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Activation of Human Factor V by Factor Xa and Thrombin†

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ABSTRACT: The activation of human factor V by factor Xa and thrombin was studied by functional assessment of cofactor activity and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by either autoradiography of ^{125}I-labeled factor V activation products or Western blot analyses of unlabeled factor V activation products. Cofactor activity was measured by the ability of the factor V/Va peptides to support the activation of prothrombin. The factor Xa catalyzed cleavage of factor V was observed to be time, phospholipid, and calcium ion dependent, yielding a cofactor with activity equal to that of thrombin-activated factor V (factor Va). The cleavage pattern differed markedly from the one observed in the bovine system. The factor Xa activated factor V subunits expressing cofactor activity were isolated and found to consist of peptides of M_r 220 000 and 105 000. Although thrombin cleaved the M_r 220 000 peptide to yield peptides previously shown to be products of thrombin activation, cofactor activity did not increase. N-Terminal sequence analysis confirmed that both factor Xa and thrombin cleave factor V at the same bond to generate the M_r 220 000 peptide. The factor Xa dependent functional assessment of ^{125}I-labeled factor V coupled with densitometric analyses of the cleavage products indicated that the cofactor activity of factor Xa activated factor V closely paralleled the appearance of the M_r 220 000 peptide. This observation facilitated the study of the kinetics of factor V activation by allowing the activation of factor V to be monitored by the appearance of the M_r 220 000 peptide (factor Xa activation) or the M_r 105 000 peptide (thrombin activation). Factor Xa catalyzed activation of factor V obeyed Michaelis-Menten kinetics and was characterized by a K_m of 10.4 nM, a k_{cat} of 2.6 min^{-1}, and a catalytic efficiency (k_{cat}/K_m) of 4.14 × 10^6 M^{-1} s^{-1}. The thrombin-catalyzed activation of factor V was characterized by a K_m of 71.7 nM, a k_{cat} of 14.0 min^{-1}, and a catalytic efficiency of 3.26 × 10^6 M^{-1} s^{-1}. This indicates that factor Xa is as efficient an enzyme toward factor V as thrombin.

Factor V circulates in plasma as a high molecular weight single-chain protein (Nesheim et al., 1979a; Esmon, 1979; Dahlback, 1980; Katzmann et al., 1981; Kane & Majerus, 1981; Mann et al., 1981) which is proteolytically cleaved to yield the coagulation cofactor factor Va (Nesheim & Mann, 1979; Esmon, 1979; Suzuki et al., 1982). Factor Va is an essential, nonenzymatic cofactor of the coagulation complex prothrombinase, the catalyst which converts prothrombin to thrombin (Owren, 1947a,b; Ware & Seegers, 1948; Murphy & Seegers, 1948; Heldebrandt et al., 1973; Suttie & Jackson, 1977). The prothrombinase complex consists of the cofactor factor Va, the serine protease factor Xa, calcium ions, and an appropriate cell membrane (Tracy et al., 1981, 1983b; Kane

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& Majerus, 1982) or phospholipid surface (Bloom et al., 1979; Pusey et al., 1982; Krishnaswamy & Mann, 1988) for proper assembly of the protein components. Factor Va is responsible for mediating the majority of the protein–protein and protein–membrane interactions required for prothrombinase assembly. By virtue of its high-affinity binding to cell membranes (Tracy et al., 1979, 1981, 1983b; Kane & Majerus, 1982) and phospholipid (Bloom et al., 1979; Higgins & Mann, 1983; Pusey et al., 1982; Krishnaswamy & Mann, 1988), factor Va constitutes at least part of the receptor for factor Xa at the membrane surface (Miletich et al., 1980; Tracy et al., 1981, 1983a, 1987; Tracy & Mann, 1983a). Factor Va is responsible also for mediating substrate interaction with the membrane-bound enzyme (Guinto & Esmon, 1984). Once assembled, the prothrombinase complex converts prothrombin to thrombin at a rate approximately 280 000-fold faster than that catalyzed by factor Xa alone (Nesheim et al., 1979b; Miletich et al., 1978). The deletion of factor Va from the complex reduces the rate of thrombin generation by 4 orders of magnitude (Nesheim et al., 1979b). The central role which factor Va assumes in complex assembly, coupled with its profound influence on the rate of thrombin formation, provides strong evidence that the activation of factor V to factor Va is a key regulatory event. This notion is underscored by the inability of the procofactor factor V to participate, to any significant degree, in thrombin generation (Nesheim et al., 1979b; Foster et al., 1983).

Several proteases have been identified which cleave factor V to yield different levels of cofactor activity. Thrombin is believed to be the most potent physiological activator of factor V and is by far the factor V activator most widely recognized with respect to coagulant and hemostatic responses (Nesheim & Mann, 1979; Esmon, 1979; Suzuki et al., 1982; Mann et al., 1988). However, since factor Va activity is absolutely required for thrombin generation, the initial activation of factor V has been the subject of some investigation. Foster and colleagues, working in the bovine system, have shown that factor Xa will activate factor V, although at a rate which is 2 orders of magnitude slower than that catalyzed by thrombin (Foster et al., 1983). However, since an early event in hemostasis may consist of the stoichiometric association of factor V and factor Xa on a membrane surface, factor V activation by factor Xa may be critical to the initial stages of thrombin formation. Other described factor V activators include platelet-associated (Tracy & Mann, 1983b; Kane et al., 1982), monocyte-associated (Tracy & Rohrbach, 1983), neutrophil-associated (Oates & Salem, 1987), and endothelial cell (Rodgers & Kane, 1986) associated proteases, plasmin (Lee & Mann, 1989), and calpain (Bradford et al., 1988). However, the extent to which these activators contribute to factor V activation in normal or pathophysiological events is poorly understood.

This report details studies of the ability of human factor Xa to activate human plasma factor V. These studies were undertaken to elucidate the relative importance of the factor Xa catalyzed versus thrombin-catalyzed activation of both solution-phase and membrane-bound factor V. They will serve also as a foundation for ongoing studies concerning the thrombin-catalyzed and factor Xa catalyzed activation of platelet-released factor V. Data are presented which indicate that factor Xa activation of human factor V yielded a factor Va molecule which expressed cofactor activity identical with that of thrombin-activated factor V and is defined also by kinetic parameters very similar to those describing thrombin activation of factor V.

Experimental Procedures

L-α-Phosphatidylcholine (hen egg) and L-α-phosphatidylserine (bovine brain) were purchased from Sigma. IODO-GEN was from Pierce Chemical Co. $Na^{125}I$ was from Amersham. The thrombin inhibitor dansylarginine *N,N*-(3-ethyl-1,5-pentanediyl)amide (DAPA)[1] was a generous gift from Dr. K. G. Mann, University of Vermont College of Medicine. Phospholipid vesicles (PCPS) composed of 75% (w/w) phosphatidylcholine and 25% (w/w) phosphatidylserine were prepared as previously described (Barenholtz et al., 1977; Bloom et al., 1979) and were usually used within 5 days of preparation.

Proteins were purified from human fresh frozen plasma. Factor V was isolated by immunoaffinity chromatography as described (Katzmann et al., 1981; Nesheim et al., 1981). Factor X and prothrombin were purified by the method of Bajaj and colleagues (Bajaj et al., 1981). Traces of protein C contamination in the factor X preparations, undetectable by gel electrophoresis, were removed with an immobilized α-human protein C IgG generously provided by Dr. William Church, University of Vermont College of Medicine. Factor X was activated with the factor X activator from Russell's viper venom as described (Jesty & Nemerson, 1976). The factor X activator was purified from Russell's viper venom as described by Kisiel and colleagues (Kisiel et al., 1976). α-Thrombin was prepared by activation of prothrombin with taipan snake venom as described (Owen & Jackson, 1973). Protein purity was confirmed using $NaDodSO_4$–polyacrylamide gel electrophoresis ($NaDodSO_4$–PAGE) with and without reduction with 5% 2-mercaptoethanol (v/v) in either 5–15% gradient or 10% slab gels according to the methods described by Laemmli (1970). Proteins were visualized by staining the gels with Coomassie brilliant blue R-250. Molecular weights and extinction coefficients, $E^{1\%}_{280nm}$, of the various proteins were taken as follows: factor V, 330 000, 9.6 (Tracy et al., 1982); factor Xa, 50 000, 11.6 (Bajaj et al., 1981); prothrombin, 72 000, 14.2 (Bajaj et al., 1981); and thrombin, 37 000, 17.4 (Mann, 1976).

Factor V was radioiodinated by using the IODO-GEN transfer technique (Fraker & Speck, 1978; Dewanjee et al., 1986). $Na^{125}I$ (2 mCi/mg of protein) was added to an IODO-GEN-coated plastic tube (10 μg of protein/μg of IODO-GEN) in 100 μL of 0.5 M phosphate buffer. Following 5 min of vortexing, the oxidized iodonium ion was transferred to a separate vessel containing factor V (0.2–0.5 mg/mL) in 0.02 M Tris/0.15 M NaCl, pH 7.4, which was incubated on ice for 5 min with occasional mixing. ^{125}I-Labeled factor V was reisolated by chromatography on phenyl-Sepharose (Nesheim et al., 1979a). Protein was then dialyzed into 10 mM Tris/borate, 1 mM $CaCl_2$/50% glycerol, pH 6.5, and stored at −20 °C. Specific radioactivities ranged from 900 to 3100 cpm/ng of protein (0.1–0.4 mol of I/mol of protein). ^{125}I-Labeled factor V retained approximately 100% activity as determined by functional assay (see below).

Analysis of Factor V Activation by $NaDodSO_4$–PAGE followed by Autoradiographic or Western Blotting Techniques. Reaction mixtures contained 50 nM factor V, 2 mM $CaCl_2$, and 20 μM PCPS vesicles in 20 mM Tris/0.15 M NaCl, pH 7.4 at 25 °C. PCPS vesicles were omitted from

[1] Abbreviations: $NaDodSO_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; DAPA, dansylarginine *N,N*-(3-ethyl-1,5-pentanediyl)amide; PCPS, phosphatidylcholine/phosphatidylserine (75:25 w/w) vesicles; IgG, immunoglobulin G.

the mixtures when thrombin was used to activate the factor V. Activation was initiated by adding factor Xa (5 nM) or thrombin (0.5–5 nM). Unlabeled factor V was used for Western blotting analyses, whereas ^{125}I factor V was used for autoradiographic analyses.

(*A*) *Western Blot Analyses.* At timed intervals, aliquots of the reaction mixtures were removed and quenched by the addition of 2–3 volumes of 0.0625 M Tris, pH 6.8, and 2% $NaDodSO_4$, heated at 100 °C for 2 min, and then subjected to $NaDodSO_4$–PAGE in 5–15% gradient slab gels as described (Laemmli, 1970). Following electrophoresis, separated proteins were transferred to nitrocellulose according to the methods of Towbin (Towbin et al., 1979). Transfer was performed at 200 V for 2 h at 4 °C. Nitrocellulose was then blocked with 0.05% Tween 20 in 20 mM phosphate-buffered saline, pH 7.0. Following conjugation with a burro anti-human factor V polyclonal antibody as the primary probe, factor V and its derived peptides were visualized using biotinylated goat α-horse IgG (Vector Laboratories) and an avidin–biotin–conjugated horseradish peroxidase complex (Vector Laboratories). Blots were developed with 4-chloro-1-naphthol and hydrogen peroxide. Electrophoresis and transfer of protein samples were accomplished with a mini-gel system (Hoefer Scientific Instruments).

(*B*) *Autoradiography.* Aliquots of activation mixtures were obtained as above and subjected to $NaDodSO_4$–PAGE as described, with an equal amount of isotope applied to each lane. Dried gels were subjected to autoradiography at −70 °C using Kodak XR-1 film and Dupont "Lightning Plus" intensifying screens.

Functional Assessment of Factor Va Activity. Factor Va activity was determined in an assay which measures the effect of factor Va on prothrombin activation through the assembly and function of the prothrombinase complex (Nesheim et al., 1979b). The formation of thrombin is continuously monitored by the change in fluorescence intensity of DAPA as it interacts with thrombin (Nesheim et al., 1979c). A very important characteristic of DAPA is its ability to eliminate feedback by the thrombin-catalyzed activation of factor V (Nesheim et al., 1979c). This permits the assaying of factor V without the interference of feedback activation by thrombin. Assay mixtures contained 1.39 μM prothrombin, 3 μM DAPA, 20 μM PCPS, and 2 mM $CaCl_2$ in 20 mM Tris/0.15 M NaCl, pH 7.4. Factor V (Va) samples removed from factor Xa catalyzed or thrombin-catalyzed activation mixtures were assayed at concentrations less than 1 nM. Reactions were initiated by addition of 1–5 nM factor Xa. Under these conditions, factor Va is the limiting component; thus, initial rates of thrombin formation are proportional to the concentration of factor Va in the assay. Fluorescence measurements were obtained routinely on a Perkin-Elmer LS-3b fluorescence spectrometer with the excitation and emission wavelengths set at 335 and 565 nM, respectively.

N-Terminal Sequence Analysis. Factor Va peptides for sequence analysis were prepared by activating approximately 1 nmol of factor V with 100 pmol of factor Xa until cofactor activity peaked. The thrombin-generated peptide was obtained by activation of factor V (0.1 mg/mL) with 5 nM thrombin for 2 min at 37 °C. Reactions were quenched by addition of glacial acetic acid (10% v/v). Following overnight dialysis against 0.2 M acetic acid, the samples were lyophilized to dryness. The samples were subjected to $NaDodSO_4$–PAGE in a 7.5% slab gel (Laemmli, 1970). Following electrophoresis, the appropriate molecular weight bands were quickly visualized with Coomassie brilliant blue R-250, excised, and electroeluted as described (Hunkapillar et al., 1983). Samples were analyzed for N-terminal sequence on an Applied Biosystems 475A gas-phase sequencer in the Given Analytical Facility, University of Vermont.

Kinetic Analysis. Kinetic analyses were facilitated by our ability to establish a direct correlation between the appearance of the M_r 220 000 peptide generated by factor Xa cleavage and the rate of change of prothrombin activation, i.e., factor Va cofactor activity. Parallel reaction mixtures contained various concentrations of prothrombin (1.39, 2.78, 4.17 μM), 7.5 μM DAPA, 20 μM PCPS, 2 mM $CaCl_2$, and 0.5 nM ^{125}I factor V in 0.02 M Tris/0.15 M NaCl, pH 7.4. The reactions were initiated by the addition of 5 nM factor Xa and were maintained at 25 °C. One reaction mixture was used for the continuous monitoring of prothrombin activation through enhanced fluorescence of the DAPA–thrombin complex. In these assays, thrombin generation would be directly related to the ability of factor Xa present in the reaction mixture to activate factor V to factor Va, resulting in the formation of a functional prothrombinase complex. Thus, the rate of change of prothrombin activation could be correlated with factor V activation to factor Va. Fluorescence intensity measurements were taken every second with an SLM 8000 photon-counting fluorescence spectrophotometer equipped with hardware and software modifications (On-Line Systems) allowing for appropriate data capture, display, and manipulation. The excitation wavelength was 280 nm (band-pass, 2 nm), and the emission wavelength was 560 nm (band-pass, 16 nm). Scattered light was minimized with a long-pass filter (Schott KV 408) in the emission beam. Coincident with the fluorescence measurements, aliquots were removed from the parallel reaction mixture every 10 s and quenched in 0.0625 M Tris, pH 6.8, and 2% $NaDodSO_4$ for $NaDodSO_4$–PAGE and densitometric analyses of the factor V/Va derived peptides produced over time.

The fluorescence intensity data were plotted as a function of time and further manipulated to obtain the rate of the reaction (dF/dt) over the entire time course of the reaction. To obtain a smooth first-derivative curve, the data set was averaged over four readings (i.e., 4 s) and then subjected to four cycles of smoothing, equivalent to a 9-channel smooth, which further reduced the amount of noise inherent in the fluorescence readings to an acceptable level. Each cycle of smoothing (3-channel smooth) performs the operation:

$$y = \tfrac{1}{4}[y(x-1)] + \tfrac{1}{2}[y(x)] + \tfrac{1}{4}[y(x+1)]$$

where y = fluorescence intensity at time = x (Bevington, 1969). The derivative values were obtained from a 3-point quadratic fit for each data point. The averaging, smoothing, and derivative programs were kindly provided by Dr. Jolyon Jesty, State University of New York at Stony Brook. When the rate of change of thrombin generation (dF/dt) was plotted versus the time-dependent appearance of the M_r 220 000 peptide as determined by densitometry, a linear correlation was obtained, indicating that the generation of this peptide could be used to assess kinetically the factor Xa activation of factor V. These data are presented and discussed in detail under Results.

The rate of activation of factor V by factor Xa was calculated from the slope of a plot of the concentration of the M_r 220 000 peptide versus time. Peptide concentration was determined by densitometric analyses of autoradiographs representing the time-dependent factor Xa activation of factor V. Reaction mixtures were maintained at 25 °C and contained varying concentrations of factor V (2.5–100 nM), 20 μM PCPS, and 2 mM $CaCl_2$ in 0.02 M Tris/0.15 M NaCl, pH

7.4. ^{125}I factor V (expressing 100% activity following activation) was present as a tracer (500–1000 cpm/μL). Reactions were initiated with 1 nM factor Xa, and aliquots were removed at various times (10-s intervals for the reactions containing low substrate concentrations, 1-min intervals for the reactions containing high substrate concentrations) and quenched as described previously. Samples were subjected to $NaDodSO_4$–PAGE, and gels were dried for autoradiography and densitometric analyses as detailed below.

Kinetic analyses of thrombin activation of factor V were accomplished in an analogous manner. Reaction conditions were as those described above with the exceptions that factor V concentrations ranged from 20 to 500 nM, thrombin was present at 0.5 nM, and analyses were done in the absence of 20 μM PCPS vesicles. Thrombin activation of factor V was monitored by the time-dependent appearance of the M_r 105 000 peptide, the NH_2-terminal-derived subunit of the factor Va molecule resulting from thrombin cleavage. Peptide concentration was determined as described above. For each activator, initial rate data were obtained for each substrate concentration, and substrate versus velocity plots were constructed. Kinetic constants were calculated from the data by weighted nonlinear least-squares regression analysis using the Fortran computer program HYPER described by Cleland (1979) and kindly provided by Dr. Sriram Krishnaswamy, University of Vermont College of Medicine.

Densitometric Analyses. Densitometric analyses were accomplished with a Microscan 1000 scanning densitometer (TRI Inc.) equipped with a solid-state linear diode array camera to digitize images through a photographic lens. Resulting digitized images contain approximately 0.25×10^6 pixels with each pixel measuring 0.16 mm × 0.16 mm. Linearity of this system is 0–2.5 absorbance units. In addition, preliminary experiments were done to ensure that the amount of radioactivity applied to the gel lanes, coupled with the time required for autoradiographic visualization, was within the linear response of the X-ray film. Data were analyzed with a 80826-based computer equipped with a math coprocessor and software which allows for either automatic or manual background subtraction and full editing capability. Data are expressed as integrated volumes for each protein band using the arbitrary density units of the scanning system.

Results

Factor Xa Mediated Cleavage of Factor V and Assessment of Cofactor Activity. The ability of factor Xa to rapidly cleave ^{125}I factor V is demonstrated in Figure 1A. Factor Xa cleaved the single-chain protein to yield primarily peptides of M_r 220 000, 150 000, and 105 000. The autoradiograph indicated that a single cleavage occurred within 10 s, generating peptides of M_r 220 000 and 150 000, followed by a second cleavage to generate an M_r 105 000 peptide. No other significant cleavages were observed after 50 min of incubation, suggesting that these peptides are the major products of the factor Xa mediated cleavage of factor V under the conditions of this study. The appearance of minor peptides ≤74 000 daltons was apparent, however, indicating the existence of cleavage sites that are less preferred. The factor V peptides generated by factor Xa appeared to be electrophoretically indistinguishable from those produced by thrombin (Figure 1B). Thrombin cleavage of factor V yielded two high molecular weight peptides of M_r 280 000 and 220 000, as well as three end products: an M_r 150 000 activation peptide and the two subunits that comprise the active cofactor, the amino-terminal-derived heavy chain (M_r 105 000) and the carboxy-terminal-derived light chain (M_r 74 000). The fragmentation pattern observed is similar to ones

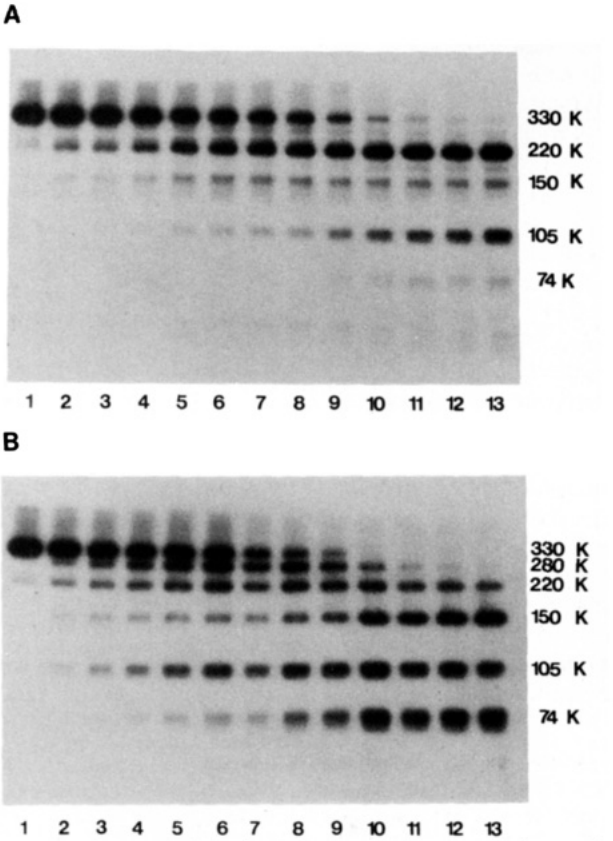

FIGURE 1: Cleavage of single-chain human factor V by factor Xa and thrombin. (A) Factor V (50 nM) in the presence of trace ^{125}I factor V (1000 cpm/μL), 20 μM PCPS, and 2 mM $CaCl_2$ in 0.02 M Tris/0.15 M NaCl, pH 7.4, was activated with 5 nM factor Xa at 25 °C. Lanes 1–4 represent aliquots removed at 0, 10, 30, and 60 s, respectively; lanes 5–13 represent aliquots removed at 2, 3, 4, 5, 10, 20, 30, 40, and 50 min, respectively. All aliquots were subjected to $NaDodSO_4$–PAGE. An equal amount of isotope was applied to each lane. Apparent molecular weights are indicated. (B) Factor V (0.33 mg/mL) and trace ^{125}I factor V (1000 cpm/μL) in 0.02 M Tris/0.15 M NaCl, pH 7.4, were activated with 5 nM (0.5 unit/mL) thrombin at 37 °C. Lanes 1–13 represent aliquots removed at 0, 10, 20, 30, 40, 50, 60, 90, 120, 180, 240, 300, and 420 s and treated as in (A). At 2 min (lane 9), thrombin was added to 20 nM. An equal amount of isotope was loaded in each lane.

described previously by Suzuki and colleagues (Suzuki et al., 1982) and Katzmann and colleagues (Katzmann et al., 1981). In contrast to thrombin cleavage of factor V, factor Xa cleavage produced neither the M_r 280 000 intermediate nor the light chain to any significant degree.

The similarity of the factor Xa mediated cleavages of factor V with the thrombin-mediated cleavages raised concerns that thrombin or prothrombin might be contaminating our protein preparations. To address this issue, factor Xa cleavage of factor V was studied in the presence of 3 μM DAPA, a potent inhibitor of thrombin (Nesheim et al., 1979c). No inhibition of cleavage was observed (data not shown), indicating that the observed cleavages were not due to contaminating thrombin since the DAPA present was sufficient to completely inhibit 1 nM thrombin.

When factor V was incubated with factor Xa in the absence of calcium ions, PCPS vesicles, or both, no cleavage of factor V was observed. These data indicate that membrane-bound factor V is the preferred substrate for this reaction as was previously demonstrated in the bovine system (Foster et al., 1983). Therefore, all studies were performed in the presence of 2 mM $CaCl_2$ and 20 μM PCPS vesicles, a concentration of vesicles sufficient to bind all of the factor V in the reaction mixtures (Higgins & Mann, 1983).

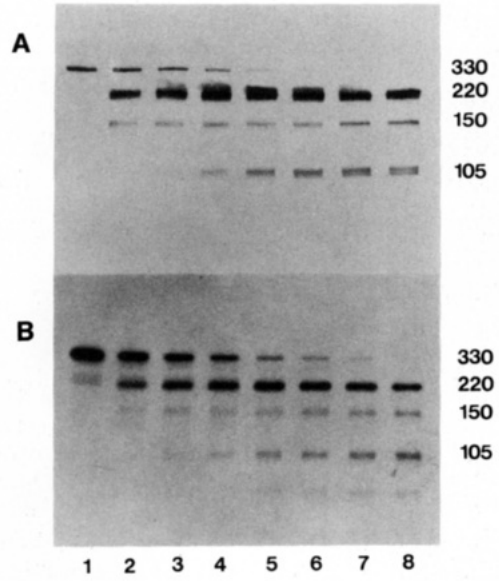

FIGURE 2: Comparison of Western blotting and autoradiographic analyses of factor V cleavage products. Factor V (50 nM) in the presence of trace ^{125}I factor V (5000 cpm/μL) was activated with 5 nM factor Xa as described in the legend to Figure 1A. Lanes 1–8 represent aliquots removed at 0, 2, 5, 10, 20, 30, 45, and 60 min, respectively. (A) Aliquots containing 150 ng of factor V were subjected to NaDodSO$_4$–PAGE and Western blot analysis. (B) Aliquots containing an equal amount of isotope were subjected to NaDodSO$_4$–PAGE and autoradiography. Apparent molecular weights are indicated. Virtually identical cleavage patterns were obtained.

To determine if the factor Xa generated peptides resulted in the expression of factor Va cofactor activity, activity measurements were made coincident with samples being withdrawn for NaDodSO$_4$–PAGE and Western blot analysis. Western blot analyses were performed to ensure that ^{125}I factor V was reacting in a manner identical with the unmodified protein. These analyses were performed with a burro anti-human factor V immunoglobulin known to react with all the thrombin-derived factor V peptides (Viskup et al., 1987) as well as with plasma factor V fragments generated during intravascular coagulation and fibrinolysis (Rubin et al., 1986). Data were obtained indicating that a Western blot analysis of a reaction mixture containing both unlabeled and ^{125}I factor V revealed an identical cleavage pattern mediated by factor Xa following autoradiography (Figure 2). Finally, the usefulness of this technique to detect cleavage of plasma-derived factor V will validate the use of this technique in future studies with platelet factor V and will allow for direct comparison between the two major pools of factor V (plasma and platelet) with regard to cleavage and activation.

Factor Va activity was assessed on the basis of its ability to serve as a cofactor for the factor Xa catalyzed activation of prothrombin (Nesheim et al., 1981) and was performed as follows. An aliquot of a reaction mixture containing factor V, factor Xa, PCPS vesicles, and $CaCl_2$ was added to a cuvette containing 1.39 μM prothrombin in 0.02 M Tris/0.15 M NaCl, pH 7.4, 2 mM $CaCl_2$, 20 μM PCPS, and 3 μM DAPA so that the cofactor added to the reaction mixture would be in limiting amounts (0.1 nM). The reactions were initiated by the addition of 5 nM factor Xa, and fluorescence was continuously monitored. Under these conditions, the initial rate of prothrombin activation is entirely dependent upon the expression of factor Va activity. Characteristic activity profiles obtained with single-chain factor V and factor V cleaved by factor Xa and/or thrombin are shown in Figure 3. The insets represent Western blot analyses of the factor V/Va peptides being assayed for cofactor activity. As shown in Figure 3A,

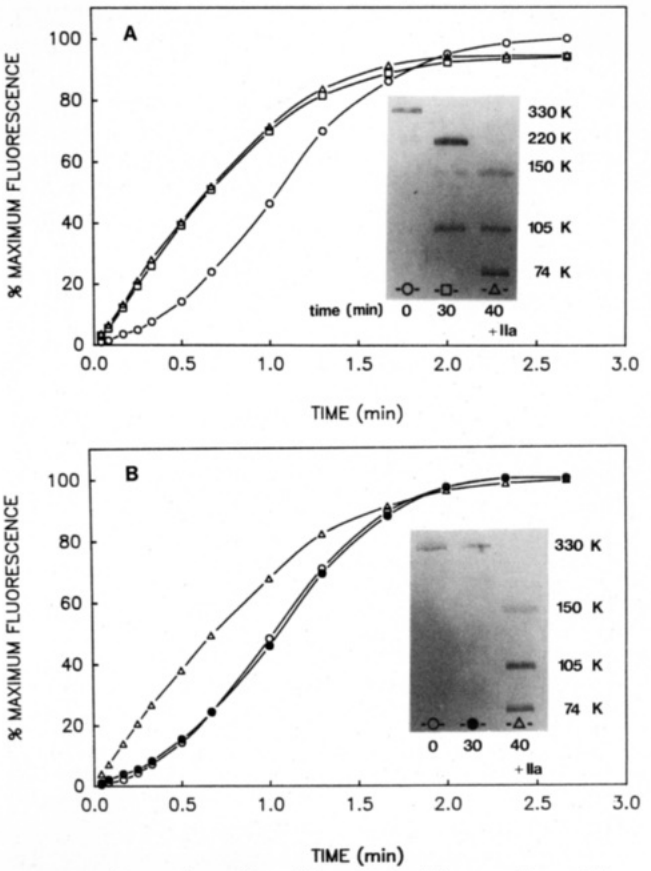

FIGURE 3: Comparison of the ability of factor Va$_{(Xa)}$ and factor Va$_{(IIa)}$ to express cofactor activity. (A) Factor V in the absence of trace label was incubated with factor Xa as described in Figure 1A. Following 30 min of incubation, 20 nM thrombin was added and incubated for an additional 10 min. Aliquots were removed at 0, 30, and 40 min and subjected to Western blotting analyses (inset) and functional assays for factor Va activity. Apparent molecular weights are indicated. (B) Factor V was incubated as described above in the absence of factor Xa. After 30 min, 20 nM thrombin was added, and aliquots were removed and processed as in (A). The percent maximum fluorescence of the thrombin–DAPA complex is plotted as a function of time of assay. Factor Va$_{(Xa)}$ and factor Va$_{(IIa)}$ expressed the same cofactor activity.

an aliquot of a reaction mixture containing 50 nM factor V was assayed prior to enzyme addition and after 30 min of incubation with 5 nM factor Xa. Thrombin was then added to the reaction mixture to 5 nM for an additional 10 min. When single-chain factor V was assayed (time = 0), a sigmoidal fluorescence tracing was obtained (open circles). It was characterized by a lag phase, followed by an increase in the rate of prothrombin activation and a plateau, due to substrate depletion. The lag seen in the early stages of such an assay is due to the inability of factor V to express cofactor activity. In other words, the initial rate of prothrombin activation is slow. However, during the course of the assay, it would appear that the factor Xa present in a 50-fold molar excess over factor V converted the cofactor to a more active species, as might be expected based on studies in the bovine system (Foster et al., 1983). This accounts for the gradual increase in the rate of prothrombin activation during the assay. These observations were verified by assay of factor V following incubation with factor Xa for 30 min at an enzyme to substrate ratio of 1:10 (time = 30 min, open squares). The fluorescence tracing showed an immediate and substantial increase in the initial rate of prothrombin activation with no apparent lag observed. As seen in the inset, factor Xa cleavage of factor V yielded the M_r 220 000, 150 000, and 105 000 peptides.

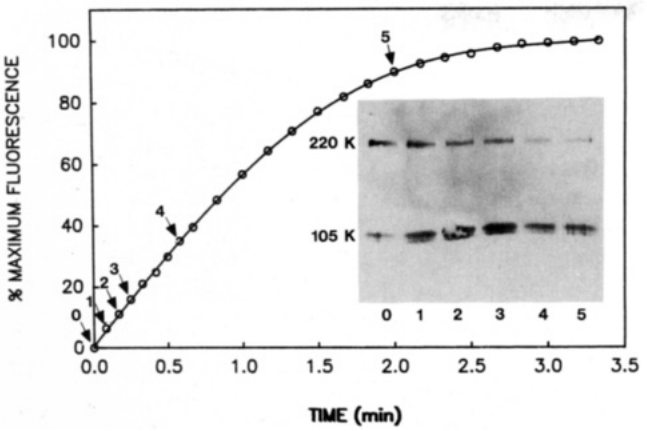

FIGURE 4: Assessment of additional cleavage of factor $Va_{(Xa)}$ during a functional assay of cofactor activity. Factor $Va_{(Xa)}$ was added to parallel functional assay reaction mixtures containing 1.39 μM prothrombin, 3 μM DAPA, 20 μM PCPS, and 2 mM $CaCl_2$ in 0.02 M Tris/0.15 M NaCl, pH 7.4. Reactions were initiated by the addition of 5 nM factor Xa. One reaction mixture was used to monitor cofactor activity through enhanced fluorescence of the DAPA–thrombin complex. Aliquots were removed from the parallel reaction mixture at the times indicated by the arrows (0–5) on the fluorescence tracing, quenched with acetic acid, dialyzed, lyophilized, and subjected to $NaDodSO_4$–PAGE and Western blot analysis (inset). Apparent molecular weights are indicated. Factor $Va_{(Xa)}$ did not undergo further cleavage during the functional assay, indicating that the initial rate of prothrombin activation observed is a true assessment of the cofactor activity of the factor $Va_{(Xa)}$ shown in lane 0.

Additional thrombin incubation resulted in the cleavage of the M_r 220 000 peptide to yield the M_r 74 000 light-chain subunit characteristic of thrombin-activated factor V. However, further incubation of the factor Xa cleaved factor V with thrombin did *not* increase its cofactor activity since virtually identical initial rates of prothrombin activation were obtained prior to and after thrombin addition (time = 40 min, open triangles). Figure 3B represents a control experiment where factor V was treated as in Figure 3A, except that factor Xa was omitted from the 30-min incubation step. As shown in the inset, the factor V did not undergo proteolysis during the 30-min incubation. Furthermore, the ability of thrombin to activate the factor V was unaffected as indicated by the Western blot analysis, since it can be seen that the initial rate of prothrombin activation following thrombin activation was the same as those shown in Figure 3A. Also, the same initial rate of prothrombin activation is observed when factor V is activated at 0 or 30 min. These data suggest that factor Xa cleaved factor V [factor $Va_{(Xa)}$][2] expressed full cofactor activity when compared to thrombin-activated factor V [factor $Va_{(IIa)}$][2] and that further cleavage to yield the light chain was not necessary for the expression of maximum cofactor activity.

Functional assays of factor $Va_{(Xa)}$ are complicated by the fact that factor Xa is required for prothrombin activation. A substantial molar excess of factor Xa over factor $Va_{(Xa)}$ could be exerting changes in the factor V during prothrombin activation not reflected by the autoradiographs or Western blot analyses prior to assay of the factor V derived peptides. It is possible that factor Xa, or traces of thrombin not complexed with DAPA, could cleave the M_r 220 000 peptide to the light chain, generating factor $Va_{(IIa)}$. Thus, the functional data obtained would not be a true reflection of the state of factor V cleavage prior to assay. To examine these possibilities, factor V was incubated with factor Xa for 20 min as described in Figure 3. An aliquot was removed and assayed for activity; the resulting fluorescence tracing is shown in Figure 4. Aliquots removed from a parallel reaction mixture at the times indicated by the arrows on the fluorescence tracing were quenched with glacial acetic acid, dialyzed, lyophilized, and subjected to $NaDodSO_4$–PAGE and Western blot analysis. As shown in the inset, the factor $Va_{(Xa)}$ was not further cleaved during the assay. In particular, no cleavage was observed in the early stages of the assay (lanes 0–3), from which the initial rate is calculated. This indicates that neither factor Xa nor traces of thrombin are cleaving the factor $Va_{(Xa)}$ during the course of the assay, providing evidence that the initial rate of prothrombin activation observed is a true assessment of the cofactor activity of the factor $Va_{(Xa)}$ depicted in lane 0. A possible explanation for the lack of factor $Va_{(Xa)}$ cleavage is that once a competent prothrombinase complex has been formed, prothrombin becomes the preferred substrate for factor Xa. This is consistent with the observations of Tracy and colleagues (Tracy et al., 1983a), who observed that factor Xa cleaves platelet-bound factor Va more slowly in the presence of prothrombin. These data collectively indicate that full cofactor activity is expressed by the M_r 220 000 and 105 000 peptides and that further cleavage is not required for maximum cofactor activity. These results are further verified by data that will be discussed later in which the appearance of the M_r 220 000 peptide parallels the increase in cofactor activity.

Table I: N-Terminal Sequence of the 220 000-Dalton Factor V Fragment

sequence determined by cDNA cloning[a]	---Thr-Phe-His-Pro-Leu-Arg-Ser-Glu---
published sequence of thrombin fragment[a]	---ND[b]-Phe-ND-Pro-Leu-Arg-ND-Glu
thrombin fragment	---ND-Phe-His-Pro-Leu-Arg-ND
factor Xa	---ND-Phe-ND-Pro-Leu-ND-Ser-Glu

[a] Jenny et al. (1987). [b] Amino acid could not be identified.

N-Terminal Sequence Analysis. The M_r 220 000 peptide generated by factor Xa cleavage of factor V appeared to be the same peptide as that generated by thrombin. To confirm this observation, N-terminal sequence analysis of the two peptides was performed. The factor Xa derived peptide and the thrombin-derived peptide were prepared for sequencing as described under Experimental Procedures. The results of the sequencing analyses are shown in Table I and compared to the amino acid sequence determined from cDNA cloning (Jenny et al., 1987; Kane et al., 1987) and the published sequence of the thrombin peptide (Jenny et al., 1987; Kane et al., 1987). Five out of seven and five out of eight amino acids from the thrombin-derived and factor Xa derived peptides, respectively, could be identified and correctly matched to the published data. These data indicate that both factor Xa and thrombin cleave factor V at the same site, Arg^{1018}-Thr^{1019}.

Isolation of Factor Xa Activated Factor V. The subunit composition of factor $Va_{(Xa)}$ was determined by subjecting factor Xa activated factor V to immunoaffinity chromatography. The monoclonal antibody used in this step is specific for an epitope on the light chain of factor Va (Katzmann et al., 1981). The rationale for using this approach is based on the following observations. First, immunoprecipitation studies indicated that this antibody reacts with the factor Xa cleaved factor V peptides (data not shown). In the presence of calcium ions, the M_r 220 000 and 105 000 peptides were immunoprecipitated, while only the M_r 220 000 peptide was immunoprecipitated in the presence of the calcium ion chelator EDTA.

[2] The nomenclature of Foster et al. (1983) described for the bovine system is used to denote human factor Xa activated factor V [factor $Va_{(Xa)}$] and thrombin-activated factor V [factor $Va_{(IIa)}$] as well.

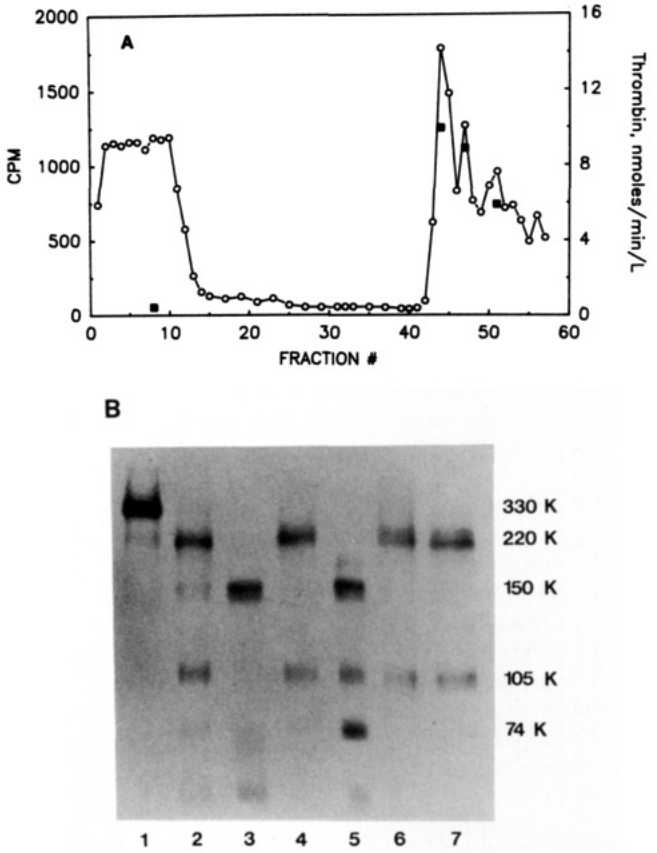

FIGURE 5: Isolation of factor $Va_{(Xa)}$ subunits. (A) A reaction mixture containing 50 nM factor V, trace ^{125}I factor V (1000 cpm/μL), 20 μM PCPS, and 2 mM $CaCl_2$ in 0.02 M Tris/0.15 M NaCl, pH 7.4, was activated with 5 nM factor Xa. Following lysis of vesicles with 0.05% Triton X-100, the reaction mixture was applied to an immunoaffinity column equilibrated with 0.02 M Tris/0.15 M NaCl, pH 7.4. The column was washed with 0.02 M imidazole, 0.1 M NaCl, and 2 mM $CaCl_2$, pH 6.5, and the bound factor V peptides were eluted with the same buffer containing 1.2 M NaCl. Fractions (0.5 mL) were collected and monitored for radioactivity (○). Selected fractions were assayed for cofactor activity (■). (B) Selected fractions were analyzed by $NaDodSO_4$–PAGE and autoradiography. Lane 1, factor V; lane 2, factor V activated with factor Xa (10:1 substrate to enzyme ratio) for 55 min; lane 3, fraction 8; lane 4, fraction 44; lane 5, fraction 44 plus thrombin (10 units/mL, 10 min at 37 °C); lane 6, fraction 47; lane 7, fraction 51. An equal amount of isotope was applied to each lane. Apparent molecular weights are indicated.

These data suggest that the M_r 220000 peptide contains the light-chain epitope recognized by the antibody. Second, the N-terminal sequence analysis presented in this report suggested also that the factor Xa derived M_r 220000 peptide contains the light-chain epitope since the thrombin-derived peptide, generated by cleavage at the same bond that factor Xa cleaves, is the light-chain precursor in thrombin activation of factor V. Together, these observations suggested that the antibody might be useful in binding the M_r 220000 peptide formed following factor Xa cleavage and that immunoaffinity chromatography would be a reasonable approach for the isolation of factor $Va_{(Xa)}$. ^{125}I factor V was activated to ^{125}I factor $Va_{(Xa)}$ as described under Experimental Procedures and was subjected to immunoaffinity chromatography in the presence of calcium ions as shown in Figure 5A. Fractions (0.5 mL) were assayed for radioactivity (open circles) so that the peptide elution could be monitored. Selected fractions were assayed for cofactor activity (filled squares) and were subjected to $NaDodSO_4$–PAGE and autoradiography (Figure 5B). Lanes 1 and 2 of Figure 5B represent the factor V prior to and after factor Xa activation, respectively. Lane 3 represents material that did not bind to the column (fraction 8), which appeared to consist mainly of an M_r 150000 peptide. There was negligible cofactor activity associated with this material (Figure 5A). The bound material was eluted from the column by increasing the ionic strength. Lanes 4, 6, and 7 represent fractions 44, 47, and 51, respectively. The eluted material consisted of two peptides—the M_r 220000 and 105000 peptides. As shown in Figure 5A, the elution of cofactor activity parallels the elution of these peptides. When a sample of fraction 44 was thrombin-activated, the M_r 220000 peptide was cleaved to the M_r 150000 and 74000 peptides (Figure 5B, lane 5); however, the cofactor activity did not increase. These data indicated that the active species generated by factor Xa activation of factor V is composed of the M_r 220000 and 105000 peptides and that these peptides express full cofactor activity.

Kinetic Analyses of Factor V Activation by Factor Xa and Thrombin. The determination of the kinetic parameters governing the activation of factor V by factor Xa was made feasible by the establishment of a direct correlation between the appearance of the M_r 220000 peptide generated by factor Xa cleavage and the expression of factor Va cofactor activity as manifested in the rate of change of prothrombin activation. ^{125}I factor V was activated by factor Xa in the presence of PCPS vesicles, calcium ions, prothrombin, and DAPA as described under Experimental Procedures and prothrombin activation monitored continuously through the enhanced fluorescence of the DAPA–thrombin complex. The rate of change of prothrombin activation (i.e., the rate of change of fluorescence of the DAPA–thrombin complex, dF/dt) in these assays (Figure 6A) reflected the ability of factor Xa present in the reaction mixture to activate the ^{125}I factor V and lead to the formation of a functional prothrombinase complex. Therefore, the rate of change of fluorescence could be correlated with the activation of ^{125}I factor V to ^{125}I factor $Va_{(Xa)}$. Coincident with the fluorescence measurements, aliquots were removed from a parallel reaction mixture every 10 s, quenched, and subjected to $NaDodSO_4$–PAGE; the factor V/Va derived peptides produced over time were analyzed densitometrically.

The fluorescence intensity data were plotted as a function of time and further manipulated to obtain the rate of the reaction (dF/dt). In order to obtain a smooth dF/dt curve, the fluorescence data required manipulation so that the noise inherent in the assay system could be minimized. The data were averaged and smoothed, and derivative curves were calculated as described under Experimental Procedures. The results of one such functional assay are shown in Figure 6A. A reaction mixture containing 2.78 μM prothrombin, 7.5 μM DAPA, 20 μM PCPS vesicles, 2 mM $CaCl_2$, and 0.5 nM ^{125}I factor V in 0.02 M Tris/0.15 M NaCl, pH 7.4, was initiated by the addition of 5 nM factor Xa. Fluorescence (F) of the thrombin–DAPA complex was monitored over time (solid line). Following smoothing of the data set, a derivative curve (dF/dt) was calculated (dotted line). The fluorescence tracing does not begin at time = 0 because the reaction was not initiated with factor Xa until 17 s after the base-line fluorescence was adjusted. The data points collected prior to initiation were deleted so that the smoothing and derivative programs could avoid the transient dip in fluorescence intensity caused by the addition of factor Xa and mixing of the reaction mixture. Cleavages observed by autoradiography occurring coincident with the fluorescence measurements are shown in Figure 6B. Lanes 1–25 represent samples removed during the first 4 min of the assay shown in Figure 6A. Densitometric analysis of the autoradiograph was performed to assess the time-dependent appearance of the M_r 220000 and 105000 peptides and determine if one or both of them correlated with cofactor activity.

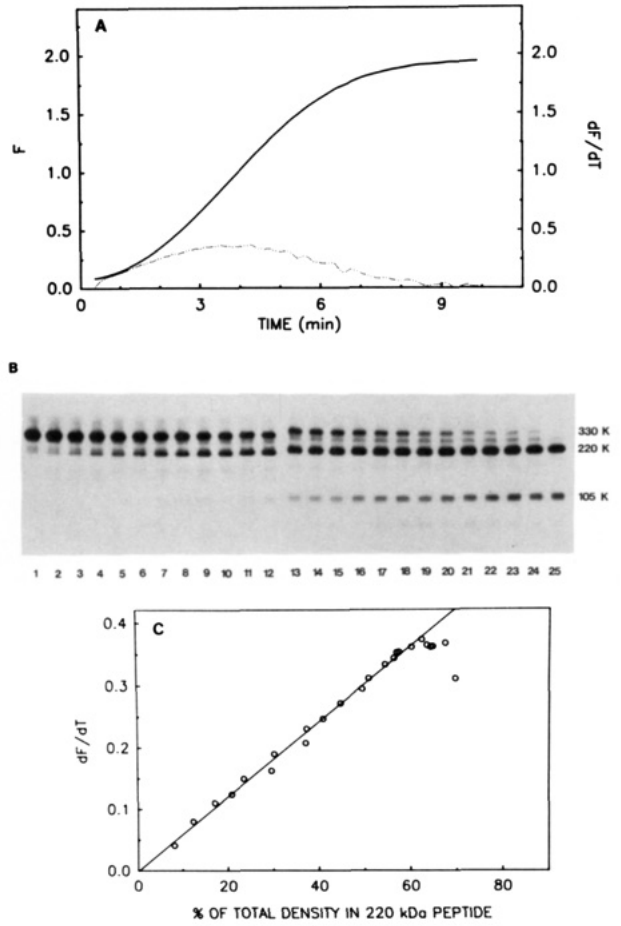

FIGURE 6: Factor V activation by factor Xa correlates with the appearance of the M_r 220000 peptide. (A) A reaction mixture containing 2.78 μM prothrombin, 7.5 μM DAPA, 20 μM PCPS vesicles, 2 mM $CaCl_2$, and 0.5 nM ^{125}I factor V in 0.02 M Tris/0.15 M NaCl, pH 7.4, was initiated by the addition of 5 nM factor Xa. The fluorescence (F) of the thrombin–DAPA complex was captured, smoothed as described under Experimental Procedures, and plotted as a function of time of assay (solid line). A derivative of this tracing (dF/dt) describing the cofactor activity during the assay was then calculated (dotted line). (B) Aliquots were removed from a parallel reaction mixture at 10-s intervals and prepared for NaDodSO$_4$–PAGE and autoradiography (lanes 1–25). An equal amount of isotope was applied to each lane. (C) The density of the M_r 220000 peptide was plotted as a function of the rate of change of prothrombin activation (dF/dt) for each time point. The correlation coefficient of the straight line drawn through the data points is 0.997, indicating a direct correlation between the appearance of the M_r 220000 peptide and the increase in factor $Va_{(Xa)}$ activity. The last five data points do not fall on the line due to depletion of the substrate, prothrombin.

The M_r 105000 peptide did not correlate with expression of cofactor activity since activity was observed prior to the appearance of the peptide (data not shown). However, a plot of the percentage of total density of the M_r 220000 peptide in each lane as a function of the slope of the fluorescence tracing showed a linear relationship (Figure 6C) up to approximately 3.2 min (lane 20 of Figure 6B). This indicated that an increase in the M_r 220000 peptide is accompanied by a corresponding increase in cofactor activity. The last five data points do not fall on the line due to depletion of the substrate, prothrombin. To verify these results, the experiments described above were performed with varying concentrations of prothrombin (1.39–4.17 μM) to prolong the onset of substrate depletion. The same results were observed (data not shown). This indicated that the generation of this peptide could be used to assess kinetically the factor Xa activation of factor V.

The kinetic parameters governing the activation of factor V by factor Xa were determined as follows. Reaction mixtures containing unlabeled factor V and trace amounts of ^{125}I factor V, ranging from 2.5 to 100 nM, in the presence of 20 μM PCPS vesicles and 2 mM $CaCl_2$ were initiated with 1.0 nM factor Xa at 25.0 °C. Aliquots were removed at timed intervals and prepared for NaDodSO$_4$–PAGE, autoradiography, and densitometric analyses as described under Experimental Procedures. The initial rates of activation of factor V by factor Xa were calculated from the slope of a plot of the concentration of the M_r 220000 peptide as a function of time. The initial rates of activation were then plotted as a function of initial factor V concentration. These data were fitted to a rectangular hyperbola (Figure 7A) using weighted nonlinear least-squares regression analysis (Cleland, 1979). The inset

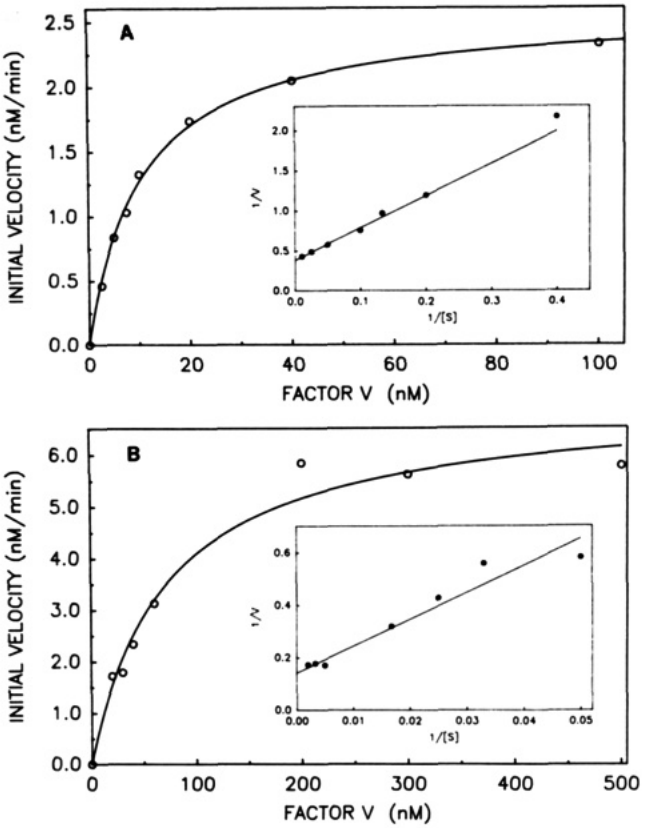

FIGURE 7: Determination of kinetic parameters governing the activation of factor V by factor Xa and thrombin. (A) Reaction mixtures containing unlabeled factor V and trace ^{125}I factor V, ranging in concentration from 2.5 to 100 nM, 20 μM PCPS vesicles, and 2 mM $CaCl_2$ in 0.02 M Tris/0.15 M NaCl, pH 7.4, were initiated by the addition of 1.0 nM factor Xa. (B) Analogous reaction mixtures containing unlabeled factor V and trace ^{125}I factor V, ranging in concentration from 20 to 500 nM, and 2 mM $CaCl_2$ in the absence of PCPS vesicles were initiated by the addition of 0.5 nM thrombin. Aliquots were removed at timed intervals and prepared for NaDodSO$_4$–PAGE, autoradiography, and densitometric analysis. Initial rates were obtained from plots of the concentration of the M_r 220000 peptide (A) or the M_r 105000 peptide (B) as a function of time; these rates were then plotted as a function of factor V concentration. The data were fitted to a rectangular hyperbola (Cleland, 1979) to obtain the kinetic constants listed in Table II. The insets are reciprocal plots of the data.

Table II: Kinetic Constants for the Activation of Human Factor V

	K_m[a] (nM ± SE)	k_{cat} (min^{-1} ± SE)	k_{cat}/K_m (M^{-1} s^{-1} × 10^{-6})
factor Xa	10.40 ± 0.6	2.6 ± 0.1	4.14
thrombin	71.7 ± 12.5	14.0 ± 0.8	3.26

[a] The kinetic constants and standard errors were obtained by nonlinear least-squares regression analysis (Cleland, 1979) of the data illustrated in Figure 7.

in Figure 7A is a reciprocal plot of the data; the line is drawn according to the constants derived from the fitting procedure and listed in Table II.

The kinetic parameters governing the activation of factor V by thrombin were determined in an analogous manner. Reaction mixtures containing concentrations of unlabeled and trace ^{125}I factor V, ranging from 20 to 500 nM, and 2 mM $CaCl_2$ were initiated with 0.5 nM thrombin at 25.0 °C. The activation of factor V was monitored by the appearance of the M_r 105 000 peptide because this peptide appeared to arise at the same time and in a parallel fashion as the M_r 220 000 peptide. The M_r 220 000 peptide was subsequently cleaved by thrombin to yield the light chain of factor $Va_{(IIa)}$, making it unsuitable as an indicator for the thrombin-catalyzed activation of factor V. Initial rates of activation were obtained and plotted as a function of initial factor V concentration as described above. Figure 7B shows the fitted data and the reciprocal plot (inset); the constants derived from the fitting procedure are listed in Table II. The data indicate that the K_m for the factor Xa catalyzed activation of factor V is 7-fold lower than the K_m for the thrombin-catalyzed activation. However, the rate constant is 5-fold lower, indicating that the catalytic efficiency (k_{cat}/K_m) of the factor Xa catalyzed activation of factor V is not significantly different than the catalytic efficiency of the thrombin-catalyzed activation of factor V.

Discussion

The data presented in this study indicate that factor Xa cleaves and activates human factor V in a time-dependent, phospholipid-dependent, and calcium ion dependent manner, yielding a cofactor that expresses functional activity equivalent to thrombin-activated factor V. Factor $Va_{(Xa)}$ was found to consist of two subunits: an amino-terminal-derived M_r 105 000 peptide and a carboxy-terminal-derived M_r 220 000 peptide. Although these peptides appear to be indistinguishable from thrombin-derived cleavage peptides, there is strong evidence to suggest that the cleavages were not catalyzed by undetected traces of thrombin. When the specific thrombin inhibitor DAPA was included in reaction mixtures containing factor V and factor Xa (10 to 1 substrate to enzyme ratio), no inhibition of cleavage was observed. However, when the same concentration of DAPA was added to a reaction mixture containing factor V and thrombin (50 to 1 substrate to enzyme ratio), cleavage was completely blocked. Furthermore, in a reaction mixture where the factor $Va_{(Xa)}$ peptides were being assessed for additional cleavages during a cofactor assay, the cleavage of the M_r 220 000 peptide to yield the light chain, readily catalyzed by thrombin (Figure 1B), was not observed (Figure 4). This indicates that DAPA is indeed blocking thrombin feedback activation of factor V, since a 2000-fold molar excess of thrombin over factor V is generated during the assay. Therefore, it is reasonable to conclude that factor Xa, and not contaminating thrombin, is catalyzing the activation of factor V in these studies.

The factor Xa catalyzed cleavages of factor V that were observed in this study are represented schematically in Figure 8A. Scission of the procofactor at position A yields peptides of M_r 220 000 and 150 000. A second cleavage at position B results in the release of an M_r 71 000 activation peptide and the amino-terminal-derived M_r 105 000 subunit. The amount of the M_r 150 000 peptide appears to remain constant in Figure 1A because its formation from cleavage of the M_r 330 000 molecule is balanced by its cleavage to form the M_r 105 000 peptide. Prolonged incubation with factor Xa leads to full cleavage of the M_r 150 000 peptide to form peptides of M_r

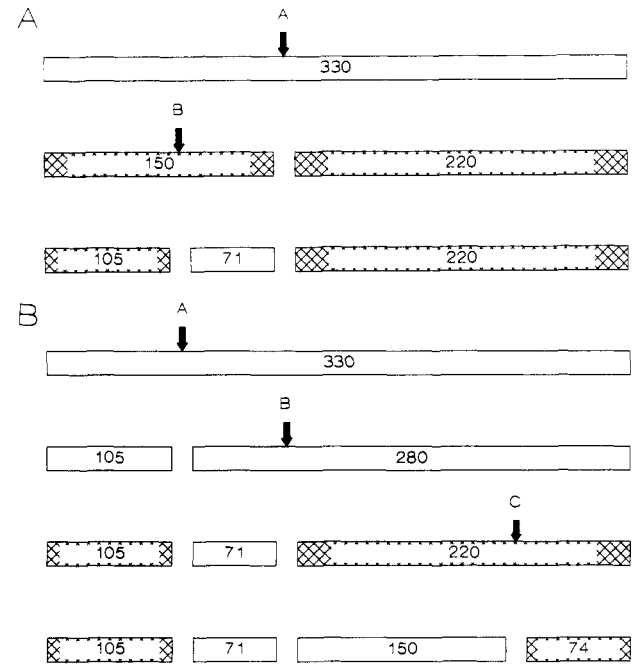

FIGURE 8: Schematic diagram of factor V cleavages mediated by factor Xa (A) or thrombin (B). The arrows indicate preferred cleavage sites. The shaded boxes represent peptides involved in the expression of cofactor activity.

105 000 and, by inference, 71 000 (data not shown), which is consistent with published activation schemes (Suzuki et al., 1982; Nesheim et al., 1981). The 71 000-dalton peptide does not stain with Coomassie blue (Suzuki et al., 1982), is not visible in Western blots, and is very poorly radioiodinated. This is presumably due to the eight potential N-linked glycosylation sites present on the peptide (Jenny et al., 1987). Since no other peptides of significant density were observed, it is inferred that these bonds are preferentially cleaved by factor Xa. Densitometric analyses coupled with cofactor activity measurements indicated that the appearance of factor Va activity was closely paralleled by cleavage of factor V at position A to yield the M_r 220 000 and 150 000 peptides (Figure 6). The ability of these two peptides to serve as a functional cofactor in the prothrombinase complex suggests that the expression of cofactor activity is dependent upon a conformational arrangement of cofactor domains that is attained, at least partially, following cleavage at position A. Since the M_r 220 000 peptide contains the light-chain domain which has been shown to mediate binding of factor Va to membrane or phospholipid surfaces and therefore the binding of factor Xa in the prothrombinase complex (Tracy et al., 1983a; Tucker et al., 1983), and the M_r 150 000 peptide contains the heavy-chain domain which has been shown to mediate the binding of prothrombin (Guinto & Esmon, 1984), it is conceivable that cleavage at position A releases conformational constraints within the factor V molecule and allows for the proper orientation of these subunits necessary for expression of cofactor activity. To illustrate their ability to function as cofactor, the two peptides shown in Figure 8A are shaded. No effects on the cofactor activity were observed following the second cleavage at position B, which generated the amino-terminal-derived M_r 105 000 peptide and an activation peptide. Since no further cleavages were required for expression of maximum cofactor activity (Figure 3), it would appear that all conformational constraints were removed and prothrombinase complex function could proceed at optimal rates. These data are in marked contrast to what was found in the bovine system, which will be discussed below.

The thrombin-catalyzed cleavages of factor V that were observed in this study are represented schematically in Figure 8B. This activation pattern is in contrast to the one described here for factor Xa cleavage of factor V, where generation of the M_r 280000 and 74000 peptides was not observed to any significant extent. The observations are in reasonably good agreement with those of Suzuki and colleagues (Suzuki et al., 1982), who also studied the thrombin-catalyzed activation of ^{125}I human factor V. They found that the expression of cofactor activity did not parallel the appearance of the M_r 105000 peptide, but rather the M_r 220000 peptide which was formed after the M_r 105000 peptide began appearing. We, however, have observed that under the conditions of our studies, cleavages at positions A and B (Figure 8B) occur virtually simultaneously, releasing the M_r 220000 and 105000 peptides in a parallel fashion. Therefore, when the kinetic analysis of thrombin-catalyzed activation of factor V was performed, we chose to use the appearance of the M_r 105000 peptide as an indicator of factor Va formation, since the M_r 220000 peptide was cleaved to yield the light chain shortly after it was formed.

The factor $Va_{(Xa)}$ cofactor activity was compared to that of factor $Va_{(IIa)}$ in Figure 3, since the subunit composition of the two cofactors differed. It was found that factor $Va_{(Xa)}$ expressed the same cofactor activity as factor $Va_{(IIa)}$, even though thrombin cleaved factor $Va_{(Xa)}$ to yield factor $Va_{(IIa)}$. The results of kinetic studies performed to compare the ability of factor Xa and thrombin to activate human factor V are summarized in Table II. The activation of factor V by factor Xa was characterized by a K_m of 10.4 nM, a k_{cat} of 2.6 min^{-1}, and a catalytic efficiency (k_{cat}/K_m) of 4.14×10^6 M^{-1} s^{-1}. The activation of factor V by thrombin was characterized by a K_m of 71.7 nM, a k_{cat} of 14.0 min^{-1}, and a catalytic efficiency of 3.26×10^6 M^{-1} s^{-1}. These results indicate that factor Xa expresses the same catalytic efficiency toward factor V as does thrombin. This suggests that the activation of factor V by factor Xa may be a relevant mechanism for the initiation of prothrombinase assembly and function in vivo, since a fully active cofactor can be generated relatively rapidly in the absence of thrombin.

A greater appreciation of these results can be obtained when they are compared to similar studies performed in other systems. The factor Xa catalyzed activation of bovine factor V described by Foster and colleagues (Foster et al., 1983) yielded a cleavage pattern distinctly different from the one described here for the human system. Cleavage of bovine factor V by factor Xa yielded intermediate peptides of M_r 162000 and 126000 and final products of M_r 94000 (heavy-chain subunit), 56000, 48000, 45000, and 30000. On the basis of these data, it is evident that the factor Xa catalyzed activation of factor V proceeds via a different set of pathways in the two species. Although bovine factor $Va_{(Xa)}$ was not further cleaved by thrombin, it also expressed the same cofactor activity as factor $Va_{(IIa)}$ (Foster et al., 1983). The catalytic efficiency of the activation of bovine factor V by factor Xa has not been reported, but it was shown that thrombin catalyzes the activation of factor V approximately 100-fold faster than factor Xa on a molar basis (Foster et al., 1983). Data listed in Table II indicate that thrombin catalyzes the activation of human factor V only 5-fold faster than factor Xa.

Studies performed in the porcine system with factor VIII:C, a cofactor in the activation of factor X by factor IXa, demonstrated that although factor Xa can activate factor VIII:C, thrombin-activated factor VIII:C possessed 3 times more relative cofactor activity (Lollar et al., 1985). The catalytic efficiencies of thrombin and factor Xa activation of porcine factor VIII:C are 5.0×10^6 and 1.1×10^6 M^{-1} s^{-1}, respectively (Lollar et al., 1985). A similar study in the human system found that thrombin-activated factor VIII:C was twice as active as factor Xa activated factor VIII:C (Neuenschwander & Jesty, 1988). Collectively, these data indicate that the factor Xa catalyzed activation of human factor V is somewhat unique in that (1) factor $Va_{(Xa)}$ is as active as factor $Va_{(IIa)}$, unlike factor Xa activated porcine or human factor VIII:C, and (2) the catalytic efficiency of factor Xa toward human factor V is equal to that of thrombin, unlike what is found in the bovine system.

Studies performed in a plasma system indicate that when heparin is present in contact-activated plasma, the thrombin-mediated feedback activation of factor V and factor VIII is inhibited (Buchanan et al., 1985; Ofosu et al., 1987, 1989). It has also been postulated that this inhibition can be circumvented by the activation of these cofactors by factor Xa (Ofosu et al., 1987). Indirect evidence exists that factor Xa effectively activates factor V and factor VIII in a plasma system when thrombin has been inhibited (Ofosu et al., 1989). This further underscores a key regulatory role which factor Xa may assume in the initiation of prothrombinase activity.

However, the role of factor Xa may be even more critical to prothrombinase activity on the surface of platelets, where platelet-released factor V is bound. Platelet factor V is released as a partially proteolyzed molecule (Viskup et al., 1987). Preliminary observations from this laboratory (Monkovic & Tracy, 1988) indicate that platelet-released factor V is more efficiently activated by catalytic amounts of factor Xa than by thrombin. When it is taken into account that platelet concentrations at sites of vascular injury are increased approximately 600-fold, it becomes evident that the role of platelet-released factor V, relative to plasma factor V, may be a critical one with regard to the support of prothrombinase complex function.

The data presented in this report indicate that factor Xa cleaves and activates human factor V to yield a cofactor as active as thrombin-activated factor V. Collectively, the data suggest that factor Xa may be an important activator of factor V in vivo at the onset of coagulation, when virtually no thrombin is available for feedback activation reactions that are required for the explosive increase in prothrombinase activity that accompanies a coagulation episode. Ongoing studies in this laboratory concerning platelet factor V and its activation by factor Xa and thrombin should extend our knowledge of prothrombinase complex assembly and function on the platelet surface.

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Registry No. Factor V, 9001-24-5; factor Xa, 9002-05-5; factor Va, 65522-14-7; thrombin, 9002-04-4; calcium, 7440-70-2.

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