Coagulation factor Va Glu-96–Asp-111: a chelator-sensitive site involved in function and subunit association

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Coagulation FVa (factor Va) accelerates the essential generation of thrombin by FXa (factor Xa). Although the noncovalent Ca^{2+} -dependent association between the FVa light and heavy subunits (FVaL and FVaH) is required for function, little is known about the specific residues involved. Previous fragmentation studies and homology modelling led us to investigate the contribution of Leu-94–Asp-112. Including prospective divalent cation-binding acidic amino acids, nine conserved residues were individually replaced with Ala in the recombinant B-domainless FVa precursor (ΔFV). While mutation of Thr-104, Glu-108, Asp-112 or Tyr-100 resulted in only minor changes to FXa-mediated thrombin generation, the functions of E96A (81 %), D111A (70 %) and D102A (60 %) mutants (where the single-letter amino acid code is used) were notably reduced. The mutants targeting neighbouring acidic residues, Asp-79 and Glu-119, had activity comparable with ΔFV , supporting the specific involvement of select residues. Providing a

basis for reduced activity, thrombin treatment of D111A resulted in spontaneous dissociation of subunits. Since FVaH and FVaL derived from E96A or D102A remained associated in the presence of Ca^{2+} , like the wild type, but conversely dissociated rapidly upon chelation, a subtle difference in divalent cation co-ordination is implied. Subunit interactions for all other single-point mutants resembled the wild type. These data, along with corroborating multipoint mutants, reveal Asp-111 as essential for FVa subunit association. Although Glu-96 and Asp-102 can be mutated without gross changes to divalent cation-dependent FVaH–FVaL interactions, they too are required for optimal function. Thus Glu-96–Asp-111 imparts at least two discernible effects on FVa coagulation activity.

Key words: calcium, factor Va, factor Xa, prothrombinase.

INTRODUCTION

FVa (factor Va) is an essential cofactor that accelerates the pro-enzyme FXa (factor Xa) at the critical point of merger between upstream branches of the blood coagulation cascade [1]. When associated with aPL (anionic phospholipid) and Ca^{2+} , FVa and FXa complex to form prothrombinase [2], which is responsible for the physiological activation of prothrombin to thrombin, the effector of clot formation.

FVa circulates as an inactive precursor, FV (factor V), which has internal homology repeats in the order $\text{NH}_2\text{-A}_1\text{-A}_2\text{-B-A}_3\text{-C}_1\text{-C}_2\text{-CO}_2$ [1]. Excision of the unique B-domain by thrombin or FXa generates FVa, a heterodimer consisting of the FVaH (FVa heavy subunit) and FVaL (FVa light subunit) derived from $\text{A}_1\text{-A}_2$ and $\text{A}_3\text{-C}_1\text{-C}_2$, respectively. Both FVaH and FVaL are required for prothrombinase function [3–5] and consequently their Ca^{2+} -dependent non-covalent association [6,7] is fundamental for haemostasis. Despite the importance of the intrasubunit association, little is known about the points of contact or how divalent cations facilitate the interaction. Based on the sequence homology between A-type domains [8], inferences have been made from molecular modelling [9,10] using the X-ray crystal structure derived from ceruloplasmin [11] as a homologous template. These models have suggested that the subunits of FVa interact exclusively through an elaborate interface between the A-domains. Approximately each half of an A-domain consists of a plastocyanin-like homology repeat [10], which are predicted by modelling to individually facilitate FVa intrasubunit contacts with a cognate A-domain. In this way, the A_1 N-terminus and A_2

C-terminus would link FVaH to the C- and N-termini of FVaL A_3 , respectively. Consistent with most residues predicted to be at the $\text{A}_1\text{-A}_3$ interface [10], FVa plasmin-mediated fragmentation studies recently excluded the absolute requirement for the N-terminal 93 residues of A_1 and the N-terminal 216 residues of A_3 [12].

The binding of Ca^{2+} to bovine FVa [13,14] and FV [15] has been quantified in the past. A single high-affinity site is present on both FVa and FV, whereas only FV appears to have an additional pair of low-affinity binding sites [15]. Occupancy of the high-affinity Ca^{2+} site has been correlated to FVa subunit association and consequent activity [7,14]. While direct evidence is lacking for the location of the Ca^{2+} -binding site, both FVaH and FVaL must be present for the cryptic site to become accessible [13]. Utilizing higher-affinity Tb^{3+} to probe for potentially weak incomplete Ca^{2+} -binding sites on individual subunits, it has been suggested that the metal ion is co-ordinated primarily with FVaH [14]. To identify the possible location of the high-affinity Ca^{2+} binding site on FV, speculations have been made based on A-domain sequence identity with coagulation FVIII (factor VIII) and ceruloplasmin and combined with a predicted location near the FVaH–FVaL interface by modelling [9]. A cluster of conserved acidic residues were identified which could co-ordinate with Ca^{2+} including Glu-96, Asp-102, Glu-108, Asp-111 and Asp-112. Using another strategy, an algorithm has been applied to the FVa homology model to deduce anionic pockets of proper size to sequester a Ca^{2+} ion [10]. This suggested a second set of residues involving Glu-148, Asn-149 and Asp-1577, or Glu-1572, Glu-1576 and Glu-1583. While the latter anionic

Abbreviations used: FVa, factor Va; FVaH, heavy subunit of FVa; FVaL, light subunit of FVa; FV, factor V; ΔFV , recombinant B-domainless FV; FXa, factor Xa; FVIII, factor VIII; PS, phosphatidylserine; PC, phosphatidylcholine; LV, large vesicles; aPL, anionic phospholipid; PEG 8000, polyethylene glycol 8000; mAb, mAb, monoclonal antibody.

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cluster cannot be excluded at this time, experimental evidence in support of the former has recently been presented [12]. The data showed that in contrast to a plasmin-mediated fragment starting at Leu-94 which remained bound to FVaL in the presence of Ca^{2+} , a similar size fragment starting at Met-110 did not. This observation indicated that the amino acids comprising Leu-94–Lys-109 may be involved in subunit association. To directly investigate the contribution to FVa function of amino acids predicted to bind a divalent cation, each highly conserved acidic residue between Leu-94 and Asp-112 was replaced by Ala using site-directed mutagenesis. In addition, two conserved neutral amino acids contained within the area of interest and the two nearest acidic residues neighbouring the targeted region were replaced. We now report that optimal prothrombinase function requires Glu-96, Asp-102 and Asp-111 through effects on divalent cation-dependent FVaH–FVaL interactions.

EXPERIMENTAL

Chemicals and reagents

Hepes, EDTA, PS (phosphatidylserine), PC (phosphatidylcholine), BSA, PEG 8000 (polyethylene glycol 8000), PMSF (all from Sigma), hirudin (Calbiochem), H-D-Phe-Pip-Arg-pNA. 2HCl (H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroaniline dihydrochloride; S-2238; Chromogenix) and Tween 20 (Fisher) were obtained commercially. Opti-MEM and Dulbecco's modified Eagle's medium/Ham's F12, Geneticin, Fungizone, gentamycin, trypsin, L-glutamine and LipofectAMINE 2000 were purchased from Gibco-BRL. TA cloning kit and QuikChange XL site-directed mutagenesis kit were from Stratagene; QIAQuick PCR purification kit and maxi-prep kit were from Qiagen. pCR3.1 vector and INV α F' competent cells were from Invitrogen. The pMT2-V plasmid encoding human FV was from the American Type Culture Collection [16]. Bio101 Gene Clean Kit was from Bio-Can. *Pfu* DNA polymerase was from Strategene, and the restriction enzymes were from Gibco-BRL and Amersham Biosciences. K293 cells were generously given by Dr W. P. Sheffield (Canadian Blood Services, McMaster University, Hamilton, Canada). Primers were synthesized at Queen's University (Kingston, Canada). Small unilamellar vesicles (average diameter, 50 nm) consisting of a 75:25 mixture of PC/PS were prepared and quantified as described in [12]. LV (large vesicles; 300–600 nm), consisting of 75:25 PC/PS, were made by extrusion using a Liposofast Basic apparatus (Avestin) as described in [17].

Proteins

Human FVa, FXa, prothrombin, thrombin, mAbs (monoclonal antibodies) specific for human FVaH (AHV-5146) and human FVaL (AHV-5112), and sheep anti-human FV polyclonal antibody (PAHFV-S) were from Haematologic Technologies (Essex Junction, VT, U.S.A.). mAb specific for FVa A₃ domain (14H12) was generated by Dr R. Lemieux (Héma-Québec, Québec City, Québec, Canada), for which in-house recombinant FVaL produced using a baculovirus expression system and purified by electroelution was the immunogen [12]. Peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) and peroxidase-conjugated goat anti-rabbit IgG (Promega), were used as secondary detection antibodies combined with the chemiluminescent detection system (ECL[®]; Amersham Biosciences) as a substrate.

Production of human B-domainless FV

As shown previously by several groups, Δ FV (recombinant FV engineered to be deficient in the B-domain), retains FXa cofactor

activity [18,19]. Since the B-domain is a large polypeptide (≈ 120 kDa) containing numerous potentially complicating post-translational modifications, Δ FV was produced for our studies using oligonucleotide overlap extension [20,21] from human FV encoded on the plasmid pMT2-V [16]. The secretory peptide and entire heavy subunit domain within FV were amplified together using the following primers: 1, 3'-CGT CGT AAT CCT TAA TCC TCG TTG TTA CCT TTG TCT-5' and 2, 5'-TAC CCA AAG CTT AGC GGG AGC AGG AAA GGA-3'. The light subunit of FV was amplified separately using the following primers: 3, 5'-GCA GCA TTA GGA ATT AGG AGC AAC AAT GGA AAC AGA-3' and 4, 5'-GAC ACT ATA AAT GAT CCG CCG GCG TAT CCA ATT T-3'.

In all cases, amplification was performed using native *Pfu* DNA polymerase to minimize errors. Successful PCR was detected by agarose gel electrophoresis. The resulting product (4.3 kb) contained a thrombin cleavage site composed of one-half the cleavage site at the end of the A₂ domain (LGIR–SFRN, using the single-letter code) and one-half the cleavage site at the beginning of the A₃ domain (WYLR–SNNG) giving LGIR–SNNG, which yielded a recombinant protein with a single novel thrombin cleavage site directly between FVaL and FVaH. PCR products were cleaned using the QIAQuick PCR purification kit, and then combined with primers 2 and 4, and re-amplified. The amplified hybrid DNA was purified using the Bio101 Gene Clean Kit. The ends of Δ FV DNA were prepared for cloning into the pCR3.1 vector by cleaving outside the FV sequence using *NorI* and *HindIII*. The ligated constructs were transformed into INV α F' competent cells. Positive clones were selected based on DNA mobility in agarose electrophoresis, PCR amplification using specific primers, and pattern of restriction digestion. DNA was extracted using a maxi-prep kit, and each clone was fully sequenced to confirm the correct sequence and absence of mutations introduced by PCR (University of Ottawa Sequencing Service, Ottawa, Canada).

Construction of Δ FV mutants

Δ FV mutants were generated using the QuikChange XL site-directed mutagenesis kit. The pCR3.1 expression vector containing the Δ FV insert was used as a template in the mutagenesis reaction. For each of the mutants, two complementary oligonucleotides with the desired mutation(s) were amplified using the following primers: D79A, 5'-GTTCACTTTAAAAATAAGGCAGCTAAGCC-CTTGAGCATCC-3'; E96A, 5'-GGTACAGTAAATTATCAGC-AGGTGCTTCTTACCTTGACCAC-3'; Y100A, 5'-ATCAGAA-GGTGCTTCTGCCCTTGACCACACATTCCCTGC-3'; D102A, 5'-AGGTGCTTCTTACCTTGCCACACATTCCCTGCG-3'; T104A, 5'-GGTGCTTCTTACCTTGACCACGCATTCCCTGCGAGAAG-3'; E108A, 5'-ACACATTCCCTGCGGCGAAGATGGACGACGCTG-3'; D111A, 5'-TCCCTGCGGAGAAGATGGCCGACGCTGTGGCTCCAG-3'; D112A, 5'-TGCGGAGAA-GATGGACGCGCTGTGGCTCCAGGC-3'; E119A, 5'-GTG-GCTCCAGGCCGAGCATACCTATGAATGGAG-3'; E96A/D102A (referred to as ED), 5'-CAGTAAATTATCAGCAGGTGCTTCTTACCTTGCCACACATTCCC-3'; E108A/D111A/D112A (referred to as EDD), 5'-CACACATTCCCTGCGGCGAAGATGGCCGCGCTGTGGCTCCAGG-3'; Y100A/T104A (referred to as YT), 5'-ATCAGAAAGGTGCTTCTGCCCTTGACCACGCATTCCCTGCGG-3'; and the insertion mutant H103-[AAAA]-T104 (referred to as A₄), 5'-CTTCTTACCTTGACCA-CGCAGCTGCTGCAACATTCCCTGCGGAG-3'.

PCR products were extracted using the maxi-prep kit and the presence of the desired mutations was confirmed by DNA sequencing. To generate the combined mutant E96A/D102A/

E108A/D111A/D112A (referred to as ED/EDD), the EDD construct was used as a template in subsequent mutagenesis reactions.

Stable expression of Δ FV and mutants

Transfection was performed using LipofectAMINE 2000 reagent according to the manufacturer's instructions, with minor modifications. Briefly, purified DNA was linearized with *ScaI*, then heated for 10 min at 55 °C to inactivate the enzyme. DNA (25 μ g) was suspended in 1.5 ml of Opti-MEM containing 2 mM CaCl_2 . LipofectAMINE 2000 (75 μ l) was suspended in the same volume of Opti-MEM containing 2 mM CaCl_2 . DNA was combined with LipofectAMINE 2000 for 20 min at room temperature, and the mixture was used to transfect 80 % confluent K293 cells. At 72 h, the media was changed to Dulbecco's modified Eagle's medium/Ham's F12 supplemented with 10 % fetal calf serum, 1 % L-glutamine, 1 % gentamycin and 1 mg/ml Geneticin. For stable expression of recombinant protein, cells were removed with trypsin/EDTA (0.25 % trypsin, 0.5 mM EDTA and 15 mM NaCl in PBS) and cultured in serum-free medium (Opti-MEM, supplemented with 1 % L-glutamine, 1 % gentamycin and 1 mg/ml Geneticin). The serum-free medium was collected 72 h after growth and centrifuged at 27 000 *g* (Beckman JA20 rotor) for 20 min at 4 °C to remove any cell debris. PMSF (Sigma) was added to conditioned media to a final concentration of 200 μ M and aliquots were stored at –80 °C until use. The amount of Δ FV secreted into media was quantified by Western blot analysis using anti-FVaL as opposed to anti-FVaH, since mutations were exclusively generated in FVaH. Mean pixel intensities from scanned blots were quantified by Northern Eclipse software (Empix) and compared with pixel intensities generated from a serial dilution of known concentrations of purified natural human FVa. As a negative control, the pCR3.1 DNA vector containing no Δ FV DNA was used to transfect K293 cells.

Binding of Δ FV and mutants to anionic phospholipid vesicles

Conditioned medium from cells containing Δ FV or mutants (0.5 nM), or mock-transfected cell supernatants, were incubated with LV (PC/PS, 75:25) in HBS (20 mM Hepes/150 mM NaCl, pH 7.4), 2 mM Ca^{2+} and 0.1 % w/v PEG 8000 at 22 °C for 10 min. The aPL-bound protein was collected by centrifugation and washed. The pellets were heated at 95 °C for 5 min in Laemmli sample buffer, subjected to SDS/PAGE (8 % gels) [28], and electrotransferred to PVDF membranes [29]. The PVDF was blocked at 22 °C for 1 h in 5 % (w/v) non-fat milk, and cleaved FV products were detected using anti-FVaL mAb. For all SDS/PAGE, the apparent molecular mass of proteins was estimated from a graph correlating the $\log_{10}$ of known molecular mass of standards (Bio-Rad) to relative band mobility (results not shown).

Proteolysis of Δ FV by thrombin

Δ FV (2 nM) was incubated with thrombin (1 unit/ml) in the presence of LV (200 μ M) and Ca^{2+} (2 mM) in HBS at 22 °C. The digests were sampled over time and heated at 95 °C for 5 min in Laemmli sample buffer. The digests were subjected to SDS/PAGE (8 % gels), stained with Coomassie Blue, or electrotransferred to PVDF. The PVDF membrane was blocked at 22 °C for 1 h in non-fat milk (5 %), and cleaved products of FV were detected by Western analysis using anti-FVaH or anti-FVaL antibodies.

Cofactor activity of Δ FV and mutants

Δ FV and mutants were tested for their ability to enhance the thrombin generation of FXa by prothrombinase. Thrombin generation was assessed chromogenically by incubating samples with 70 μ l of 0.4 mM S2238 chromogenic substrate. Saturating concentrations of FXa (2 nM), phospholipid vesicles (200 μ M), prothrombin (1.4 μ M) and conditioned medium containing an equal concentration of either Δ FV or Δ FV mutants in HBS/ Ca^{2+} (HBS with 2 mM Ca^{2+}) and 0.1 % PEG 8000 were added at 22 °C for 10 min. Thrombin activity was followed spectrophotometrically by monitoring the change in A_{405} using a Spectramax kinetic microplate reader (Molecular Devices).

FVaH–FVaL dissociation

Δ FV and mutants (0.5 nM) were cleaved with thrombin (1 unit/ml) for 30 min on LV as described above except that prior to thrombin treatment, the vesicles were briefly centrifuged and the pellet was resuspended in HBS/ Ca^{2+} so that only Δ FV or mutants bound to LV were assayed. To inhibit residual thrombin activity, the sample was treated with excess hirudin (10 units/ml) prior to sedimentation. The pelleted vesicles were resuspended, and incubated at 22 °C for selected times in HBS/ Ca^{2+} , or in HBS with EDTA (10 mM; HBS/EDTA) to allow FVaH dissociation. At each time, the vesicles were centrifuged and washed in HBS/ Ca^{2+} and the pellet was subjected to SDS/PAGE (7 % gels), transferred to PVDF and visualized with anti-FVaH or anti-FVaL.

RESULTS

Production and mutation of Δ FV

Using plasma-derived human FVa, we recently reported that Leu-94–Lys-109 within the A₁ domain appears to play a critical part in the FVaL and FVaH Ca^{2+} -dependent association. To investigate the role of acidic amino acids within this and the adjacent region predicted to be involved in the Ca^{2+} -dependent FVa subunit association, nine amino acids in Δ FV were individually changed to Ala (Figure 1, ▼). Based on ceruloplasmin homology modelling, five acidic amino acids previously reasoned to have an appropriate orientation to bind Ca^{2+} [9] were selected for mutation, including Glu-96, Asp-102, Glu-108, Asp-111 and Asp-112. Tyr-100 and Thr-104 were also mutated because they are highly conserved for unknown reasons in the A₁ domains of ceruloplasmin, FVIII and FV from all species of known sequence. As specificity controls, the first acidic amino acid on either side of the implicated 94–112 segment, Asp-79 and Glu-119, were substituted with alanine. The combined functional effects of these single residues were also investigated by producing four multipoint mutants, also depicted in Figure 1. The functional effect of a large disruption in the 94–112 region was evaluated by inserting four alanines (A₄) between His-103 and Thr-104 in a final mutant. Δ FV and the 14 mutants secreted into serum-free culture medium were used for all studies. In each case, Western blots revealed a non-reducible protein with an estimated molecular mass of $\approx$ 170 kDa, using either commercial monoclonal anti-FV antibodies (Haematologic Technologies; AHFV-5112 and AHFV-5146), or a mAb produced by our laboratory against baculovirus-expressed human FV light subunit (14H12). No anti-FV-reactive band was observed in the supernatant of cells transfected with the null expression vector lacking the FV sequence (mock).

The concentration of secreted recombinant protein was measured by digital analysis of Western blots using anti-FVaL mAb. The pixel density of the major band at $\approx$ 170 kDa for Δ FV and

Figure 1 Human FV residues selected for mutagenesis

Amino acids Asp-79–Glu-119 are shown. Amino acids Leu-94–Met-109 implicated in the Ca^{2+} -dependent FVaH–FVaL association by previous fragmentation studies from our laboratory [12] are shown in a shaded box. Five acidic residues implicated by homology modelling to constitute a potential Ca^{2+} -binding site [9] are highlighted by black boxes. The residues selected for single-point mutation to Ala (Asp-79, Glu-96, Tyr-100, Asp-102, Thr-104, Glu-108, Asp-111, Asp-112 and Glu-119) are indicated by ▼. Multiple substitutions to Ala (Glu-96/Asp-102, Glu-108/Asp-111/Asp-112, Tyr-100/Thr-104, Glu-96/Asp-102/Glu-108/Asp-111/Asp-112), are connected above or below the sequence. A final mutant was constructed in which four alanines (A_4) were inserted between His-103 and Thr-104 as depicted by △.

Figure 2 Secretion and aPL binding of ΔFV and mutants

(A) Media from either mock-transfected cells, or cells transfected with ΔFV or the indicated mutant were incubated with LV (200 μM) at 22 °C for 10 min in HBS/ Ca^{2+} . (B) As in (A), except that following incubation with LV, aPL-bound protein was collected by centrifugation and washed. Protein was detected following separation on SDS/PAGE (8% gels), transferred to PVDF membrane and detected with anti-FVaL mAb.

each mutant was determined using Northern Eclipse software and compared with a linear standard curve generated using purified natural human FVa. A known amount of purified natural FVa was included on each gel as an internal control for normalizing blot intensity. Using this method, it was found that secretion of ΔFV and the various mutants into media was similar, indicating that mutation had no significant effect on expression levels. The average concentration of the ΔFV mutants, under these conditions, ranged from 2.3 to 3.6 nM, which was similar to the average concentration of 3.1 nM for ΔFV . For each clone, a small variable amount of a lower-molecular-mass antigen was detectable at ≈ 100 kDa when using the FVaH-specific antibody (Figure 2A), which was not included to determine concentration. To confirm the digital quantification of ΔFV and mutants, identical amounts calculated using the derived concentrations were compared. As can be seen in Figure 2(A), the resulting band intensities did not differ appreciably. This was also true when other antibodies directed at FVaH or FVaL were used (results not shown), which supported the use of these relative concentrations to compare ΔFV and mutants in functional experiments.

Binding of ΔFV mutants to anionic phospholipid

All binding of FV and FVa to aPL has been previously attributed to the region containing FVaL [22,23] and involves the C_2 domain

Figure 3 Prothrombinase activity of ΔFV and mutants

Thrombin generation catalysed by prothrombinase was measured in a reaction mixture containing FXa (2 nM), small unilamellar vesicles (200 μM), prothrombin (1.4 μM) and conditioned serum-free media containing various concentrations of ΔFV or mutant in HBS/ Ca^{2+} /PEG 8000 at 22 °C. Following a 10 min incubation, the reaction was stopped with EDTA (15 mM) and thrombin generation was measured chromogenically using S2238 as a substrate. Means $\pm$ S.D. from triplicates are shown.

[24,25]. We therefore anticipated that mutation of ΔFV in the A_1 domain would be inconsequential to the interaction with aPL. To confirm that no unforeseen alterations in the recombinant proteins occurred that would have gross effects on aPL binding, equal antigenic concentrations of recombinant proteins were incubated with LV (75:25 PC/PS) and the protein remaining bound after washing was analysed by Western blotting. These data showed that there was no evident alteration in the capacity of the single or multipoint mutants to bind excess aPL-containing LV compared with ΔFV (Figure 2B). Under these conditions, the amount of ΔFV antigen in the supernatant after centrifugation of LV was not detectable (results not shown), indicating that each molecule of ΔFV contained a functional aPL-dependent binding domain. Furthermore, the association with LV was Ca^{2+} -independent and required aPL (results not shown), consistent with the properties of plasma-derived FV.

Prothrombinase activity of ΔFV and mutants

The prothrombinase cofactor function of ΔFV is demonstrated in Figure 3, contrasting the negligible thrombin generation that was observed when mock-transfected cell supernatant was assayed for FVa cofactor activity. The latter not only demonstrated the dependence of the experiment on added ΔFV , but also that the cells do not endogenously secrete constituents into culture media that contribute to prothrombin activation. On a molar basis, the observed increase in thrombin generation due to ΔFV was $\approx 25\%$ of that mediated by an equal concentration of FVa purified from plasma (results not shown), which is comparable with reports by other laboratories using recombinant FV [18,19].

To investigate a potential role for FV residues Glu-96, Asp-102, Glu-108, Asp-111 and Asp-112 in prothrombinase function, the effect of substituting each to alanine was first evaluated and compared with ΔFV . The simultaneous substitution of all five acidic residues to Ala (ED/EDD) resulted in nearly complete reduction (98%) of ΔFV activity, which strongly supported a functional role for this region of FV (Figure 3). To minimize allosteric changes that may accompany the large variation of

Figure 4 Thrombin-mediated conversion of Δ FV to FVa

Δ FV (2 nM) was treated with thrombin (1 unit/ml) in the presence of LV (200 μ M) and Ca^{2+} (2 mM) in HBS at 22 °C. At the indicated time (min) aliquots were withdrawn from the mixture and the reaction stopped by heating in SDS-containing Laemmli sample buffer. Samples were separated by SDS/PAGE (8 %). The protein was transferred to PVDF and detected with anti-FVaL (A) or anti-FVaH (B).

charge due to the five mutations in ED/EDD and to localize individual residues contributing to function, the respective single-point mutants were prepared. When compared with Δ FV, the largest effects on prothrombinase activity occurred when Glu-96 > Asp-111 > Asp-102 were mutated. At 2.2 nM (the concentration where differences appeared to be maximal), substitution to Ala resulted in 81, 70 and 60 % reduction, respectively. Moderate reductions in activity were observed for Thr-104 (35 %), Glu-108 (30 %) and Asp-112 (25 %). In contrast, relatively inconsequential effects were observed for substitution of Tyr-100 (16 %), Asp-79 (7 %) and Glu-119 (0 %) by Ala. The latter two mutations (Asp-79 and Glu-119) are the nearest acidic residues neighbouring the predicted Ca^{2+} -sensitive region in the A₁ domain (i.e. Leu-94–Asp-112). The finding that these have limited or no involvement in Δ FV function serves as a negative control for mutagenesis and supports the specific contribution of select residues.

Effect of Δ FV mutation on the FVaH–FVaL interaction

Having observed that at least Glu-96, Asp-102 and Asp-111 play a significant part in Δ FV function, we next investigated whether the Ca^{2+} -dependent non-covalent interaction between FVaH and FVaL was affected. An experiment was designed to take advantage of the requirement for FVaL to anchor FVaH to aPL [22,24]. To first establish the conditions needed for complete thrombin-mediated proteolysis and to ascertain that Δ FV exhibited a typical cleavage pattern, a time course of thrombin-mediated Δ FV cleavage was performed in the presence of aPL-containing vesicles and Ca^{2+} (Figure 4). SDS/PAGE showed that the single-chain Δ FV was completely converted by 1 unit/ml thrombin within 20 min into the expected FVaL ($\approx$ 74/71 kDa) and FVaH ($\approx$ 105 kDa) subunits. All Δ FV mutants were comparably proteolysed by 20 min of incubation with the same concentration of thrombin (results not shown), which established the experimental conditions for complete FVaH and FVaL generation and suggested that the targeted residues are unlikely to be involved Δ FV or mutant recognition by thrombin.

To determine whether the observed decreases in prothrombinase activity due to mutation of Δ FV correlated to enhanced dissociation of FVaH–FVaL, equal antigenic concentrations of the Δ FV mutants were equilibrated with excess aPL-containing LV

Figure 5 Effect of Δ FV mutation on FVaH–FVaL association

Δ FV and mutants (0.5 nM) bound to aPL in HBS/ Ca^{2+} /PEG 8000 were preincubated with thrombin (1 unit/ml) for 20 min at 22 °C. Proteolysis was stopped with hirudin (10 units/ml), and Ca^{2+} (2 mM) or EDTA (15 mM) in HBS/PEG 8000 was added. The aPL-bound protein was collected by centrifugation and washed in HBS/ Ca^{2+} /PEG 8000 over 3 h. All reactions were stopped by heating in SDS-containing Laemmli sample buffer. Protein remaining bound to aPL was separated on SDS/PAGE (7 % gels), transferred to PVDF and probed with anti-FVaH (AHV-1546) mAb by Western analysis. Band intensities were quantified and represented in arbitrary units at 1, 2 and 3 h of incubation with Ca^{2+} (A) or EDTA (B) for Δ FV and each mutant.

so that the same amount of each variant was bound to LV at time zero and undetectable amounts were contained in solution (as Figure 2B). Each mixture was treated with thrombin to achieve complete conversion to FVa (as Figure 4) and the amount of each subunit bound to washed LV was probed with either a FVaH- or FVaL-specific antibody at 1 h intervals. FVaL has been well established as the exclusive aPL-binding subunit and, for each mutant, remained associated with aPL for the 3 h duration of the experiment regardless of the presence of Ca^{2+} (results not shown). This observation confirmed that approximately the same number of molecules of FVa are generated for each Δ FV mutant and that the efficiency of LV sedimentation does not change over the time-course.

While the amount of FVaL remaining bound to aPL was unaffected by mutation, this was not the case for FVaH. To illustrate these qualitative changes, the Western blot band intensity of FVaH associated with LV at 1, 2 and 3 h post thrombin treatment, is presented in Figure 5 for each mutant. In the presence of Ca^{2+} , Figure 5(A) shows that FVaH derived from Δ FV remained bound to aPL throughout the experiment, which is known to require interaction with FVaL [22,24]. Consistent with earlier observations made with FVa purified from human plasma [12], inclusion of an excess of chelator (EDTA) in the incubation mixture resulted in slow dissociation of FVaH derived from Δ FV (Figure 5B). In sharp contrast, simultaneous substitution of all five acidic amino acids in the ED/EDD mutant caused complete dissociation of FVaH from FVaL prior to the first sampling at 1 h, regardless of the presence of Ca^{2+} . The spontaneous and rapid dissociation of FVa subunits provides an explanation for the nearly complete loss of prothrombinase function observed for the ED/EDD mutant (Figure 3).

The specific amino acids contributing to the spontaneous dissociation of FVaH from FVaL due to ED/EDD mutation were assigned using our panel of nine single-point mutants. Figure 5 shows that only substitution of Asp-111 by Ala was sufficient to give rise to the phenotype observed for ED/EDD. In the presence of 2 mM Ca^{2+} and the absence of chelator, the FVaH derived from all other single-point ΔFV mutants behaved like ΔFV , without exhibiting dissociation from FVaL over the duration of the experiment. This is interesting because at least mutation of Glu-96 and Asp-102 caused significant (i.e. > 60 %) loss of function (Figure 3), which were conducted at the same divalent cation concentration. Therefore a property of FVa must be affected that is required for FXa cofactor activity, but which is independent of subunit dissociation.

Speculating that altered co-ordination of divalent cations may explain reduced activity without subunit dissociation for E96A and D102A, we followed FVaH dissociation in the presence of a chelator. In contrast to FVa subunits derived from ΔFV , which dissociated only 70 % after 3 h in the presence of EDTA, mutation of Glu-96 or Asp-102 resulted in nearly complete FVaH dissociation prior to 1 h (Figure 5B). When Asp-79, Tyr-100, Thr-104, Glu-108, Asp-112 or Glu-119 was substituted for Ala, insignificant effects on the apparent rate of chelator-induced FVa dissociation were observed. These data suggest that mutation of Glu-96 or Asp-102 changes the susceptibility of FVa-bound divalent cations to chelation by EDTA.

Effect of multipoint mutation on ΔFV

The indicated functional contribution of FV residues spanned by Leu-94–Asp-112 was further mapped by generating multipoint mutants consisting of Ala substitutions at adjacent acidic residues, Glu-108, Asp-111 and Asp-112 (EDD) or Glu-96 and Asp-102 (ED). Since only mutation of Asp-111 induced spontaneous dissociation of FVaL and FVaH, this was not combined with Glu-96 or Asp-102 to determine if the phenotypes observed for the single-point mutants persisted. Figure 6(A) shows that the activity of EDD and ED at 2.2 nM was significantly decreased by 78 and 85 % respectively compared with ΔFV . Upon conversion of EDD to FVa by thrombin, a rapid loss of FVaH from FVaL bound to aPL-containing LV was observed in the absence (Figure 6B) or presence (Figure 6C) of chelator, similar to D111A. In the presence of Ca^{2+} (Figure 6B) the same experiment showed that the rate that ED-derived FVaH dissociated from FVaL was slower than from D111A, but nevertheless significantly faster than E96A or D102A, indicating a cumulative effect. Chelator-mediated subunit dissociation observed for ED was rapid, as for E96A, D102A, D111A or ED/EDD. These data support the single-point mutant experiments suggesting that the Leu-94–Asp-112 region participates in FVa function loosely divisible according to C-terminal and N-terminal effects.

To further explore the functional contribution of the conserved neutral amino acids within Leu-94–Asp-112, a combined mutant consisting of Tyr-100 and Thr-104 was produced (YT), which reduced prothrombinase activity by 65 % at 2.2 nM (Figure 6A). Western blot analysis of LV-bound FVaH derived from YT (Figures 6B and 6C) was similar to that observed for mutation of Glu-96 or Asp-102, with a marginally slower dissociation of FVaH in the presence of chelator. Since individual mutation of either Tyr-100 or Thr-104 more closely resembled ΔFV , we investigated whether a gross mutation within Leu-94–Asp-112 was sufficient to confer the rapid chelator-dependent dissociation of FVaH characteristic of a mutation at Glu-96 or Asp-102. Four alanines (A_4) were consequently inserted on the N-terminal side of Thr-104. This resulted in a 94 % reduction of prothrombinase

Figure 6 Effect of multipoint ΔFV mutation on prothrombinase activity and FVaH–FVaL association

(A) Prothrombinase activity of multipoint ΔFV mutants and FVaH–FVaL association after treatment with thrombin was performed as described in Figures 3 and 5, respectively. Panels (B) and (C) correspond to treatments in Figures 5(A) and 5(B) respectively.

function (Figure 6A). Like the single-point Glu-96 and Asp-102 mutants, accelerated FVaH dissociation from FVaL was observed after conversion to FVa in the presence of chelator (Figure 6C), while in the presence of divalent cations, the subunit interaction was stable over the 3 h duration of the experiment (Figure 6B). Thus a substantial insertion causing nearly complete loss of FVa cofactor function was insufficient to mediate spontaneous FVaH–FVaL dissociation. This observation adds further support for a specific contribution of Asp-111 to the association of FVa subunits, and a divisible contribution of the region spanning Glu-96–Thr-104 to prothrombinase function.

DISCUSSION

It has been well established that the coagulation cofactor function of FVa requires the interaction of its subunits, FVaH and FVaL, which associate reversibly in a divalent cation-dependent manner [7,27]. Although several metal ions have been identified that can facilitate subunit association [6,7], the relative concentration of these suggests that Ca^{2+} is the probable physiological mediator. Despite the importance of the FVaH–FVaL interaction, little is

known about the specific residues involved in divalent cation binding or in subunit contact. Their experimental identification has been complicated by the requirement for both subunits in formation of the single Ca^{2+} -binding site [13]. Recently, two plasmin-mediated FVaH fragments of indistinguishable mobility on SDS/PAGE (≈ 30 kDa) were reported. These differ overtly in their ability to interact with FVaL [12]. One starting at Leu-94 forms a Ca^{2+} -dependent complex with FVaL. The other, beginning only sixteen residues away at Met-110, is unable to maintain an interaction with FVaL. This chance observation identified Leu-94–Lys-109 as a plausible Ca^{2+} -sensitive region involved in FVa subunit association. Interestingly, predictions made earlier from homologous protein modelling that combined the X-ray structure of ceruloplasmin and the sequence identity of FVIII, suggested five acidic amino acids included in Glu-96–Asp-112 of FV which could be involved in Ca^{2+} binding [9]. Because of the coincidental overlap of FV functional regions involving Ca^{2+} deduced by data [12] and theory [9], the present report addresses the hypothesis that Leu-94–Asp-112 is a divalent cation-sensitive functional region within FV.

The data shown here were derived from introduction of nine single-point and five multipoint mutations into ΔFV (wild-type B-domainless) and strongly support the region spanning Glu-96–Thr-104 and, by a different mechanism Asp-111 alone, as being essential for optimal FVa-dependent activation of prothrombin by FXa. In the presence of supplemented Ca^{2+} and potentially other trace divalent cations that may be contained in culture media, replacement of individual residues with Ala mediated a wide range of reduced activity compared with ΔFV , in the order E96A (81%), D111A (70%), D102A (60%), T104A (35%), E108A (30%), D112A (25%), Y100A (16%), D79A (7%) and E119A (0%). D79A and E119A, the nearest acidic amino acid substitutions outside the immediate segment of interest, did not differ appreciably from ΔFV . The lack of functional involvement of amino acids Asp-79 and Glu-119 supports the specificity of effects seen by replacement of the other amino acids.

To explain how mutation within Glu-96–Asp-111 may weaken FVa function, gross effects on the FVaH–FVaL association were investigated. FVa subunits derived from ΔFV remained associated in the presence of Ca^{2+} over 3 h. Consistent with a role in subunit interaction and justifying the observed decrease in prothrombinase activity, all single-point or multipoint mutants that included D111A (i.e. EDD and ED/EDD) resulted in rapid dissociation of FVaH from FVaL even in excess Ca^{2+} . However, the apparent FVaH–FVaL interaction was not affected by any other mutant studied in the presence of divalent cations. Hypothesizing that subtle divalent cation-binding changes may account for the functional effects observed for other mutants, chelator-induced FVaH–FVaL dissociation was followed. Under these conditions, FVaH derived from ΔFV was only 70% dissociated from FVaL after 3 h, comparable with natural purified FVa [12]. Although no different from ΔFV in the presence of Ca^{2+} , nearly complete chelator-dependent release of FVaH from FVaL was observed prior to the first sampling at 1 h for the mutants exhibiting prothrombinase activity reduced by $> 60\%$, including E96A, D102A, YT and A₄. These data suggest that disruption of Glu-96–Thr-104 affects the co-ordination of metal ions required for optimal function, causing increased susceptibility to chelation and consequent rapid chelator-induced dissociation of FVaH–FVaL. When combined, our observations implicate the relatively small region encompassing Glu-96–Asp-111 in two key FVa functions: intrasubunit association and a separable cofactor effect on FXa.

At this time, the specific FVa function(s) altered by mutation of residues within Glu-96–Thr-104 is unknown. To accelerate

FXa, FVa must participate simultaneously in a number of binary macromolecular interactions. An effect of ΔFV mutation on aPL binding can be excluded because all experiments were conducted at an excess of aPL-containing vesicles so that differences would be negligible. Regardless, it is doubtful that mutation of FVaH affected aPL binding since this function of FV/FVa has been assigned exclusively to FVaL by independent laboratories [22–24]. Similarly, effects of ΔFV mutation on conversion to FVa by thrombin cannot be excluded, but are unlikely to be the basis for our findings since experiments were conducted under conditions that facilitated complete conversion of the procofactor to FVa for all mutants. To unambiguously investigate contributions of Glu-96–Thr-104 to FVa–FXa or FVa–prothrombin interactions, or other as yet poorly understood catalytic effects on FXa activity, direct experiments using purified mutants are required. Although mutation of Asp-111 or Glu-96–Thr-104 appears to result in distinguishable FVa functional phenotypes, spontaneous subunit dissociation induced by the former would preclude detection of the functional changes characteristic of the latter. We consequently favour Asp-111 as being required with Glu-96–Thr-104 to concertedly facilitate optimal FVa function.

Aside from Ca^{2+} being essential to link FVaH to FVaL, the precise role of metal ions in FVa function is unclear. Experiments conducted with the E96A or D102A mutants suggest for the first time that changes in divalent cation sequestration can correlate to reduced prothrombinase function, but without observed effects on the FVaH–FVaL association. Therefore metal ions mediate FVa function in addition to the non-covalent complex between subunits. While Asp-111 was demonstrated by our studies to be essential for FVa subunit association, it is unknown whether it constitutes part of the Ca^{2+} -binding site, a fundamental FVaH–FVaL contact point or both. On the other hand, the finding that mutations within Glu-96–Thr-104 only increase the apparent rate of FVaH–FVaL dissociation due to chelation, implies participation of these residues mainly in divalent cation binding and not direct subunit interactions. This apparently distinct metal ion role in FVa subunit association and cofactor function has similarity to the interesting picture emerging for the homologous pro-cofactor, FVIII, which requires Ca^{2+} to adopt a functional conformation [28] but a different divalent cation for subunit association [29]. It should be noted that the contrasting Ca^{2+} -dependence of FVa and FVIII subunit association may reflect unknown differences between the metal-ion-requirement of the active cofactor compared with the respective pro-cofactor, especially since FV is a single polypeptide, whereas FVIII is a non-covalent heterodimer.

In addition to interactions with Ca^{2+} , FV [30] and FVIII [31] have a single Cu^{2+} -binding site with homology to ceruloplasmin, the major copper binding protein in plasma [11]. Cu^{2+} has been shown to enhance the affinity between FVIII subunits by two orders of magnitude [29], while functional studies concerning a role for Cu^{2+} have not been reported for either FV or FVa. Only one of six homologous Cu^{2+} binding sites in ceruloplasmin is identifiable in FV and FVIII [9,10]. This corresponds to residues His-85 in FVaH, and His-1815 and His-1817 in FVaL. In the three-dimensional model of FVa [9,10], these neighbour the chelator-sensitive functional site identified in the present work, Glu-96–Asp-111. Therefore it is tempting to speculate that Ca^{2+} and Cu^{2+} may bind adjacent to each other in FVa, and residues within Glu-96–Asp-111 may differentially affect the function of both.

The present results identify Glu-96–Asp-111 in the coagulation factor V A₁ domain as a novel site involved in prothrombinase cofactor activity. Contained within this region, is the first individual residue implicated in FVa intrachain association, Asp-111. While Glu-96 and Asp-102 are also required for

optimal function, unlike Asp-111 their mutation does not result in gross changes to divalent cation-dependent FVaH–FVaL interactions. While the precise role in FVa function is not yet known, Glu-96 or Asp-102 substitution to Ala enhances susceptibility to chelation, indicating a role for divalent cations in FVa function that is separable from subunit association.

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