Functional Properties of Recombinant Factor V Mutated in a Potential Calcium-Binding Site†

Kristoffer W. Sørensen,‡,§ Gerry A. F. Nicolaes,§ Bruno O. Villoutreix,|| Tomio Yamazaki,‡,⊥ Guido Tans,§ Jan Rosing,§ and Björn Dahlbäck*,‡

Department of Clinical Chemistry, Lund University, Malmö University Hospital, Malmö, Sweden, Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht, The Netherlands, INSERM U428, University of Paris V, 4 Avenue de l'Observatoire, Paris, France, and Department of Hemostasis and Thrombosis, Clinical Research Center, Nagoya Medical Center, Nagoya, Japan

Received November 27, 2003; Revised Manuscript Received March 12, 2004

ABSTRACT: Activated coagulation factor V (FVa) is a cofactor of activated factor X (FXa) in prothrombin activation. FVa is composed of a light chain (LC) and a heavy chain (HC) that are noncovalently associated in a calcium-dependent manner. We constructed a recombinant FV Asp111Asn/Asp112Asn mutant (rFV-NN) to abolish calcium binding to a potential calcium-binding site in FVa in order to study the specific role of these residues in the expression of FVa activity. Whereas thrombin-activated recombinant FV wild type (rFV-wt) presented with stable FVa activity, incubation of rFV-NN with thrombin resulted in a temporary increase in FVa activity, which was rapidly lost upon prolonged incubation. Loss of FVa activity was most likely due to dissociation of HC and LC since, upon chromatography of rFVa-NN on a SP-Sepharose column, the HC did not bind significantly to the resin whereas the LC bound and could be eluted at high ionic strength. In contrast, rFVa-wt adhered to the column, and both the HC and LC coeluted at high ionic strength. In the presence of phospholipid vesicles, the loss of rFVa-NN activity was partially prevented by FXa, active site inhibited FXa, and prothombin in a dose-dependent manner. We conclude that the introduced amino acid substitutions result in a loss of the high-affinity (calcium-dependent) interaction of the HC and LC of FVa. We propose that the introduced substitutions disrupt the calcium-binding site in FV, thereby yielding a FV molecule that rapidly loses activity following thrombin-catalyzed activation most likely via dissociation of the HC and LC.

Activated human blood coagulation factor V (FVa) is the essential nonenzymatic cofactor in activated factor X (FXa) catalyzed activation of prothrombin that occurs during blood coagulation (*1*). The presence of FVa, calcium ions, and a procoagulant phospholipid surface increases the catalytic efficiency (k_{cat}/K_m) of FXa toward prothrombin more than 300000-fold (*2*, *3*), which in effect renders prothrombin activation subject to downregulation via inactivation of FVa through the activated protein C pathway (reviewed in refs *1* and *4*).

Factor V (FV), the precursor of FVa, is a single chain plasma glycoprotein with a domain structure (A1-A2-B-A3-C1-C2) similar to that of factor VIII (FVIII) (*5*−*7*). Activation of FV is catalyzed by thrombin and occurs through proteolysis of specific peptide bonds resulting in dissociation of the B-domain and formation of the FVa heterodimer composed of a heavy chain (A1-A2) and light chain (A3-C1-C2) (*8*). The FVa heavy chain (HC) and light chain (LC) are noncovalently associated in a divalent metal ion-dependent manner (*8*−*11*).

FV and FVa have been reported to bind the metal ions calcium and copper (*8*, *9*, *12*), but the binding sites for these ions remain to be identified. One high-affinity calcium-binding site has been reported in bovine FV ($K_d < 1.1 \times 10^{-8}$ M) and in bovine FVa [$K_d = (24 \pm 4.0) \times 10^{-6}$ M] (*8*), whereas the isolated bovine HC and LC have been reported not to bind calcium (*10*). The calcium ion in FV and FVa readily exchanges with calcium ions in solution and is removable with chelating agents such as EDTA, which cause dissociation of the FVa heterodimer and a rapid loss of FVa activity (*8*, *9*). Functional FVa can be reconstituted from chelated FVa or isolated HC and LC by the addition of calcium ions (*8*, *10*).

The FV A-domains share ∼40% amino acid sequence identity with the copper-binding plasma protein ceruloplasmin (CP) (*5*−*7*). The resolution of the crystal structure of CP (*13*) has facilitated comparative modeling of the FV A-domains and analysis of the three-dimensional model for potential calcium-binding sites (*14*, *15*). Protein-bound calcium ions are often coordinated by five or more oxygen atoms present in residues adjacent in the primary protein

† This work was supported by Marie Curie Training Grant QLK5-CT-2000-60007 (K.W.S.), Grant 902-26-227 from the Dutch Organization for Scientific Research (NWO, G.A.F.N.), grants from the Swedish Research Council (07143), Söderberg's Foundation, Österlund's Foundation, and Påhlsson's Trust, and research funds from Malmö University Hospital.

* To whom correspondence should be addressed. Phone: (+46) 40-331501. Fax: (+46) 40-337044. E-mail: bjorn.dahlback@klkemi.mas.lu.se.

‡ Lund University.

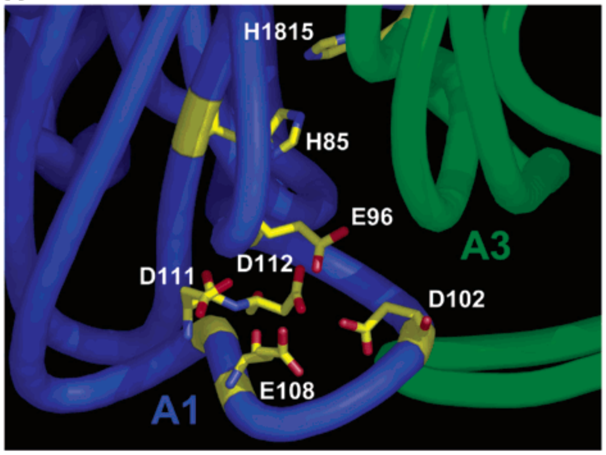
§ Cardiovascular Research Institute Maastricht.

|| University of Paris V.

⊥ Nagoya Medical Center.

10.1021/bi0361362 CCC: $27.50
Published on Web 04/24/2004

A



B

```
                           1    1    1  11             22                 3   32    3
Human FV:          93  KLSEGASYLDHTFPAEKMDDAVAP  146  SHENLIED  1570  QRETDIEDSDDIPEDT
Bovine FV:         93  KFSEGASYSDHTLPMEKMDDAVAP  146  SYVNLVED  1561  QSD----DVDYVPEDT
Murine FV:         92  KFSEGASYADHTFPAERKDDAVAP  145  SYENLTQD  1530  QSEMDHEDTGHTPKDT
Porcine FV:        93  KFAEGASYPDHTFLVEKMDDAVAP  146  SYENLIQD  1608  QRE----DIDDVPEHT
Human FVIII:      107  KASEGAEYDDQTSQREKEDDKVFP  160  SHVDLVKD  1714  SSPHVLRNRAQSGSVP
Murine FVIII:     108  KASEGDEYEDQTSQMEKEDDKVFP  161  SHVDLVKD  1684  TS-HVLRNRYQSDNVP
Porcine FVIII:    108  KSSEGAEYEDHTSQREKEDDKVLP  161  SHVDLVKD  1496  ESPRALRNRAQNGEVP
P. textilis FV:    85  KWSEGSSYSDGTSDVERLDDAVPP  138  SHVNMVRD   805  KSQ-----VRSRAAKT
D. rerio FV:      112  KQSEGSLYFDNTSLLEKNDDVIQP  165  SHFDIVRD  1466  QRR--LDKTAQNERPT
Human CP:         109  KEHEGAIYPDNTTDFQRADDKVYP  162  SHIDAPKD        ----------------
```

FIGURE 1: Potential calcium-binding site in human FV. (A) Polypeptide Cα trace (ribbons) of the A1 domain (blue) and A3 domain (green) of the human FV A-domain model rendered with the software PyMol (*37*). The residues potentially involved in calcium binding in the A1 domain (Glu96, Asp102, Glu108, Asp111, and Asp112) and the proposed copper-binding site (His85 and His1815) are presented as sticks with the following atom coloring code: oxygen, red; carbon, yellow; nitrogen, blue. (B) Amino acid sequence alignment of human FV (NCBI accession number P12259) and FVIII (P00451), bovine FV (Q28107), murine FV (NP_032002) and FVIII (Q06194), porcine FV (AAG28381) and FVIII (P12263), human CP (P00450), *P. textilis* pseutarin C nonenzymatic subunit (*30*), and *Danio rerio* FV-like protein (CAC94896). Multiple amino acid sequence alignment was performed using CLUSTAL W (version 1.83). Amino acid numbering refers to the position in the (predicted) mature protein. The numbers above the sequence indicate the residues suggested to be involved in the potential calcium-binding sites in the human FV A1 domain (1), the A1 and A3 domain (2), and the A3 domain (3).

structure with an average oxygen−calcium distance of ∼2.4 Å (*16*). On the basis of the FV A-domain model a potential calcium-binding site has been suggested to involve some or all of the following residues: Glu96, Asp102, Glu108, Asp111, and Asp112, which are located in the A1 domain at the A1−A3 domain interface (see Figure 1) (*14*). Different calcium-binding sites have been proposed (*15*), one involving residues Glu148, Asn149 in the A1 domain, and Asp1577 in the A3 domain and the other comprising Glu1572, Glu1576, and Glu1583 in the A3 domain (see Figure 1B).

A protein sequence alignment of FV and FVIII from several different species as well as two FV-like proteins and human CP is presented in Figure 1B. The residues proposed to be involved in the potential calcium-binding site in the FV A1 domain (region 96−112) are completely conserved in all aligned sequences except CP. In an attempt to abolish calcium binding to this potential calcium-binding site we have used site-directed mutagenesis to substitute Asp for Asn at positions 111 and 112 in human FV, thereby removing two side-chain oxygen atoms from the proposed binding site.

Assessment of expressable FVa activity of the recombinant human FVa Asp111Asn/Asp112Asn (rFVa-NN) and wild type (rFVa-wt) was used to study the stability of the FVa heterodimer in solution and in the presence of components of the prothrombinase complex. Dissociation of the FVa heterodimer was probed using radiolabeled recombinant FV and ion-exchange chromatography followed by immunoprecipitation, SDS−PAGE,[1] and quantification of the HC and LC by autoradiography.

From the data presented we conclude that the amino acid substitutions introduced in a highly conserved potential calcium-binding site result in loss of the high-affinity (calcium-dependent) interaction of the HC and LC of FVa. Because the substitutions are structurally tolerated, we propose that they disrupt the calcium-binding site in FV, thereby yielding a FV molecule that rapidly loses activity following thrombin-catalyzed activation most likely via dissociation of the HC and LC.

EXPERIMENTAL PROCEDURES

Materials. Hepes and ovalbumin were purchased from Sigma, and acid-free bovine serum albumin was obtained from ICN Biomedicals (Aurora, OH). Natural L-α-phosphatidylcholine (chicken egg) and L-α-phosphatidylserine (porcine brain) extracts were from Avanti Polar Lipids (Pelham, AL). DMEM, OptiMEM GlutaMax, and Cys/Met-free DMEM were purchased from Gibco (Paisley, Scotland). Redivue Pro-Mix ^{35}S cell labeling mix, PD-10 columns, and protein A−Sepharose were from Amersham (Uppsala, Sweden). Fast-flow SP-Sepharose, diethylaminoethyl- (DEAE-) dextran, and Hoefer electrophoresis equipment were purchased from Pharmacia (Uppsala, Sweden). Restriction enzymes *Sfi*I and *Xcm*I were from New England Biolabs (Beverly, MA), and DNA ligation kit 2 was from Takara Shuzo Co. (Otsu, Japan). The chromogenic substrates S2238 and S2765 were kindly provided by Chromogenix (Milano, Italy), and the micro-BCA protein assay reagent kit was from Pierce (Rockford, IL). 1,5-DNS-GGACK was obtained from Calbiochem (San Diego, CA), and PefaBloc TH was purchased from Kordia Laboratory Supplies (Leiden, The Netherlands). Prestained molecular weight standards were from Bio-Rad (Hercules, CA).

Proteins. Human prothrombin was purified according to the method described by Stenflo for bovine prothrombin (*17*) and activated with the Taipan snake venom (*18*). Thrombin was purified by chromatography on SP-Sepharose essentially as described by Lundblad et al. (*19*). Recombinant hirudin and human FXa were purchased from Kordia Laboratory Supplies (Leiden, The Netherlands), and 1,5-DNS-GGACK-FXa (DEGR-FXa) was obtained as described previously (*20*). Molar thrombin and FXa concentrations were determined by active site titration with *p*-NPGB as described previously (*21*, *22*).

Mutagenesis of Human FV cDNA. The recombinant FV variant Asp111Asn/Asp112Asn was created by PCR-based site-directed mutagenesis of the eukaryotic expression vector pMT2-V using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two complementary oligonucleotides with the desired mutation were used, the sequence of

[1] Abbreviations: BSA, bovine serum albumin; DEGR-FXa, factor Xa inhibited with 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone; *p*-NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate hydrochloride; PAGE, polyacrylamide gel electrophoresis; S2238, D-Phe-(pipecolyl)-Arg-pNA.

the sense oligo being 5′-GCGGAGAAGATGAACAACGCT-GTGGCTCCAGG-3′. A 2.0 kb restriction fragment was isolated after digestion with *Sfi*I and *Xcm*I and used to replace the corresponding wild-type fragment in the pMT2-V vector. The presence of only the desired mutation was confirmed by DNA sequencing.

Transient Expression and Radiolabeling of Recombinant FV. Recombinant FV was transiently expressed in COS-1 cells using the DEAE-dextran method described previously (*23*, *24*) with minor modifications. Briefly, cells were transfected for 5 h in a mixture containing 2 μg/mL expression vector in DMEM with 0.5 mg/mL DEAE-dextran, 100 mM Tris (pH 7.3), and 2 mM glutamine. Forty-eight hours after transfection the culture medium was replaced with serum-free OptiMEM GlutaMax supplemented with 2.5 mM $CaCl_2$, 0.1 mg/mL BSA, 50 IU/mL penicillin, and 50 μg/mL streptomycin. The conditioned medium was harvested 72 h after transfection and centrifuged at 1500*g* for 5 min to remove cell debris. FV expression levels were determined using a commercial FV ELISA kit (Kordia Laboratory Supplies, Leiden, The Netherlands) according to the manufacturer's instructions. FV antigen levels were read from a calibration curve made with pooled human plasma assuming that full plasma contains 7 μg/mL FV.

Radiolabeling of recombinant FV was done as described above except that 48 h after transfection the culture medium was replaced with Cys/Met-free DMEM supplemented with 50 μCi/mL [^{35}S]Met and [^{35}S]Cys, 2 mM glutamine, 2.5 mM $CaCl_2$, 0.1 mg/mL BSA, 50 IU/mL penicillin, and 50 μg/mL streptomycin.

Factor Va Assay. Factor Va activity was determined by quantification of the effect of FVa on the rate of FXa-catalyzed prothrombin activation as described previously (*25*). Briefly, prothrombin activation was allowed to proceed for a fixed period of time (usually 1 min) at 37 °C in a reaction mixture containing 0.5 μM prothrombin, a limiting amount of FVa ($\leq$20 pM), 5 nM FXa (or other amounts if indicated in the figure legend), 40 μM phospholipid (10:90 molar ratio of phosphatidylserine to phosphatidylcholine), 0.5 mg/mL ovalbumin, 150 mM NaCl, and 2 mM $CaCl_2$ in 25 mM Hepes (pH 7.9). Where mentioned, the reaction mixture contained a reversible thrombin inhibitor (2 μM PefaBloc TH) to prevent FV activation by thrombin generated in the FVa assay mixture. The rates of prothrombin activation were determined with the chromogenic substrate S2238 (*3*).

Ion-Exchange Chromatography of FVa. Conditioned medium containing radiolabeled recombinant FV was concentrated 5-fold on a Vivaspin 100 kDa molecular mass cutoff concentrator (Vivascience, Gloucestershire, U.K.) and activated with 50 nM α-thrombin for 20 min at 37 °C. The sample was loaded on a SP-Sepharose resin (1 mL) equilibrated in 25 mM Hepes (pH 7.5) containing 10 mM $CaCl_2$ (buffer A). The resin was washed with five column volumes of buffer A containing 0.1 mg/mL BSA (buffer B) and eluted with five column volumes of buffer B containing 1 M NH_4Cl.

Immunoprecipitation of FVa. To quantify the relative amounts of HC and LC in fractions from the ion-exchange chromatography described above, FV was immunoprecipitated essentially as described previously (*26*). Briefly, all column fractions (1 mL) were supplemented with 0.5% Nonidet P-40 (v/v) and 0.1% SDS (w/v), and the ionic strength was adjusted to $I = 0.322$ M. Samples were added to protein A−Sepharose and excess amounts of an in-house-generated rabbit anti-human FV polyclonal antibody (no. 8806) sufficient to precipitate all FV in each sample. Following overnight incubation at 4 °C with end-over-end turning the samples were centrifuged at 20000*g* for 1 min. Precipitates were washed three times with 10 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40 (v/v), and 0.1% SDS (w/v), after which bound protein was eluted by boiling for 10 min in 3 × SDS sample buffer containing 2% β-mercaptoethanol. Samples were centrifuged at 20000*g* for 1 min, and the supernatants were subjected to 7.5% SDS−PAGE. A PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the software ImageQuant were used to quantify the radioactivity of the bands on the gels.

Table 1: Expression Levels and Functional Analysis of Recombinant FV[a]

FV construct	antigen (ng/mL)	rate of prothrombin activation: FV activation in the FVa assay (s^{-1})	rate of prothrombin activation: FV activation in solution (s^{-1})
wild type	514 ± 11	93.0 ± 5.9	106 ± 7.2
Asp111Asn/Asp112Asn	494 ± 32	41.6 ± 1.5	3.5 ± 0.2

[a] FV concentrations were determined using an ELISA, and FVa activities were determined using the chromogenic FVa assay as described in Experimental Procedures. FVa activity is expressed as moles of prothrombin activated per second per mole of FVa calculated assuming 1 ng/mL ~ 3.03 pM FV. FVa activity was measured following FV activation either in the presence of all FVa assay components (i.e., in the presence of 2 mM $CaCl_2$, 5 nM FXa, 40 μM phospholipid, and 0.5 μM prothrombin) or in solution in which case FV was incubated with 10 nM α-thrombin for 10 min at 37 °C in 25 mM Hepes (pH 7.9), 150 mM NaCl, 5 mM $CaCl_2$, and 5 mg/mL BSA, after which FVa activity was determined in the FVa assay. Data are averages ± the standard deviation of five independent expressions.

RESULTS

Expression and Characterization of the Recombinant FV Mutant. To study the functional properties of FV mutated in a potential calcium-binding site, rFV-NN and rFV-wt were transiently expressed in COS-1 cells. Antigen levels in conditioned medium collected 72 h after transfection were determined by ELISA and are presented in Table 1. The expression level of both rFV-wt and rFV-NN was approximately 500 ng/mL ($n = 5$), indicating that the introduced amino acid substitutions do not impair the intracellular processing and secretion of the mutant FV molecule and suggest proper folding of the mutant molecule.

FVa activity was determined after activation of rFV with thrombin and quantification of the effect of rFVa-wt or rFVa-NN on the rate of FXa-catalyzed prothrombin activation. For both rFVa-NN and rFVa-wt, prothrombin activation was consistently found to be a linear function of time throughout the period (20 min) during which FVa activity was monitored in the FVa assay (data not shown). However, for rFVa-NN we observed a marked difference in FVa activity, depending on whether FV activation was performed by a 10 min incubation with thrombin in a separate calcium-containing activation mixture or whether activation occurred in the FVa assay mixture (i.e., in the presence of FXa, prothrombin, calcium ions, and phospholipid vesicles). Table 1 shows the

rates of prothrombin activation obtained for both conditions of FV activation. The FVa activity of rFVa-wt activated in solution (106 ± 7.2 s^{-1}) was similar to the activity obtained after activation in the FVa assay (93.0 ± 5.9 s^{-1}), and the turnover numbers obtained are in good agreement with the turnover number (100 s^{-1}) on record (*27*). In contrast, the FVa activity of rFVa-NN following 10 min of activation in solution was 30-fold lower (3.5 ± 0.2 s^{-1}) whereas this activity was 45% of wild type (41.6 ± 1.5 s^{-1}) when activation of rFV-NN was allowed to occur in the FVa assay.

Time Course of rFV Activation in Solution. The observation that rFVa-NN has substantially reduced FVa activity following activation in solution prompted us to investigate the time course of FV activation in solution. To this end rFV-NN and rFV-wt were activated using a low concentration of thrombin, and the generation of FVa activity with time was monitored at various time points by transferring aliquots from the activation mixture into the FVa assay mixture. In this experiment 2 μM reversible thrombin inhibitor PefaBloc TH was present in the FVa assay mixture to prevent activation of rFV-wt or rFV-NN by thrombin generated during the assay. As can be seen from Figure 2A, incubation with thrombin caused an increase in FVa activity for both rFV-wt and rFV-NN. However, whereas the FVa activity of rFV-wt gradually increased to reach a stable plateau after 15 min of activation, corresponding to complete rFV activation, FVa activity of rFV-NN increased only transiently during the first 2 min of activation and then rapidly declined. Furthermore, we observed that the profile of thrombin-catalyzed rFV-NN activation in solution was dependent on the concentration of thrombin used for activation and that the maximal FVa activity determined increased with increasing concentrations of thrombin (see Figure 2B). As can be seen from Figure 2B, the rate of rFV-NN activation as well as the rate at which rFVa-NN activity is lost is enhanced by increasing concentrations of thrombin present in the activation mixture. In a control experiment the irreversible thrombin inhibitor hirudin was added to the activation mixture before activation of rFV-wt or rFV-NN was complete. The addition of hirudin completely blocked further activation of rFV-wt (Figure 2C, inset), indicating that thrombin activity toward rFV was efficiently inhibited after addition of hirudin to the activation mixture. The inhibition of thrombin by hirudin was confirmed using the thrombin-specific chromogenic substrate S2238 (data not shown). Addition of an equal amount of hirudin likewise inhibited further activation of rFV-NN, but it did not prevent the loss of rFVa-NN activity (Figure 2C). From this, combined with the fact that HC and LC fragments similar to those obtained with rFVa-wt are formed (see below), we conclude that the observed loss of rFVa-NN activity does not result from further cleavage of rFVa-NN by thrombin.

Dissociation of the rFVa Heterodimer. To assess the structural integrity of the rFVa-NN heterodimer following thrombin-catalyzed activation in solution, radiolabeled rFVa was subjected to cation-exchange chromatograpy on a SP-Sepharose column in the presence of calcium. As can be seen in Figure 3, thrombin-catalyzed activation of rFV-wt (Figure 3A, lane 1) and rFV-NN (Figure 3B, lane 1) gave rise to activation fragments of identical molecular mass with the B-domain (150 kDa), HC (105 kDa), and LC (74 kDa) being clearly visible. As shown in Figure 3A,C the LC of rFVa-wt adhered completely to the column, and the majority of the HC coeluted with the LC at high salt (Figure 3A, lanes 8 and 9). Likewise, the LC of rFVa-NN adhered completely to the resin (Figure 3B,D), but the majority of the rFVa-NN

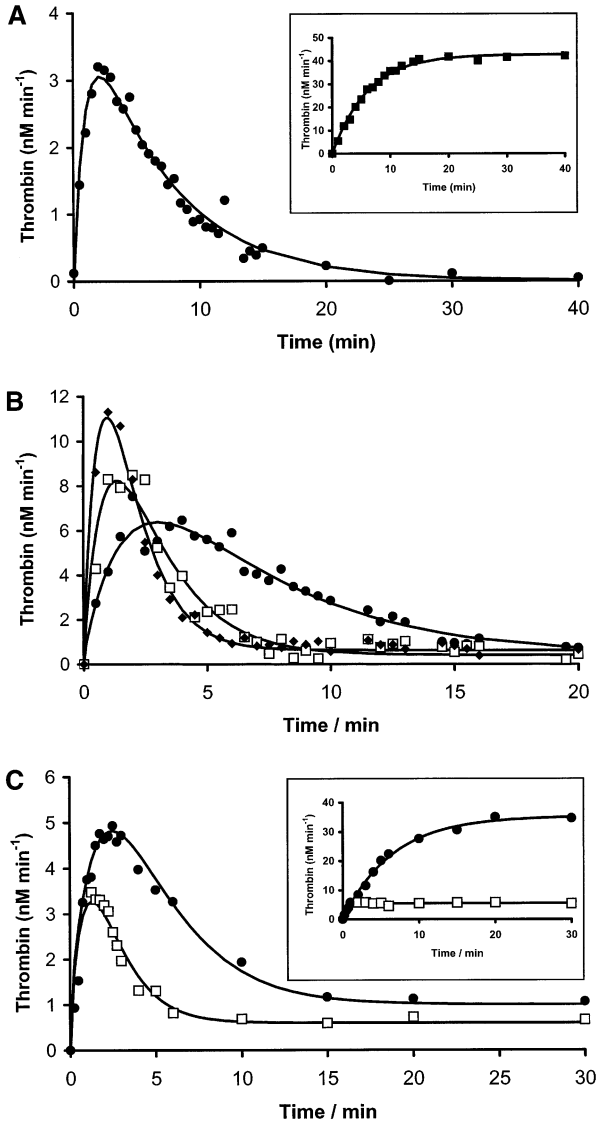


FIGURE 2: Time course of thrombin-catalyzed rFV activation. (A) rFV-NN (149 pM) and rFV-wt (144 pM, inset) were activated by 0.5 nM human α-thrombin at 37 °C in 25 mM Hepes (pH 7.9), 150 mM NaCl, 5 mM $CaCl_2$, and 5 mg/mL BSA. At the indicated time points samples of the rFV-NN (●) and rFV-wt (■, inset) activation mixtures were diluted 10-fold into FVa assay mixtures containing 2 μM PefaBloc TH and 0.5 nM FXa, and the rates of prothrombin activation were quantified. The plotted data are from one experiment representative of three independent time courses of rFV activation. (B) rFV-NN (156 pM) was activated by 0.5 nM (●), 1.0 nM (□), or 2.0 nM (▲) human α-thrombin at 37 °C in 25 mM Hepes (pH 7.9), 150 mM NaCl, 5 mM $CaCl_2$, and 5 mg/mL BSA. At the indicated time points the FVa activity present in the rFV-NN activation mixtures was determined as described above. (C) rFV-NN (149 pM) and rFV-wt (144 pM, inset) were activated by 0.8 nM human α-thrombin at 37 °C in 25 mM Hepes (pH 7.9), 150 mM NaCl, 5 mM $CaCl_2$, and 5 mg/mL BSA. Following 65 s of activation aliquots of the activation mixtures were diluted 2-fold into 2 nM hirudin (□) or 0.8 nM human α-thrombin (●) in 25 mM Hepes (pH 7.9), 150 mM NaCl, 5 mM $CaCl_2$, and 5 mg/mL BSA. At the indicated time points the FVa activity present in the rFV-NN activation mixtures (corrected for 2-fold dilution) was determined as described above.

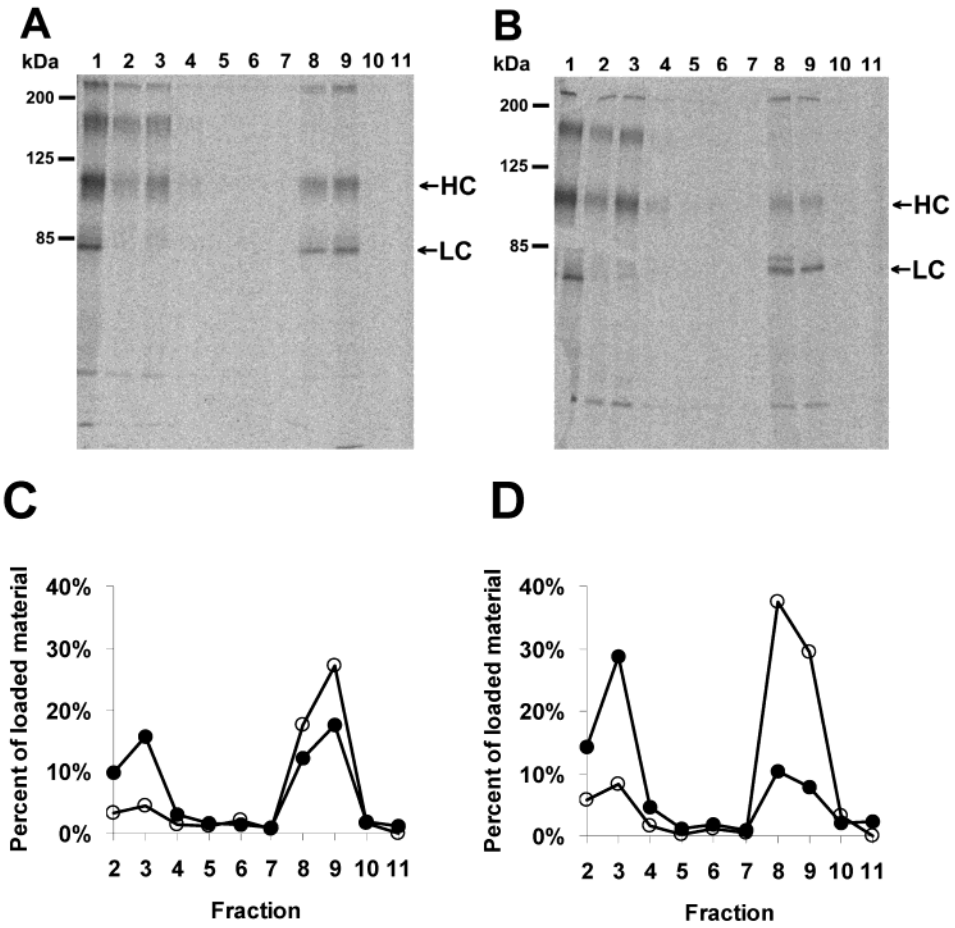


FIGURE 3: Copurification of FVa HC and LC in the presence of calcium ions. (A, B) Radiolabeled rFV-wt (A) and rFV-NN (B) were activated by thrombin and chromatographed on a SP-Sepharose column as described in Experimental Procedures. Fractions were immunoprecipitated, and precipitated proteins were separated by 7.5% SDS−PAGE and visualized by autoradiography. Activated FV was loaded in lane 1, the five wash fractions were loaded in lanes 2−6, and lanes 7−11 contain the five elution fractions. (C, D) The radioactivity of the 105 kDa fragment (HC, ●) and the 74 kDa fragment (LC, ○) of rFVa-wt (C) and rFVa-NN (D) from the gels in (A) and (B) were quantitated as described in Experimental Procedures and expressed as a percent of the radioactivity in the starting material (lane 1). In the case of both rFVa-wt and rFVa-NN, thrombin adhered to the column and coeluted with the LC at high salt.

HC was recovered in the wash fractions (Figure 3B, lanes 2 and 3) with a smaller fraction coeluting with the LC at high salt (Figure 3B, lanes 8 and 9). Since SP-Sepharose only binds the LC, copurification of HC and LC in the case of rFVa-wt indicates that the HC remained associated with the LC in the presence of calcium ions. In contrast to rFVa-wt, the HC and LC of rFVa-NN are to a large extent recovered in separate fractions. This shows that the rFVa-NN heterodimer dissociates despite the presence of calcium ions.

Stability of rFVa-NN in the Presence of Phospholipid and FXa or Prothrombin. Prothrombin activation in the presence of rFVa-NN was consistently found to be a linear function of time in the FVa assay, indicating that the rFVa-NN heterodimer was stable in the presence of FXa, prothrombin, and phospholipid. To identify the stabilizing component(s), we activated rFV-NN in the presence of phospholipid and various concentrations of FXa (Figure 4A), active site inhibited FXa (DEGR-FXa, Figure 4B), or prothrombin (Figure 4C,D). FVa activity was determined at different time points using the FVa assay as described. Increasing concentrations of FXa partially prevented the loss of rFVa-NN activity, but even the highest concentration of FXa (5 nM) did not completely stabilize the activity of rFVa-NN (Figure 4A). Approximately 40% of rFVa-NN activity was lost 19.5 min after activation in the presence of 5 nM FXa whereas a similar fraction of activity was lost only 1.5 min after activation in the absence of FXa. A similar protection of the loss of activity of thrombin-activated rFVa-NN was observed when the same experiment was performed with DEGR-FXa (Figure 4B). This indicates that the active site of FXa is not required for stabilization of rFVa-NN.

In the presence of phospholipid, prothrombin also (partially) prevented the loss of rFVa-NN activity in a dose-dependent manner (Figure 4C), and this effect of prothrombin was also observed in the presence of phospholipid and 5 nM DEGR-FXa (Figure 4D). However, the concentrations of prothrombin required to partially prevent the loss of rFVa-NN activity were much higher than the concentrations of FXa required for a comparable stabilization. Furthermore, the stabilizing effect of FXa and DEGR-FXa on rFVa-NN activity was only observed in the presence of phospholipid vesicles, but phospholipid by itself did not prevent the loss of rFVa-NN activity (data not shown).

DISCUSSION

Several reports have shown that the association of FVa HC and LC to form functional FVa is dependent on calcium

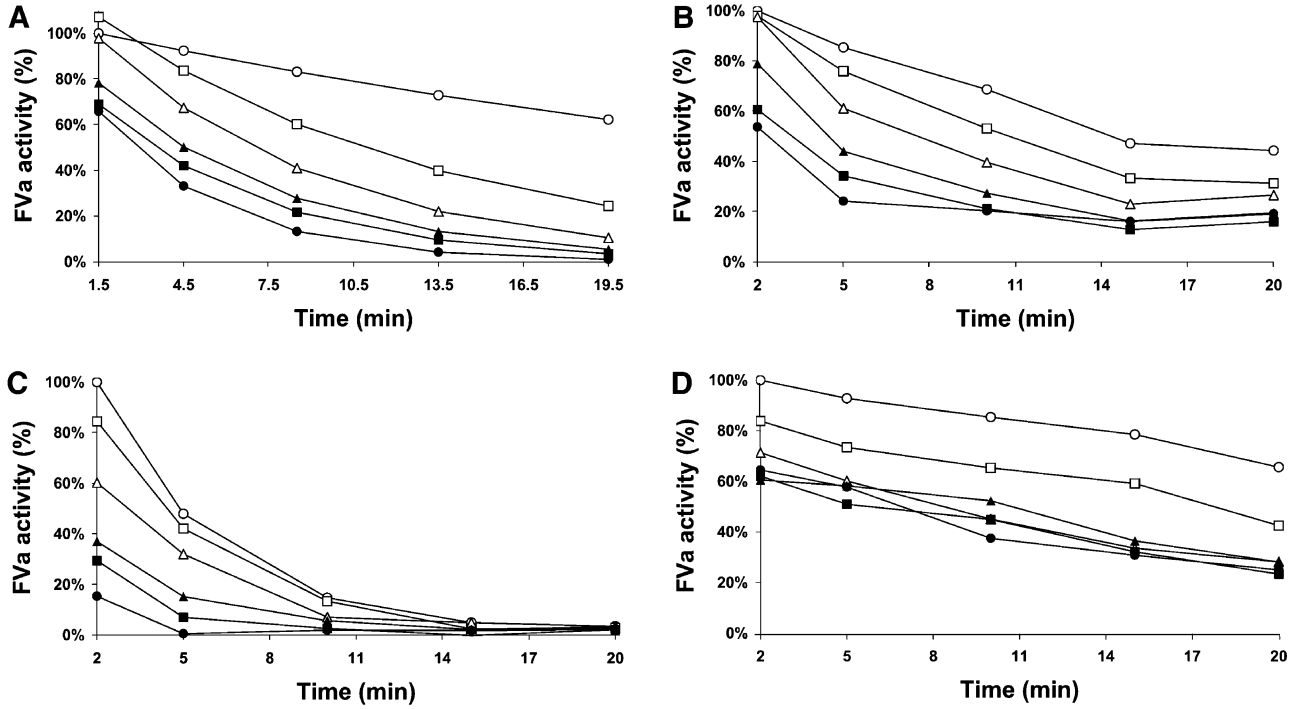


FIGURE 4: Stability of rFVa-NN activity in the presence of phospholipid and FXa or prothrombin. rFV-NN (156 pM) was incubated with 40 μM phospholipid (10:90 molar ratio of phosphatidylserine to phosphatidylcholine) and various concentrations of FXa (A), DEGR-FXa (B), prothrombin (C), or prothrombin and 5 nM DEGR-FXa (D) exactly 1 min prior to activation with 10 nM α-thrombin at 37 °C. At the indicated time points (after activation) samples were diluted 10-fold into the FVa assay, and FVa activity was determined by quantifying the rates of prothrombin activation as described. The concentrations of FXa (A) or DEGR-FXa (B) were 5 nM (○), 1 nM (□), 0.5 nM (△), 0.2 nM (▲), 0.1 nM (■), and no FXa or DEGR-FXa (●). The concentrations of prothrombin in (C) were 5 μM (○), 2 μM (□), 1 μM (△), 0.5 μM (▲), 0.1 μM (■), and no prothrombin (●). In (D) the concentrations of prothrombin used were 1 μM (○), 0.5 μM (□), 0.2 μM (△), 0.1 μM (▲), 0.05 μM (■), and no prothrombin (●). FVa activity is expressed as a percentage of the activity determined after 1.5 (A) or 2 min (B−D) of activation.

ions (*8*−*11*). The experiments presented in this paper were designed to gain insight into the functional consequences of disrupting a potential calcium-binding site in FV (*28*).

On the basis of the theoretical model of the FV A-domains (*14*) residues Glu96, Asp102, Glu108, Asp111, and Asp112 have been proposed to form a calcium-binding site within the A1 domain, next to the A1−A3 interface. It was suggested, on the basis of structural analysis of the FVa model, that calcium would lock the conformation of this region in a manner that would allow favorable interactions with the FV A3 domain while, in the absence of calcium, some structural changes in amino acid side chains and backbone atoms would take place, creating clashes with the FVa LC. Considering the present data discussed below and data on record (*29*), this seems to be the most likely scenario. As can be seen from the sequence alignment (Figure 1B) all mentioned residues are completely conserved in all presently available protein sequences of FV and the homologous FVIII as well as in FV-like proteins from zebra fish and the eastern brown snake *Pseudonaja textilis* (*30*). This indicates an evolutionarily conserved function/importance of these residues. Furthermore, it was recently shown that the Leu94 to Lys109 region of the FV A1 domain plays a critical role in the calcium-dependent association of the HC and LC (*31*).

To abolish calcium binding to the potential calcium-binding site in FV, we substituted Asp residues at positions 111 and 112 for Asn in a recombinant FV double mutant. The rationale behind this approach is that the Asn residue is the amino acid with a side-chain structure most similar to that of Asp but with one side-chain oxygen atom less as compared to Asp. The structural similarity of Asn and Asp minimizes alteration of the overall backbone structure as caused by the mutagenesis. The expression level of the rFV-NN double mutant is similar to that of the recombinant wild type (see Table 1), which indicates that the introduced amino acid substitutions do not impair the intracellular processing and secretion of the mutant FV molecule (i.e., if the mutations were to destabilize rFV or lead to conformational changes, lower expression levels would most likely have been observed).

The activity of rFVa-NN varies markedly depending on the conditions used for FV activation. Upon activation in the presence of all components of the prothrombinase complex rFVa-NN has 45% of the activity of the recombinant wild type (see Table 1). Furthermore, the activity of rFVa-NN under these conditions was stable throughout the period (20 min) during which prothrombin activation was monitored. These observations show that rFVa-NN has at least 45% of the activity of rFVa-wt and that the introduced amino acid substitutions are not incompatible with FVa activity per se. However, a 10 min thrombin-catalyzed FV activation in the absence of other prothrombinase components yields a rFVa-NN molecule with less than 4% of the activity of rFVa-wt. Time course analysis of thrombin-catalyzed FV activation in solution shows that incubation of rFV-NN with thrombin does cause an increase in FVa activity which is rapidly lost upon prolonged incubation.

A possible explanation for the observed loss of rFVa-NN activity upon prolonged incubation with thrombin is that thrombin inactivates rFVa-NN. However, as shown in Figure

2C, the loss of rFVa-NN activity occurs despite complete inhibition of thrombin activity by hirudin. Furthermore, the activation fragments of rFVa-wt and rFVa-NN are of identical molecular mass as judged by gel electrophoresis (Figure 3A,B, lanes 1), suggesting that the amino acid substitutions present in rFV-NN do not introduce novel thrombin cleavage sites. Finally, in the FVa assay (i.e., in the presence of all components of the prothrombinase complex) the activity of rFVa-NN was stable despite the generation of high concentrations of thrombin (up to 250 nM) in the reaction mixture. On the basis of these observations it is highly unlikely that thrombin is responsible for the observed loss of rFVa-NN activity. The increased rate of loss of rFVa-NN activity observed with increasing concentrations of thrombin in the activation mixture (Figure 2B) can be explained as follows: at high thrombin concentrations there is a fast accumulation of rFVa-NN that subsequently rapidly dissociates into isolated HC and LC, whereas at low thrombin concentrations activation is much slower and the loss of FVa activity is partially compensated by ongoing activation of rFV-NN.

Using ion-exchange chromatography of metabolically radiolabeled rFV, we observed that thrombin-catalyzed activation of rFV-NN results in dissociation of the rFVa-NN HC and LC despite the presence of calcium ions. In addition, we found that, in the presence of phospholipid, FXa and active site inhibited FXa are capable of stabilizing the activity of the rFVa-NN heterodimer in a dose-dependent manner. A possible explanation for the stabilizing effect of FXa is that FXa binds both the HC and LC of rFVa-NN and thereby prevents the loss of activity. In the presence of phospholipid, prothrombin also stabilized the activity of the rFVa-NN heterodimer in a dose-dependent manner. A possible explanation for this stabilizing effect is that prothrombin binds the HC of rFVa-NN, while both prothrombin and the LC of rFVa-NN are bound to the phospholipid surface. This model would explain why high concentrations of prothrombin are required for stabilization of rFVa-NN, since dissociation of the prothrombin−HC dimer from the lipid-bound LC is still possible, whereas this is not the case for FXa which binds to the HC and LC simultaneously. Furthermore, the interaction of prothrombin with the FVa HC is of much lower affinity than the interaction of FXa with FVa. Together, these observations indicate that the mutant rFVa molecule most likely loses activity due to dissociation of the HC and LC complex.

During the preparation of this paper Zeibdawi and colleagues (*29*) published a characterization of several recombinant FV mutants harboring one or more Ala substitutions in the region Asp79 to Glu119. They observed that Ala substitution at position 111 (Asp111Ala) or 112 (Asp112Ala) resulted in 70% and 25% reduction in FVa activity, respectively. Furthermore, in the case of Asp111Ala the reduced FVa activity was accompanied by dissociation of the FVa HC and LC. Although direct comparisons are complicated by the different choice of amino acid substitution, we think the observations by Zeibdawi et al. for the single substitution mutants are in good agreement with the results reported here for the double substitution to Asp111Asn/Asp112Asn in rFV-NN. However, a direct comparison of activities reported by Zeibdawi et al. for the single FV mutants and our rFV-NN is hampered by our finding that these activities depend on the experimental conditions at which FV is activated, i.e., the time of activation (Figure 2A), the thrombin concentration (Figure 2B) and the presence of prothrombinase components in the activation mixture (Figure 4A), and the temperature at which rFV-NN is activated (data not shown).

To our knowledge there are no reports in the literature of patients carrying mutations in any of the mentioned residues (Glu96, Asp102, Glu108, Asp111, and Asp112) in FV. However, patients with missense mutations in the corresponding residues in FVIII have been identified. Three patients carrying substitutions in FVIII corresponding to FV positions 96 (Glu110Val in FVIII), 102 (Asp116Gly in FVIII), and 108 (Glu122Lys in FVIII) all suffered from severe bleeding tendencies and had functional FVIII levels below 1% (antigen levels were not determined) (*32*−*34*). One patient carrying a substitution in FVIII corresponding to position 112 in FV (Asp126His in FVIII) suffered from a moderate bleeding tendency and had 2% functional FVIII and 19% FVIII antigen (*35*). These data show that the Glu110 to Asp126 region of FVIIIa is important for FVIIIa function in vivo and support the observation that the corresponding region in FV is important for FVa function. Recently, the importance of the Glu110 to Asp126 region in FVIIIa was further demonstrated by Wakabayashi and colleagues (*36*) using several recombinant FVIII mutants, in which individual residues in the region were substituted for alanine. They observed that several residues in this region of FVIIIa (including Asp125 and Asp126, which correspond to residues Asp111 and Asp112 in FV, respectively) are part of a calcium-binding site required for expression of cofactor activity. These observations support the hypothesis of the corresponding region in FV to be part of a functionally important calcium-binding site.

In summary, the introduced Asp111Asn/Asp112Asn substitution in a potential calcium-binding site in FV yields a molecule that can be activated by thrombin but rapidly loses FVa activity most likely due to dissociation of the HC and LC. However, in the presence of all components of the prothrombinase complex the activity of the rFVa-NN heterodimer is stable. Furthermore, in the presence of phospholipid vesicles the rFVa-NN heterodimer is partially stabilized by both FXa and prothrombin in a dose-dependent manner. The stabilizing effect of FXa on the rFVa-NN heterodimer is likely due to structural features of FXa as the effect is independent of the enzymatic activity of FXa. In the experiments performed rFV-NN has a phenotype that is consistent with what is expected of a FV molecule that cannot bind calcium. We thus hypothesize that the introduced amino acid substitutions disrupt the calcium-binding site in FV. Further studies, including direct experiments on calcium binding, are required in order to quantify the effect of the introduced amino acid substitutions on calcium binding to FV.

ACKNOWLEDGMENT

We gratefully acknowledge Ing-Marie Persson for purification of prothrombin and thrombin. We thank Drs. István Balogh and Olof Axler for helpful discussions and critical reading of the manuscript.

REFERENCES

1. Dahlback, B. (2000) Blood coagulation, *Lancet 355*, 1627−1632.
2. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) The contribution of bovine Factor V and Factor Va to the activity of prothrombinase, *J. Biol. Chem. 254*, 10952−10962.
3. Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., and Hemker, H. C. (1980) The role of phospholipids and factor Va in the prothrombinase complex, *J. Biol. Chem. 255*, 274−283.
4. Esmon, C. T. (2000) Regulation of blood coagulation, *Biochim. Biophys. Acta 1447*, 349−360.
5. Jenny, R. J., Pittman, D. D., Toole, J. J., Kriz, R. W., Aldape, R. A., Hewick, R. M., Kaufman, R. J., and Mann, K. G. (1987) Complete cDNA and derived amino acid sequence of human factor V, *Proc. Natl. Acad. Sci. U.S.A. 84*, 4846−4850.
6. Church, W. R., Jernigan, R. L., Toole, J., Hewick, R. M., Knopf, J., Knutson, G. J., Nesheim, M. E., Mann, K. G., and Fass, D. N. (1984) Coagulation factors V and VIII and ceruloplasmin constitute a family of structurally related proteins, *Proc. Natl. Acad. Sci. U.S.A. 81*, 6934−6937.
7. Kane, W. H., and Davie, E. W. (1986) Cloning of a cDNA coding for human factor V, a blood coagulation factor homologous to factor VIII and ceruloplasmin, *Proc. Natl. Acad. Sci. U.S.A. 83*, 6800−6804.
8. Esmon, C. T. (1979) The subunit structure of thrombin-activated factor V. Isolation of activated factor V, separation of subunits, and reconstitution of biological activity, *J. Biol. Chem. 254*, 964−973.
9. Hibbard, L. S., and Mann, K. G. (1980) The calcium-binding properties of bovine factor V, *J. Biol. Chem. 255*, 638−645.
10. Guinto, E. R., and Esmon, C. T. (1982) Formation of a calcium-binding site on bovine activated factor V following recombination of the isolated subunits, *J. Biol. Chem. 257*, 10038−10043.
11. Laue, T. M., Johnson, A. E., Esmon, C. T., and Yphantis, D. A. (1984) Structure of bovine blood coagulation factor Va. Determination of the subunit associations, molecular weights, and asymmetries by analytical ultracentrifugation, *Biochemistry 23*, 1339−1348.
12. Mann, K. G., Lawler, C. M., Vehar, G. A., and Church, W. R. (1984) Coagulation Factor V contains copper ion, *J. Biol. Chem. 259*, 12949−12951.
13. Zaitseva, I., Zaitseva, V., Card, G., Moshkov, K., Bax, B., Ralph, A., and Lindley, P. (1996) The X-ray structure of human serum ceruloplasmin at 3.1 Å: nature of the copper centres, *J. Biol. Inorg. Chem. 1*, 15−23.
14. Villoutreix, B. O., and Dahlback, B. (1998) Structural investigation of the A domains of human blood coagulation factor V by molecular modeling, *Protein Sci. 7*, 1317−1325.
15. Pellequer, J. L., Gale, A. J., Getzoff, E. D., and Griffin, J. H. (2000) Three-dimensional model of coagulation factor Va bound to activated protein C, *Thromb. Haemostasis 84*, 849−857.
16. Anfinsen, C. B., Edsall, J. T., Eisenberg, D., and Richards, F. M. (1991) *Advances in Protein Chemistry*, Vol. 42, Academic Press, San Diego, CA.
17. Stenflo, J. (1976) A new vitamin K-dependent protein. Purification from bovine plasma and preliminary characterization, *J. Biol. Chem. 251*, 355−363.
18. Owen, W. G., and Jackson, C. M. (1973) Activation of prothrombin with *Oxyuranus scutellatus scutellatus* (Taipan snake) venom, *Thromb. Res. 3*, 705−714.
19. Lundblad, R. L., Uhteg, L. C., Vogel, C. N., Kingdon, H. S., and Mann, K. G. (1975) Preparation and partial characterization of two forms of bovine thrombin, *Biochem. Biophys. Res. Commun. 66*, 482−489.
20. Rosing, J., Hoekema, L., Nicolaes, G. A., Thomassen, M. C., Hemker, H. C., Varadi, K., Schwarz, H. P., and Tans G. (1995) Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C, *J. Biol. Chem. 270*, 27852−27858.
21. Chase, T., and Shaw, E. (1969) Comparison of the esterase activities of trypsin, plasmin, and thrombin on guanidinobenzoate esters. Titration of the enzymes, *Biochemistry 8*, 2212−2224.
22. Smith, R. L. (1973) Titration of activated bovine Factor X, *J. Biol. Chem. 248*, 2418−2423.
23. Kaufman, R. J. (1990) Vectors used for expression in mammalian cells, *Methods Enzymol. 185*, 487−511.
24. Thorelli, E., Kaufman, R. J., and Dahlback, B. (1998) The C-terminal region of the factor V B-domain is crucial for the anticoagulant activity of factor V, *J. Biol. Chem. 273*, 16140−16145.
25. Nicolaes, G. A. F., Tans, G., Thomassen, M. C. L. G. D., Hemker, H. C., Pabinger, I., Varadi, K., Schwarz, H. P., and Rosing, J. (1995) Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C, *J. Biol. Chem. 270*, 21158−21166.
26. Yamazaki, T., Nicolaes, G. A., Sørensen, K. W., and Dahlback, B. (2002) Molecular basis of quantitative factor V deficiency associated with factor V R2 haplotype, *Blood 100*, 2515−2521.
27. Tans, G., Rosing, J., Thomassen, M. C., Heeb, M. J., Zwaal, R. F., and Griffin, J. H. (1991) Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles, *Blood 77*, 2641−2648.
28. Sørensen, K. W., Nicolaes, G. A. F., Villoutreix, B. O., Yamazaki, T., Tans, G., Rosing, J., and Dahlbäck, B. (2003) Functional Properties of recombinant Factor V mutated in a potential calcium-binding site, *J. Thromb. Haemostasis 1* (Suppl. 1), OC297.
29. Zeibdawi, A. R., Grundy, J. E., Lasia, B., and Pryzdial, E. L. G (2003) Coagulation factor Va Glu96-Asp111: A chelator-sensitive site involved in function and subunit association, *Biochem. J.* (doi: 10.1042/BJ20031205).
30. Rao, V. S., Swarup, S., and Kini, R. M. (2003) The nonenzymatic subunit of pseutarin C, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom, shows structural similarity to mammalian coagulation factor V, *Blood 102*, 1347−1354.
31. Zeibdawi, A. R., and Pryzdial, E. L. (2001) Mechanism of factor Va inactivation by plasmin. Loss of A2 and A3 domains from a Ca^{2+}-dependent complex of fragments bound to phospholipids, *J. Biol. Chem. 276*, 19929−19936.
32. Maugard, C., Tuffery, S., Aguilar-Martinez, P., Schved, J. F., Gris, J. C., Demaille, J., and Claustres, M. (1998) Protein truncation test: detection of severe haemophilia a mutation and analysis of factor VIII transcripts, *Hum. Mutat. 11*, 18−22.
33. Becker, J., Schwaab, R., Moller-Taube, A., Schwaab, U., Schmidt, W., Brackmann, H. H., Grimm, T., Olek, K., and Oldenburg, J. (1996) Characterization of the factor VIII defect in 147 patients with sporadic hemophilia A: family studies indicate a mutation type-dependent sex ratio of mutation frequencies, *Am. J. Hum. Genet. 58*, 657−670.
34. Theophilus, B. D., Enayat, M. S., Williams, M. D., and Hill, F. G. (2001) Site and type of mutations in the factor VIII gene in patients and carriers of haemophilia A, *Haemophilia 7*, 381−391.
35. Liu, M., Murphy, M. E., and Thompson, A. R. (1998) A domain mutations in 65 haemophilia A families and molecular modelling of dysfunctional factor VIII proteins, *Br. J. Haematol. 103*, 1051−1060.
36. Wakabayashi, H., Freas, J., Zhou, Q., and Fay, P. J. (2004) Residues 110−126 in the A1 Domain of Factor VIII Contain a Ca^{2+} Binding Site Required for Cofactor Activity, *J. Biol. Chem. 279*, 12677−12684.
37. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA (http://www.pymol.org).

BI0361362