activation by the low levels of thrombin in the extravascular tissue and relatively sensitive to APC transported from the blood, perhaps via the endothelial cell receptor. Further experiments will be necessary to test this proposal.

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REFERENCES

1. Kane, W. H., and Davie, E. W. (1988) *Blood 71*, 539−555.
2. Kalafatis, M., Swords, N. A., Rand, M. D., and Mann, K. G. (1994) *Biochim. Biophys. Acta 1227*, 113−129.
3. Bakker, H. M., Tans, G., Thomassen, M. C. L. G. D., Yukelson, L. Y., Ebberink, R., Hemker, H. C., and Rosing, J. (1994) *J. Biol. Chem. 269*, 20662−20667.
4. Monkovic, D. D., and Tracy, P. B. (1990) *J. Biol. Chem. 265*, 17132−17140.
5. Bradford, H. N., Annamalai, A., Doshi, K., and Colman, R. W. (1988) *Blood 71*, 388−394.
6. Allen, D. H., and Tracy, P. B. (1995) *J. Biol. Chem. 270*, 1408−1415.
7. Rodgers, G. M., Cong, J. Y., Goll, D. E., and Kane, W. H. (1987) *Biochim. Biophys. Acta 929*, 263−270.
8. Samis, J. A., Garrett, M., Manuel, R. P., Nesheim, M. E., and Giles, A. R. (1997) *Blood 90*, 1065−1074.
9. Lee, C. D., and Mann, K. G. (1989) *Blood 73*, 185−190.
10. Esmon, C. T. (1980) in *The regulation of Coagulation* (Mann, K. G., and Taylor, F. B., Jr., Eds.) pp 137−143, Elsevier/North-Holland, New York.
11. Kalafatis, M., Henderson, S. C., Camire, R. M., Hassan, M. I., and Mann, K. G. (1995) *Blood 86*, 281a (Abstract).
12. Kalafatis, M., Rand, M. D., and Mann, K. G. (1994) *J. Biol. Chem. 269*, 31869−31880.
13. Esmon, C. T., and Schwarz, H. P. (1995) *Trends Cardiovasc. Med. 5*, 141−148.
14. Dahlbäck, B. (1995) *Blood 85*, 607−614.
15. Carson, S. D., and Brozna, J. P. (1993) *Blood Coagulation Fibrinolysis 4*, 281−292.
16. Osterud, B., and Rapaport, S. I. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5260−5264.
17. Silverberg, S. A., Nemerson, Y., and Zur, M. (1977) *J. Biol. Chem. 252*, 8481−8488.
18. Nakagaki, T., Foster, D. C., Berkner, K. L., and Kisiel, W. (1991) *Biochemistry 30*, 10819−10824.
19. Pedersen, A. H., Lund-Hansen, T., Bisgaard-Frantzen, H., Olsen, F., and Petersen, L. C. (1989) *Biochemistry 28*, 9331−9336.
20. Neuenschwander, P., and Morrissey, J. H. (1992) *J. Biol. Chem. 267*, 14477−14482.
21. Le Bonniec, B. F., Guinto, E. R., and Esmon, C. T. (1992) *J. Biol. Chem. 267*, 6970−6976.
22. Esmon, C. T., Esmon, N. L., Le Bonniec, B. F., and Johnson, A. E. (1993) *Methods Enzymol. 222*, 359−385.
23. Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem. 249*, 594−605.
24. Neuenschwander, P. F., Bianco-Fisher, E., Rezaie, A. R., and Morrissey, J. H. (1995) *Biochemistry 34*, 13988−13993.
25. Kane, W. H., and Majerus, P. W. (1981) *J. Biol. Chem. 256*, 1002−1007.
26. Smirnov, M. D., Safa, O., Regan, L., Mather, T., Stearns-Kurosawa, D. J., Kurosawa, S., Rezaie, A. R., Esmon, N. L., and Esmon, C. T. (1998) *J. Biol. Chem. 273*, 9031−9040.
27. Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem. 249*, 594−605.
28. Le Bonniec, B. F., and Esmon, C. T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7371−7375.
29. Bloom, J. W., Nesheim, M. E., and Mann, K. G. (1979) *Thromb. Res. 15*, 595−599.
30. Laemmli, U. K. (1970) *Nature 227*, 680−685.
31. Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307−310.
32. Thorelli, E., Kaufman, R. J., and Dahlbäck, B. (1997) *Eur. J. Biochem. 247*, 12−20.
33. Suzuki, K., Dahlbäck, B., and Stenflo, J. (1982) *J. Biol. Chem. 257*, 6556−6564.
34. Nemerson, Y. (1968) *J. Clin. Invest. 47*, 72−80.

35. Krieg, U. C., Isaacs, B. S., Yemul, S. S., Esmon, C. T., Bayley, H., and Johnson, A. E. (1987) *Biochemistry 26*, 103−109.
36. Jenny, R. J., Tracy, P. B., and Mann, K. G. (1994) in *Haemostasis and Thrombosis* (Bloom, A. L., Forbes, C. D., Thomas, D. P., and Tuddenham, E. G. D., Eds.) pp 465−476, Churchill Livingstone, Edinburgh.
37. Butenas, S., Ribarik, N., and Mann, K. G. (1993) *Biochemistry 32*, 6531−6538.
38. Neuenschwander, P. F., Branam, D. E., and Morrissey, J. H. (1993) *Thromb. Haemostasis 70*, 970−977.
39. Keller, F. G., Ortel, T. L., Quinn-Allen, M. A., and Kane, W. H. (1995) *Biochemistry 34*, 4118−4124.
40. Thorelli, E., Kaufman, R. J., and Dahlbäck, B. (1998) *Thromb. Haemostasis 80*, 92−98.
41. Thorelli, E., Kaufman, R. J., and Dahlbäck, B. (1998) *J. Biol. Chem. 273*, 16140−16145.
42. Coughlin, S. R. (1994) *Semin. Hematol. 31*, 270−277.
43. Kim, S., and Nelson, P. G. (1998) *J. Biol. Chem. 273*, 11923−11929.
44. Tsao, B. P., Fair, D. S., Curtiss, L. K., and Edgington, T. S. (1984) *J. Exp. Med. 159*, 1042−1057.
45. Le, D. T., Borgs, P., Toneff, T. W., Witte, M. H., and Rapaport, S. I. (1998) *Am. J. Physiol. Heart Circ. Physiol. 274*, H769−H776.
46. Xu, J., Qu, D., and Esmon, C. T. (1998) *Vasc. Biol. '98 Meet.* (Abstract).
47. Couet, J., Li, S., Okamoto, T., Scherer, P. E., and Lisanti, M. P. (1997) *Trends Cardiovasc. Med. 7*, 103−110.
48. Tracy, P. B., Rohrbach, M. S., and Mann, K. G. (1983) *J. Biol. Chem. 258*, 7264−7267.

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