Proteolysis of Factor V by Cathepsin G and Elastase Indicates That Cleavage at Arg^{1545} Optimizes Cofactor Function by Facilitating Factor Xa Binding†

Rodney M. Camire,‡ Michael Kalafatis,§ and Paula B. Tracy*

Department of Biochemistry, University of Vermont, College of Medicine, Burlington, Vermont 05405

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ABSTRACT: The single-chain procofactor factor V is cleaved by thrombin (FVa_{IIa}) at Arg^{709}, Arg^{1018}, and Arg^{1545} and by a variety of other proteases to generate a cofactor species with various levels of cofactor function. Having demonstrated previously that monocyte-bound forms of cathepsin G and elastase cleave and activate factor V, studies were initiated here using purified proteins to probe factor V structure/function. Electrophoretic, Western blotting, and amino-terminal sequence analyses revealed that cathepsin G cleaves factor V at several sites (Phe^{1031}, Leu^{1447}, Tyr^{1518}, and potentially Tyr^{696}), ultimately generating an amino-terminal 103 kDa heavy chain and a carboxy-terminal 80 kDa light chain (FVa_{CG}). Elastase also cleaves factor V at several sites (Ile^{708}, Ile^{819}, Ile^{1484}, and potentially Thr^{678}), generating a cofactor species, FVa_{HNE}, with an amino-terminal 102 kDa heavy chain and a carboxy-terminal 90 kDa light chain. Incubation of FVa_{IIa} with either cathepsin G or elastase resulted in cleavage within the heavy chain, releasing peptides of ∼2000 and ∼3000 Da, respectively, generating $FVa_{IIa/CG}$ and $FVa_{IIa/HNE}$. The functional activity of each cofactor species was assessed either by clotting assay or by employing a purified prothrombinase assay using saturating amounts of factor Xa. Significant differences in cofactor function were observed between the two assay systems. Whereas FVa_{IIa}, FVa_{CG}, $FVa_{IIa/CG}$, FVa_{HNE}, and $FVa_{IIa/HNE}$ all had similar cofactor activities in the purified prothrombinase assay, FVa_{CG} and FVa_{HNE} had no cofactor activity in the clotting-based assay, and $FVa_{IIa/CG}$ and $FVa_{IIa/HNE}$ had ∼30−35% clotting activity relative to FVa_{IIa}. These disparate results led us to examine the binding interactions of these cofactors with the various prothrombinase components. Kinetic analyses indicated that FVa_{IIa} ($K_{d(app)} = 0.096$ nM), $FVa_{IIa/CG}$ ($K_{d(app)} = 0.244$ nM), and $FVa_{IIa/HNE}$ ($K_{d(app)} = 0.137$ nM) bound to membrane-bound factor Xa much more effectively than FVa_{CG} ($K_{d(app)} = 1.46$ nM) and FVa_{HNE} ($K_{d(app)} = 0.818$ nM). In contrast, studies of the activated protein C (APC)-catalyzed inactivation of each of the factor V(a) species indicated that they were all equivalent substrates for APC with no differences observed in the rate of inactivation or the cleavage mechanism, suggesting that APC interacts with the light chain at a site distinct from factor Xa. The K_m values for prothrombin, as well as the k_{cat} values for each of the FV(a) species, were all similar (∼0.25 μM and ∼1900 min^{-1}). In addition, kinetic analyses indicated that whereas FVa_{CG} and FVa_{HNE} exhibited a slightly reduced ability to interact with phospholipid vesicles (∼2−3-fold), the remaining FV(a) species assembled equally well on this surface. Collectively, these data indicate that FVa_{CG} and FVa_{HNE} have a diminished capacity to support factor Xa binding; however, cleavage at Arg^{1545} and removal of the extended B-domain in these cofactors restore near-total factor Xa binding. Thus, cleavage at Arg^{1545} optimizes cofactor function within prothrombinase by facilitating factor Xa binding to membrane-bound FVa.

After vascular injury, the expression of tissue factor on damaged subendothelial cells initiates the coagulation response (*1*, *2*). Generation of a platelet−fibrin plug and propagation of this response require the proper assembly and function of the prothrombinase complex. This enzymatic complex is composed of the serine protease factor Xa bound to the nonenzymatic cofactor factor Va on a cellular membrane surface in the presence of Ca^{2+} ions (*3*, *4*). In addition to binding the substrate prothrombin, factor Va provides at least part of the receptor for factor Xa on cellular surfaces (*5*) and also increases the catalytic efficiency of factor Xa (*3*). Factor Va is an essential component of prothrombinase such that its deletion reduces the rate of prothrombin activation by 4 orders of magnitude (*4*). The importance of the cofactor is further underscored by the massive hemorrhage and lethality associated with factor V deficiency both in clinical situations and in an in vivo mouse model, respectively (*6*, *7*).

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* To whom correspondence should be addressed. Phone: (802) 656-1995. Fax: (802) 862-8229. Email: ptracy@salus.med.uvm.edu.

‡ Present address: Division of Hematology, 310B Abramson Research Center, The Children's Hospital of Philadelphia, 34th St. and Civic Center Blvd., Philadelphia, PA 19104.

§ Present address: Department of Chemistry, Science Building, 2351 Euclid Ave., Cleveland State University, Cleveland, OH 44115.

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The central role which factor Va assumes in the prothrombinase complex indicates that both its activation and its inactivation must be key regulatory events in maintaining the normal hemostatic balance. It is well recognized that activated protein C (APC)[1] is the principle down-regulator of factor Va, cleaving the cofactor at Arg^{306}, Arg^{506}, and Arg^{679}, leading to dissociation of the A2 domain (*8*, *9*). Conditions that slow the rate of this reaction (such as factor V^{Leiden}) can lead to venous thrombosis (*10*, *11*). Several laboratories have studied the activation of single-chain plasma factor V (A1-A2-B-A3-C1-C2 domains; amino acids 1−2196), with thrombin being the most recognized activator, cleaving the procofactor at Arg^{709}, Arg^{1018}, and Arg^{1545}, generating factor Va_{IIa} (*12*−*15*). Factor Va_{IIa} is a heterodimer composed of an amino-terminal 105 kDa heavy chain (A1-A2 domains; amino acids 1−709) associated via Ca^{2+} ions to the carboxy-terminal 74/71 kDa light chain (A3-C1-C2 domains; amino acids 1546−2196) (*12*, *16*). While the binding affinities of factor Va_{IIa} for factor Xa (*4*, *17*), prothrombin (*18*), phospholipid vesicles (*19*, *20*), and platelets (*21*, *22*) are well characterized, information regarding the molecular events and interactions leading to full expression of cofactor function is lacking. Elucidating how novel protease-derived cofactors mediate protein−protein and protein−phospholipid interactions within prothrombinase will provide valuable information regarding structure/function relationships of factor V.

In addition to thrombin, several proteases cleave and activate human factor V, generating a cofactor species with variable amounts of activity. For example, our laboratory has demonstrated that human factor Xa activates human plasma factor V in a membrane- and Ca^{2+}-dependent manner following cleavage at or near Arg^{709} and at Arg^{1018} (*23*). Processing by other proteases includes an activator from Russell's viper venom (RVV-V) (*24*), calpain (*25*), plasmin (*26*), platelet proteases (*22*, *27*), *Naja naja oxiana* venom (*28*), venoms of snakes belonging to the genus *Elapidae* (*29*), and meizothrombin (*30*) as well as elastase (*31*−*34*) and cathepsin G (*32*, *33*).

Cathepsin G and elastase are serine proteases found in the azurophilic granules of polymorphonuclear (PMN) leukocytes and are thought to be involved in the degradation of a variety of connective tissue proteins including elastin, collagen, and proteoglycans which may lead to pulmonary emphysema, and rheumatoid arthritis as well as atherosclerosis (*35*). Several studies have demonstrated that these enzymes cleave a variety of coagulation proteins (*36*−*39*) including factor V at the monocyte surface (*32*). The significance of these reactions in normal hemostasis is questionable since both cathepsin G and elastase are under strict regulation by plasma protease inhibitors (*40*). However, these proteases may play a significant role in thrombin generation at extravascular tissue sites where they may be protected, or in certain clinical situations where extensive depletion of protease inhibitors occurs. Interestingly, cathepsin G and elastase may also be involved in platelet activation events. For example, cathepsin G has been shown to be a potent platelet agonist, inducing extensive aggregation, shape change, Ca^{2+} mobilization, and α and dense granule exocytosis, and stimulation of phospholipase C/protein kinase C pathways and elastase may enhance some of these responses (*41*−*44*). Thus, these proteases not only are involved in the processes of wound repair, host defense, extracellular tissue remodeling, and inflammation but also may be involved in the regulation of thrombin generation through cleavage of clotting factors and activation of platelets.

The current study was undertaken to examine how the cathepsin G- and elastase-derived cofactors fully assemble and function within prothrombinase in terms of their interactions with factor Xa, phospholipid, and prothrombin. Results from these studies not only provide insight into factor V structure/function relationships but also provide a molecular explanation of why cleavage at Arg^{1545} within factor V is an essential step in achieving full cofactor function.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Tris(hydroxymethyl)aminomethane (Tris-base), L-α-phosphatidyl-L-serine (bovine brain), L-α-phosphatidylcholine (egg yolk), Tween-80, Tween-20, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), heparin (bovine lung), and glycine were purchased from Sigma (St. Louis, MO). Sodium chloride, ethylenediaminetetraacetic acid tetrasodium salt (EDTA), calcium chloride dihydrate, and poly(ethylene glycol)-8000 (PEG-8000) were purchased from J. T. Baker (Phillipsburg, NJ). The thromboplastin reagent (Simplistic Excel) used in the clotting assays was purchased from Organon Teknika Corp. (Durham, NC). Human neutrophil elastase, human neutrophil cathepsin G, Boc-Ala-Ala-Pro-Ala-pNA (elastase substrate II), Suc-Ala-Ala-Pro-Phe-pNA (cathepsin G substrate), and hirudin (recombinant) were purchased from Calbiochem (La Jolla, CA). L-Ala-L-Ala-L-Pro-L-Val-chloromethyl ketone (elastase inhibitor) was purchased from Bachem (Torrance, CA). Z-Gly-Leu-Phe-chloromethyl ketone (cathepsin G inhibitor) was purchased from Enzyme Systems Products (Dublin, CA). The chromogenic substrate S-2238 was purchased from Chromogenix (Mölndal, Sweden). Poly(vinylidene difluoride) (PVDF) membranes were purchased from Millipore Corp. (Bedford, MA). Pure nitrocellulose membrane sheets (0.45 μm) were purchased from Bio-Rad (Hercules, CA). The chemiluminescent substrate, Luminol, and Reflection autoradiography film were purchased from DuPont NEN Research Products (Boston, MA). The fluorescent α-thrombin inhibitor dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) (*45*) and human APC were gifts from Haematologic Technologies Inc. (Essex Junction, VT). Phospholipid vesicles composed of 75% (% w/w) phosphatidylcholine and 25% (% w/w) phosphatidylserine (PCPS) were prepared as previously described (*46*). The concentration of the phospholipid vesicles was determined by phosphorus assay (*47*).

Preparation of Proteins. All coagulation proteins were of human origin and purified from fresh frozen plasma. Plasma-derived factor V was isolated by immunoaffinity

[1] Abbreviations: APC, activated protein C; Tris-base, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid tetrasodium salt; PEG-8000, poly(ethylene glycol)-8000; DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; PCPS, phosphatidylcholine/phosphatidylserine vesicles (75%:25%); SDS−PAGE, sodium dodecyl sulfate−polyacylamide gel electrophoresis; HRP, horseradish peroxidase; RVV-V, factor V activator from Russell's viper venom; PVDF, poly(vinylidene difluoride); PMN, polymorphonuclear.

chromatography as described by Katzmann et al. (*48*). Factor X and prothrombin were purified as described (*49*). Factor X was activated with the factor X activator purified from Russell's viper venom (*50*) and purified on benzamidine−Sepharose as described (*51*). α-Thrombin was prepared by activation of prothrombin with taipan snake venom as described (*52*). All proteins used were >95% pure as judged by sodium dodecyl sulfate−polyacylamide gel electrophoresis (SDS−PAGE) before and after disulfide bond reduction according to the method of Laemmli (*53*). Molecular weights and extinction coefficients ($E^{1\%}_{280\ nm}$) of the various proteins used were taken as follows: prothrombin 72 000, 14.2 (*49*); thrombin 37 000, 17.4 (*54*); factor V 330 000, 9.6 (*55*); factor Xa 50 000, 11.6 (*49*).

Generation of Protease-Activated Factor V(a) Species. The various factor V(a) species were generated following incubation of single-chain human plasma factor V (300 nM) with α-thrombin, elastase, or cathepsin G. The nomenclature and the preparation of the factor V(a) species are as follows: factor Va_{IIa}, human factor V incubated with α-thrombin (0.5 NIH unit/mL, 5 nM) at 37 °C for 10 min, followed by the addition of hirudin (15 nM); factor Va_{HNE}, human factor V incubated with elastase (2 nM) at ambient temperature for 50 min followed by the addition of L-Ala-L-Ala-L-Pro-L-Val-chloromethyl ketone (0.5 μM); factor Va_{CG}, human factor V incubated with cathepsin G (2 nM) at ambient temperature for 50 min followed by the addition of Z-Gly-Leu-Phe-chloromethyl ketone (1 μM); factor $Va_{IIa/HNE}$, factor Va_{IIa} incubated with elastase (2 nM) at ambient temperature for 50 min followed by the addition of L-Ala-L-Ala-L-Pro-L-Val-chloromethyl ketone (0.5 μM); factor $Va_{IIa/CG}$, factor Va_{IIa} incubated with cathepsin G (2 nM) at ambient temperature for 50 min followed by the addition Z-Gly-Leu-Phe-chloromethyl ketone (1 μM). Control experiments indicated that addition of protease inhibitors had no effect when carried over into purified prothrombinase assays or clotting assays.

Determination of Factor V(a) Cofactor Activity by Clotting Assay. In some experiments, the cofactor activity of each of the factor V(a) species was measured at selected time intervals by clotting assay using factor V deficient plasma (immunodepleted). In a typical assay, 50 μL of factor V deficient plasma was mixed with an equal volume of the sample to be analyzed. To start the assay, 100 μL of the thromboplastin reagent (which contains Ca^{2+}) was added while manually rocking the tube at 37 °C. The assay end point was determined by visualization of the fibrin clot. A standard curve using normal human plasma (dilution of normal human plasma from 1:4 to 1:512) was established prior to each experiment and was expressed as log of factor Va activity in units/mL versus clot time. Clot times for a particular series of experiments were compared to the standard curve, and factor Va activity was expressed as units per milligram of factor Va protein, where 1 unit of factor V activity is defined as that amount of factor V contained in 1 mL of human plasma.

Functional Binding Studies: Measurements of Rates of Thrombin Generation Using S-2238. In experiments such as those detailed in Figure 5, functionally defined apparent dissociation constants ($K_{d(app)}$) for factor Xa binding to factor V(a)−PCPS were inferred from an assay measuring α-thrombin generation at various factor V(a) concentrations. Assay mixtures contained PCPS vesicles (20 μM), DAPA (3 μM), factor Xa (15 pM), and the various factor V(a) species (30−0.0097 nM) in 20 mM HEPES, 0.15 M NaCl, 5 mM $CaCl_2$, 0.01% Tween-80, pH 7.4. DAPA was included in all experiments to prevent feedback reactions catalyzed by α-thrombin generated during the assay. This mixture was allowed to incubate at ambient temperature for 5 min, and the reaction was initiated with prothrombin (1.4 μM). Similar experiments were performed holding factor V(a) at a fixed limiting concentration (15 pM) and titrating factor Xa (30−0.0097 nM) (Table 1). Samples of the reaction mixture were removed at various time intervals (0, 0.5, 1, 1.5, and 2 min) and diluted 3-fold in 20 mM HEPES, 0.15 M NaCl, 50 mM EDTA, 0.1% PEG-8000, pH 7.4 (quench buffer), to quench the reaction. The α-thrombin concentration in each sample was determined using the chromogenic substrate S-2238 (0.4 mM, final). The change in absorbance at 405 nm was monitored using a Molecular Devices V_{max} spectrophotometer and compared to an α-thrombin standard curve (0−50 nM) prepared daily using purified α-thrombin. The initial rate of α-thrombin generation in the various assay mixtures under these conditions is linear, and no more than 10% of the substrate prothrombin was consumed during the course of the assay.

The kinetic parameters of factor Xa-catalyzed prothrombin activation ($K_{m(app)}$ and V_{max}) with each of the factor V(a) species were determined by measuring the rate of thrombin formation at varying prothrombin concentrations. Assay mixtures contained PCPS vesicles (20 μM), DAPA (3 μM), various factor Va species (10 pM), and various concentrations of prothrombin (5 μM to 0.05 μM), in 20 mM HEPES, 0.15 M NaCl, 5 mM $CaCl_2$, 0.01% Tween-80, pH 7.4. This mixture was allowed to incubate at ambient temperature for 5 min, and the reaction was initiated with factor Xa (30 nM). At selected time intervals, samples were removed from the reaction mixture and placed in quench buffer. α-Thrombin generation was monitored as described above using the chromogenic substrate S-2238 (0.4 mM, final). Using these nominal concentrations of factor Xa and factor Va in the presence of saturating PCPS vesicles (20 μM), >90% of the added factor Xa is complexed with each of the factor Va species. Kinetic constants ($K_{m(app)}$ and V_{max} for prothrombin) were obtained by fitting the data to the Michaelis−Menten equation using nonlinear least-squares regression analysis with the graphing program Prism version 2.0 (Graphpad, San Diego, CA). The assay described above was also performed with limiting factor Xa (15 pM) in the presence of saturating factor Va (30 nM).

The ability of each of the factor V(a) species to assemble on PCPS vesicles was also assessed kinetically (Table 1). Varying amounts of PCPS vesicles (100−0.003 μM) were added to each of the factor V(a) species (30 nM), DAPA (3 μM), and prothrombin (2 μM). The reaction was initiated upon addition of factor Xa (15 pM). At selected time intervals, samples were removed from the reaction mixture and placed in quench buffer. α-Thrombin generation was monitored as described above using the chromogenic substrate S-2238 (0.4 mM, final). The kinetic parameters were obtained by fitting the data to a single-site binding isotherm using nonlinear least-squares regression analysis with the program Prism.

APC-Catalyzed Inactivation of the Protease-Activated Factor V(a) Species. Each of the factor V(a) species was generated as described above and brought to a final concentration of 20 nM. Following addition of 20 μM PCPS, the reaction was initiated upon addition of 0.1 nM APC. At selected time points, samples of the reaction mixture were removed, and residual cofactor activity was monitored in a purified prothrombinase assay (1.4 μM prothrombin, 3 μM DAPA, 20 μM PCPS, 10 nM factor Xa, and factor V(a) >0.1 nM). The rate of thrombin generation was monitored with S-2238 as described above. At the same time intervals that each of the factor V(a) species was monitored for residual cofactor activity, samples were removed and prepared for SDS−PAGE, followed by Western blotting analyses as described below.

Kinetic Data Analyses. Functionally defined apparent dissociation constants ($K_{d(app)}$) for factor Xa binding to factor V(a)−PCPS or factor V(a) binding to factor Xa−PCPS were obtained from plots of the rate of thrombin generation as a function of the factor V(a) or factor Xa concentration. The apparent dissociation constant ($K_{d(app)}$) of factor Xa binding to membrane-bound factor V(a) is given by the following equation:

$$K_d = \frac{[Va]_F[Xa]_F}{[Va{\cdot}Xa]} \quad (1)$$

where $[Va]_F$ and $[Xa]_F$ are the concentrations of free factor Va and factor Xa. Experimental data were then fit to eq 2 which is the quadratic solution for eq 1 (*51*), using nonlinear least-squares regression analysis with the graphing program Prism:

$$[Xa]_{bound} = \{[[nXa]_T + [Va] + K_d] - \sqrt{[[n[Xa]_T] + [Va] + K_d]^2 - [4n[Xa]_T[Va]]}\}/2[Va] \quad (2)$$

assuming that n = moles of factor Xa bound per mole of factor Va at saturation and $v_{obs} = V_0 + [Xa_{bound}V_\infty]$, where v_{obs} is the observed catalytic rate, V_0 is the catalytic rate at $[Xa]_T = 0$, and V_∞ is the catalytic rate at infinite factor Xa concentration (i.e., when the fixed concentration of factor Va is saturated with factor Xa).

Gel Electrophoresis and Western Blotting. Samples of various factor V(a) reaction mixtures were prepared for SDS−PAGE by addition of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.001% bromophenol blue (final concentrations). Following heating at 95 °C for 3 min, SDS−PAGE was performed on 5−15% linear gradient slab gels according to the method of Laemmli (*53*). Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 and destained with 18% methanol/9% acetic acid. Alternatively, the various factor V(a) species were transferred to nitrocellulose using electroblotting techniques as described by Towbin et al. (*56*). Transfer was performed at 500 mA for 2 h at 4 °C (*23*). Nitrocellulose was blocked with 5% nonfat dry milk in 20 mM Tris-base, 0.15 M NaCl, 0.05% Tween-20 at pH 7.4. The factor Va antigen was probed with a mouse anti-human factor Va_{IIa} heavy-chain IgG monoclonal antibody (αHF-Va_{HC}#17) (*57*, *58*) or a mouse anti-human factor Va_{IIa} light-chain IgG monoclonal antibody (αHFVa_{LC}#9) (*57*). The secondary antibody used was a horse anti-mouse IgG coupled to horseradish peroxidase (HRP; Southern Biotechnologies, Birmingham, AL). Detection of factor V(a) was performed by enhanced chemiluminescence (Western Blot Chemiluminescence Detection Kit, DuPont NEN, Boston, MA) by exposure of blots (5−30 s) to Reflection autoradiography film developed in a Kodak M35A X-OMAT processor.

Amino-Terminal Sequence Analysis. The amino-terminal sequence of the peptides from PVDF membranes was determined using automatic Edman degradation on an Applied Biosystems 475A protein sequencing system in the laboratory of Dr. Alex Kurosky (University of Texas, Medical Branch at Galveston) (*59*).

RESULTS

Cathepsin G- and Elastase-Mediated Cleavage of Human Factor V: Assessment of Cofactor Activities Using a Clotting-Based Assay. Having demonstrated previously that monocyte-bound forms of cathepsin G and elastase cleave and activate factor V (*32*), structure/function studies were performed to define the cleavage mechanism responsible for generating functional cofactor species. Purified plasma-derived factor V was incubated with cathepsin G, elastase, or thrombin, and the time-dependent change in cofactor activity was monitored in a clotting assay using factor V-deficient plasma. Incubation of factor V with thrombin to generate factor Va_{IIa} resulted in a 15-fold increase in its clotting activity (Figure 1A, closed circles and inset). In marked contrast, cathepsin G and elastase generated cofactor species, factor Va_{CG} and factor Va_{HNE}, respectively, which expressed minimal clotting activity relative to that observed with factor Va_{IIa} (Figure 1A, factor Va_{CG}, closed squares; factor Va_{HNE}, closed triangles, inset). A transient increase in clotting activity was observed in the early phase of the reaction (~2−3-fold); however, this activity was lost such that at the end of the time course the cofactor activity remaining was equivalent to that observed with unactivated factor V.

At the same time intervals that each of the factor V(a) species were analyzed for cofactor function, samples were withdrawn and analyzed by SDS−PAGE to visualize any cleavage products formed. Despite only a small and transient increase in cofactor activity, cathepsin G and elastase both cleaved the single-chain procofactor at multiple residues, yielding several high molecular mass intermediates (>200 kDa) as well as other polypeptides ranging in molecular mass from 160 kDa to 80 kDa (Figure 1B,C). Although the end products comprising factor Va_{CG} (Figure 1B, 103 and 80/78 kDa) and factor Va_{HNE} (Figure 1C, 102 and 90/88 kDa) had minimal cofactor activity in a clotting-based assay, these polypeptides are very similar in molecular mass to the heavy and light chains of factor Va_{IIa} (105 and 74/71 kDa). These data collectively indicate that full expression of cofactor activity in a clotting-based assay requires specific cleavages within distinct regions of factor V which thrombin can effectively mediate, but which cathepsin G and elastase cannot.

Characterization of the Cathepsin G- and Elastase-Mediated Cleavage Sites in Factor V. To begin to understand why a particular cofactor species expressed a certain level of activity, it was necessary to define the order and location of the cleavage sites within the procofactor to

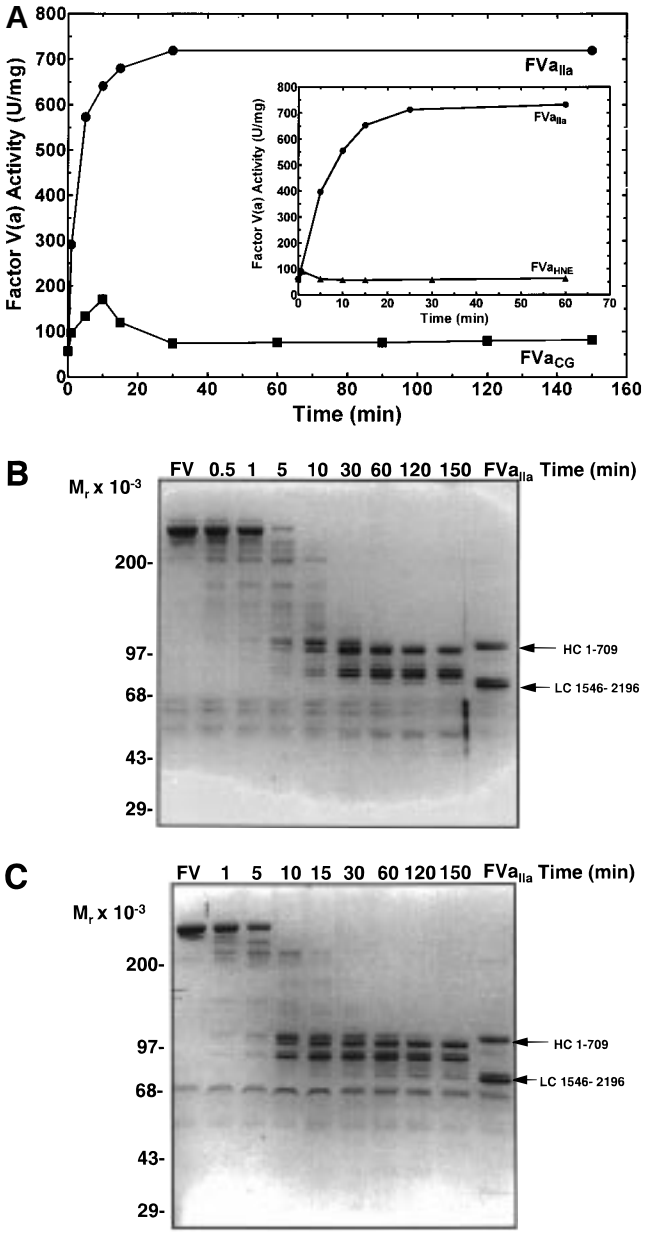


FIGURE 1: Cleavage and activation of human factor V by cathepsin G and elastase. Panel A: Purified human plasma-derived factor V (300 nM) in 20 mM HEPES/0.15 M NaCl/5 mM $CaCl_2$/0.01% Tween-80, pH 7.4, was incubated with either thrombin (5 nM, ●), cathepsin G (2 nM, ■), or elastase (2 nM, inset, ▲) at ambient temperature. At the time points indicated, samples were withdrawn from the reaction mixtures, and the resulting cofactor activities were monitored in a clotting assay using FV-deficient plasma as described under Experimental Procedures. At the same time intervals, samples were removed and analyzed by 5−15% linear gradient SDS−PAGE (6 μg of protein/lane) followed by staining with Coomassie Brilliant Blue R-250 as shown in panels B (FV plus cathepsin G) and C (FV plus elastase). The time of incubation of FV with either enzyme is indicated above each lane, and the molecular weight markers are shown on the left of the gels. FV and FVa_{IIa} respectively were placed in the first and the last lanes of both gels as controls. HC = heavy chain; LC = light chain.

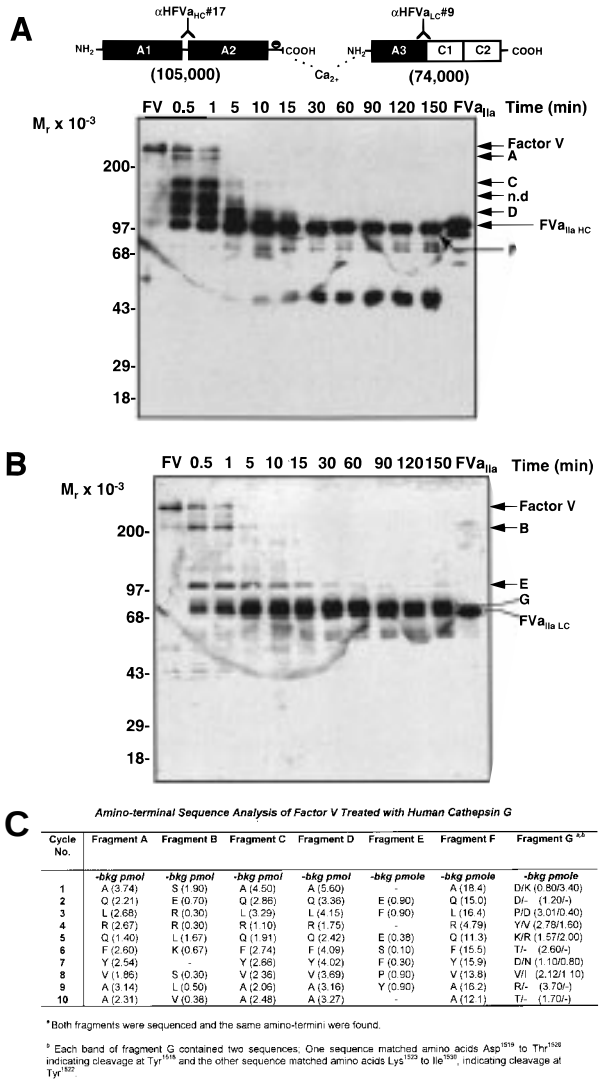
establish product/precursor relationships. Factor V was subjected to limited proteolysis with either cathepsin G or elastase, followed by amino-terminal sequence analyses on the isolated fragments. Precursor−product relationships were also established using Western blotting techniques with two monoclonal antibodies: one directed against the factor Va_{IIa} heavy chain ($\alpha HFVa_{HC}$#17) (*57*, *58*), the other directed against the factor Va_{IIa} light chain ($\alpha HFVa_{LC}$#9) (*57*).

Western blotting techniques along with amino-terminal sequence analyses of fragments A−G (Figure 2) indicated that cathepsin G cleaved factor V at Phe^{1031}, Leu^{1447}, and Tyr^{1518} and at several other unidentified sites. Cleavage at these sites resulted in the generation of an amino-terminal 103 kDa heavy chain (fragment F) and a carboxy-terminal 80/78 kDa light chain (fragment G). Based on the Western blotting results (Figure 2A), it is evident that the factor Va_{CG} heavy chain or fragment F is derived from fragment D. Since both of these fragments are derived from the amino-terminal region of factor V and contain the same amino terminus, we were unable to determine the exact position of this cleavage site. However, based on preferred cleavage sites for cathepsin G and the molecular mass of the factor Va_{CG} heavy chain (103 kDa), we predict that the cleavage occurs at or near Tyr^{696}. As shown in Figure 2B, fragment G migrates as a doublet and is derived from the carboxy-terminal region of factor V. Each fragment of the doublet was sequenced, and both bands contained the same amino terminus (Figure 2C), indicating that the difference in molecular mass of these bands must be restricted either to the carboxy-terminal region or to differences in posttranslational modifications. Therefore, from these results we can conclude that factor Va_{CG} is composed of a 103 kDa heavy chain (amino acids 1−696) derived from the amino-terminal portion of factor V and an 80/78 kDa light chain (amino acids 1519−2196) doublet derived from the carboxy-terminal portion of factor V.

A similar experimental approach was taken to locate the specific cleavage sites within factor Va_{HNE}, and the data are presented in Figure 3. Based on Western blotting analyses (Figure 3A,B) and amino-terminal sequence analyses of fragments A, B, C, D, and E (Figure 3C), our results indicate that elastase cleaved factor V initially at Ile^{708}, followed by cleavages at Ile^{819} and Ile^{1484}. Cleavage at these sites resulted in the generation of 105 and 90/88 kDa fragments. The 105 kDa polypeptide is an intermediate, and is further cleaved toward the carboxy-terminal end of the molecule to generate the 102 kDa factor Va_{HNE} heavy chain (Figure 3A). The precise location of this cleavage site is unknown; however, based on the molecular mass of the fragment and the sequence specificity of elastase, we predict that cleavage occurs at either Thr^{678} or Ala^{677}. Therefore, factor Va_{HNE} is composed of a 102 kDa heavy chain (amino acids 1−678/677) derived from the amino-terminal portion of factor V and a 90/88 kDa light chain doublet (amino acids 1485−2196) derived from the carboxy-terminal portion of factor V.

Regulation of Factor Va_{IIa} Cofactor Activity by Cathepsin G and Elastase. Both cofactor species differ slightly from factor Va_{IIa} in that they lack ∼15−30 amino acids from the carboxy-terminal region of their heavy chains and have an additional 30−60 amino acids on their light chains. Experiments were performed in order to investigate the effects these modified heavy and light chains had on cofactor function.

Incubation of factor Va_{IIa} with either cathepsin G or elastase resulted in a substantial decrease in cofactor function until the clotting activity of each new cofactor (factor $Va_{IIa/CG}$, Figure 4A, closed circles; or factor $Va_{IIa/HNE}$, inset, closed diamonds) reached a plateau of ∼30% of the initial clotting



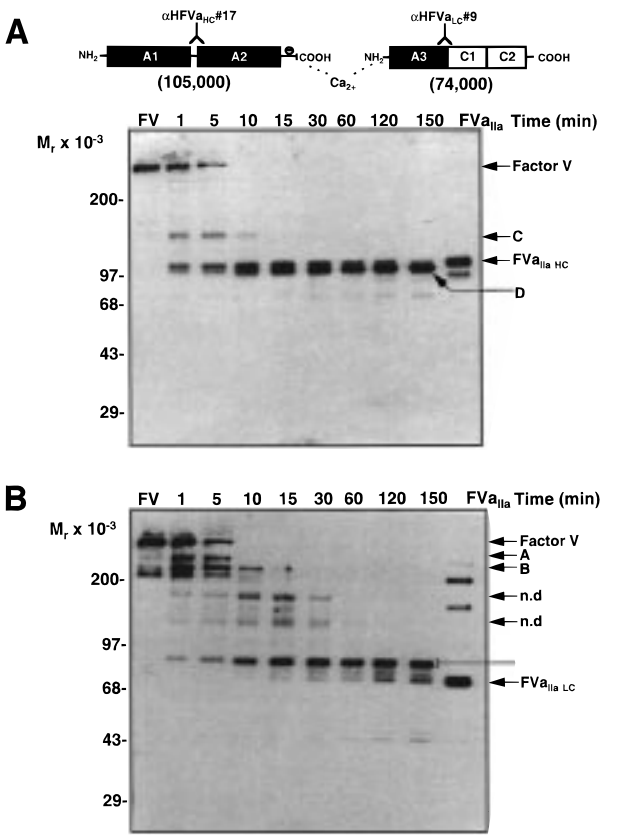
C *Amino-terminal Sequence Analysis of Factor V Treated with Human Cathepsin G*

Cycle No.	Fragment A	Fragment B	Fragment C	Fragment D	Fragment E	Fragment F	Fragment G [a,b]
	-bkg pmol	*-bkg pmol*	*-bkg pmol*	*-bkg pmol*	*-bkg pmole*	*-bkg pmole*	*-bkg pmole*
1	A (3.74)	S (1.90)	A (4.50)	A (5.60)	-	A (18.4)	D/K (0.80/3.40)
2	Q (2.21)	E (0.70)	Q (2.86)	Q (3.36)	E (0.90)	Q (15.0)	D/- (1.20/-)
3	L (2.68)	R (0.30)	L (3.29)	L (4.15)	F (0.90)	L (16.4)	P/D (3.01/0.40)
4	R (2.67)	R (0.30)	R (1.10)	R (1.75)	-	R (4.79)	Y/V (2.78/1.60)
5	Q (1.40)	L (1.67)	Q (1.91)	Q (2.42)	E (0.38)	Q (11.3)	K/R (1.57/2.00)
6	F (2.60)	K (0.67)	F (2.74)	F (4.09)	S (0.10)	F (15.5)	T/- (2.60/-)
7	Y (2.54)	-	Y (2.86)	Y (4.02)	F (0.30)	Y (15.9)	D/N (1.10/0.80)
8	V (1.86)	S (0.30)	V (2.36)	V (3.69)	P (0.90)	V (13.8)	V/I (2.12/1.10)
9	A (3.14)	L (0.50)	A (2.06)	A (3.16)	Y (0.90)	A (16.2)	R/- (3.70/-)
10	A (2.31)	V (0.36)	A (2.48)	A (3.27)	-	A (12.1)	T/- (1.70/-)

[a] Both fragments were sequenced and the same amino-termini were found.

[b] Each band of fragment G contained two sequences. One sequence matched amino acids Asp^{1519} to Thr^{1526} indicating cleavage at Tyr^{1518} and the other sequence matched amino acids Lys^{1529} to Ile^{1535}, indicating cleavage at Tyr^{1527}.

FIGURE 2: Western blotting and amino-terminal sequence analyses of cathepsin G-derived factor V(a) fragments. Plasma-derived factor V (300 nM) in 20 mM HEPES/0.15 M NaCl/5 mM $CaCl_2$/0.01% Tween-80, pH 7.4, was incubated with cathepsin G (2 nM) at ambient temperature. At selected time intervals (indicated above each lane), samples (50 ng/lane) were removed from the reaction mixture and analyzed by 5−15% linear gradient SDS−PAGE followed by transfer to nitrocellulose for Western blotting analyses. Panel A: Factor V(a) fragments derived from cathepsin G cleavage were probed with an α-heavy chain monoclonal antibody (αHFVa$_{HC}$#17) (*57*, *58*). Panel B: The blot shown in panel A was stripped of the primary and secondary antibodies by incubation with 0.1 M glycine/0.5 M NaCl, pH 2.8, overnight at ambient temperature followed by incubation with 62.5 mM Tris/HCl, pH 6.8, 2% SDS, and 10 mM β-mercaptoethanol for 1 h at 50 °C. The blot was then reprobed with an α-light chain monoclonal antibody (αHFVa$_{LC}$#9) (*57*). The molecular weight markers are indicated at the left of each blot. The letters to the right of the blots represent fragments which were subjected to amino acid sequence analysis (see panel C). The schematic in panel A represents regions in factor Va which the monoclonal antibodies recognize. Panel C: ∼150 pmol of factor V was subjected to limited proteolysis with cathepsin G (2 nM, 25 min). The resulting mixture was then analyzed by 4−8% linear gradient SDS−PAGE followed by transfer to a PVDF membrane (data not shown). The resulting fragments (representative fragments shown in panels A and B) were subjected to 10 cycles of amino-terminal sequence analysis as described under Experimental Procedures. The numbers in parentheses indicate the minus background picomoles of amino acid at a given cycle. The dash indicates that we were unable to determine which amino acid was present at a given cycle.



C *Amino-terminal Sequence Analyses of Factor V Treated with Human Neutrophil Elastase*

Cycle No.	Fragment A [a]	Fragment B [b]	Fragment C	Fragment D [c]	Fragment E [c]
	-bkg pmol	*-bkg pmol*	*-bkg pmol*	*-bkg pmol*	*-bkg pmol*
1	A/R (2.40/0.50)	A/R (0.90/1.56)	A (7.01)	A (12.7)	V (3.50)
2	Q/S (1.58/1.00)	Q/L (0.40/1.78)	Q (3.84)	Q (9.21)	G (2.70)
3	L/F (0.82/0.10)	L/L (1.80)	L (5.26)	L (11.3)	L (2.87)
4	R/R (0.80)	R/- (0.90/-)	R (0.75)	R (3.24)	S (1.00)
5	Q/N (1.00/0.60)	Q/L (0.30/1.37)	Q (5.00)	Q (9.11)	K (0.90)
6	F/S (0.70/1.30)	F/G (0.30/0.50)	F (3.69)	F (8.11)	D (2.29)
7	Y/S (1.19/0.50)	Y/A (0.10/0.50)	Y (4.34)	Y (9.61)	G (0.70)
8	V/L (0.87/0.20)	V/- (0.10/-)	V (3.49)	V (8.94)	T (1.30)
9	A/N (0.40/0.50)	A/E (0.10/0.97)	A (3.80)	A (7.71)	D (2.90)
10	-/Q (-/0.30)	-/F (-/0.30)	A (5.74)	A (9.38)	Y (0.90)

[a] Based on amino acid sequence analysis, this fragment contains two sequences. One matched amino acids Ala^1 to Ala^9 representing the amino terminus of factor V and the other matched amino acids Arg^{709} to Gln^{718}, indicating cleavage at Ile^{708}.

[b] Based on amino acid sequence analysis, this fragment contains two sequences. One matched amino acids Ala^1 to Ala^9 representing the amino terminus of factor V and the other matched amino acids Arg^{820} to Phe^{829}, indicating cleavage at Ile^{819}.

[c] Both fragments were sequenced and the same amino-termini were found.

FIGURE 3: Western blotting and amino-terminal sequence analyses of elastase-derived factor V(a) fragments. Plasma-derived factor V (300 nM) in 20 mM HEPES/0.15 M NaCl/5 mM $CaCl_2$/0.01% Tween-80, pH 7.4, was incubated with elastase (2 nM) at ambient temperature and analyzed as detailed for the cathepsin G-treated factor V in Figure 2. Panel A: Elastase-derived fragments probed with αHFVa$_{HC}$#17. Panel B: Elastase-derived fragments probed with αHFVa$_{LC}$#9. The letters to the right of the blots represent fragments which were subjected to amino acid sequence analysis (see panel C). Panel C: ∼150 pmol of factor V was subjected to limited proteolysis with elastase (2 nM, 25 min). The resulting mixture was analyzed as described in Figure 2.

activity. Electrophoretic and amino-terminal sequence analyses (data not shown) indicated that both cathepsin G and elastase cleaved factor Va$_{IIa}$ such that peptides of ∼2000 and ∼3000 Da, respectively, were released from the carboxy-terminal region of the heavy chain (Figure 4B), correlating with an ∼60−70% loss in clotting activity. These results directly implicate this region of the heavy chain as essential for maintaining full cofactor function in this assay system.

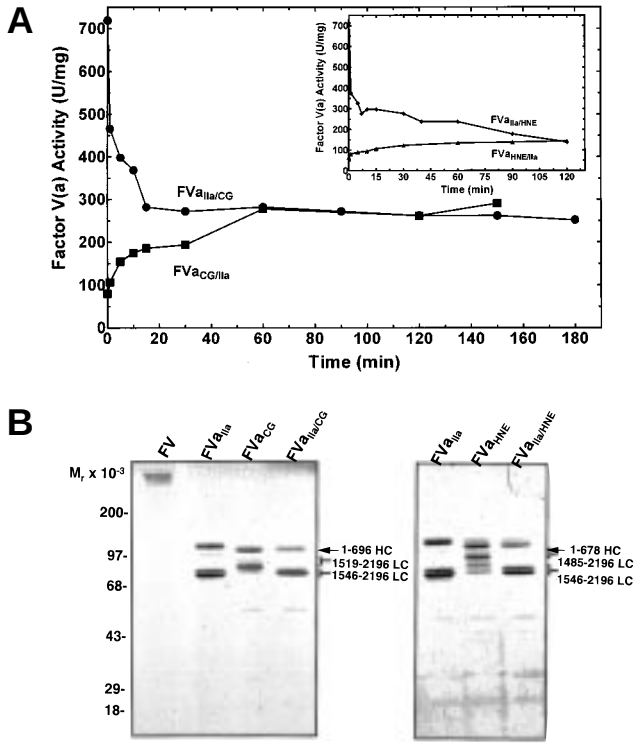


FIGURE 4: Effect of protease cleavage on the cofactor function of factor Va_{IIa}, factor Va_{CG}, and factor Va_{HNE}. The various factor V(a) species in 20 mM HEPES/0.15 M NaCl/5 mM $CaCl_2$/0.01% Tween-80, pH 7.4, were incubated with the following proteases at ambient temperature: factor Va_{IIa} was incubated with cathepsin G (2 nM, ●) or with elastase (2 nM, inset, ◆); factor Va_{CG} was incubated with thrombin (5 nM, ■); and factor Va_{HNE} was incubated with thrombin (5 nM, inset, ▲). Panel A: At the time points indicated, samples were withdrawn from the reaction mixtures, and the resulting cofactor activities were monitored in a clotting assay using factor V-deficient plasma as described under Experimental Procedures. Panel B: Each of the factor V(a) species (6 μg of protein/lane) was analyzed by 4−12% linear gradient SDS−PAGE followed by staining with Coomassie Brilliant Blue R-250. The molecular weight markers are indicated at the left of the gel. The amino acid numbers corresponding to the cathepsin G- or elastase-derived factor V(a) fragments are indicated at the right of the gels.

We next examined how the extra 30−60 amino acids at the amino terminus of the light chain altered cofactor function by incubating factor Va_{CG} or factor Va_{HNE} with thrombin. Electrophoretic and amino-terminal sequence analyses (data not shown) indicated that thrombin cleaved the factor Va_{CG} and factor Va_{HNE} light chains at Arg^{1545} to generate new light chains which migrated at ~74/71 kDa. As expected, these cofactor species (factors $Va_{CG/IIa}$ and $Va_{HNE/IIa}$) migrated on an SDS−PAGE gel in an identical fashion to factors $Va_{IIa/CG}$ and $Va_{IIa/HNE}$ (data not shown). Furthermore, generation of these cofactors resulted in a 3−5-fold increase in cofactor function such that their clotting activities correlated very well to those observed with factor $Va_{IIa/CG}$ and factor $Va_{IIa/HNE}$ (Figure 4A, factor $Va_{CG/IIa}$, closed squares; factor $Va_{HNE/IIa}$, closed triangles, inset), consistent with their identical subunit compositions. These results indicate that specific cleavage at Arg^{1545} in factor Va_{CG} and factor Va_{HNE} by thrombin is an important step in achieving partial cofactor function in a clotting-based assay. Thus, the attached B region of the cathepsin G- and elastase-treated cofactors appears to provide an inhibitory constraint such that these cofactors cannot participate fully in the assembly and function of prothrombinase under these assay conditions.

Assessment of the Cofactor Activity of the Protease-Activated Factor V(a) Species Using a Purified Component Assay. Results detailed above were performed in a complex plasma-based assay system which not only has a multitude of components but also generates only limiting amounts of factor Xa (<10 pM) during the assay (*60*), and uses a relatively undefined source of phospholipid. To understand at the molecular level why cathepsin G- or elastase-treated factor V and factor Va_{IIa} have diminished cofactor activities, we used purified human proteins and synthetic phospholipid vesicles to test the ability of the various factor V(a) species to assemble and function within prothrombinase. Using synthetic PCPS vesicles (20 μM), physiological concentrations of prothrombin (1.4 μM), rate-saturating amounts of factor Xa (10 nM), and limiting amounts of factor V(a) (0.5 nM), essentially identical rates of prothrombin activation were observed for each of the cofactor species analyzed, ~1800 nM IIa min^{-1} nM $prothrombinase^{-1}$, (data not shown). These results indicate that by using saturating amounts of factor Xa, maximal cofactor function can be achieved, suggesting that (1) depending on the assay system employed, very different levels of cofactor function may be observed and (2) the cathepsin G- and elastase-derived cofactors must be very sensitive to assay conditions that do not optimize cofactor−protein or cofactor−phospholipid interactions. Thus, assay conditions should be able to be chosen to effectively optimize functional differences between these cofactor species and factor Va_{IIa}.

Kinetic Analyses Defining the Binding Interactions between Each Factor V(a) Species and Factor Xa, PCPS, and Prothrombin. To gain additional insight into the functional differences observed with each cofactor species in the various assay systems employed, kinetic experiments were designed to examine cofactor interactions with factor Xa, phospholipid vesicles, and prothrombin. The apparent dissociation constant for complex formation between membrane-bound factor Xa and each of the membrane-bound cofactors was inferred from kinetic experiments in which the initial steady-state rate of prothrombin activation at fixed amounts of factor Xa and PCPS vesicles was determined as a function of the factor V(a) concentration. As shown in Figure 5, the apparent dissociation constants ($K_{d(app)}$) of factor Va_{IIa} (0.096 nM) and factor $Va_{IIa/CG}$ (0.244 nM) for membrane-bound factor Xa were nearly identical. In marked contrast, 10-fold more factor Va_{CG} ($K_{d(app)}$ = 1.46 nM) was required to obtain similar rates of prothrombin activation. Similar results were obtained when each of the factor V(a) species were held constant (15 pM) and initial steady-state rates of prothrombin activation were determined as a function of the factor Xa concentration (0.01−30 nM; Table 1). Thus, the decreased rate of prothrombin activation observed with limiting amounts of factor Va_{CG} or factor Xa appears to result from a reduced capacity of this cofactor to interact with factor Xa and not PCPS vesicles. To confirm these findings, kinetic experiments were performed such that rates of prothrombin activation were monitored as a function of the PCPS vesicle concentration. While factor Va_{CG} exhibited a slightly reduced ability to interact with PCPS vesicles (~2-fold increase in $K_{1/2}$, Table 1), factor $Va_{IIa/CG}$ incorporated equally well into prothrombinase relative to factor Va_{IIa} (Table 1),

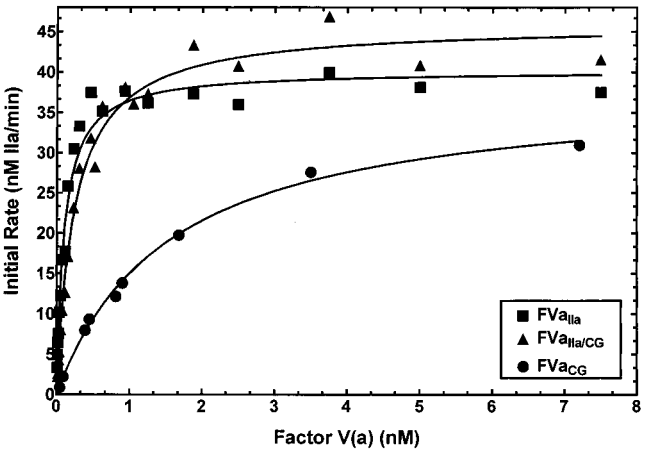


FIGURE 5: Comparison of the cofactor activities of factor Va_{IIa}, factor Va_{CG}, and factor $Va_{IIa/CG}$ in assay systems consisting of purified components. Initial rates of prothrombin activation were determined at ambient temperature at a fixed limiting concentration of factor Xa (15 pM) in 20 mM HEPES/0.15 M NaCl/5 mM $CaCl_2$/0.01% Tween-80 buffer containing 20 μM PCPS, 3 μM DAPA, and varying concentrations of the indicated factor V(a) species (factor Va_{IIa}, ■; factor $Va_{IIa/CG}$, ▲; factor Va_{CG}, ●). The reaction was initiated by addition of 1.4 μM prothrombin, and rates of thrombin generation were determined as described under Experimental Procedures. In each experiment, the titration was carried out to 30 nM factor V(a); however, for graphical purposes the data are limited to 7.5 nM cofactor concentration. The solid lines represent a nonlinear least-squares regression fit of the data as described under Experimental Procedures. The apparent dissociation constant ($K_{d(app)}$) derived from each of the titrations is listed in Table 1.

indicating no significant defect in phospholipid binding assessed by analyses of the kinetics of prothrombin activation.

Similar experimental protocols were used to investigate the ability of factors Va_{HNE} and $Va_{IIa/HNE}$ to interact with factor Xa. Just as factor Va_{CG} had a reduced ability to interact with factor Xa, so did factor Va_{HNE} (Table 1). Additionally, factor Va_{HNE} had a slightly reduced ability to interact with PCPS vesicles (~3-fold increase in $K_{1/2}$), while no significant defects in phospholipid binding were observed for factor $Va_{IIa/HNE}$ (Table 1). Thus, factor Va_{CG} and factor Va_{HNE}, both of which have extended light chains, have a reduced capacity to interact with factor Xa. This diminished binding to factor Xa appears to be fully corrected in this assay system by removing the extended 30−60 amino acids from the light chain upon cleavage at Arg^{1545} by thrombin (factor $Va_{IIa/CG}$ or factor $Va_{IIa/HNE}$). These results indicate that the importance of the cleavage site at Arg^{1545} is, at least in part, to facilitate maximum cofactor binding to factor Xa within prothrombinase, and optimization of this interaction requires removal of B domain fragments from the light chain.

We also determined the kinetic parameters of prothrombin activation (K_m and k_{cat}) for each of the factor V(a) species. Conditions were chosen such that each of the membrane-bound cofactor species was saturated with membrane-bound factor Xa. Figure 6 depicts the initial steady-state rate of thrombin formation obtained for factor Va_{IIa}, factor Va_{CG}, and factor $Va_{IIa/CG}$ as a function of the prothrombin concentration. The reactions obeyed Michaelis−Menten kinetics, and the binding isotherms were fit to the Michaelis−Menten equation using nonlinear least-squares regression analysis. Membrane-bound factor Xa−Va_{IIa}, Xa−Va_{CG}, and Xa−$Va_{IIa/CG}$ complexes were characterized by an apparent K_m for prothrombin of approximately 0.2 μM and a k_{cat} of approximately 1800−2200 nM IIa formed min^{-1} nM prothrombinase^{-1}. Similar results were obtained with each of the elastase-derived cofactors (Table 2). Interestingly, and in contrast to the clotting-based assay, factors $Va_{IIa/CG}$ and $Va_{IIa/HNE}$ consistently expressed higher k_{cat} values, suggesting that these cofactor species, at least in a purified component assay and under conditions in which all of the membrane-bound cofactor is bound to factor Xa, are more active cofactors than factor Va_{IIa}, an observation which currently lacks a molecular explanation.

APC-Catalyzed Inactivation of the Protease-Activated Factor V(a) Species. Previous reports, investigating the binding interactions between membrane-bound factor Va_{IIa} and APC, indicate that APC binds to the light chain of the cofactor (*61*). Since factor Va_{CG} and factor Va_{HNE} have extended light chains and a reduced ability to interact with factor Xa, we determined if these different light chains altered APC function. Reaction conditions were chosen such that each of the protease-activated factor V(a) species was held constant at 20 nM (the ~K_m of factor Va_{IIa} for APC (*62*)) and the reaction was initiated with catalytic amounts of APC (0.1 nM). Following the addition of APC, residual cofactor activity was monitored in a purified prothrombinase assay employing synthetic phospholipid vesicles, and the fragments derived from APC-catalyzed cleavage of factor V(a) were monitored by SDS−PAGE, followed by Western blotting analyses. No significant differences in either assay system were observed for each of the cofactor species (data not shown). These results indicate that although APC binds to the light chain of factor Va_{IIa} (*61*), it most likely does so at a site distinct from factor Xa, since each of the cofactor species examined were equivalent substrates for APC. Furthermore, cleavage at Arg^{1545} in factor V does not appear to be required for APC functional interactions.

DISCUSSION

The generation of an active factor V(a) cofactor species which functions effectively in the prothrombinase complex is absolutely essential for maintaining the normal hemostatic balance. Results from this study extend previous investigations of the cathepsin G- and elastase-mediated activation of factor V by (1) elucidating the specific cleavage sites in factor V mediated by cathepsin G and elastase, thus defining the specific factor V(a) fragments generated by these enzymes, (2) defining kinetic parameters associated with each of the factor V(a) species, (3) demonstrating that the cofactor activity observed is dependent upon the assay systems employed, and (4) providing insight into the significance of the thrombin-catalyzed cleavage at Arg^{1545}. We were able to demonstrate that although factors Va_{CG} and Va_{HNE} have subunit compositions resembling factor Va_{IIa}, they are poor cofactors with respect to their ability to interact with factor Xa. The inability of these cofactors to effectively bind factor Xa results from the extended 30−60 amino acids of the B domain which remains attached to the amino-terminal end of their light chains. We were also able to demonstrate that each of the cofactor species were similar substrates for APC, indicating that cleavage at Arg^{1545} is not required for APC functional activity against the cofactor.

Table 1: Comparison of the Functional Properties of the Various Factor V(a) Species

	FVa_{IIa}	FVa_{HNE}	$FVa_{IIa/HNE}$	FVa_{CG}	$FVa_{IIa/CG}$
subunit composition (HC/LC)[a]	105/74	102/90	102/74	103/80	103/74
$K_{d(app)\ Xa-V(a)}$ (nM)[b]	0.096 ± 0.009	0.818 ± 0.029	0.137 ± 0.010	1.459 ± 0.104	0.244 ± 0.022
$K_{d(app)\ Xa-V(a)}$ (nM)[c]	0.073 ± 0.003	0.949 ± 0.135	0.127 ± 0.016	1.453 ± 0.069	0.116 ± 0.016
$K_{1/2\ IIase}$ (μM)[d]	0.378 ± 0.048	1.168 ± 0.099	0.307 ± 0.037	0.591 ± 0.117	0.345 ± 0.045

[a] The subunit compositions of the cofactor species are given in kilodaltons; HC = heavy chain; LC = light chain. [b] Apparent dissociation constant for the membrane-bound factor Xa−V(a) complex determined at a limiting factor Xa concentration. [c] Apparent dissociation constant for the membrane-bound factor Xa−V(a) complex determined at a limiting factor V(a) concentration. [d] Half-maximal prothrombinase complex assembly for factor V(a)−factor Xa association on the phospholipid surface; see Experimental Procedures.

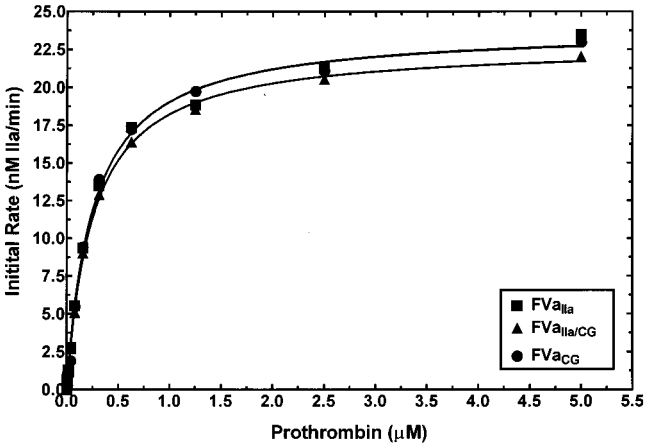


FIGURE 6: Determination of kinetic parameters for the various factor V(a) species. Initial rates of prothrombin activation were determined at ambient temperature at a fixed limiting concentration of factor Xa (10 pM) in 20 mM HEPES/0.15 M NaCl/5 mM $CaCl_2$/0.01% Tween-80 buffer containing 20 μM PCPS, 3 μM DAPA, and saturating amounts of the various factor V(a) species (30 nM) (factor Va_{IIa}, ■; factor $Va_{IIa/CG}$, ▲; factor Va_{CG}, ●). The reaction was initiated upon addition of varying concentrations of prothrombin (0.05−5.0 μM), and rates of thrombin generation were determined as described under Experimental Procedures. The solid lines represent nonlinear least-squares regression fits of the data to the Michaelis−Menten equation. K_m and V_{max} are listed in Table 2.

Assay Conditions Dictate the Amount of Cofactor Activity Expressed by Factor Va_{CG} and Factor Va_{HNE}. Results presented in Figure 1A demonstrate that factor Va_{CG} and factor Va_{HNE} have essentially no cofactor activity relative to that observed with factor Va_{IIa} in a clotting-based assay. In marked contrast, when conditions are chosen in an assay consisting of purified components such that >90% of the factor V(a) is complexed with factor Xa, no difference in cofactor function was observed between any of the cofactor species. These results support previous observations in the literature. One study investigating the activation of factor V by elastase using a one-stage assay with limiting amounts of factor Xa (*31*) demonstrated only a 2−3-fold increase in cofactor activity. In contrast, our laboratory (*32*) and others (*34*) have demonstrated that factor Va_{HNE} exhibits a similar level of cofactor activity compared with factor Va_{IIa} when employing saturating amounts of factor Xa.

The Carboxy-Terminal Region of the Factor Va_{IIa} Heavy Chain Is Essential for Expression of Maximum Cofactor Activity. Using a protease purified from the venom of *Naja naja oxiana*, which cleaves factor Va_{IIa} at Asp^{683}, generating FVa_{NO} (101 kDa heavy chain and 74/71 kDa light chain), Bakker et al. were able to demonstrate using limiting concentrations of factor Xa or prothrombin, that this novel cofactor has a 10-fold and a 4-fold reduced affinity for those proteins, respectively (*28*). Although factors $Va_{IIa/CG}$ and $Va_{IIa/HNE}$ have similar subunit compositions relative to factor Va_{NO}, we did not observe any significant defect in factor Xa binding or interactions with prothrombin. In addition, we have previously demonstrated using a protease purified from the venom of *Naja naja nigricollis*, which cleaves factor Va_{IIa} at Asp^{697} to generate factor $Va_{IIa/NN}$ (100 kDa heavy chain and 74 kDa light chain), that this cofactor species binds factor Xa with a similar affinity to that observed with factor Va_{IIa} (*63*). Defining the final cleavage sites in the heavy chains of factor $Va_{IIa/CG}$ and factor $Va_{IIa/HNE}$ (speculated to be Tyr^{696} and Thr^{678}, respectively) will be helpful in sorting out these apparent discrepancies. It is interesting to note, however, that factors $Va_{IIa/CG}$, $Va_{IIa/HNE}$, and $Va_{IIa/NN}$ (*63*) have less than optimal cofactor activities in a clotting-based assay (~35% relative to FVa_{IIa}; Figure 4). Although this apparent inconsistency remains to be explained, this observation may result from the inability of these cofactors to optimally bind to tissue thromboplastin used in the clotting assay. However, based on the current studies, binding of these cofactors to synthetic phospholipid vesicles is not compromised. An additional explanation is that the cathepsin G- and elastase-derived cofactors are more susceptible to APC-catalyzed inactivation during propagation of the clotting assay. However, we were unable to demonstrate an increase or decrease in APC sensitivity with each of the cofactors in an assay comprised of purified components. Because of the complex nature of the clotting assay, it is difficult to predict whether each of the factor V(a) species may respond differently to APC or other proteases in that setting. Thus, our data together with those on record (*28*, *63*) indicate that the acidic carboxy-terminal region (~30 amino acids) in the factor Va_{IIa} heavy chain is important for full expression of cofactor function.

Table 2: Kinetic Constants for Prothrombinase Assembly and Function

	FVa_{IIa}	FVa_{HNE}	$FVa_{IIa/HNE}$	FVa_{CG}	$FVa_{IIa/CG}$
subunit composition (HC/LC)[a]	105/74	102/90	102/74	103/80	103/74
K_m (μM)[b]	0.253 ± 0.016	0.173 ± 0.014	0.201 ± 0.013	0.253 ± 0.019	0.250 ± 0.010
k_{cat} (min^{-1})[b]	1964 ± 35	1815 ± 40	2319 ± 42	1867 ± 37	2296 ± 52

[a] The subunit compositions of the cofactor species are given in kilodaltons; HC = heavy chain; LC = light chain. [b] Kinetic parameters (K_m and k_{cat}) of prothrombin activation by the membrane-bound factor Xa−V(a) complexes.

Cleavage at Arg^{1545} Optimizes Factor Va Cofactor Function within Prothrombinase. Both factors Va_{CG} and Va_{HNE} have an extended ~30−60 amino acids of the B domain attached to the amino-terminal regions of their light chains (Figure 1B,C). Cleavage at Arg^{1545} and removal of these fragments optimize factor Xa binding within prothrombinase as determined in a purified prothrombinase assay (Table 1). Two recent studies began to address the importance of the thrombin and factor Xa cleavage sites within factor V through use of recombinant DNA techniques (*64*, *65*). Investigating the thrombin cleavage sites, these studies concluded that cleavage at both Arg^{709} and Arg^{1545} was required for full expression of cofactor function, whereas cleavage at Arg^{1018} enhanced the rate of cleavage at Arg^{1545} (*64*, *65*). In addition, it was also demonstrated using RVV-V that isolated cleavage at Arg^{1545} was sufficient for full activation of factor V (*64*). Investigating the factor Xa cleavage sites, Thorelli et al. concluded that cleavage at Arg^{1018} was not sufficient for full expression of factor Va cofactor activity (*65*). These results are in contrast to our previous investigations of factor Xa cleavage of factor V which stated cleavage at Arg^{1018} was sufficient for complete activation of factor V (*23*). Based on the results of the present study, insight into these apparent inconsistencies can be explored. Both studies, concluding that cleavage at Arg^{1545} is important for full expression of cofactor function, employed picomolar concentrations of both factor V(a) (~20 pM) and factor Xa (~20−60 pM) in their respective prothrombinase assays (*64*, *65*). Using these nominal concentrations of enzyme and cofactor, the rate of thrombin generation will be very sensitive to changes in the membrane-bound factor Va−factor Xa dissociation constant. Thus, under those limiting protein concentrations, a factor V molecule that cannot be cleaved at Arg^{1545} will appear to have a reduced cofactor activity relative to that observed with factor Va_{IIa}. Alternatively, in our previous investigations (*23*), nanomolar concentrations of factor Xa (1−5 nM) and factor V(a) (~0.5 nM) were employed; therefore, rates of thrombin generation will be less sensitive to changes in the membrane-bound factor V(a)−factor Xa dissociation constant. Thus, using protein concentrations which approach saturation of the membrane-bound factor V(a)−factor Xa complex will lead to different conclusions about the importance of cleavage at Arg^{1545}, since in those assay systems the rates of prothrombin activation will not significantly differ between factor Va_{IIa} and factor V(a) which cannot be cleaved at Arg^{1545}. This is perhaps best illustrated in Figure 5 and Table 1. Employing assay conditions that were less than optimal for factor Va_{CG} and factor Xa (i.e., limiting conditions) results in significant differences in the rate of prothrombin activation relative to that observed with factor Va_{IIa}. However, employing conditions that approach saturation of the membrane-bound FVa_{CG}−factor Xa complex results in essentially the same rate of prothrombin activation as that observed with factor Va_{IIa}. Thus, we can conclude that at limiting factor Xa−factor Va concentrations, such as during the initiation of coagulation (*66*), cleavage at Arg^{1545} and removal of any B domain fragment are important for full cofactor function. However, once higher concentrations of factor Xa become available, possibly during the propagation phase of coagulation (*66*), cleavage at Arg^{1545} may be less important.

Physiological Significance of the Cathepsin G- and Elastase-Mediated Cleavage of Factor V. Because of the high concentrations of plasma protease inhibitors such as α_1-anti-protease inhibitor, α_2-macroglobulin and α_1-antichymotrypsin, cathepsin G and elastase would most likely be present only in very small amounts (<nM) in plasma. However, recent studies have suggested that these proteases are present in an active form, resistant to protease inhibitors, on the surface of unstimulated and stimulated human neutrophils (*67*) and on monocytes (*32*). These results suggest that at extravascular tissue sites or at other sequestered sites, cathepsin G and elastase may be very effective in regulating thrombin generation through the activation of factor V. Further support for the significance of these reactions comes from the observation that platelets and PMN leukocytes can interact with one another through the α-granule protein P-selectin (*68*). P-selectin-mediated PMN leukocyte−platelet adhesion events may result in a sequestered microenvironment between cell membranes protecting both cathepsin G and elastase from protease inhibitors, allowing for activation of platelet-derived factor V/Va released from platelet α-granules. The physiological significance of these events remains to be established. However, it is intriguing to note not only that cathepsin G will activate factor V but also that this protease can also activate platelets (*68*), potentially leading to the release of platelet-derived factor V/Va. Thus, these various cellular contacts may provide a unique environment to allow proteases such as cathepsin G and elastase to regulate thrombin generation through activation of clotting factors and platelets. However, these same molecular processes if left unregulated may also lead to thrombosis, inflammation, and atherosclerosis.

Summary. The data presented in this report indicate that both cathepsin G and elastase cleave factor V to generate cofactor species with variable amounts of cofactor activity, depending on the assay system employed and the amounts of factor Xa in that assay system. In addition, we were able to demonstrate that at limiting concentrations of factor V(a) and factor Xa, cleavage at Arg^{1545} optimizes cofactor function within prothrombinase. Our results also support the notion that at least one of the functions of the B domain is to prevent expression of factor V(a) activity prior to proteolysis by blocking factor Xa binding to the light chain of factor Va, where we have previously identified a factor Xa binding site in the A3 domain (*69*). Thus, assessment of the cleavage requirements for the generation of a factor V(a) species which expresses full activity appears to be dependent upon how that cofactor molecule interacts with the various constituents of prothrombinase, and these apparent requirements will depend on the assay system employed.

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